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Ekspresja chemokiny CCL18 oraz jej receptorów CCR8 i PITPNM3 w glejaku wielopostaciowym. Znaczenie w procesie nowotworzenia

Glioblastoma multiforme – expression and impast of the CCL18, CCR8, and PITPNM3 on the process of oncogenesis

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Aneks

Oświadczenia współautorów

WYKAZ WAŻNIEJSZYCH STOSOWANYCH SKRÓTÓW

ACKR2	ang. *atypical chemokine receptor 2*, atypowy receptor chemokiny 2
AMAC-1	ang. *alternative macrophage activation-associated C-C chemokine 1*
ATRX	ang. *a-thalassemia/mental-retardation-syndrome-X-linked*, Zespół alfa talasemia / *niepełnosprawność intelektualna sprzężona z* chromosomem X
BMI	ang. *body mass index*, wskaźnik masy ciała
CAF	ang. *cancer-associated fibroblasts*, fibroblasty związane z rakiem
CBTRUS	ang. *Central Brain Tumor Registry of the United States*, Centralny Rejestr Guzów Mózgu w Stanach Zjednoczonych
CCL	ang. *CC chemokine ligands*, ligandy chemokinowe
CCR	ang. *chemokine receptors*, receptory chemokinowe
CDKN2A/B	ang. *cyclin-dependent kinase inhibitor 2A*, cyklinozależne inhibitory kinazy 2A
CNS	ang. *central nervous system*, ośrodkowy układ nerwowy
CNV	ang. *copy number variations*, wariancje genów
CoCl2	ang. *cobalt chloride*, chlorek kobaltu
CTCL	ang. *cutaneous T-cell lymphoma*, chłoniak skórny T-komórkowy
CUN	ang. *central nervous system*, ośrodkowy układ nerwowy
CXCR4	ang. *CXC motif chemokine receptor 4*, receptor chemokin C-X-C typu 4
DC	ang. *dendritic cells*, komórki dendrytyczne
DC-CK1	ang. *dendritic cell derived C-C chemokine 1*, chemokina C-C pochodząca z komórek dendrytycznych 1
DLBCL	ang. *diffuse large B cell lymphoma*, chłoniak rozlany z dużych komórek B
DPPIV	ang. *dipeptidyl peptidase IV*, peptydaza dipeptydylowa IV
EBV	ang. *Epstein–Barr virus*, wirus *Epstein-Barr*
EGF	ang. *epidermal growth factor*, nabłonkowy czynnik wzrostu
EGFR	ang. *epidermal growth factor receptor*, receptor nabłonkowego czynnika wzrostu
EMT	ang. *epithelial-mesenchymal transition*, transformacja epitelialno-mezenchymalna
ERK	ang. *extracellular signal-regulated kinases*, kinaza regulowana sygnałem zewnątrzkomórkowym
MAPK	ang. *mitogen-activated protein kinases*, kinaza białkowa aktywowana mitogenem
FGFR1	ang. *fibroblast growth factor receptor 1*, receptor czynnika wzrostu fibroblastów 1
GBM	ang. *glioblastoma multiforme*, glejak wielopostaciowy

GH	ang. *growth hormone,* hormon wzrostu
GOF	ang. g*ain of funtion,* wzmocnienie funkcji
GPR75	ang. G-*protein-coupled receptor 75,* receptor sprzężony z białkiem G
HbA1c	ang. *glycated hemoglobin, hemoglobina glikowana*
HPFS	ang. *Health Professionals Follow-Up Study,* badanie kontrolne pracowników ochrony zdrowia
HRE	ang. *hypoxia-response elemenents*, sekwencja promotorowa związana z *niedotlenieniem*
HTLV-1	ang. *human T cel leukemia virus type 1,* wirus ludzkiej białaczki z komórek T
IARC	ang. *International Agency for Research on Cancer,* Międzynarodowa Agencja Badań nad Rakiem
ICP	ang. *intracranial pressure*, ciśnienie śródczaszkowe
IDH	ang. *isocitrate dehydrogenase*, dehydrogenaza izocytrynianowa
IGF	ang. *insulin-like growth factor,* insulinoopodobny czynnik wzrostu
IGFBP-2	ang. *insulin-like growth factor binding protein-2,* insulinopodobny czynnik wzrostu wiążący białko-2
JEM	ang. *job exposure matrix*
JmjC	ang. *jumonji C family*
KSHV	ang. *Kaposi sarcoma-related human herpes virus-8,* mięsak Kaposiego związany z zakażeniem *ludzkim wirusem opryszczki* typu *8*
LARC	ang. *liver activation regulated chemokine*, chemokina regulowana aktywacją wątroby
LOH	ang. *loss of heterozygosity*
MAPK	ang. *mitogen-activated protein kinase,* kinaza aktywowana mitogenami
MDSC	ang. *myeloid-derived suppressor cells*, komórki supresorowe pochodzenia mieloidalnego
MEC	ang. *mucosae-associated epithelial chemokine*
MGMT	ang. *O-6-methylguanine DNA methyltransferase* - metylotransferaza DNA O-6-metyloguaninowa
MIP-1α	ang. *macrophage inflammatory protein-1α,* białko zapalne makrofagów-1α
MIP-3α	ang. *macrophage inflammatory protein-3α,* białko zapalne makrofagów-3α
MIP-4	ang. *macrophage inflammatory protein 4,* białko zapalne makrofagów 4
MRI	ang. *magnetic resonance imaging,* rezonans magnetyczny
MSC	ang. *mesenchymal stem cells*, mezenchymalne komórki macierzyste
NDMA	ang. *N-nitrosodimethylamine, N-nitrozodimetyloamina*
NEC	ang. *not elsewhere classified, niesklasyfikowane*
NHS	ang. *Nurses' Health Study*
NHSII	ang. *Nurses' Health Study II*

Nir1 ang. *N-terminal domain interacting receptor 1*

NK ang. *natural killer T*, komórki naturalnej cytotoksyczności

NLPZ *ang. non-steroidal anti-inflammatory drugs*, niesteroidowe leki przeciwzapalne

NOS ang. *not otherwise specified,* nie określono

OS ang. *overall survival*, całkowity czas przeżycia

PAHs ang. *polycyclic aromatic hydrocarbons*, wielopierścieniowe węglowodory aromatyczne

PARC ang. *pulmonary and activation regulated chemokine*

PD-L1 ang. *programmed death-ligand 1, zaprogramowane* białko *śmierci-ligand 1*

PFS ang. *progression-free survival*, czas przeżycia wolny od progresji

p-GBM ang. *pediatric glioblastoma multiforme,* pediatryczny glejak wielopostaciowy

PI3K ang. *phosphatidylinositol 3-kinase, kinaza 3-fosfatydyloinozytolu*

PITPNM3 ang. *phosphatidylinositol transfer membrane-associated protein 3,* białko przenoszące fosfatydyloinozytol związane z błoną 3

PTEN ang. *phosphatase and tensin homolog, homolog fosfatazy i tensyny*

PYK2 ang. *protein tyrosine kinase,* białkowa kinaza tyrozynowa

RAC2 ang. *Ras-related C3 botulinum toxin substrate 2*

SNPs ang. *single nucleotide polymorphisms*, polimorfizm pojedynczych nukleotydów

STAT3 ang. *transducer and activator of transcription 3, przekaźnik* sygnału i *aktywator transkrypcji 3*

TAC ang. *tumor-associated cells*, komórki związane z nowotworem

TADC ang. *tumor-associated dendritic cells, komórki dendrytyczne związane z nowotworem*

TAM ang. *tumor associated macrophages*, makrofagi związane z nowotworem

TAN ang. *tumor-associated neutrophils,* neutrofile związane z nowotworem

TECK ang. *thymus-expressed chemokine,* chemokina ulegająca ekspresji w grasicy

TERT ang. *telomerase reverse transcriptase,* odwrotna transkryptaza telomerazy

TET ang. *ten-eleven translocation*

***TGF*-β1** ang. *transforming growth factor β1,* transformujący czynnik wzrostu *beta*

TIL ang. *tumor-infiltrating lymphocytes, limfocyty* infiltrujące nowotwór

TP53 ang. *tumor protein 53*

TSNA ang. *tobaccospecific nitrosamines*, nitrozoaminy specyficzne dla tytoniu

VEGF ang. *vascular endothelial growth factor*, czynnik wzrostu śródbłonka naczyniowego

VEGF-A	ang. *vascular endothelial growth factor A*, czynnik wzrostu śródbłonka naczyniowego A
VEGF-C	ang. *vascular endothelial growth factor C*, czynnik wzrostu śródbłonka naczyniowego C
vMIP-I	ang. *viral macrophage inflammatory protein*-I, białko zapalne makrofagów-I
vMIP-II	ang. *viral macrophage-inflammatory protein-II*, białko zapalne makrofagów-II
WHO	ang. *World Health Organization*, Światowa Organizacja Zdrowia

1. WSTĘP

1.1. CHARAKTERYSTYKA GLEJAKA WIELOPOSTACIOWEGO

Glejak wielopostaciowy (GBM, ang. *glioblastoma multiforme*) jest jednym z najbardziej agresywnych nowotworów złośliwych. GBM jest też najczęstszym złośliwym pierwotnym guzem mózgu oraz centralnego układu nerwowego (CUN), stanowiącym 14.5% wszystkich guzów oraz 48.6% złośliwych guzów CUN [1].

Glejak wielopostaciowy wywodzi się z linii astrocytarnej [2] i jest najbardziej złośliwym typem nowotworu wśród glejaków (IV stopień). Trudno jednoznacznie określić częstość występowania GBM, bowiem różni się on w zależności od analizowanych raportów. Niektóre publikacje donoszą, że częstość występowania GBM wynosi 3.19 przypadków na 100 000 osobo/lat [3] zaś inne, że 4.17 na 100 000 osób [4]. Zapadalność w populacji pediatrycznej (0–18 r.ż.) wynosi 0.85 na 100 000, a pediatryczny glejak wielopostaciowy (p-GBM, ang. *pediatric glioblastoma multiforme*) odpowiada za 3–15% pierwotnych guzów mózgu [5] wśród tej grupy wiekowej, mimo że pierwotne guzy centralnego układu nerwowego są drugim najczęstszym rodzajem nowotworu u dzieci, a najczęstszym wśród guzów litych [6]. Ostrom et al. [7] opisują zachorowalność w populacji 0–19 lat w USA po dostosowaniu do wieku na poziomie 0.18 (95% CI 0.16–0.19) na 100 000.

1.2. KLASYFIKACJA GLEJAKA WIELOPOSTACIOWEGO

Międzynarodowym standardem nazewnictwa oraz diagnostyki glejaków jest klasyfikacja Światowej Organizacji Zdrowia (WHO, ang. *World Health Organization*), zgodnie z którą, GBM jest nowotworem o IV stopniu złośliwości.

Klasyfikacja glejaków polega na ocenie stopnia złośliwości, który określany jest na podstawie kryteriów histopatologicznych. Zgodnie z nimi wyróżniono 4 rodzaje tego nowotworu [8]:

- **Glioblastoma, IDH-wildtype** (ang. *isocitrate dehydrogenase- wildtype*) – (90% przypadków), rozwijający się *de novo* i w grupie wiekowej około 60 lat;

- **Glioblastoma, IDH-mutant** (10% przypadków) – jest to wtórny GBM rozwijający się zwykle u młodszych pacjentów na podłożu glejaków o większym zróżnicowaniu (stopień I–III według klasyfikacji WHO) – wiąże się z lepszym rokowaniem [9];
- **Gliobastoma NOS** (ang. *not otherwise specified)* – status mutacji IDH nie może być określony ze względu na brak materiału histologicznego lub molekularnego do badania;
- **NEC** (ang. *not elsewhere classified*) – czwarta kategoria wyodrębniona w ostatnich latach. W tym typie GBM niezbędne oznaczenia dotyczące klasyfikacji nowotworu zostały wykonane, ale ich wynik nie pozwala na dopasowanie nowotworu do kategorii z podziału WHO z 2016. Taka sytuacja może zajść w przypadku różnic między cechami klinicznymi, histologicznymi, immunohistologicznymi i genetycznymi nowotworu. Istnieje również możliwość istnienia nowej podjednostki GBM, o nieznanej kombinacji cech, jeszcze nie sklasyfikowanej w podziale WHO.

Warto zauważyć, że klasyfikacja glejaków wprowadzona w 2016 r. zawiera nie tylko histologiczną klasyfikację nowotworu, lecz również zmiany molekularne w obrębie komórek [10].

Coraz bardziej dostępna możliwość profilowania nowotworów i użycie metod machine-learningu pozwala na trafne przewidywanie rokowania i odpowiedzi na konkretne metody leczenia [11]. Nowe kryteria i nomenklatura wprowadzona w najnowszej klasyfikacji WHO wzmacnia trend genotypowania GBM. Gwiaździak (ang. *astrocytoma*) stopnia II, II lub IV zastąpił nowotwory wcześniej klasyfikowane jako gwiaździak rozlany (ang. *diffuse astrocytoma*), gwiaździak anaplastyczny (ang. *anaplastic astrocytoma*), lub glejak wielopostaciowy (ang. *glioblastoma*). Oprócz badania histopatologicznego istotną rolę zaczęły odgrywać: status delecji cyklinozależnego inhibitora kinazy 2A/B (*CDKN2A/B*, ang. *cyclin-dependent kinase inhibitor kinazy 2A/B*), mutacja promotora odwrotnej transkryptazy telomerazy (*TERT*, ang. *telomerase reverse transcriptase*), amplifikacja genu receptora nabłonkowego czynnika wzrostu *(EGFR, ang. epidermal growth factor receptor*) oraz jednoczesne zyskanie dodatkowego chromosomu 7 z utratą chromosomu 10 (+7/–10) [12].

1.3. CHARAKTERYSTYKA CHEMOKIN

Podrodzina CC (β-chemokiny) chemokin jest grupą chemotaktycznych cytokin 1–28 znanych jako ligandy chemokinowe (CCL, ang. *motif chemokine ligands*). Dzielą one wspólny element budowy pod postacią N-końcowej domeny CC, a numery w nazwach zostały nadane w kolejności ich odkrycia [13]. Właściwa liczba chemotaktycznych chemokin wynosi 27, ponieważ nazwy CCL9 i CCL10 określają tę samą chemokinę. Powyższe 27 chemokin są ligandami dla 10 receptorów – CC (CCR, ang. *motif chemokine receptors*) numerowanych od 1 do 10. Tak jak w przypadku reszty chemokin, chemokiny chemotaktyczne są kluczowe dla funkcjonowania układu odpornościowego [13]. Mimo swojego działania przeciwnowotworowego CC wykazują również wpływ pronowotworowy i odgrywają ważną rolę w onkogenezie.

1.4. LECZENIE GLEJAKA WIELOPOSTACIOWEGO

Glejak wielopostaciowy pozostaje niewyleczalnym nowotworem [14]. Celem postępowania medycznego jest ustalenie rozpoznania oraz wydłużenie i poprawa jakości życia chorego. Ostateczne rozpoznanie stawia się na podstawie obowiązkowego badania histopatologicznego i badań molekularnych [15]. Większość pacjentów jest leczona terapią kilku modalności. Ważnym elementem prowadzenia leczenia jest leczenie objawowe (objawy występujące w mechanizmie miejscowego ucisku i dysfunkcji ośrodków – epilepsja, ubytki neurologiczne, wodogłowie, podwyższone ciśnienie śródczaskowe (ICP, ang. *intracranial pressure*). Leczenie chirurgiczne pozostaje podstawowym elementem opieki nad pacjentem. Rozlewanie się guza sprawia trudność w odróżnieniu masy komórek nowotworowych od otaczającej zdrowej tkanki. Z tego powodu mimo uzyskania pełnej makroskopowej resekcyjności następuje wznowa miejscowa.

Niepowodzenie w uzyskaniu czystych marginesów chirurgicznych wynika z przypuszczalnej progresji komórek nowotworowych wzdłuż włókien neuronów bez zmian makroskopowych, co nie daje szansy operatorowi na uzyskanie mikroskopowej resekcyjności i wyleczenie pacjenta samym zabiegiem operacyjnym. W terapii GBM wykorzystywana jest również chemio- oraz radioterapia.

Obecnie nie są prowadzone testy przesiewowe wykrywający GBM przed wystąpieniem objawów klinicznych. Najczęściej wykorzystywaną metodą diagnostyczną dla GBM jest rezonans magnetyczny [16].

Mediana całkowitego czasu przeżycia (OS, ang. *overall survival*) chorych na GBM jest niska i wynosi tylko 15 miesięcy [1].

1.5. CHARAKTERYSTYKA KOMÓREK ZWIĄZANYCH Z NOWOTWORAMI

Mikrośrodowisko guza nowotworowego nie jest jednolite i zawiera wiele typów komórek. Składa się z patologicznych komórek nowotworowych oraz prawidłowych komórek odpornościowych. Liczne komórki układu odpornościowego obejmują makrofagi związane z nowotworem (TAM, ang. *tumor associated macrophages*), komórki dendrytyczne (DC, ang. *dendritic cells*), limfocyty T, komórki naturalnej cytotoksyczności (NK ang. *natural killer T*), komórki supresyjne pochodzące z linii mieloidalnej (MDSC, ang. *myeloid-derived suppressor cells*) i komórki tuczne. Odgrywają one ważną rolę w progresji nowotworu.

Szczególną uwagę zwracają TAM, które z uwagi na ekspresję markerów przeciwzapalnych (np. interleukina 10) przypominają makrofagi M-2. Podczas rozwoju nowotworu TAM przechodzą przez okres zmiany z fenotypu z M-1 na M-2.

Komórki dendrytyczne (DC, ang. *dendritic cell*) pochodzą ze szpiku i są rozlokowane w prawie wszystkich tkankach organizmu. Rola tych komórek w rozwoju nowotworu pozostaje kontrowersyjna. Badania *in vivo* oraz *in vitro* wskazują zarówno na ich działanie pro- jak i antynowotworowe.

Termin MDSC został zaproponowany w 2007 roku, uwzględnia opis grupy niedojrzałych komórek szpikowych odnajdywanych w warunkach patologicznych [17].

Limfocyty T mimo swojej zdolności do niszczenia komórek z powodu niskiej aktywności, wyczerpania i wieku mogą nie zatrzymać rozwoju guza nowotworowego. Ich aktywność może być obniżona z powodu cytokin, chemokin oraz enzymów produkowanych przez TAM.

Komórki NK są częścią nieswoistej odpowiedzi immunologicznej. Zdolność do niszczenia komórek nowotworowych przez NK jest osłabiana przez czynniki wydzielane przez guz nowotworowy.

Komórki tuczne wywodzą się z hematopoetycznych komórek szpiku i biorą udział w remodelingu tkanek, gojeniu się ran oraz angiogenezie. Poprzez wydzielanie czynników zapalnych oraz rozkładanie chemokin oraz cytokin mają właściwości pro- oraz przeciwzapalne.

Fibroblasty związane z nowotworem (CAF, ang. *cancer-associated fibroblasts*) są heterogenną grupą aktywowanych fibroblastów, wydzielającą czynniki mające wpływ na rozwój guza, występowanie przerzutów oraz oporność na chemioterapeutyki. Mimo że większość badań sugeruje pronowotworowe działanie CAF, niektóre publikacje wskazują zdolność CAF do hamowania rozoju nowotworów na wczesnym etapie [18].

1.6. PRZESŁANKI DO PODJĘCIA BADAŃ

Mimo licznych prac dotyczących GBM oraz wpływu chemokin na onkogenezę brakuje w piśmiennictwie prac dotyczących wpływu komórek nowotworowych na zmiany ekspresji chemokin wytwarzanych przez komórki związane z nowotworem, np. przez TAM [19], MDSC [20] i fibroblasty związane z rakiem (CAF, ang. *cancer-associated fibroblasts)* [21]. Opisanie tych zależności z uwzględnieniem specyficznych warunków panujących w obrębie guza nowotworowego (hipoksja, zmniejszona dostępność substancji odżywczych) może prowadzić do lepszego zrozumienia mechanizmów onkogenezy oraz umożliwić opracowanie nowych terapii i leków o nowym punkcie uchwytu.

2. CEL PRACY

Celem badań była próba wyjaśnienia roli chemokin i ich receptorów w interakcji komórek glejaka wielopostaciowego z komórkami towarzyszącymi nowotworom w warunkach panujących w guzie.

Szczegółowe cele obejmowały:

- określenie udziału CCL18 i jej receptorów CCR8 i PITPNM3 w procesie nowotworzenia w glejaku wielopostaciowym,
- określenie wpływu warunków panujących w guzie na proces nowotworzenia,
- określenie wpływu płci chorych na progresję nowotworu,
- przegląd epidemiologii glejaka wielopostaciowego, czynników ryzyka i czynników ochronnych zachorowania na glejaka wielopostaciowego.

3. MATERIAŁ I METODY

3.1. WYBÓR MODELU BADAWCZEGO

W prezentowanych badaniach wykorzystano hodowlę ludzkich komórek glejaka (glioblastoma astrocytoma U-87 MG), którą poddano działaniu czynników odzwierciedlających warunki panujące w guzie (hipoksja i niedobor składników odżywczych). Zbadano ekspresję CCL18 oraz jej receptorów CCR8 i PITPNM3 w komórkach oraz w strefie guza i w okolicy okołoguzowej GMB pobranych w trakcie operacji.

3.2. MATERIAŁ BADAWCZY

Wykorzystano następujące materiały:

- guzy nowotworowe pobrane od chorych operowanych z powodu glejaka wielopostaciowego w Klinice Neurochirurgii i Neurochirurgii Dziecięcej PUM Samodzielnego Publicznego Szpitala Klinicznego Nr 1 im. Prof. Tadeusza Sokołowskiego Pomorskiego Uniwersytetu Medycznego w Szczecinie,
- linię komórkową ludzkich komórek glejaka (glioblastoma astrocytoma (U-87 MG),
- medyczne bazy danych w wersji elektronicznej.

3.3. METODY

3.3.1. BADANIA *IN VITRO*

Hodowlę komórkową ludzkich komórek glejaka [glioblastoma astrocytoma (U-87 MG), pochodzących z European Collection of Authenticated Cell Cultures (ECACC)] prowadzono w medium EMEM z dodatkiem 10% (v/v) inaktywowanej płodowej surowicy bydlęcej (FBS), zawierającej 2 mM L-glutaminy, 1 mM pirogronianu sodu, 1% aminokwasów, 100 U/mL penicyliny i 100 µg/mL streptomycyny. Hodowlę prowadzono w inkubatorze, w warunkach 37°C,

95% wilgotności i 5% CO_2. Komórki U-87 MG zostały rozdzielone na 6-cio studzienkowe płytki z gęstością 20 tys. komórek/cm^2 w kompletnym medium. Po 72 godzinnej inkubacji (zagęszczenie komórek wynoszące 70–80%) komórki zostały przemyte 3 krotnie podgrzanym do 37°C roztworem soli fizjologicznej buforowanej fosforanem (ang. *phosphate buffered saline*, PBS). Następnie komórki były hodowane przez kolejne 24 godziny w różnych warunkach celem przeanalizowania wpływu warunków panujących w guzie [stanu niedotlenienia (hipoksji) i niedoboru składników odżywczych)] na ekspresję genów *CCL18, CCR8 i PITPNM3*.

Cechą guza GBM jest obecność struktur zwanych pseudopalisadami [22]. Struktury te wykazują niedotlenienie i niedobór składników odżywczych związany z dużą odległością lub zablokowaniem światła naczyń krwionośnych przebiegających przez pseudopalisadę. Aby przeanalizować wpływ warunków w pseudopalisadach na komórki GBM, zbadano wpływ warunków panujących w guzie na ekspresję ww. genów. W tym celu komórki U-87 MG potraktowano chlorkiem kobaltu ($CoCl_2$) (200 mM), powszechnie stosowanym w eksperymentach w celu wywołania hipoksji [23]. Ponadto, aby odzwierciedlić warunki niedoboru składników odżywczych hodowlę prowadzono w warunkach pożywki o niskim stężeniu L-glutaminy (0.2 mM), bez pirogronianu sodu, w obecności glukozy (5.5 mM) w pożywce. Po 24 godzinach inkubacji komórki poddano działaniu trypsyny (0.25% trypsin-EDTA solution). Po odwirowaniu (25°C, 300 G, 5 min), supernatant odrzucono, a osad przepłukano PBS i odwirowano ponownie w celu przygotowania do analizy ekspresji genów.

3.3.2. BADANIA GUZÓW POBRANYCH OD PACJENTÓW

Próby guzów GMB do badań pochodziły od 28 pacjentów (16 mężczyzn i 12 kobiet) ze zdiagnozowanym GMB. Kryterium wykluczenia był wiek poniżej 18 roku życia oraz zdiagnozowany guz inny niż GMB. Na badania uzyskano zgodę Komisji Bioetycznej (nr KB-0012/96/14). Po stwierdzeniu guza w badaniach neuroobrazowych pacjenci byli kwalifikowani do operacji z użyciem neuronawigacji w znieczuleniu ogólnym. Zakres resekcji zależał od topografii guza.

W badaniach obrazowych możliwe było wydzielenie i pobranie do badań 3 stref morfologicznych guza nowotworowego:

- rdzeń guza (ang. *non-enhancing tumor core*, TC) zlokalizowany w centrum guza,
- rozrastająca się strefa guza (ang. *enhancing tumor region*, ET),
- okolica okołoguzowa, pośrednia między guzem a zdrowymi tkankami (ang. *peritumoral area*, PA).

Strefa okołoguzowa została uznana za kontrolę w przeprowadzonych badaniach.

3.3.3. BADANIA Z ZASTOSOWANIEM ŁAŃCUCHOWEJ REAKCJI POLIMERAZY Z OBRAZOWANIEM W CZASIE RZECZYWISTYM (QRT-PCR)

Badanie ekspresji genów *CCL18, CCR8, PITPNM3* w guzach pobranych od pacjentów i w komórkach U-87 MG wykonano metodą łańcuchowej reakcji polimerazy z obrazowaniem w czasie rzeczywistym (qRT-PCR). W pierwszym etapie badań wyizolowano całkowity RNA, następnie wykonano odwrotną transkrypcję RNA na komplementarny DNA (cDNA). W ostatnim etapie przeprowadzono łańcuchową reakcję polimerazy z obrazowaniem w czasie rzeczywistym (qPCR) z wykorzystaniem znaczników fluorescencyjnych ilości amplifikowanych cDNA i specyficznych starterów dla genów CCL18, CCR8, PITPNM3 i genu kontrolnego dehydrogenazy glicerolo-3-fosforanowej (*GAPDH*). Całkowity RNA ekstrahowano z 50–100 mg próbek guza przy użyciu zestawu RNeasy Lipid Tissue Mini Kit oraz z 300 tys. komórek przy użyciu zestawu RNeasy Mini Kit. cDNA przygotowano z 1 µg całkowitego komórkowego RNA w 20 µl objętości reakcji przy użyciu zestawu do syntezy cDNA FirstStrand i starterów oligo-dT. Ilościową ocenę poziomów mRNA przeprowadzono przy użyciu analizatora RT-PCR w czasie rzeczywistym ABI 7 500 Fast z odczynnikiem Power SYBR Green PCR Master Mix. Analizę przeprowadzono w warunkach 95°C (15 s), 40 cykli w 95°C (15 s) i 60°C (1 min). W tych warunkach zamplifikowano tylko jeden produkt PCR. Każdą próbkę analizowano w dwóch powtórzeniach, a średnie wartości Ct wykorzystano do dalszej analizy. Względna ekspresja genów w poszczególnych próbach została określona metodą ΔΔCT, z wykorzystaniem *Gapdh* jako genu referencyjnego o konstytutywnej ekspresji.

3.3.4. BADANIA PROLIFERACJI KOMÓREK

Celem zbadania wpływu CCL18 na proliferację glejaka, komórki U-87 MG wysiano na 96-dołkowej płytce z gęstością 20 tys. komórek/cm2 w kompletnej pożywce. Po 24 godzinach inkubacji pożywkę usunięto i komórki przemyto ciepłym PBS. Następnie do studzienek dodano pełną pożywkę z 10 ng/ml, 20 ng/ml lub 50 ng/ml CCL18. Komórki inkubowano przez 48 godzin. Następnie do każdej studzienki dodano 20 µl 3-(4,5-dimetylotiazol-2-ilo)-2,5-difenylotetrazolu (MTT) (5 µg/µl) i inkubowano przez 2 godziny w inkubatorze. W następnym etapie dokładnie usunięto pożywkę hodowlaną i do każdej studzienki dodano 150 µl sulfotlenku dimetylu (DMSO) i inkubowano w ciemności przez 10 min. Absorbancję każdego dołka mierzono przy użyciu czytnika mikropłytek (EZ Read 2000, Biochrom, Polska) przy długości fali 590 nm.

3.3.5. BADANIA KONFLUENCJI KOMÓREK

Celem zbadania konfluencji komórek glejaka w w.w warunkach wykonano mikrofotografie komórek U-87 MG wybarwionych hematoksyliną z użyciem mikroskopu (Leica DM5000B), a następnie poddano analizie za pomocą oprogramowania ImageJ Fiji (Johannes Schindelin, Albert Cardona, Mark Longair, Benjamin Schmid i inni, https://imagej.net/software/fiji/downloads, wersja 1.2, dostęp 20 września 2021 r.).

3.3.6. BADANIA MIGRACJI KOMÓREK

Celem zbadania wpływu CCL18 na migrację glejaka, komórki U-87 MG zostały posiane na płytkach 6-cio studzienkowych z gęstością 20 tys./cm^2 i hodowane w warunkach hipoksji oraz w warunkach kontrolnych. Następnie komórki przepłukano PBS. Komórki inkubowano z medium suplementowanym CCL18 w stężeniach 10 ng/mL, 20 ng/mL i 50 ng/mL lub w warunkach kontroli przez 16 godzin. Następnie przemyto komórki PBS i dodano roztworu trypsyny z EDTA (0.25%) do momentu rozdzielenia komórek. Następnie dodano pełne medium i odwirowano (25°C, 300 G, 5 min). Odwirowane komórki zostały umieszczone w medium zawierającym 1% albuminę surowicy bydlęcej (BSA). W każdej studzience

przeliczono liczbę komórek i odpowiadające objętości mieszaniny komórek wraz z medium bez FBS a z 1% BSA zostały dodane do górnej komory Nunc™ Polycarbonate Cell Culture Inserts w wielostudzienkowych płytkach (8.0 UM PC) uzyskując 1×10^5 komórek zawieszonych w medium bez FBS lecz z 1% BSA. 750 µL medium z 20% FBS zostało dodane do dolnej komory po czym inkubowano płytki przez 8h. Kolejnym krokiem było ufiksowanie komórek, które przekroczyły błonę. W tym celu komory zostały przemyte dwukrotnie PBS, a następnie umocowanie przez dodanie roztworu formaldehydu (4%, pH 6.9; 3min, 25°C). Komory zostały przemyte dwukrotnie PBS, dodano 100% metanolu do obu komór i inkubowano przez 20 min w 25°C. Kolejny raz przemyto dwukrotnie komory PBS. Następnie komórki zostały wybarwione hematoksyliną. Górną warstwę komórek usunięto zostawiając tylko komórki, które przemieściły się przez błonę. Liczbę komórek w każdej próbce zbadano przy użyciu mikroskopu (Leica DMi1, KAWA.SKA, Polska).

3.3.7. BADANIA IMMUNOHISTOCHEMICZNE

Celem zbadania koekspresji CCL18 i będącego markerem makrofagów – CD68 w guzie GMB wycinki glioblastoma zostały utrwalone w 10% formalinie a następnie przemyte czystym etanolem, natępnie czystym etanolem z ksylenem (1:1) i ksylenem. Po wysyceniu tkanek w ciekłej parafinie, preparaty zostały uformowane w bloczki parafinowe. Z pomocą mikrotomu (Microm HM340E) uzyskano serie przekrojów grubości 3–5 µm, które następnie ułożono na pokrytych polysiną szkiełkach mikroskopowych. Przekroje glioblastoma zostały następnie odparafinowane w ksylenie i uwodnione w malejących stężeniach etanolu.

W celu ekspozycji epitopów do badań immunohistochemicznych odparafinowane i uwodnione sekcje zostały dwukrotnie przegotowane w mikrofalówce (700 W dwukrotnie po 5 min) w Target Retrieval Solution. Po ostudzeniu i przemyciu PBS endogenna peroksydaza została dezaktywowane roztworem 3% perhydrolu w metanolu, a następnie wycinki inkubowano przez noc w temperaturze 4°C z przeciwciałami przeciwko: CD68 (w rozcieńczeniu 1:5000), CCL18 (w rozcieńczeniu 1:250). Aby zwizualizować kompleksy antygen-przeciwciało został użyty system Dako LSAB + System-HRP (DakoCytomation, K0679),

oparty na reakcji awidyna-biotyna-peroksydaza chrzanowa z diaminobenzydyną jako chromogenem. Skrawki zostały przemyte wodą destylowaną i wybarwione hematoksyliną. W ujemnej kontroli próbki były przetwarzane bez obecności pierwszorzędowych przeciwciał. Pozytywne barwienie określono mikroskopowo (Leica DM5000B) poprzez wizualną identyfikację brązowej pigmentacji.

3.3.8. ANALIZA STATYSTYCZNA

Analizę statystyczną przeprowadzono przy użyciu programu Statistica PL 13.0 (StatSoft, Polska).

W obliczeniach statystycznych wykorzystano:

1. **Test Shapiro-Wilka** do oceny zgodności rozkładu uzyskanych wartości zmiennych z rozkładem normalnym.
2. **Test ANOVA rang Kruskala-Wallisa** do zbadania istotności różnic cech demograficznych i podstawowych badanej grupy pod względem płci.
3. **Test U Manna-Whitneya** do oceny istotności różnic między badanymi grupami.
4. **Test rang Wilcoxona** do oceny istotności różnic między strefami guza i w badaniach *in vitro.*
5. **Współczynnik korelacji rang Spearmana** dla oceny siły korelacji między ekspresją badanych genów a strefą guza.

Różnice między grupami uznawano za statystycznie istotne przy poziomie istotności $p < 0,05$.

4. OMÓWIENIE PUBLIKACJI STANOWIĄCYCH PODSTAWĘ PRACY DOKTORSKIEJ

4.1. PRZEGLĄD EPIDEMIOLOGII, CZYNNIKÓW RYZYKA I CZYNNIKÓW OCHRONNYCH ZACHOROWANIA NA GLEJAKA WIELOPOSTACIOWEGO

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4.1.1. PATOGENEZA GLEJAKA WIELOPOSTACIOWEGO

Glejak wielopostaciowy jest nowotworem o agresywnym przebiegu. Ze względu na liczne odmienności, zarówno genetyczne jak i epigenetyczne, charakteryzuje się zróżnicowanym przebiegiem i często występującą opornością na terapię. W ciągu ostatnich 30 lat rokowanie pacjentów wzrosło niewiele w porównaniu z innymi nowotworami [24]. Aby opracować metody terapeutyczne o zadowalającej skuteczności konieczne są dalsze badania na temat zmian molekularnych GBM oraz analiza jego epidemiologii.

Najczęstszym zajętym przez glejaka wielopostaciowego płatem jest płat czołowy [25], zdecydowanie częściej lokalizuje się w okolicy nadnamiotowej [26] niż podnamiotowej. Najrzadszymi lokalizacjami GBM są pień mózgu oraz móżdżek.

Współczesny postęp technologii genomicznej pozwolił na lepsze zrozumienie kluczowych zmian molekularnych wyzwalających GBM. Statusy opisanych poniżej markerów molekularnych niosą ze sobą informacje prognostyczne, predykcyjne, ułatwiające różnicowanie poszczególnych typów nowotworów i dają nadzieje na zrozumienie progresji nowotworu i stworzenie terapii celowanych:

a) **Mutacja *ATRX*** **(**ang. *a-thalassemia/mental-retardation-syndrome-X-linked*): Gen *ATRX* znajdujący się na Xq21.1 koduje białko biorące współudział w szlaku rearanżacji chromatyny [27]. Mutacje ATRX występują w około 57% wtórnych glejaków wielopostaciowych, występują częściej w glejakach z obecną

mutacją *IDH* niż w typie dzikim (71% vs sporadycznie) [8] i współwystępują z mutacjami *IDH1* oraz *TP53* [28]. Mutacje *ATRX* są pozytywnymi czynnikami prognostycznymi [29].

b) **Mutacja promotera *TERT*** (ang. *Telomerase Reverse Transcriptase*): gen *TERT* koduje telomerazę, a mutacja promotora genu *TERT* skutkuje zwiększoną aktywnością telomerazy i wydłużeniem telomerów, co sugeruje, że utrzymanie obecności telomerów jest niezbędne dla tworzenia się guzów mózgu [30]. Dwie najczęstsze mutacje promotora *TERT* to C228T oraz C250T, zlokalizowane odpowiednio na 124 oraz 146 parze zasad kodującej ten promotor [31]. Mutacje te mogą prowadzić do nawet czterokrotnego zwiększenia ekspresji *TERT* [32]. Mutacje *TERT* występują nawet do 80% w glioblastoma [32]. Rola mutacji *TERT*p jako czynnika prognostycznego nie została jednomyślnie określona.

c) **Mutacja *TP53*** (ang. *Tumor protein P53*): gen *TP53* jest zlokalizowany na ludzkim chromosomie w locus 17p13.1. Funkcjonalne białko p53 jest homotetramerem, pełniącym kluczową rolę w sieci regulacyjnej kontrolującej proliferację, przeżycie, integralność genomu oraz inne funkcje komórek. Obecność mutacji *TP53* jest powiązana z progresją GBM [33]. Inaktywacja p53 glejaka wielopostaciowego koreluje z większą inwazyjnością [34], zmniejszoną apoptozą komórek [35] i zwiększoną proliferacją [36]. Linie komórkowe obarczone mutacją inaktywującą p53 wykazują większą oporność na chemioterapeutyki uszkadzające DNA (ang. *deoxyribonucleic acid*), takie jak cisplatyna [35]. Mimo, że mutacje *TP53* korelują ze złą prognozą w innych nowotworach [37], w glioblastoma nie stanowią wartości prognostycznej [36]. Mutacje *TP53* występują częściej w IDH-mutant GBM niż IDH-wildtype GBM (81% vs. 27%) [8]. Mutacja *TP53* typu GOF (ang. *Gain of Funtion*) skutkuje powstaniem produktu o nowej funkcji lub zmienionej ekspresji, w przypadku *TP53* w GBM prowadzi do zwiększenia złośliwości komórek przez nasilenie ich proliferacji, migracji, inwazji, tworzenia przerzutów, oporności na leki, niestabilności genomu oraz nasilenia ich przeżywalności [38]. Wang, Xiang et al. [39] podają, że mutacje typu GOF wiążą się z gorszym całkowitym przeżyciem oraz że zmniejszają wrażliwość GBM na temozolomid przez zwiększenie ekspresji MGMT [39].

d) **Mutacja BRAF V600E** (ang. *v-raf murine sarcoma viral oncogene homolog B1*): BRAF jest częścią szlaku kinaz RAS-RAF-MEK-ERK-MAP. Ten precyzyjnie regulowany szlak odpowiada za wzrost komórki, a mutacje nadające konstytutywną aktywność kinazie BRAF skutkują niekontrolowaną proliferacją komórek i powstawaniem nowotworów. Mutacja V600E polega na podmianie waliny w pozycji 600 łańcucha tego białka na glutaminian. W literaturze częstość występowania wszystkich mutacji BRAF w GBM ocenia się na 2–6%. Mutacja BRAF V600E może być dogodnym punktem uchwytu efektywnej personalizowanej terapii przeciwnowotworowej inhibitorami kinaz, co znajduje potwierdzenie w publikowanych opisach przypadku (ang. *case-report*), jak na przykład odpowiedź kliniczna na vemurafenib będący inhibitorem kinazy BRAF w 3 pediatrycznych przypadkach glejaka o wysokim stopniu złośliwości z mutacją BRAFV600E [40].

e) **Mutacja GATA4**: GATA4 jest czynnikiem transkrypcyjnym z rodziny GATA6, uznawanym za gen supresorowy. W zdrowych astrocytach nie wpływa na wzrost komórek. Seria badań przeprowadzonych przez Agnihotri et al. [41] wykazała, że w 94/163 ludzkich GBM komórki nowotworowe utraciły ekspresję GATA4, GATA4 hamuje transformację do GBM *in vitro* i *in vivo*, a re-ekspresja GATA4 w komórkach GBM uwrażliwia je na działanie temozolomidu, bez względu na status mutacji MGMT *(ang. O-6-methylguanine-DNA methyltransferase).* Mimo potrzeby przeprowadzenia kolejnych badań rola GATA4 w odpowiedzi na temozolomid sugeruje przydatność statusu mutacji GATA4 jako biomarkeru predykcyjnego.

f) **Mutacja FGFR1** (ang. *Fibroblast Growth Factor Receptor 1*)**:** rodzina białek FGFR jest grupą receptorów transbłonowych o funkcji kinazy tyrozynowej. Dokładny wpływ sygnalizacji przez FGFR na aspekty patobiologiczne poszczególnych nowotworów pozostaje nieznany [42]. Najsilniejsze dowody wskazują, że mutacja FGFR1 przyczynia się do złego rokowania w GBM i sygnalizacja przez ten szlak wiąże się ze zwiększoną radiooopornością, inwazyjnością i fenotypem komórek macierzystych nowotworu [43].

g) **Mutacja EGFR** (ang. *epidermal growth factor receptor):* receptor nabłonkowego czynnika wzrostu jest receptorem o aktywności kinazy tyrozynowej, aktywowanym przez nabłonkowy czynnik wzrostu

(EGF, ang. *epidermal growth factor*). EGFR promuje proliferację komórkową przez aktywację szlaków kinazy aktywowanej mitogenami (MAPK, ang. *mitogen-activated protein kinases*) oraz *kinazy 3-fosfatydyloinozytolu (*PI3K-Akt, ang. *phosphatidylinositol 3-kinase*) [44]. Amplifikacja EGFR, obserwowana w około 40% przypadków glejaka wielopostaciowego [45], przez część autorów jest wiązana z gorszym rokowaniem, jednak wyniki badań nie są w tej kwestii jednoznaczne [46–50]. Amplifikacja EGFR występuje częściej w *IDH-wild type* GBM niż w *IDH-mutant* GBM [8]. Duże zainteresowanie badaczy przyciągnęła mutacja EGFRvIII, która jest najpopularniejszą mutacją EGFR, która prowadzi do konstytutywnej aktywacji EGFR, co skutkuje aktywacją dalszych szlaków kinaz tyrozynowych.

h) **Metylacja promotora MGMT** (ang. *O-6-methylguanine DNA methyltransferase*): gen *MGMT* znajduje się na chromosomie 10q26 i koduje białko odpowiedzialne za naprawę DNA przez usuwanie grupy alkilowej z pozycji O^6 guaniny, ważnego miejsca alkilacji DNA. Obecność metylacji promotora MGMT jest pozytywnym czynnikiem predykcyjnym [51]. Pacjenci z obecną metylacją promotera MGMT mają dłuższe całkowite przeżycie [51]. Autorzy badania Hegi et al. [52] sugerują przydatność oznaczania statusu metylacji promotora MGMT w celu identyfikacji pacjentów, którzy mogą odnieść korzyści z włączenia temozolomidu do standardowej radioterapii w porównaniu z samą radioterapią. Użycie O6-BG (ang. *O6-benzylguanine*), inhibitora MGMT, przywraca wrażliwość na temozolomid linii komórkowych [53].

i) **Mutacja PTEN** (ang. *phosphatase and tensin homolog*)**:** gen *PTEN* jest genem supresorowym, znajdującym się na 10q23. Jego mutacja, czyli utrata heterozygotyczności (LOH, ang. *Loss of heterozygosity*) lub metylacja zaburza szlaki wykorzystujące 3-kinazę fosfatydyloinozytolu (PI3K, ang. *phosphatidylinositol 3-kinase*) i występuje w przynajmniej 60% przypadków GBM [54]. Utrata funkcji PTEN z powodu mutacji lub LOH jest związana ze słabym rokowaniem GBM. Według Koul [54] utrata ekspresji PTEN świadczy o progresji wysoko złośliwego nowotworu – PTEN jest obecne w większości nowotworów o niskim stopniu złośliwości (ang. *low-grade*). Brito et al. [55] podają że delecja PTEN w GBM *IDH-wild* type wiąże się z dłuższym całkowitym przeżyciem.

4.1.2. CZYNNIKI WPŁYWAJĄCE NA ROZWÓJ GLEJAKA WIELOPOSTACIOWEGO

Przeglad piśmiennictwa wskazuje, że istnieje wiele zmiennych wpływającym na rozwój GBM należą do nich np.:

a) **Wiek** – jest istotnym czynnikiem wpływającym na rozwój różnych chorób, w tym nowotworów. Wiele badań potwierdza, że także w przypadku GBM wiek znacząco wpływa na częstość jego występowania. Zdecydowana większość przypadków zachorowań dotyczy osób powyżej 40 roku życia. Potwierdzają to badania Kai et al. [56] w których wykazano, że dla 47.9% badanych wiek rozpoznania GBM wynosił ≥ 65 roku życia, zaś dla 46.3% między 40–64 lata. W badaniach Tian et al. zdecydowanie więcej (55.2%) przypadków glejaka zdiagnozowano wśród respondentów między 41–60 rokiem życia. Na podstawie raportu Centralnego Rejestru Guzów Mózgu w Stanach Zjednoczonych (CBTRUS, ang. *Central Brain Tumor Registry of the United States*) z 2013 r., 2017 czy 2020 r. zaobserwowano, że częstość występowania GBM wzrasta wraz z wiekiem, osiągając szczyt w wieku 75–84 lat i spada po 85 roku życia. Glioblastoma (GBM) jest najbardziej agresywnym glejakiem rozlanym z linii astrocytów, który pozostaje guzem nieuleczalnym, a w większości przypadków mediana przeżycia wynosi mniej niż 15 miesięcy. W badaniu Lam et al. [26] mediana przeżywalności wyniosła 20 miesięcy, z czego 46.9% badanych przeżywało do 2 lat od rozpoznania. Z kolei Fabbro-Peray et al. [4]. Gittelman et al. [57] czy Ostrom et al. [58] zaobserwowali, że najczęściej pacjenci żyją do roku od rozpoznania.

b) **Miejsce zamieszkania** – Barker et al. [59] opisali w 1976 roku, że zachorowalność na glioma w południowej części Anglii o wartości 3.94 na 100 000, która była mniejsza w dużych obszarach miejskich. Z kolei Walker et al. [60] opisali mniejszą śmiertelność podczas pierwszych 5 tygodni od postawienia diagnozy GBM w grupie mieszkańców zamieszkujących obszary wiejskie w porównaniu do mieszkańców miast. Ponadto większą śmiertelność przez pierwsze 1.5 roku od diagnozy GBM zaobserwowano u osób o niskich dochodach w porównaniu do grupy o wysokich dochodach.

c) **Status materialny respondentów** – w badaniu przeprowadzonym w USA przez Cote et al. [61] zachorowalność na glejaki była wyższa w hrabstwach o wyższym statusie socjoekonomicznym w porównaniu z hrabstwami o niższym statusie socjoekonomicznym. Hrabstwa o wysokim statusie socjoekonomicznym charakteryzowały się niższą śmiertelnością z powodu glejaków. Różnice w zachorowalności oraz śmiertelności autorzy wiążą z rasą oraz statusem socjoekonomicznym a nie różnicą między miejskim a wiejskim obszarem zamieszkania.

4.1.3. CZYNNIKI OCHRONNE ORAZ CZYNNIKI RYZYKA ZACHOROWANIA NA GLEJAKA WIELOPOSTACIOWEGO

Do czynników ochronnych, które należy wymienić biorąc pod uwagę rozwój schorzeń nowotworowych, w tym GBM, należą:

a) **Płeć oraz hormony** – przegląd piśmiennictwa potwierdza protekcyjne działanie żeńskich hormonów płciowych na rozwój nowotworów z grupy glejaków. Zaobserwowano zwiększone ryzyko rozwoju glioma u kobiet z późną pierwszą miesiączką i późną menopauzą [48]. Ponadto odnotowano zmniejszone ryzyko rozwoju glioma u kobiet stosujących doustną hormonalną antykoncepcję lub hormonalną terapię zastępczą, jednak czas stosowania terapii hormonalnej nie miał znaczenia [48]. Opisano także brak wpływu podawania egzogennych estrogenów na ryzyko rozwoju nowotworów z grupy glejaków [62]. Wszystkie dotychczasowe badania wskazują na wyższą (od 1.12 do 2.59) częstość występowania GBM u mężczyzn niż u kobiet [63].

b) **Niesteroidowe leki przeciwzapalne i paracetamol** – mimo istnienia przesłanek molekularnych świadczących o protekcyjnym działaniu niesteroidowych leków przeciwzapalnych (NLPZ) na rozwój GBM brak jednoznacznego potwierdzenia ich w piśmiennictwie. Wyniki badań są często niejednoznaczne ze względu na obecność wielu potencjalnych czynników zaburzających, natomiast badania prospektywne ze względu na brak odpowiedniej liczebności chorych na GBM. Badanie kliniczno-kontrolne przeprowadzone w latach 2007–2010 w grupie 517 chorych na GBM i 400 uczestnikach w grupie kontrolnej wykazało odwrotną zależność

między korzystaniem z NLPZ przynajmniej 6 miesięcy i ryzykiem zachorowania na GMB (OR = 0.68; 95% CI 0.49–0.96) [64]. Jednak w piśmiennictwie można znaleźć również publikacje przemawiające za brakiem korelacji między stosowaniem NLPZ a ryzykiem glejaka. Prospektywne badanie zainicjowane w 1995 roku w grupie 302 767 mieszkańców USA autorstwa Daugherty et al. [65], wykazało wystąpienie 341 przypadków glejaków w tej grupie, w tym 264 GBM. Regularne stosowanie aspiryny (powyżej dwóch razy w tygodniu) nie miało związku z ryzykiem wystąpienia GBM w porównaniu do jej niestosowania. Z kolei badanie przeprowadzone na podstawie *Danish Cancer Registry* i rejestru recept w dużej grupie chorych (n = 2 688) i grupie kontrolnej (n = 18 848) w latach 2000–2009 również nie wykazało korelacji między stosowaniem NLPZ a ryzykiem wystąpienia glejaka. Natomiast Bruhns et al. [66] nie wykazali statystycznie istotnej różnicy w przeżywalności u chorych stosujących i niestosujących NLPZ w terapii.

c) **Inne substancje medyczne** – wśród substancji wpływających na GBM możemy wyróżnić kannabinoidy. Większość z nich wiąże się z receptorami kannabinoidowymi sprzężonymi z białkami G, CB1 i CB2, które działają jako agoniści lub odwrotni agoniści. Co istotne, w nowotworach GBM stwierdzono ekspresję receptorów specyficznych dla kannabinoidów (CB1 i CB2), równo w liniach komórkowych GBM, w komórkach guza pierwotnego *ex-vivo* pochodzących od pacjentów z GBM i *in situ*, w biopsjach tkanek GBM. Zaobserwowano, że glejaki o wysokim stopniu złośliwości, do których należy GBM, wyrażają wysoki poziom CB2, a ekspresja tego białka dodatnio koreluje ze stopniem złośliwości [67]. Mimo powyższych obserwacji potrzeba więcej badań na temat wpływu kannabinoidów na rokowanie, jakość życia chorych i potencjalne działanie ochronne przed zachorowaniem na GBM.

d) **Inne leki:**

- ✓ Leki antyhistaminowe

  Metaanaliza przeprowadzona przez Xie et al. [68] nie wykazała wydłużenia całkowitego czasu przeżycia (OS, ang. *overall survival*) oraz czasu przeżycia wolnego od progresji (PFS, ang. *progression-free*

survival) u osób stosujących statyny. W pracy Ferris et al. [64] przyjmowanie statyn wykazało znamiennie statystyczną odwrotną zależność między długością terapii i ryzykiem glioma. Ponadto Scheurer et al. [69] zaobserwowali OR: 0.89 (95% CI: 0.63, 1.25) GBM u stosujących leki antyhistaminowe.

- ✓ Statyny

  W metaanalizie przeprowadzonej przez Xie et al. [83] nie zaobserwowano wydłużenia całkowitej przeżywalności u osób stosujących statyny. W badaniu Ferrisa et al. [74] przyjmowanie statyn wiązało się ze znamiennie statystycznie odwrotną zależnością między czasem trwania terapii statynami a ryzykiem wystąpienia glejaka. W metaanalizie przeprowadzona przez Rendoma et al. [84] opisano działanie statyn hamujące wzrost glejaka poprzez różne mechanizmy in vitro. Jednakże, efekty te nie były statystycznie istotne pod względem częstości występowania glejaka i całkowitej przeżywalności chorych.

e) **Atopia** – jest kolejnym czynnikiem mającym wpływ na ryzyko zachorowania na nowotwory z grupy glejaków. Badania wykazały odwrotny związek między atopią, a ryzykiem zachorowania. Metaanaliza przeprowadzona w 2007 roku przez Linos et al. [70] wśród 3450 pacjentów chorujących na glejaki przedstawia łączone ryzyko względne zachorowania na glioza u osób z atopią, która w porównaniu do osób bez historii atopii wynosiła dla alergii, astmy i wyprysku odpowiednio 0.61 (95% CI = 0.55 to 0.67), 0.68 (95% CI = 0.58 to 0.80) i 0.69 (95% CI = 0.58 to 0.82). Schwartzbaum et al. opisali również protekcyjny wpływ polimorfizmów genów powiązanych z występowaniem astmy na ryzyko wystąpienia GBM [71]. Mechanizm odpowiedzialny za te zależności nie został jednoznacznie ustalony. Przypuszczalnym wyjaśnieniem tego zjawiska jest występująca zwiększona wrażliwość układu odpornościowego. Wpływ alergii na rozwój nowotworu zachodzi nie tylko w przypadku glejaków, ale również raka trzustki. Co ciekawe niektóre przeciwciała z grupy IgE skierowane przeciwko alergenom reagują krzyżowo z antygenami glejaków [93], a niektórzy autorzy donoszą o odwrotnej korelacji między stężeniem przeciwciał w osoczu a ryzykiem zachorowania na glioma [72].

Do czynników ryzyka zachorowania na GBM należą:

a) **Palenie produktów tytoniowych i nitrozaminy** – nie zostało ono jednoznacznie powiązane ze zwiększonym ryzykiem rozwoju glejaka wielopostaciowego [73] i glioma [74]. Ze względu na zróżnicowane wyniki badań, kolejne próby ustalenia korelacji lub jej braku są pożądane. Dotyczy to szczególnie dymu papierosowego, w przypadku którego udowodniono wpływ na ryzyko rozwoju chorób nowotworowych niektórych narządów. Zawarte w dymie papierosowym mutageny takie jak: nitrozoaminy specyficzne dla tytoniu (TSNA, ang. *tobaccospecific nitrosamines*) oraz wielopierścieniowe węglowodory aromatyczne (PAHs, ang. *polycyclic aromatic hydrocarbons*) przenikają barierę krew-mózg [75], co potencjalnie może mieć wpływ na rozwój nowotworów centralnego układu nerwowego [76]. Nitrozaminy, które mogą pochodzić z palenia wyrobów tytoniowych, ale również z reakcji azotanów i azotynów używanych w żywności, w produktach mięsnych: szynkach, bekonie, kiełbasach. *N-nitrozodimetyloamina* (NDMA, ang. *N-nitrosodimethylamine*) jest jedną z najczęściej występujących nitrozamin w żywności [77]. NDMA jest silnym karcynogenem zdolnym wywoływać nowotwory w modelach zwierzęcych [78]. Azotany obecne w pokarmach trafiając do układu trawiennego są wchłaniane do krwi, a następnie wydzielane do śliny. W następstwie przełykania azotany trafiają do żołądka, gdzie w kwaśnym środowisku przekształcają się w nitrozaminy [79]. Badanie w grupie chorych zdiagnozowanych w Izraelu między 1987 i 1991 wykazało, że związki N-nitrozoaminy nie były bezpośrednio powiązane z guzami mózgu [80]. W badaniu opublikowanym przez Michaud et al.[81] w grupie spożywającej najwięcej przetworzonych produktów mięsnych i grupie narażonej na azotany ryzyko zachorowania na glioma nie było zwiększone i wynosiło odpowiednio (RR: 0.92; 95% CI: 0.48, 1.77) i (RR: 1.02; 95% CI: 0.66, 1.58). Metaanaliza Saneei et al. [82] zawierająca dane 18 badań obserwacyjnych nie wykazała związku między spożywaniem przetworzonego czerwonego mięsa a zwiększoną częstością występowania glioma.

b) **Pochodzenie etniczne** – istnieje ograniczony związek między poszczególnymi grupami etnicznymi, a ryzykiem rozwoju GBM. Ostrom et al. [83] podają 2.97 razy większą zachorowalność na GBM u rasy kaukaskiej

w porównaniu do azjatyckiej oraz 1.99 razy większą u rasy kaukaskiej niż u afroamerykanów. W badaniu przeprowadzonym w 2006 roku przez Fukushima et al. [84] porównało mutacje występujące w pierwotnych glejakach wielopostaciowych w grupie Japończyków z mutacjami występującymi w grupie Szwajcarów opisanej przez Ohgaki et al. [85].

c) **Promieniowanie jonizujące** – jest uznanym czynnikiem ryzyka wielu nowotworów. Bezpośrednie uszkodzenie materiału genetycznego lub wytworzenie wolnych rodników w sąsiedztwie nici DNA skutkuje zwiększoną częstością występowania mutacji w obrębie materiału genetycznego komórek. Ponieważ kontrolowane badania kliniczne na temat wpływu promieniowania na kancerogenezę są niemożliwe do przeprowadzenia ze względów etycznych, badania kliniczno-kontrolne odgrywają główną rolę w opisywaniu tego zjawiska. Ron et al. [86] już w 1988 powiązał dawki 1–2 Gy ze zwiększonym ryzykiem guzów neuronalnych. Przegląd piśmiennictwa dokonany przez Bowers et al. [87] w 2013 roku udokumentował 8.1–52.3 razy większe ryzyko zachorowania na nowotwór centralnego układu nerwowego po radioterapii głowy z powodu guza OUN w dzieciństwie w porównaniu do ogólnej populacji, proporcjonalne do dawki. Gold et al. [88] potwierdził zwiększone ryzyko zachorowania na kolejny guz mózgu po radioterapii pierwszego. Większość badań dotyczących przeprowadzania badania tomografią komputerowa (TK) w dzieciństwie i ryzyka rozwoju glioma nie wykazało zwiększonego ryzyka, oprócz badania opisującego 1 nadmiarowy guz mózgu na 10 000 pacjentów w okresie 10 lat po ekspozycji na 1 skan TK [89].

d) **Urazy głowy** – z powodu opisanych anegdotycznych przypadków rozpoznania nowotworów ośrodkowego układu nerwowego (*CNS*, ang. *central nervous system*) (nie tylko GBM) po przebytym urazie głowy, przeprowadzono dalsze badania na temat urazów głowy jako czynnika etiologicznego nowotworów mózgu, uzyskując mieszane rezultaty. Niestety dostępne badania są dosyć ograniczone. Udowodnienie ciągu przyczynowo skutkowego jest w tym przypadku bardzo trudne. W badaniu przeprowadzonym na duńskiej populacji, u pacjentów po urazie głowy nie rozpoznawano częściej glejaków w porównaniu do ogólnej populacji Duńskiej. Z kolei w badaniu kliniczno-kontrolnym dotyczącym oceny występowania oponiaków oraz glejaków

po urazie głowy udokumentowano wyższe ryzyko występowania oponiaków, ale niższe ryzyko występowania glejaków (OR = 1.2; 95% CI: 0.9–1.5 dla jakiegokolwiek urazu; OR = 1.1; 95% CI: 0.7–1.6) [90].

e) **Otyłość** – tkanka tłuszczowa posiada wiele funkcji w organizmie człowieka. Oprócz magazynowania związków odżywczych pod postacią tłuszczy spełnia rolę wydzielniczą. W obrębie tkanki tłuszczowej następuje między innymi produkcja estrogenów [91] oraz substancji prozapalnych [92]. Z tych względów tkanka tłuszczowa może mieć potencjalny wpływ na rozwijanie się nowotworów, w tym glejaka wielopostaciowego. Aktualne dane sugerują, że niski wskaźnik masy ciała (BMI, ang. *body mass index* < 18.5 kg/m^2) w wieku 21 lat jest związany z niższym ryzykiem rozwoju glejaków (glioma) w późniejszych latach życia, jednak wyniki były istotne statystycznie tylko w grupie kobiet [93]. Z kolei Moore et al. [94] wykazali, że pacjenci, którzy w wieku 18 lat byli otyli (BMI 30.0–34.9 kg/m^2) mieli prawie cztery razy większe ryzyko zachorowania na glejaka niż osoby, które w wieku 18 lat miały BMI między 18.5–24.9 kg/m^2 (RR = 3.74; 95% CI = 2.03–6.90; P_{trend} = 0.003). W badaniu Kaplan et al. [80] zwiększona konsumpcja tłuszczy i cholesterolu była odwrotnie proporcjonalna do częstości występowania glioma (wysokie spożycie tłuszczy OR = 0.45; 95% CI: 0.20–1.07; wysokie spożycie cholesterolu: OR = 0.38; 95% CI: 0.14–1.01). Cote et al. [95] zaobserwowali odwrotną zależność pomiędzy hiperlipidemią, a glioma. Badanie w Izraelu w grupie chorych zdiagnozowanych między 1987 i 1991 wykazało zależność między występowaniem glioma i oponiaków, a dietą bogato białkową (OR = 1.94; 95% CI: 1.03–3.63) [80]. Seliger et al. [96] wykazał w swoich badaniach spadek ryzyka zachorowania na GBM u osób chorujących na cukrzycę (OR = 0.69; 95% CI = 0.51–0.94). Spadek ryzyka był najwyraźniej zaznaczony u mężczyzn chorujących powyżej 5 lat lub ze słabą kontrolą glikemii (*hemoglobina glikowana,* HbA1c ≥ 8). Z kolei efekt niższego ryzyka GBM był nieobecny u kobiet (OR = 0.85; 95% CI = 0.53–1.36).

f) **Wzrost** – mimo, że wysoki wzrost jest związany z większą częstością zachorowania na niektóre nowotwory [97] to dokładny mechanizm tego zjawiska nie jest wyjaśniony. Prawdopodobnie bierze w nim udział szlak związany z insulinoopodobnym czynnikiem wzrostu (IGF, ang. *insulin-like*

growth factor) i hormonem wzrostu (GH, ang. *growth hormone*), który determinuje przyrost i końcowy wzrost u ludzi. W ponad 80% guzów glioblastoma zachodzi nadekspresja insulinopodobnego czynnika wzrostu wiążącego białko-2 (IGFBP-2, ang. *insulin-like growth factor Winding protein-2*), jednego z biomarkerów złośliwości GBM [98]. W guzach o niższej agresywności IGFBP-2 jest zwykle niewykrywalny i pojawia się wraz z progresją nowotworu [99]. W pracy opublikowanej przez Moore et al. [94] ryzyko zachorowania na glejaka wśród wysokich osób (powyżej 190 cm) było dwa razy większe niż wśród osób o wzroście poniżej 160 cm (wielowymiarowe ryzyko względne [RR] = 2.12; 95% przedział ufności [CI] = 1.18–3.81; P_{trend} = 0.006). Z kolei badanie przeprowadzone przez Little et al. [100] nie powiązało wzrostu osób dorosłych z ryzykiem rozwinięcia glioma.

g) **Metale** – monografia Międzynarodoej Agencji Badań nad Rakiem (IARC, ang. *International Agency for Research on Cancer*) wymienia kadm, związki kadmu, związki chromu i związki niklu jako substancje kancerogenne dla ludzi, a ołów nieorganiczny jako potencjalnie kancerogenny. Żaden z tych czynników nie został uznany za czynniki powiązane z nowotworami mózgu. Zdolność niektórych metali ciężkich do przenikania bariery krew-mózg oraz wnikania drogą nerwów węchowych [101] skłania do bliższego zbadania ich wpływu na ryzyko rozwoju GBM. W pracy badającej zawodową ekspozycję (JEM, ang. *job exposure matrix*) na poszczególne metale nie zaobserwowano zwiększonego ryzyka zachorowania na glejaki i zawodową ekspozycją na chrom, nikiel i ołów wśród 2.8 miliona mężczyzn zatrudnionych w 1970 roku (n = 3 363 przypadków glioma). Parent et al. [102] donoszą o zwiększonej zachorowalności na glioma w związku z zawodową ekspozycją na arszenik, rtęć i produkty naftowe. Nie wykazali jednak zwiększonego ilorazu szans wystąpienia glioma dla spawaczy narażonych na ołów i kadm oraz opary spawalnicze [102]. Badania sugerują, że ołów może pokonywać barierę krew-mózg i odkładać się w tkance nerwowej [103]. Mechanizm, w którym ołów wywołuje nowotwory mózgu jest niejasny. Proponowanym wyjaśnieniem jest hamowanie syntezy i naprawy DNA oraz wchodzenie w interakcje z białkami supresorowymi. Ołów może również wywoływać stres oksydacyjny, indukować apoptozę i wpływać na niektóre

szlaki sygnałowe [103]. Ahn et al. [104] w metaanalizie donosi o zwiększonym ryzyku złośliwych nowotworów mózgu związanym z ekspozycją na ołów. Rajaraman et al. [105] nie zaobserwowali zależności między ekspozycją na ołów, a ryzykiem glioma. Bhatti et al. [106] zbadali potencjalną kancerogenność ołowiu przez analizę modyfikacji efektu polimorfizmów pojedynczych nukleotydów (SNPs ang. *single nucleotide polymorphisms*) w obrębie genów związanych funkcjonalnie ze stresem oksydacyjnym. Badanie obejmowało 494 osoby z grupy kontrolnej, 176 chorych na glejaka wielopostaciowego i 134 chorych na oponiaka, którzy zostali poddani ocenie pod kątem narażenia zawodowego na ołów. Polimorfizmy genów *RAC2* (ang. *Ras-related C3 botulinum toxin substrate 2*) oraz *GPX1* (ang. *glutathione peroxidase 1*) znamiennie modyfikowały zależność między skumulowaną ekspozycją na ołów a ryzykiem GBM.

h) **Czynniki odżywcze, chemikalia i pestycydy** – martwica tkanki mózgowej związana z rozwojem GBM prowadzi do uwolnienia triacylogliceroli, czemu może towarzyszyć uwolnienie toksyn współmagazynowanych w bogatej w fosfolipidy tkance nerwowej [107]. W badaniu z 1992 roku wykorzystującym dane z *Canadian National Cancer Incidence Database i Provincial Cancer Registries*, Morrison et al. [108] stwierdzili statystycznie istotny związek między ryzykiem zgonu z powodu GBM a zwiększonym narażeniem na emisje paliw/olejów. Ponadto autorzy zasugerowali odwrotne powiązania spożycia cholesterolu i tłuszczu z ryzykiem wystąpienia guza mózgu [80]. Kuan et al. [109] stwierdzili brak lub słabe powiązania między składnikami odżywczymi lub wzorcami żywieniowymi a ryzykiem wystąpienia glejaka. Nie znaleźli tendencji do zmniejszania ryzyka glejaka wraz ze wzrostem spożycia owoców ogółem, owoców cytrusowych i błonnika oraz zdrową dietą.

i) **Kawa i herbata** – produkty te mogą mieć potencjalne działanie chroniące przed nowotworami. Obecność antyoksydantów, w tym polifenoli, kwasu kawowego, diterpenów i związków heterocyklicznych [110] mogłaby dawać podstawy molekularne do takiego stwierdzenia. Badanie przeprowadzone przez Kang et al. [111] donosi o zahamowaniu wzrostu komórek GBM *in vitro* po ekspozycji na kofeinę, przez inhibicję podtypu 3 receptora trisfosforanu inozytolu. Huber et al. [112] w swoich badaniach wykazali podwyższone

stężenie białka MGMT (ang. *O-6-methylguanine-DNA methyltransferase)* w wątrobach szczurów po ekspozycji na diterpeny (kahweol i cafestol). Badania dotyczące wpływu kawy oraz herbaty na ryzyko zachorowania na glioma są jednak niejednoznaczne. Holick et al. [113] w badaniu z 2010 roku donosi o odwrotnej zależności między spożyciem kofeiny a ryzykiem glioma wśród mężczyzn, ale nie wśród kobiet. Z kolei Dubrow et al. [114] wykazuje w badaniach prowadzonych wśród 545 771 respondentów brak zmniejszenia ryzyka glioma wraz ze zwiększonym spożyciem kawy oraz herbaty. W badaniach realizowanych na kohorcie populacji brytyjskiej (2 201 249 osobo-lat i 364 przypadków GBM), Creed et al. [115] zaobserwowali odwrotną zależność między spożyciem herbaty, a ryzykiem glioma, która była istotna statystycznie dla wszystkich glioma oraz dla GBM u mężczyzn. W tym samym roku Cote et al. [116] (6 022 741 osobo-lat; 362 przypadków GBM) opublikowali pracę na podstawie danych z *Nurses' Health Study* (NHS), *Nurses' Health Study II* (NHSII) i *Health Professionals Follow-Up Study* (HPFS). Autorzy nie zaobserwowali zależności między spożyciem kawy a ryzykiem glioma, ale zanotowali graniczną odwrotną zależność między spożyciem herbaty, a ryzykiem glioma dla połączonej kohorty mężczyzn i kobiet HR (ang. *hazard ratio)* dla > 2 kubków/dzień w porównaniu do < 1 kubka/tydzień. Michaud et al. [117] zaobserwowali statystycznie znamienną odwrotną zależność między spożyciem kawy, a ryzykiem glioza u grupy spożywającej od 100 ml kawy i herbaty dziennie w porównaniu do grupy spożywającej mniej niż 100 ml kawy i herbaty dziennie.

j) **Spożycie alkoholu** – Właściwości alkoholu etylowego determinują jego zdolność do pokonywania bariery krew-mózg. Z tego powodu wpływa on bezpośrednio na komórki gleju. Ponadto alkohol jest znanym i udokumento-wanym czynnikiem ryzyka rozwoju wielu nowotworów [118]. Większe dawki alkoholu prowadzą do powstania jego metabolitu – aldehydu octowego, którego właściwości neurokarcynogenne zostały opisane w badaniach na zwierzętach [119]. W produktach alkoholowych znajdują się również nitrozaminy wywołujące guzy mózgu u zwierząt [120]. Mimo tego badanie przeprowadzone przez Qi et al. obejmujące 19 metaanaliz nie wykazało związku między konsumpcją alkoholu a zwiększonym ryzykiem zachorowania na glejaki [121].

k) **Sen i melatonina** – Samatic et al. [122] nie zaobserwowali związku między długością snu a ryzykiem zachorowania na glejaki. Oreskovic et al. [123] opisali mechanizmy mające efekt pronowotworowy związane z zaburzeniami snu, między innymi obniżenie poziomu antyoksydantów, immunosupresja, zmiany metaboliczne, niedobór melatoniny, obniżenie zdolności kognitywnych oraz zmiany epigenetyczne. Powyższe zmiany prowadzą do pogorszenia prognozy chorych ze złośliwymi guzami mózgu i potencjalnie nasilają progresję nowotworów. Guzy mózgu prowadzą również do występowania zaburzeń snu.

l) **Stan zapalny** – Należy zaznaczyć, że u zdrowych ludzi zachodzą mutacje mogące potencjalnie prowadzić do rozwoju nowotworów, w tym glioblastoma. Istnieją jednak mechanizmy, które w normalnych warunkach umożliwiają ochronę organizmu przed chorobą. Układ immunologiczny jest w stanie niszczyć nieprawidłowe komórki [124] a na wczesnym etapie rozwoju nawet guzy. Stan zapalny mobilizuje układ odpornościowy i ułatwia ten proces [125]. Z drugiej strony, przewlekły stan zapalny może prowadzić do zwiększenia ilości mutacji i pobudznia onkogenezy [126]. Najlepiej poznanymi czynnikami związanymi z zapaleniem w kontekście glejaka wielopostaciowego są interleukiny 1 oraz 6, a także czynnik martwicy nowotworów alfa (TNF α). TNF α jest cytokiną biorącą udział w kierowaniu ogólnoustrojową reakcją zapalną [127]. Może wykazywać działanie zarówno przeciwnowotworowe jak i pronowotworowe. Może pobudzać angiogenezę zwiększając aktywność EGFR [128], nasilając immunosupresję [129] oraz zmniejszając ekspresję genu supresorowego *PTEN* [130].

   Interleukina 1 może indukować w astrocytach i komórkach gleju ekspresję czynników nasilających angiogenezę oraz inwazję nowotworu, jak na przykład VEGF. Zwiększa również stężenie miR-155 w komórkach gleju, mikroRNA zaangażowanego w powstawanie nowotworów na tle stanu zapalnego [131]. IL-1α jest podejrzewana o udział w patogenezie nowotworów, brak jednak dowodów dotyczących jej roli w rozwoju glejaka wielopostaciowego. W zależności od rodzaju nowotworu wykazuje właściwości pro oraz przeciwnowotworowe [132].

Środowisko glejaka wielopostaciowego jest dotknięte przewlekłym procesem zapalnym. TAM (ang. *tumor-associated macrophages*) stanowią dużą część komórek nienowotworowych guza i produkują znaczące ilości interleukiny 6 [133]. Inteleukina 6 promuje w GBM regenerację, inwację oraz angiogenezę.

m) **Promieniowanie elektromagnetyczne** – wraz z popularyzacją urządzeń elektronicznych, takich jak mikrofalówka i telefony komórkowe, kontrowersyjnym tematem stał się wpływ ekspozycji na fale elektromagnetyczne i ryzyko rozwinięcia guzów CUN. Ewentualny wpływ telefonów na rozwój nowotworów pozostaje nierozstrzygnięty z powodu zróżnicowanych wyników badań, względnie krótkiego czasu od rozpowszechnienia smart fonów oraz licznych czynników zaburzających badania. W zbiorczej analizie szwedzkich badań kliniczno-kontrolnych przeprowadzonych przez Hardell oraz Carlberg [148], u osób, które korzystały z telefonów komórkowych ponad 25 lat iloraz szans (OR) zachorowania na glioma wynosił 3.0. Z kolei Villeneuve et al. [135] sugerują, że brak zwiększenia zachorowalności na glioma wraz z coraz większą popularyzacją telefonów komórkowych przemawia za brakiem związku przyczynowo skutkowego. W badaniu opublikowanym w 2010 [136] grupa, która korzystała z telefonu komórkowego przynajmniej raz na tydzień w okresie pół roku miała niższe ryzyko zachorowania na glioma niż grupa, która nigdy nie korzystała z telefonu komórkowego. Z drugiej strony osoby najbardziej narażone pod względem skumulowanej ekspozycji miały o 40% większe ryzyko zachorowania na glejaka niż osoby nigdy nie korzystające z telefonu komórkowego.

4.2. CHEMOKINY CC W GUZIE NOWOTWOROWYM: BADANIE PRZEGLĄDOWE WŁAŚCIWOŚCI PRONOWOTWOROWYCH ORAZ ANTYNOWOTWOROWYCH RECEPTORÓW CCR5, CCR6, CCR7, CCR8, CCR9, CCR10 I ICH LIGANDÓW

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Środowisko guza nie jest jednolite i składa się z obszarów o różnych właściwościach. Najważniejszy z nich to obszar dotknięty przewlekłą hipoksją [137], charakteryzujący się nagromadzeniem makrofagów związanych z nowotworem (TAM, *ang. tumor-associated macrophages*) [138], limfocytów regulatorowych T (ang. *Treg*) [139] i komórek supresorowych pochodzących ze szpiku (MDSC, ang. *myeloid-derived suppressor cells*) [140]. Funkcje pronowotworowe tych zrekrutowanych komórek są wzmocnione przez panującą w mikrośrodowisku guza przewlekłą hipoksję [141] i zakwaszenie środowiska guza [142], które nasila oporność komórek nowotworowych na leczenie przeciwnowotworowe oraz na działanie układu immunologicznego[143]. W strefach hipoksji chemokiny wykazują działanie tylko pronowotworowe, mimo ich wcześniej wspomnianej dualnej charakterystyki. Jednakże, podczas skutecznej reakcji przeciwnowotworowej układu immunologicznego te same chemokiny mogą wykazywać działanie przeciwnowotworowe [144].

Wiedza na temat działania przeciwnowotworowego i pronowotworowego poszczególnych chemokin pozwala przewidzieć następstwa poszczególnych działań terapeutycznych i poprawić ich skuteczność. Przykładem może być zastosowanie radioterapii, które prowadzi do zwiększonej ekspresji poszczególnych chemokin (np. CCL2 i CCL5) co skutkuje zrekrutowaniem TAM i Treg [145]. Ma to efekt pronowotworowy i osłabia skuteczność radioterapii. Z drugiej strony te same chemokiny (CCL2 i CCL5) przejawiają działanie przeciwnowotworowe powodując infiltrację guza nowotworowego limfocytami (TIL, ang. *tumor-infiltrating lymphocytes*) o charakterze przeciwnowotworowym [146]. Z tego powodu radioterapia zastosowana przed immunoterapią może być bardziej skuteczna [147].

4.2.1. CCL5

Chemokina CCL5 (znana również jako RANTES) jest ligandem receptorów CCR5 [148], CCR3 [149] i CCR1 [13]. Po uwolnieniu CCL5 poza komórkę, z końca N CD26/DPPIV (ang. *dipepidylo peptydaza IV*) odcina 2 aminokwasy, zwiększając tym powinowatość CCL5 do CCR5 i zmniejszając powinowatość CCL5 do CCR1 i CCR3 [150]. Działanie CCL5 jest osłabiane przez ACKR2/D6 (ang. *atypical chemokine receptor 2*), który zmniejsza stężenie tej chemokiny [151]. CCL5 jest również ligandem dla GPR75 (ang. G-*protein-coupled receptor 75),* którego ekspresja zachodzi w komórkach nerwowych mózgowia [152].

Cytokiny prozapalne zwiększają ekspresję CCL5, co skutkuje zwiększeniem liczby komórek układu immunologicznego w miejscu zapalenia [153], np. zależna od CCR3 chemotaksja eozynofilii w astmie. Oś CCL5→CCR5 indukuje i nasila cytotoksyczne właściwości przeciwnowotworowych komórek TIL w obrębie guza [154]. CCL5 jest odpowiedzialna za infiltrację guza przez komórki NK [155], konwencjonalne komórki dendrytyczne typu 1 [154], limfocyty pomocnicze T typu 1 [146] i limfocyty cytotoksyczne typu 1 (Tc1) [142]. Z tego powodu niektórzy autorzy postulują, że należałoby używać immunoterapii po terapii zwiększającej ekspresję CCL5 w guzie, aby zwiększyć infiltrację guza przez komórki odpornościowe [156]. Ponadto genom HHV-8 wywołujący mięsaka Kaposiego posiada vMIP-II (ang. *viral macrophage-inflammatory protein-II*) [157], będący antagonistą CCR5. Komórki zainfekowane przez wirusa wydzielając vMIP-II zmniejszają infiltrację guza przez TIL. vMIP-II indukuje również rekrutację limfocytów Th2 przez CCR3 i CCR8, co zmniejsza aktywność limfocytów cytotoksycznych [158].

Z drugiej strony CCL5 posiada również właściwości pronowotworowe, a jej zwiększona ekspresja występuje w raku piersi [159], glejak wielopostaciowym [160] i raku wątrobowokomórkowym [161]. Ekspresja CCL5 zachodzi w następujących komórkach guza: komórkach nowotworowych [162], CAF [21], MSC [163], MDSC [20], TAM [164] i TIL [165]. Ekspresja CCL5 w śródbłonku naczyń limfatycznych gra ważną role w powstawaniu niszy premetastatycznej [166].

CCL5 zwiększa proliferację komórek nowotworowych [161]. Aktywując szlak sygnałowy Wnt/B-catenin→STAT3 (ang. *transducer and activator of transcription 3*) indukuje samoodnowę komórek macierzystych raka prostaty [164]. Podobnie jak inne

chemokiny, CCL5 powoduje migrację, inwazję i EMT (ang. *epithelial-to-mesenchymal transition*) komórek nowotworowych przez swój receptor [161], z udziałem szlaków Hedgehog [167], Wnt/β-catenin [164] i Akt/PKB→nuclear factor κB (NF-κB) [168]. Indukcja migracji wywołanej przez CCL5 jest związana ze zwiększoną sekrecją zewnątrzkomórkowych metaloproteinaz do matrix przez komórki nowotworowe. Dodatkowo, CCL5 zwiększa oporność komórek nowotworowych na apoptozę i leki przeciwnowotworowe poprzez aktywację szlaków Akt/PKB→NF-kB i STAT3 [165]. Zwiększa to ekspresję PD-L1 (ang. *programmed death-ligand 1*) przez komórki nowotworowe i ochrania przed limfocytami T cytotoksycznymi.

CCL5 wpływa nie tylko na komórki nowotworowe, ale również na komórki związane z nowotworami. Rekrutuje MDSC [169], MSC [170], TAM [171] i limfocyty T pomocnicze 17 (Th17) [172] do niszy guza. CCL5 przez CCR5 bierze udział w rekrutacji i zwiększeniu aktywności Treg w niszy nowotworu [173].

CCL5 bierze również udział w angiogenezie, co związane jest ze zwiększeniem ekspresji czynnika wzrostu śródbłonka naczyniowego (VEGF, ang. *Vascular endothelial growth)* w komórkach nowotworowych i śródbłonka naczyń przez aktywację CCR1 i CCR5 [174]. CCL5 może powodować różnicowanie macierzystych komórek nowotworowych w komórki śródbłonka jak np. w modelu raka jajnika [175]. Produkcja tej chemokiny jest również pobudzana przez wirus *Epsteina-Barr* (EBV, ang. *Epstein–Barr virus*) w komórkach raka jamy nosowagardłowej, co prowadzi do angiogenezy [176]. Może również pośrednio wywoływać limfangiogenezę przez wzrost ekspresji czynnika wzrostu śródbłonka naczyniowego (VEGF-C, ang. *vascular endothelial growth factor C*), jak zaobserwowano w komórkach chrzęstniakomięsaka [177].

4.2.2. CCL3 i CCL4

CCL3, znana również jako MIP-1α (ang. *macrophage inflammatory protein-1α*) oraz CCL4, MIP-1 β (ang. *macrophage inflammatory protein-1 β*) są chemokinami o działaniu prozapalnym. U ludzi występują również dodatkowe wariacje CNV (ang. *copy number variations*) genów *CCL3 i CCL4*, powstałe w procesie duplikacji, znane jako odpowiednio *CCL3L i CCL4L* [178]. Produkty tych dodatkowych genów mają taką samą sekwencję aminokwasów jak CCL3 i CCL4 oraz pełnią takie same funkcje.

CCL3 i CCL4 zostały zawarte w badaniu z uwagi na swoje właściwości przeciwnowotworowe i rolę w zapoczątkowaniu odpowiedzi immunologicznej. CCL3 jest ligandem dla receptorów CCR1 i CCR5 [179], a CCL4 wchodzi w interakcje z CCR5 [148] oraz ze słabą powinowatością z CCR1 [180]. Cytokiny te rekrutują komórki dendrytyczne, neutrofile, monocyty makrofagi, komórki NK i limfocyty T do miejsca zapalenia [181]. CCL3 jest odpowiedzialna za prawidłową funkcję limfocytów T CD8+ a CCL4 odpowiada za prawidłową funkcję limfoctów T CD4+ [182]. Zdolność rekrutowania komórek odpornościowych nadaje CCL3 i CCL4 potencjał w terapiach przeciw-nowotworowych [183].

Aktywna odpowiedź immunologiczna w obrębie nowotworu prowadzi do produkcji CCL3 i CCL4 przez limfocyty B [184] i bazofile [185]. Te uwolnione cytokiny działają chemotaktycznie dla przeciwnowotworowych komórek TIL z obecnymi receptorami CCR1 i CCR 5 [183, 186–189]. Funkcje przeciw-nowotworowe, zarówno CCL3 jak i CCL4, mogą zostać osłabione przez występujące w tkance nowotworowej [13] katepsyny D [190].

CCL3 i CCL4 wykazują również działanie pronowotworowe, a ich ekspresja została zaobserwowana w komórkach związanymi z nowotworem i w komórkach nowotworowych. W obrębie guza chemokiny te pobudzają wzrost nowotworu i są produkowane przez TAM [191], MDSC [192] i MSC [163]. Ekspresja CCL4 może również zachodzić w neutrofilach związanych z nowotworem (TAN, ang. *tumor-associated neutrophils*) [193], a ekspresja CCL3 w komórkach nowotworowych [194]. Odpowiedź immunologiczna aktywując β-kateninę zmniejsza ekspresję CCL3 w komórkach nowotworowych [195]. W przewlekłej białaczce szpikowej i szpiczaku mnogim zachodzi ekspresja zarówno CCL3 jak i CCL4 [196]. CCL3 i CCL4 hamuje aktywność osteoblastów a zwiększa aktywność osteoklastów i tym samym prowadzi do resorpcji kości [197]. Dodatkowo produkcja CCL3 przez komórki ostrej białaczki szpikowej prowadzi do zahamowania erytropoezy [198]. Hipoksja szpiku kostnego zwiększa ekspresję CCR1 w komórkach szpiczaka mnogiego [199], a aktywacja receptora CCR1 przez CCL3 wpływa na funkcję receptora chemokin C-X-C typu 4 (CXCR4, ang. *CXC motif chemokine receptor 4*) i prowadzi do wyrzutu komórek szpiczaka mnogiego do krwi. Podwyższone stężenie CCL3 w osoczu krwi jest związane

z gorszą prognozą pacjentów z chłoniakiem rozlanym z dużych komórek B (DLBCL, ang. *diffuse large B cell lymphoma)* [200], pozawęzłowym chłoniakiem z komórek NK/T [201], oraz szpiczakiem mnogim [202].

Zarówno CCL3 jak i CCL4 mogą rekrutować Treg poprzez receptory CCR1 i CCR5. CCL3 może brać udział w rozwoju ostrej białaczki szpikowej poprzez rekrutację Treg do podścieliska krwiotwórczego. CCL4 może również przyciągać Treg do niszy nowotworu, jak w przypadku czerniaka [20]. Zarówno CCL3 i CCL4 mogą wywołać rekrutację MDSC do niszy guza za pośrednictwem CCR5 [203]. CCL3 [204] i CCL4 [205] poprzez oddziaływanie na CCR5 pełnią ważną rolę w funkcjonowaniu nowotworu i w początkowym okresie formowania się niszy metastatycznej w kościach poprzez rekrutację CAF.

Mimo, że CCL3 i CCL4 wykazują właściwości przeciwnowotworowe powodując infiltrację nowotworu przez cytotoksyczne TIL, mogą również wspomagać rozwój nowotworu działając bezpośrednio na komórki nowotworowe. CCL3 nasila proliferację komórek nowotworowych [206]. W komórkach szpiczaka mnogiego CCL3 aktywuje szlaki PI3K→Akt/PKB→mTOR i ERK (ang. *extracellular signal-regulated kinase*) i MAPK (ang. *mitogen-activated protein kinase)* i wywołuje lekooporność [196]. W ostrej białaczce limfoblastycznej leki przeciwnowotworowe zwiększają ekspresję CCL3 i CCL4 co prowadzi do lekooporności tych komórek nowotworowych [207].

CCL3 poprzez CCR5 powoduje migrację i inwazję komórek nowotworowych [208]. Jest to związane między innymi z aktywacją szlaków PI3K→Akt/PKB jak i ERK i MAPK [194]. Zarówno CCL3 jak i CCL4 mogą brać udział w tworzeniu przerzutów. CCL3 wydzielane przez komórki guza zwiększa ekspresję CCL2, CCL7 i CCL8 w płucach i mózgu [209], co prowadzi do umiejscawiania się przerzutów właśnie w tych narządach. Komórki nowotworowe po przedostaniu się do krwi zostają otoczone płytkami krwi, które chronią je przed działaniem komórek NK [210]. Płytki krwi wydzielają wiele czynników, włączając w to CCL3, które wspomagają komórki nowotworowe [211]. Po dotarciu do innego narządu CCL3 bierze udział w formowaniu niszy przerzutu. Komórki nowotworowe z pomocą osi CCL2→CCR2 [212] rekrutują makrofagi, które wydzielają CCL3. CCL3 zatrzymuje makrofagi w obrębie niszy przerzutu przez autokrynne działanie na receptor CCR1. Z kolei w obrębie kości zwiększona ekspresja CCL3

w monocytach pochodzących ze szpiku kostnego spowodowana jest wydzielaniem przez komórki nowotworowe nabłonkowego czynnika wzrostu (EGF, ang. *epidermal growth factor*). Powoduje to różnicowanie monocytów do osteoklastów [213] i remodeling kości wokół ognisk przerzutów. CCL3 [194] i CCL4 [214] zwiększają ekspresję czynnika wzrostu śródbłonka naczyniowego (*VEGF*, ang. *vascular endothelial growth factor*) i pośrednio wywołują angiogenezę. CCL4 zwiększa również ekspresję VEGF-C, co prowadzi do limfangiogenezy i powstawania przerzutów w węzłach chłonnych [215].

4.2.3. CCR6 i CCL20

CCL20 znana również jako LARC (ang. *liver activation regulated chemokine)* lub MIP-3α (ang. *macrophage inflammatory protein-3α* lub ang. *Exodus-1*) jest chemokiną prozapalną, będącą ligandem CCR6 [216]. Odgrywa kluczową rolę dla prawidłowego funkcjonowania komórek dendrytycznych, limfocytów T i limfocytów B [216], które posiadają CCR6. CCL20 jest produkowana przez limfocyty Th17 i odpowiada za ich prawidłowe funkcjonowanie [217]. Odgrywa również kluczową rolę w procesie ontogenezy – rekrutuje Treg i Th17 do niszy guza, wywołuje angiogenezę, migrację komórek nowotworowych i przejście epitelialno-*mezenchymalnego* (EMT, ang. *epithelial-mesenchymal transition).*

Wiele nowotworów przebiega ze zwiększoną ekspresją CCL20: rak piersi [159], rak wątrobowokomórkowy [218] i rak trzustki [219]. Ekspresja CCL20 zachodzi nie tylko w komórkach nowotworowych [220], ale również TAM [221]. Komórki nowotworowe wykazują również ekspresję CCR6. Z tego powodu w przypadku niektórych nowotworów komórki mogą stymulować swoją proliferację i migrację poprzez autokrynną aktywację osi CCL20→CCR6 [220]. Równocześnie CCL20 stymuluje angiogenezę aktywując CCR6 na powierzchni komórek śródbłonka naczyń [222]. Aktywacja CCR6 na komórkach nowotworowych może również prowadzić do zwiększenia ekspresji VEGF w ich obrębie i tym samym przyczyniać się do angiogenezy [223].

Główną funkcją CCL20 w obrębie nowotworu jest rekrutacja limfocytów Treg i Th17 do ogniska guza [224]. Pozwala to komórkom nowotworowym na ucieczkę spod nadzoru immunologicznego. CCL20 rekrutuje również komórki TAM [225]

poprzez CCR6. Mimo że CCL20 rekrutuje również komórki dendrytyczne niosące ze sobą właściwości przeciwnowotworowe [226], efekt netto działania CCL20 jest pronowotworowy.

Oprócz działania polegającego na rekrutacji komórek do ogniska nowotworu, CCL20 zwiększa również proliferację komórek nowotworowych [227], ich migrację oraz inwazję [228] i pobudza EMT [221]. Jest również współodpowiedzialna za wzorce przerzutowania do poszczególnych narządów – wysoka ekspresja CCL20 w wątrobie skutkuje częstym lokalizowaniem się w tym narządzie przerzutów, których komórki wykazują się wysoką ekspresją CCR6 [229]. CCR6 jest również ważnym czynnikiem w powstawaniu przerzutów do płuc u pacjentów z rakiem piersi [230] oraz przerzutów do nadnerczy u chorych z rakiem płuca [231]. Jednakże, ekspresja CCL20 w płucach i nadnerczach jest niska [232]. To wskazuje, że ta chemokina bierze w tych przypadkach jedynie współudział w indukcji migracji komórek nowotworowych. Ekspresja CCR6 na komórkach chłoniaków nie-Hodgkina B-komórkowych prowadzi do umiejscawiania się komórek chłoniaków na błonach śluzowych [233]. Oś CCL20→CCR6 odgrywa ważną rolę w przypadku osteolitycznych ognisk w przebiegu szpiczaka wielopostaciowego [234].

4.2.4. CCR7, CCL19 i CCL21

Do najważniejszych funkcji CCL19 znanej również jako EBI1, ELC (ang *ligand chemokine),* MIP-3 (ang. *macrophage inflammatory protein-3* lub *Exodus*-3) i CCL21 znanej również jako SLC (ang. *secondary lymphoid tissue chemokine, 6Ckine* lub *Exodus-2*) należy sprowadzanie limfocytów T do węzłów chłonnych [235]. Za ten proces odpowiada CCR7 [236], który jest receptorem dla tych chemokin. Z tego powodu zwiększona ekspresja CCL19 i CCL21 w komórkach nowotworowych ma działanie przeciwnowotworowe za sprawą cytotoksycznych TIL [237].

W przypadku komórek nowotworowych posiadających CCR7 oś CCL19/CCL21→CCR7 może mieć właściwości pronowotworowe. Hipoksja [238] i prostaglandyna E2 (PGE2) [239] zwiększają ekspresję CCR7 na komórkach nowotworowych. CCR7 nasila proliferację [240] i właściwości charakterystyczne dla macierzystych komórek nowotworowych [241]. W raku płaskonabłonkowym

przełyku aktywacja CCR7 prowadzi do zwiększonej angiogenezy poprzez aktywacje NF-κB i w konsekwencji zwiększenia ekspresji czynnika wzrostu śródbłonka naczyniowego-A (VEGF-A, ang. *Vascular endothelial growth factor A*) [242]. Dla odmiany CCL19 hamuje angiogenezę w komórkach raka jelita grubego poprzez zwiększenie ekspresji miR-206, które z kolei hamuje szlak ERK MAPK→HIF-1→VEGF-A [243]. W komórkach nowotworowych głowy i szyi [244] i komórkach raka płaskonabłonkowym przełyku [245] aktywacja CCR7 zwiększa ekspresję VEGF-C i VEGF-D (czynników odpowiedzialnych za limfangiogenezę) [242]. Aktywacja CCR7 na komórkach nowotworowych powoduje EMT i migrację komórek nowotworowych [241]. Komórki nowotworowe przebywające w naczyniach krwionośnych i limfatycznych są chronione przed *anoikis* dzięki CCR7 [246].

Niektóre białaczki, jak na przykład przewlekła białaczka limfocytowa B-komórkowa, wykazuje ekspresję CCR7 [247]. Prowadzi to do przyciągania tych komórek do wtórnych narządów limfatycznych. W białaczkach ekspresja CCR7 jest ważnym czynnikiem prognostycznym. CCR7 odgrywa ważną rolę w rozsiewie chłoniaków nieziarniczych [248]. Im wyższa ekspresja CCR7 tym gorsza prognoza chorych z rozlanym chłoniakiem z dużych komórek B [249]. Ekspresja CCR7 została również powiązana z przerzutami raka piersi do skóry [230].

Działanie osi CCL19/CCL21→CCR7 na komórki nowotworowe wpływa również na kompozycję komórkową guza nowotworowego. Zwiększona ekspresja CCL19 i CCL21 prowadzi do nacieku przeciwnowotworowych komórek TIL i poprawy prognozy u wielu pacjentów nowotworowych [250]. Mechanizmy wymykania się spod kontroli immunologicznej nowotworu zmniejszają ekspresję CCL19 i CCL21 w komórkach nowotworowych [195]. W określonych warunkach CCL19 i CCL21 mogą brać udział w rekrutacji Treg do ogniska nowotworu [251]. Rekrutacja *Treg* do ogniska guza nowotworowego skutkuje hamowaniem prawidłowej odpowiedzi przeciwnowotworowej układu immunologicznego.

4.2.5. CCR8

CCL1 (znana również jako I-309) była pierwszą zidentyfikowaną cytokiną wydzielaną przez ludzkie aktywowane limfocyty T [252]. CCL1 jest ligandem dla tylko jednego receptora – CCR8 [253]. Jest też uznana za cytokinę związaną

z limfocytami Th2 ze względu na ekspresję jej receptora (CCR8) na ich powierzchni, co odróżnia je od limfocytów Th1 nieposiadających CCR8 [254]. Ta właściwość odgrywa kluczową rolę w patogenezie astmy [255]. Oś CCL1→CCR8 jest odpowiedzialna za przyciąganie limfocytów do zdrowej skóry i odgrywa ważną rolę w fizjologii tej tkanki [256].

Zwiększona ekspresja CCL1 występuje w białaczkach wywołanych wirusami np. HTLV-1 (ang. *human T cel leukemia virus type 1)* [257]. Podobne właściwości do tej chemokiny wykazuje również wirusowe białko zapalne makrofagów-I (vMIP-I, ang. *viral macrophage inflammatory protein*-I) – homolog CCL1, wirusowe białko ulegające ekpresji w komórkach nowotworowych transformowanych przez wirusa opryszczki (ang. *herpes virus*) [258]. Innym przykładem wydzielania vMIP-I jest mięsak Kaposiego związany z zakażeniem *ludzkim wirusem opryszczki* typu *8* (KSHV, ang. *Kaposi sarcoma-related human herpes virus-8)* [259]. Rekrutacja limfocytów Th2 przez vMIP-II i receptory CCR3 i CCR8 redukuje aktywność limfocytów cytotoksycznych [158].

Ekspresja CCL1 w raku piersi nie różni się od tej w zdrowej tkance [159]. W komórkach raka wątrobowokomórkowego w badaniu przeprowadzonym przez Shin et al. (2002) ekspresja MIP-1 była bardzo słaba lub nie zachodziła [260]. Z kolei Wiedemann et al. (2019) wykazali zwiększoną ekspresję CCL1 w zrębie nowotworu i tkankach okolicy guza [261].

W glejakach ekspresja CCL1 jest słabsza niż w zdrowej tkance mózgu [160]. W przypadku nowotworów litych CCL1 jest produkowane przez CAF [262], TAM [263], komórki szpikowe CCR8–CD11b+ [264] i Treg [265]. Ekspresja CCL1 została również zaobserwowana w komórkach macierzystych raka piersi [266], pęcherza moczowego [264] i raka nerkowokomórkowego [264].

CCL1 działa na nowotwory antyapoptotyczne oraz indukuje lekooporność działając na CCR8 aktywujący kaskadę kinaz regulowanych sygnałem zewnątrzkomórkowym (ERK, ang. *extracellular signal-regulated kinases*) → kinazy białkowe aktywowane mitogenem (MAPK, ang. *mitogen-activated protein kinases*) [262]. Ma to szczególne znaczenie w oporności na apoptozę białaczki T komórkowej u dorosłych [267] i chłoniakach T komórkowych u myszy [268]. CCL1 stymuluje również proliferację [269] i powoduje migrację komórek raka

pęcherza moczowego [270]. Ekspresja CCL1 w węzłach chłonnych powoduje tworzenie przerzutów w ich obrębie, z powodu obecności receptora CCR8 na komórkach nowotworowych, które dostały się do układu limfatycznego [271]. Proces ten ma duże znaczenie przy rozsiewie czerniaka złośliwego, który charakteryzuje się zwiększoną ekspresją CCR8 na swoich komórkach [271]. Oś CCL1→CCR8 nie jest jedynym mechanizmem biorącym udział w rozsiewie nowotworów w układzie limfatycznym. Biorą w nim udział również CCL10 i CCL21 działające na CCR7 [272].

Oprócz działania wyłącznie na komórki nowotworowe CCL1 aktywuje CCR8 komórek śródbłonka naczyń i wywołuje angiogenezę [259]. CCL1 odgrywa również rolę w rekrutacji komórek szpikowych CCR8–CD11b+ [264] i Treg [266] do niszy guza. CCL1 może brać udział w konwersji limfocytów T CD4+ do Treg [265]. W procesie zależnym od transformujący czynnik wzrostu *beta* (*TGF*-β1 ang. *transforming growth factor β1*), który zwiększa ekspresję CCL1 w limfocytach CD4+, zachodzi autokrynna, zależna od CCL1 konwersja limfocytów T CD4+ do Treg. CCL1 wspiera również immunosupresyjne działanie limfocytów Treg w niszy guza [265], co odgrywa kluczową rolę w interakcji macierzystych komórek nowotworu i CAF z Treg [270]. CCL1 zwiększa w MDSC ekspresję interleukiny 6 (IL-6), mającej działanie prozapalne w mikrośrodowisku nowotworu [264].

4.2.6. CCL18

CCL18 znana również jako PARC (ang. *pulmonary and activation regulated chemokine*), AMAC-1 (ang. *alternative macrophage activation-associated C-C chemokine 1*), DC-CK1 (ang. *dendritic cell derived C-C chemokine 1*) i MIP-4 (ang. *macrophage inflammatory protein 4*) jest produkowana przez komórki dendrytyczne, szczególnie w centrach rozrodczych węzłów chłonnych. CCL18 jest odpowiedzialna za przyciąganie „naiwnych" limfocytów T do komórek dendrytycznych [273], co zapoczątkowuje odpowiedź immunologiczną. CCL18 ma również działanie przeciwzapalne i jest markerem dla makrofagów M2 [274].

W guzie nowotworowym TAM są głównym źródłem CCL18 [191] która poprzez aktywację receptorów CCR8 oraz PITPNM3 (ang. *phosphatidylinositol transfer protein 3)* powoduje przejście epitelialno-*mezenchymalne* i migrację

komórek [275]. CCL18 zwiększa również ekspresję markerów nowotworowych komórek macierzystych [276], poprzez działanie na receptor PITPNM3 komórek śródbłonka naczyniowego bierze udział w angiogenezie [277] i wpływa na komórki nienowotworowe niszy guza nowotworowego. Działając jako cytokina immunosupresyjna powoduje przekształcanie makrofagów do fenotypu M2 [274] i poprzez receptory PITPNM3 rekrutuje dziewicze limfocyty CD4+ do niszy guza [278], które następnie różnicują się do Treg. CCL18 rekrutuje niedojrzałe komórki dendrytyczne do niszy guza [279]. Dodatkowo chemokina ta bierze udział w różnicowaniu niedojrzałych komórek dendrytycznych do komórek dendrytycznych związanych z nowotworem (TADC, ang. *tumor-associated dendritic cells*) [280]. Inną funkcją CCL18 w obrębie nowotworu jest udział w międzykomórkowej komunikacji zależnej od zewnątrzkomórkowych pęcherzyków [281]. CCL18 łączy się z glikozaminoglikanami obecnymi na pęcherzykach zewnątrzkomórkowych, co pozwala na przechowanie tych pęcherzyków na powierzchni komórek z obecnym receptorem CCR8. Wykazano dotychczas, że CCL18 zmniejsza proliferację ostrej białaczki limfoblastycznej B komórkowej [282] i chłoniaka skórnego T-komórkowego (CTCL, ang. *cutaneous T-cell lymphoma*), raka niedrobnokomórkowego płuc [20], a zwiększa proliferację komórek glejaka [283] i raka płaskonabłonkowego jamy ustnej [284].

4.2.7. CCR9 i CCL25

CCL25 znana również jako TECK (ang. *thymus-expressed chemokine*) jest ligandem dla tylko jednego receptora – CCR9 [285]. Chemokina ta odgrywa ważną rolę w prawidłowym funkcjonowaniu grasicy [286]. Z uwagi na ekspresję CCL25 w układzie pokarmowym przyciąganie limfocytów do tkanek tego układu jest jedną z głównych funkcji osi CCL25→CCR9 [287]. Ma to duże znaczenie w pełnieniu funkcji odpornościowej śluzówki jelit i żołądka. Prowadzi również do lokalizowania się w tej tkance przerzutów komórek nowotworowych z obecną ekspresją receptora CCR9 [288]. Oś CCL25→CCR9 jest również odpowiedzialna za lokalizowanie się rozlanych chłoniaków z dużych komórek i chłoniaka grudkowego do układu pokarmowego [289].

CCL25 jest produkowana przez komórki raka piersi [290], raka trzustki [291] i komórki związane z nowotworem (np. komórki gwiaździste trzustki) [292]. CCL25 zwiększając ekspresję metaloproteinaz bierze udział w migracji i inwazji komórek nowotworowych [293–295]. Powoduje również przejście epitelialno-*mezenchymalnego* komórek nowotworowych [290]. W raku jelita grubego aktywacja receptora CCR9 hamuje migrację komórek nowotworowych [296]. Aktywacja CCR9 jest również związana z opornością na apoptozę i lekoopornością, co się łączy z aktywacją szlaku Akt/PKB→PI3K [297]. Dodatkowo oś CCL25→CCR9 stymuluje proliferację komórek nowotworowych [291].

Nie ma danych na temat wpływu CCL25 na rekrutację komórek przyczyniających się do rozwoju nowotworu i angiogenezy [298]. CCL25 ma działanie przeciwnowotworowe poprzez powodowanie infiltracji guza przez wykazujące ekspresję CCR9 cytotoksyczne TIL [299]. Fakt zwiększenia ekspresji VEGF-C i VEGF-D po aktywacji osi CCL25→CCR9 w komórkach raka niedrobnokomórkowego płuca wskazuje na działanie limfagiogenne CCL25 [295]. CCL25 może również rekrutować MDSC do niszy guza, na co wskazuje publikacja Sun et al. na temat endometriozy [300].

4.2.8. CCR10, CCL28 i CCL27

CCL28 znana również jako MEC (ang. *mucosae-associated epithelial chemokine*) jest chemokiną niezbędną dla prawidłowej funkcji immunologicznej śluzówek [301]. Może aktywować 2 receptory – CCR3 [302] i CCR10 [303]. Inną chemokiną aktywującą CCR10 jest również CCL27 [13] i z tego powodu ma podobne działanie do CCL28. CCL27 wywołuje migrację limfocytów CD3+ i CD4+ [304]. CCL28 i CCL27 z pomocą CCR10 wywołują migrację limfocytów T i limfocytów B, szczególnie tych produkujących IgA [305]. CCL27 jest produkowana przez keratynocyty i z tego powodu występuje głównie w skórze [301] a CCL28 jest produkowana głównie w błonach śluzowych [301]. Z tego powodu chemokiny te przyciągając komórki układu odpornościowego do błon śluzowych i nabłonków odgrywają ważną rolę w reakcjach immunologicznych przeciwko mikroorganizmom. CCL28→CCR3 pobudza również migrację eozynofilii [302] i odgrywa ważną rolę w rozwoju alergii.

CCL27 pełni również rolę wewnątrzkomórkową. W mechanizmie alternatywnego splicingu z genu CCL27 powstaje białko PESKY [306], czyli białko jądrowe ulegające ekspresji między innymi w tkance oka, mózgu i jąder. PESKY zmienia ekspresję genów związanych z aktynowym cytoszkieletem i prowadzi do migracji oraz zmiany morfologii komórek [307]. CCL27 zawiera również sekwencję lokalizacji jądrowej i po internalizacji receptora CCR10 połączonego z tą chemokiną CCL27 jest transportowana do jądra komórkowego, gdzie pełni funkcję podobną do PESKY [307].

Ekspresja CCL28 jest zmniejszona w raku piersi [308], raku jelita grubego [309] i glejaku wielopostaciowym [160]. Z kolei ekspresja CCL27 jest zmniejszona w raku endometrium [310], raku podstawnokomórkowym [311] i raku kolczystokomórkowym [311]. Ta obserwacja sugeruje, że te chemokiny, przynajmniej w początkowym stadium rozwoju nowotworu mają działanie przeciwnowotworowe. Potwierdzają to badania na temat przeżywalności pacjentów z różnymi typami raka piersi [312]. Z kolei zwiększona ekspresja CCL27 poprawia prognozę pacjentów z czerniakiem złośliwym skóry [313]. CCL28 i CLL27 biorą udział w odpowiedzi przeciwnowotworowej układu immunologicznego powodując infiltrację guza przeciwnowotworowymi komórkami NK i wraz ze zwiększoną ekspresją CCL27 i CCL28 w guzie poprawia się rokowanie chorych [310]. Z tego powodu terapie genowe które zwiększają ekspresję tych 2 chemokin mają działanie przeciwnowotworowe [314].

Mimo tego wyniki licznych badań *in vitro* przemawiają za pronowotworowym działaniem CCL28 i CCL27. Z tego powodu niektórzy autorzy sugerują, że CCL28 może wykazywać działanie lokalne, szczególnie w strefie hipoksji [315]. CCL28 i CCL27 stymulują proliferację i mają działanie antyapoptotyczne na komórki nowotworowe [316]. Badania *in vitro* wykazują spadek migracji i EMT raka kolczystokomórkowego pod wpływem CCL28 [317], ale efekty były różne w stosunku do innych nowotworów. CCL28 zwiększa migrację komórek raka wątrobowokomórkowego [318]. Aktywacja CCR10 przez CCL27 lub CCL28 skutkuje migracją komórek raka piersi [316] i komórek glejaka wielopostaciowego [319]. Po dotarciu do układu krwionośnego komórki nowotworowe umiejscawiają się w narządach o wysokiej ekspresji chemokin dla których posiadają receptory. Z tego powodu ekspresja CCR10 na komórkach nowotworowych zwiększa prawdopodobieństwo lokalizacji przerzutów w skórze, w której zachodzi produkcja CCL28 i CCL27 [320].

Oprócz wpływu na komórki nowotworowe CCL28 wywiera również działanie na nienowotworowe komórki niszy guza. W szczególności bierze udział w rekrutacji limfocytów Treg [139] a w przypadku gruczolakoraka przewodowego trzustki w rekrutacji komórek gwiaździstych związanych z nowotworem [321]. Aktywując CCR3 komórek śródbłonka naczyń CCL28 wywołuję angiogenezę [322]. Zarówno CCL28 jak i CCL27 aktywując CCR10 śródbłonka naczyń limfatycznych wywołują limfangiogenezę [323].

Rola poszczególnych chemokin w procesach nowotworowych jest stosunkowo dobrze znana. Jednak niewiele wiadomo o interakcjach między komórkami w guzie, w szczególności o bezpośrednim lub pośrednim wpływie komórek nowotworowych na komórki nienowotworowe i interakcjach między komórkami nienowotworowymi.

Szczególnie interesujące są indukowane przez komórki nowotworowe zmiany w ekspresji chemokin wytwarzanych przez komórki związane z rakiem, np. przez TAM [192], MDSC [20] i CAF [21]. W guzie komórki rakowe nie są izolowane, ale wchodzą w interakcje z komórkami związanymi z nowotworem, dlatego też komórki te (np. TAM, TIL, MDSC, CAF) powinny być częściej badane w celu odkrycia nowych mechanizmów w guzie. Niestety istnieje niewiele narzędzi badawczych, które umożliwiają ocenę interakcji między komórkami związanymi z rakiem, a rzeczywistymi komórkami nowotworowymi. Najbardziej godne uwagi są wspólne hodowle komórek rakowych i komórek związanych z rakiem [324], a także wykorzystanie kondycjonowanej pożywki z komórek rakowych do hodowli komórek nienowotworowych [325]. Zrozumienie tych interakcji pozwoli na rozwój nowych podejść terapeutycznych, zwłaszcza jeśli zostaną odkryte mechanizmy uniwersalne w jednym typie nowotworu lub nawet wspólne dla wszystkich chorób nowotworowych

4.3. EKSPRESJA CCL18 W TKANCE GLEJAKA WIELOPOSTACIOWEGO I TKANCE GUZA

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4.3.1. WPŁYW CHEMOKIN NA ONKOGENEZĘ GLEJAKA WIELOPOSTACIOWEGO

Jednym z kluczowych aspektów umożliwiających lepsze zrozumienie onkogenezy glejaka wielopostaciowego jest wzajemna komunikacja komórek guza z komórkami podścieliska oraz komórkami immunologicznymi. Jedną z głównych ról w tym procesie odgrywają chemokiny, będące grupą ok. 50 cytokin chemotaktycznych. Zaburzenie równowagi w stężeniu chemokin może mieć działanie proonkogenne. Wśród efektów zachwiania stężeń chemokin zalicza się angiogenezę, migrację, inwazję, przerzutowanie komórek nowotworu oraz rekrutowanie komórek związanych z nowotworem (TAC, ang. *tumor-associated cells*) do ogniska guza [326].

Jedną z głównych chemokin produkowanych przez makrofagi związane z nowotworem (TAM, ang. *tumor-associated macrophages)* i wydzielaną do mikro środowiska guza jest CCL18 (ang. *CC motif chemokine ligand 18*) [327] osiągająca 70 krotnie większe stężenia w tkance guza w porównaniu do grupy kontrolnej [160]. CCL18 jest wydzielane w mniejszych ilościach również przez komórki GBM [328] oraz komórki innych nowotworów (komórki raka niedrobnokomórkowego płuca [329] i czerniaka [330]).

Najlepiej poznanym receptorem CCL18 jest PITPNM3 (ang. *phosphatidylinositol transfer membrane-associated protein)* znany również jako Nir1 (ang. *N-terminal domain interacting receptor 1*) wchodzący w interakcję z PYK2 *(*ang. *protein tyrosine kinase*) [331]. Aktywacja tego receptora prowadzi do migracji komórek nowotworowych w wielu nowotworach. Kolejnym, równie ważnym receptorem aktywowanym przez CCL18 jest CCR8 (ang. *CC motif chemokine receptor 8*). W raku pęcherza moczowego aktywacja tego receptora prowadzi

do migracji i inwazji komórek nowotworu, zwiększonej ekspresji VEGF i rozwinięcia sieci naczyń limfatycznych [332]. Receptor ten i jego rola pronowotworowa nie jest jednak wystarczająco dobrze poznana.

Równie ważnym kierunkiem badań nad onkogenezą jest zrozumienie wpływu hipoksji na proces powstawania nowotworów [333]. Odpowiedź komórkowa na hipoksję wiąże się z aktywacją białek czynnika indukowanego hipoksją 1 (HIF-1, ang. *hypoxia-inducible factor 1)* oraz HIF-2 [334] oraz zmniejszenia ich degradacji [335]. Prowadzi to do indukcji ekspresji VEGF [336]. Hipoksja wywołuje odpowiedź w ekspresji genów bez udziału czynników HIF. Dzieje się to w mechanizmie metylacji DNA i histonów przez zmniejszenie aktywności enzymów zależnych od tlenu, odpowiednio TET (ang. *ten-eleven translocation*) i JmjC (ang. *Jumonji C family*). Znaczenie CCL18 zostało relatywnie dobrze opisane w modelach nowotworowych, w przeciwieństwie do jej receptorów.

4.3.2. Ekspresja CCL18 w guzach glejaka wielopostaciowego jest wyższa w stosunku do obszaru okołoguzowego

Analiza wyników badań wykazała, że ekspresja CCL18 w rdzeniu i obszarze rozrastającym się guza była ponad siedmiokrotnie wyższa niż w tkance okolicy guza ($p = 0.044$; rdzeń guza vs. obszar okołoguzowy, $p = 0.018$). Ekspresja PITPNM3 i CCR8 nie różniła się istotnie statystycznie między rdzeniem, obszarem rozrastającym się guza, a okolicznymi tkankami ($p > 0.05$). Ekspresja CCL18 w guzach GBM pochodzących od kobiet była istotnie statystycznie wyższa w obszarze rozrastającym się i rdzeniu guza w porównaniu do tkanki okołoguzowej. Ekspresja PITPNM3 u kobiet była niższa w rdzeniu guza w porównaniu do obszaru rozrastania się guza i tkanek okolicy guza. Ekspresja PITPNM3 w rdzeniu guza u kobiet była niższa niż u mężczyzn ($p = 0.0011$). Ekspresja CCR8 w rdzeniu guza u mężczyzn była niższa niż w tkance okołoguzowej ($p = 0.0033$), podobnie jak w przypadku guzów pobranych od kobiet ($p = 0.0077$).

4.3.3. HIPOKSJA ZWIĘKSZA EKSPRESJĘ CCL18 I RECEPTORA DLA TEJ CHEMOKINY: PITPNM3 W KOMÓRKACH U-87 MG

$CoCl_2$ (zastosowany jako czynnik wywołujący hipoksję) zwiększył ekspresję CCL18 ($p < 0.0001$), PITPNM3 ($p < 0.0001$) i VEGF ($p = 0.002$) w linii komórkowej U-87 MG. Warunki hipoksji nie miały jednak wpływu na zmiany w ekspresji CCR8. Zastosowanie podłoża ubogo odżywczego nie miało wpływu na ekspresję CCL18 ani jej receptory PITPNM3 i CCR8 ($p > 0.05$), ale wpłynęło na obniżenie ekspresji VEGF ($p = 0.0007$) w komórkach U-87 MG. Stężenia w zakresie 10 ng/mL – 50 ng/mL CCL18 nie wpłynęły na proliferację komórek U-87 MG ($p > 0.05$). Stężenia CCL18 w zakresie od 10 ng/mL do 50 ng/mL wpłynęły na zwiększenie migracji komórek U-87 MG ($p < 0.01$). Przy wartości stężenia 10ng/mL CCL18 migracja komórek wzrosła o 36%, a przy wartości 50 ng/mL wzrosła trzykrotnie. Efekt ten urósł do czterokrotnie większej migracji komórek po preinkubacji komórek w roztworze $CoCl_2$ imitującym warunki hipoksji.

W regionach guza odpowiadającym hipoksji i upośledzonego zaopatrzenia w substancje odżywcze uwidoczniono liczne makrofagi zdolne do syntezy i uwalniania CCL18 do przestrzeni zewnątrzkomórkowej. Powyższe wyniki wskazują na zwiększoną ekspresję CCL18 w GBM i są zgodne z dostępnym piśmiennictwem. CCL18 odgrywa ważną rolę w chorobach nowotworowych i jej stężenie jest odwrotnie proporcjonalne do przeżywalności chorych. Poniższa publikacja jest pierwszą analizującą ekspresję receptorów dla CCL18 takich jak PITPNM3 i CCR8 w GBM. Z tego względu wyników przedstawionych w pracy nie można porównać do podawanych w piśmiennictwie. W badanych guzach GBM ekspresja PITPNM3 pozostała niezmieniona. U kobiet ekspresja CCL18 w guzie była niższa niż w tkankach okolicy guza.

Wpływ receptora PITPNM3 na procesy nowotworowe najlepiej zbadano w modelach raka piersi, które wykazały podwyższoną ekspresję tego receptora [337], a także niedrobnokomórkowego raka płuca [331] i raka płaskonabłonkowego jamy ustnej [338]. Istnieją doniesienia, że receptor ten może odgrywać ważną rolę w rozwoju niektórych nowotworów.

Dane dostępne w *Human Protein Atlas* [339] wskazuje, że zwiększona ekspresja PITPNM3 w komórkach nowotworu jest związana z gorszą prognozą chorujących na raka piersi. Ta sama zależność może być zaobserwowana u chorych na glejaki [340].

Z tych powodów wydaje się, że PITPNM3 może odgrywać ważną rolę w rozwoju GBM. Różnice w ekspresji CCL18 i jej receptorów u kobiet i mężczyz mogą również tłumaczyć większą zachorowalność i śmiertelność mężczyzn chorujących na GBM [341] jednakże w piśmiennictwie brak jest dotychczas dowodów na rolę jaką może odgrywać płeć w ekspresji PITPNM3.

4.3.4. Wpływ niedotlenienia, stresu oksydacyjnego i stanu niedoboru składników odżywczych na ekspresję i funkcję CCL18

W przeprowadzonych badaniach model warunków hipoksji (z zastosowaniem $CoCl_2$) został uzyskany z powodzeniem, na co wskazuje wzrost ekspresji VEGF – marker hipoksji [342]. Użycie podłoża ubogo- odżywczego spowodowało obniżenie ekspresji VEGF, zgodnie z danymi dostępnymi w piśmiennictwie [343]. Charakterystyka wzrostu GBM sprawia, że w obrębie guza powstają strefy o upośledzonym zaopatrzeniu w składniki odżywcze i tlen. Oba czynniki – hipoksja i ograniczona dostępność składników odżywczych mają wpływ na ekpresję VEGF.

W opisywanej pracy warunki beztlenowe spowodowały zwiększenie ekspresji CCL18 w hodowli komórek linii U-87 MG. Warto zaznaczyć, że wpływ hipoksji na ekspresję CCL18 może się różnić w zależności od charakteru komórek. Komórki nienowotworowe, na przykład komórki dendrytyczne [344], monocyty [345] i makrofagi [346] reagują na hipoksję zmniejszoną ekspresją CCL18. Jest to związane ze zmniejszoną aktywnością JMJD3 (ang. *Jumonji domain-containing protein-3*), co skutkuje zwiększoną metylacją promotora CCL18. Promotor CCL18 nie zawiera sekwencji promotorowej związanej z *niedotlenieniem* (HRE, ang. *hypoxia-response element*) i przez to nie jest aktywowany przez HIF-1 or HIF-2 [344]. Huang [322] opisuje zmniejszenie ekspresji CCL18 w warunkach hipoksji w komórkach gruczolakoraka płuca, jednak obserwowany efekt nie był istotny statystycznie.

Wyniki przeprowadzonych badań wskazują na zwiększenie ekspresji PITPNM3 w warunkach hipoksji, co znajduje odzwierciedlenie w piśmiennictwie. PITPNM3 jest genem ulegającym większej ekspresji w warunkach hipoksji. Olbryt et al. [347] potwierdzili to w liniach komórkowych PC-3 raka prostaty oraz SK-OV-3 raka jajnika.

W naszych badaniach warunki hypoksji nie wpłynęły na ekspresję CCR8 w komórkach U87. Obecnie brak jest dostępnych danych o wpływie tych warunków na ekspresję badanego receptora. Jednak wydaje się, że hypoxia nie wpływa na ekspresję CCR8, a potwierdzeniem tej tezy mogą być wyniki otrzymane w analizie całego transkryptonu przy użyciu (mikromacierzy) microarrays z wykorzystaniem trzech linii komórkowych, w których nie wykazano wpływu hipoksji na ekspresję CCR8 [347].

4.3.5. Wpływ CCL18 na proliferację komórek U-87 MG

W przeprowadzonych badaniach nie wykazano wpływu CCL18 na proliferację linii komórkowej U-87 MG. Wynik ten jest w części zgodny z wynikami badań dostępnymi w piśmiennictwie, które wskazują na brak pobudzenia proliferacji przez CCL18 w liniach komórkowych przewodowego raka trzustki BxPC-3 i PANC-1 [348], MGC-803 raka żołądka i GES-1 komórek nabłonka żołądka [349]. Jednakże, działanie CCL18 wydaje się zależne od zastosowanego modelu badawczego. Ma et al. (2019) [283] oraz Wang et al. (2016) [283] wykazali bowiem zwiększoną proliferację, odpowiednio komórek glioblastoma linii U-251 oraz komórek raka piersi linii komórkowych MDA-MB-231 i MCF-7. Wydaje się prawdopodobne, że wpływ CCL18 na proliferację zależy od ekspresji jej receptorów oraz innych białek odgrywających rolę w pobudzaniu proliferacji przez tę chemokinę,

4.3.6. Wpływ CCL18 na migrację komórek U-87 MG

W przeprowadzonych badaniach CCL18 indukowało migrację komórek linii U-87 MG. Obserwacja ta jest zgodna z wynikami badań w dostępnym piśmiennictwem opisującym migrację i inwazję komórek komórek raka piersi [337], raka pęcherza moczowego [332], komórek raka wątroby [350], niedrobnokomórkowego raka płuca [331] i raka prostaty [351]. Nasze badania jako pierwsze wykazało nasilenie procesu migracji komórek nowotworowych w warunkach hipoksji. Pozwoli to lepiej zrozumieć proces progresji nowotworu, szczególnie glioblastoma, gdzie obszary dotknięte hipoksją indukują migrację komórek nowotworowych.

5. WNIOSKI

1. W guzie glejaka wielopostaciowego ekspresja cytokiny CCL18 ulega zwiększeniu, zmieniona jest również ekspresja jej receptorów CCR8 i PITPNM3.
2. W mikrośrodowisku guza glejaka wielopostaciowego objętym hipoksją dochodzi do nagromadzenia makrofagów. Charakterystyka warunków panujących w tym obszarze guza prowadzi do różnicowania tych komórek do fenotypu, który charakteryzuje się zwiększoną ekspresją CCL18.
3. Zwiększona ekspresja CCL18 oraz receptora PITPNM3 w guzie nowotworowym prowadzi do pobudzenia migracji komórek nowotworowych. Mechanizm ten może przyczynić się do inwazji GBM i prowadzi do pogorszenia prognozy chorych.
4. Różnice w ekspresji CCL18 i jej receptorów u kobiet i mężczyz mogą tłumaczyć większą zachorowalność i śmiertelność mężczyzn chorujących na GBM.
5. Chemokiny prezentują właściwości pro- oraz przeciwnowotworowe w zależności od czynników współistniejących. Z tego powodu zwiększona ekspresja danej chemokiny nie zawsze jednoznacznie wskazuje na pogorszenie lub poprawę prognozy chorych na nowotwór. Chemokiny powodują rekrutację różnych komórek do niszy nowotworu i migrację oraz inwazję komórek nowotworowych.
6. Ze względu na istotne różnice w raportach epidemiologicznych, niemożliwe okazało się porównanie niektórych danych np. dotyczących zachorowalności na GBM z uwzględnieniem zmienności geograficznej. Analiza danych zebranych w pracy, potwierdza jednocześnie, że GBM jest najbardziej złośliwym pierwotnym guzem mózgu, który zdecydowanie częściej występuje u mężczyzn.
7. Przeanalizowane czynniki ryzyka wystąpienia glejaka, jak i czynniki o działaniu protekcyjnym pozwalają na stwierdzenie, że pomimo niepokojącego trendu dotyczącego zachorowalności na GBM, nadal trudno bezpośrednio określić przyczyny jego występowania. Dotychczasowe dane literaturowe sugerują, że ryzyko zachorowania na GBM jest większe u mężczyzn, osób otyłych oraz zwiększa się wraz z wiekiem i wzrostem. Dostępne dane nie pozwalają jednoznacznie określić, jaki wpływ na rozwój glejaka ma stosowanie używek, niektórych leków (np. NLPZ) czy stosowanie telefonów komórkowych.

6. STRESZCZENIE

Wstęp: Glejak wielopostaciowy (GMB) jest jednym z najbardziej agresywnych nowotworów złośliwych. GBM jest też najczęstszym złośliwym pierwotnym guzem mózgu oraz centralnego układu nerwowego. Mimo wielu badań dotyczących GBM oraz wpływu chemokin na onkogenezę w dostępnym piśmiennictwie brakuje prac dotyczących wpływu komórek nowotworowych w GMB na zmiany ekspresji chemokin wytwarzanych przez komórki związane z nowotworem, w tym szczególnie makrofagi. Opisanie tych zależności z uwzględnieniem specyficznych warunków panujących w obrębie guza nowotworowego (hipoksja, zmniejszona dostępność substancji odżywczych) może prowadzić do lepszego zrozumienia mechanizmów onkogenezy oraz umożliwić opracowanie nowych terapii i leków.

Celem badań było przedstawienie roli chemokin i ich receptorów w interakcji komórek glejaka wielopostaciowego z komórkami towarzyszącymi nowotworom w warunkach panujących w guzie. Szczegółowe cele obejmowały: określenie udziału CCL18 i jej receptorów CCR8 i PITPNM3 w procesie nowotworzenia w GMB, określenie wpływu warunków panujących w guzie na proces nowotworzenia, określenie wpływu płci chorych na progresję nowotworu, przegląd epidemiologii glejaka wielopostaciowego, czynników ryzyka i czynników ochronnych zachorowania na GMB. W pracy przedstawiono także aktualne dane z piśmiennictwa naukowego dotyczące epidemiologii glejaka wielopostaciowego, omówiono działanie chemokin 1–28 i ich receptorów w kontekście nowotworów oraz opisano udział CCL18 i jej receptorów CCR8 i PITPNM3 w procesie nowotworzenia w GMB.

Materiał i metody badań: Badania przeprowadzono z wykorzystaniem guzów nowotworowych pobranych od chorych operowanych z powodu glejaka wielopostaciowego oraz linię komórkową ludzkich komórek glejaka (glioblastoma astrocytoma (U-87 MG), którą poddano działaniu czynników odzwierciedlających warunki panujące w guzie (hipoksja i niedobór składników odżywczych). Analizie poddano również dane z medycznych baz danych w wersji elektronicznej.

Ekspresję CCL18 oraz jej receptorów CCR8 i PITPNM3 w komórkach oraz w guzie i w okolicy okołoguzowej GMB zbadano za pomocą metody

łańcuchowej reakcji polimerazy z obrazowaniem w czasie rzeczywistym (qRTPCR). Przeprowadzono badania immunohistochemiczne guza GMB oraz badania proliferacji i migracji komórek U-87 MG pod wpływem inkubacji z CLL18.

Wyniki i wnioski: Chemokiny wykazują właściwości pro- oraz przeciwnowotworowe w zależności od czynników współistniejących. Z tego powodu zwiększona ekspresja danej chemokiny nie zawsze jednoznacznie wskazuje na pogorszenie lub poprawę rokowania dla chorych na GMB. Chemokiny powodują rekrutację różnych komórek do niszy nowotworu i migrację oraz inwazję komórek nowotworowych.

W przeprowadzonych badaniach w guzie glejaka wielopostaciowego ekspresja cytokiny CCL18 uległa zwiększeniu, zmieniona była również ekspresja jej receptorów CCR8 i PITPNM3. Stwierdzono również różnice w ekspresji CCL18 i jej receptorów u kobiet i mężczyzn, które mogą tłumaczyć większą zachorowalność i śmiertelność mężczyzn chorujących na GBM. W rejonie guza glejaka wielopostaciowego objętym hipoksją doszło do nagromadzenia makrofagów. Charakterystyka warunków panujących w tym obszarze guza prowadziła do różnicowania tych komórek do fenotypu M2, który charakteryzuje się zwiększoną ekspresją CCL18. Zwiększona ekspresja CCL18 oraz PITPNM3 w guzie nowotworowym skutkowała pobudzeniem migracji komórek nowotworowych. Mechanizm ten może przyczyniać się do inwazji/rozsiewu GBM i prowadzi do pogorszenia rokowania dla chorych.

Przeanalizowane czynniki ryzyka wystąpienia glejaka, jak i czynniki o działaniu protekcyjnym pozwalają na stwierdzenie, że pomimo niepokojącego trendu dotyczącego zachorowalności na GBM, nadal trudno bezpośrednio określić przyczyny jego występowania. Dotychczasowe dane sugerują, że ryzyko zachorowania na GBM jest większe u mężczyzn, osób otyłych oraz zwiększa się wraz z wiekiem i wzrostem. Dostępne dane nie pozwalają jednoznacznie określić, jaki wpływ na rozwój glejaka ma stosowanie używek, niektórych leków (np. NLPZ) czy stosowanie telefonów komórkowych.

7. ABSTRACT

Introduction: Glioblastoma multiforme (GBM) is one of the most aggressive malignancies, and the most common malignant primary tumour of the brain and central nervous system. Despite many studies on GBM and the influence of chemokines on oncogenesis, there is a lack of work in the available literature on the effect of cancer cells in GMB on changes in the expression of chemokines produced by tumour-associated cells, particularly macrophages. Describing these relationships with consideration of specific conditions within the tumour (hypoxia, reduced nutrient availability) may lead to a better understanding of the mechanisms of oncogenesis and enable the development of new therapies and drugs.

The aim of this study was to describe the role of chemokines and their receptors in the interaction of glioblastoma multiforme cells with tumour associated cells under tumour conditions. Specific objectives included: determination of the contribution of CCL18 and its receptors CCR8 and PITPNM3 to tumourigenesis in GMB, determination of the influence of tumour conditions on tumourigenesis, determination of the influence of patient gender on tumour progression, review of the epidemiology of glioblastoma multiforme, risk factors and protective factors for GMB. The publication presents current data from the scientific literature on the epidemiology of glioblastoma multiforme, discusses the action of chemokines 1–28 and their receptors in the context of cancer, and describes the involvement of CCL18 and its receptors CCR8 and PITPNM3 in the tumourigenesis process in GMB.

Study material and methods: The study was conducted using tumour samples taken from patients operated on for glioblastoma multiforme, and human glioblastoma astrocytoma cell line (U-87 MG) treated to reflect tumour conditions (hypoxia and nutrient deprivation). Data from electronic medical databases were also analysed.

The expression of CCL18 and its receptors (CCR8 and PITPNM3) in cells and in the tumour and peri-tumoral area of the GMB was examined by real-time imaging polymerase chain reaction (qRTPCR). Immunohistochemical studies of the GMB tumour and studies of U-87 MG cell proliferation and migration under incubation with CLL18 were performed.

Results and conclusion: Chemokines exhibit pro- and anti-tumour properties depending on concomitant factors. Therefore, increased expression of a particular chemokine does not always unequivocally indicate a worsened or improved prognosis for GMB patients. Chemokines induce the recruitment of different cells into the tumour niche, and the migration and invasion of tumour cells.

In our study, the expression of the cytokine CCL18 in glioblastoma multiforme was increased, and the expression of its receptors CCR8 and PITPNM3 was also altered. Differences in the expression of CCL18 and its receptors in men and women were also found, which may explain the higher morbidity and mortality in men with GBM. There was an accumulation of macrophages in the hypoxic region of the region of tumour. The characteristics of the conditions in this tumour area led to the differentiation of these cells to the M2 phenotype characterised by increased CCL18 expression. Increased expression of CCL18 and PITPNM3 in the tumour resulted in stimulation of tumour cell migration. This mechanism may contribute to GBM invasion/dissemination and leads to a poorer patient's prognosis.

After analysis of the risk factors, as well as factors with a protective effect for glioma, we concluded that, despite the worrying trend in the incidence of GBM, it is still difficult to directly identify the causes of GBM. Data date suggest that the risk of GBM is higher in men, obese individuals and increases with age and height. The available data do not make it clear what impact the use of stimulants, certain drugs (e.g. NSAIDs) or the use of mobile phones has on the development of glioma.

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Review

Epidemiology of Glioblastoma Multiforme–Literature Review

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Simple Summary: Glioblastoma multiforme (GBM) is one of the most aggressive malignancies, accounting for 14.5% of all central nervous system tumors and 48.6% of malignant central nervous system tumors. The median overall survival (OS) of GBM patients is only 15 months. The aim of this review was to provide an overview of the epidemiology of GBM and factors that may have a significant impact on the risk of GBM.

Abstract: Glioblastoma multiforme (GBM) is one of the most aggressive malignancies, with a median overall survival of approximately 15 months. In this review, we analyze the pathogenesis of GBM, as well as epidemiological data, by age, gender, and tumor location. The data indicate that GBM is the higher-grade primary brain tumor and is significantly more common in men. The risk of being diagnosed with glioma increases with age, and median survival remains low, despite medical advances. In addition, it is difficult to determine clearly how GBM is influenced by stimulants, certain medications (e.g., NSAIDs), cell phone use, and exposure to heavy metals.

Keywords: glioblastoma multiforme; epidemiology; risk factor; protective factor; brain tumor; central nervous system

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1. Introduction

Glioblastoma multiforme (GBM) is one of the most aggressive malignancies and also the most common malignant primary tumor of the brain and central nervous system, accounting for 14.5% of all central nervous system tumors and 48.6% of malignant central nervous system tumors [1]. The median overall survival (OS) of GBM patients is low, at only 15 months [1,2].

GBM originates from astrocytic glial cells [3], and is the higher-grade malignant glioma (grade IV). It is difficult to clearly define the incidence of GBM as it varies depending on the report, from 3.19 cases per 100,000 person-years [4,5] to 4.17 per 100,000 person-years [6]. The incidence in the pediatric population (0–18 years) is 0.85 per 100,000, where pediatric glioblastoma multiforme (p-GBM) accounts for 3–15% of primary brain tumors [7–12] in this age group, although primary tumors of the central nervous system are the second-most-common type of cancer in children and the most common among solid tumors in children [13]. Ostrom et al. [14] presented an age-adjusted incidence rate of 0.18 (95% CI 0.16–0.19) per 100,000 people in the 0–19-year-old US population.

Due to the limited number of publications on the epidemiology of Glioblastoma multiforme, we have relied, in many respects, on works on glioma tumors, which include GBM. These studies cannot be put on par with studies describing GBM alone, but they allow us to approximate and build on existing knowledge potentially relevant to GBM.

2. Classification of GBM

The international standard for the nomenclature and diagnosis of gliomas is the World Health Organization (WHO) classification, in which GBM is a grade-IV cancer.

The fourth WHO classification of gliomas from 2016 is based on the degree of malignancy, as determined by histopathological criteria, in which four types of this neoplasm have been distinguished [15]:

- **Glioblastoma, *isocitrate dehydrogenase* (*IDH*)** wildtype (90% of cases), developing de novo at about 60 years of age;
- **Glioblastoma, *IDH*-mutant** (10% of cases), secondary GBM that usually develops in younger patients with gliomas of higher differentiation (WHO grades I-III); it carries a significantly better prognosis than wildtype *IDH* [16];
- **Glioblastoma not otherwise specified (NOS)**, the *IDH* mutation status could not be determined due to a lack of histological or molecular material for testing;
- **Not-elsewhere-classified (NEC) Glioblastoma**, fourth category distinguished in recent years. The necessary determinations regarding the classification of the tumor have been made, but the results do not allow matching the tumor to any of the aforementioned categories of the 2016 WHO division. This situation may occur in the case of discrepancies between the clinical, histological, immunohistological, and genetic features of the tumor. There is also the possibility that a GBM subunit exists with an unknown combination of features that is not yet classified in the WHO division.

It is worth noting that the 2016 classification of gliomas takes into account not only the histological classification of the tumor, but also the molecular changes within the cells [17].

The mutation of *IDH1* and *IDH2* reduces the affinity of enzyme isocitrate dehydrogenase for the standard substrate, isocitrate, and increases its affinity towards alpha-ketoglutarate. This in turn results in the conversion of alpha-ketoglutarate to the oncometabolite 2-hydroxyglutarate, a compound implicated in the inhibition of cell differentiation via a competitive blockade of α-ketoglutarate-dependent dioxygenases. This triggers the blockade of dioxygenase functions—DNA and histone demethylation [18]—and leads to the activation of hypoxia-inducible factor 1 (HIF-1) [19]. It is now recognized that these epigenetic changes are mainly involved in the formation of low-grade gliomas [18]. Further transformation of low-grade gliomas with an IDH mutation to secondary GBM requires more genetic alterations, such as the amplification of epidermal growth factor receptor (EGFR) and lost expression of the phosphatase and tensin homolog (PTEN) [20].

The trend of GBM genotyping is expanded in the new WHO classification. Increasingly widespread molecular profiling and the use of machine learning methods lead to the more accurate determination of prognoses and responses to specific treatments [21]. The discovery of new mutations present in GBM offers the potential for the creation of new drugs with new targets, and the association of mutations within tumors with different clinical courses will facilitate the diagnosis and prediction of disease severity. In 2017, the formation of cIMPACT-NOW (Consortium to Inform Molecular and Practical Approaches to central nervous system (CNS) Tumor Taxonomy) was announced to evaluate and recommend changes to the WHO classification of brain tumors [22].

The latest criteria and nomenclature introduced by the WHO in 2021 continue to strengthen the role of molecular genetics in the diagnosis of GBM. IDH-mutant tumors that could previously be classified as diffuse astrocytoma, anaplastic astrocytoma, or glioblastoma are currently considered a single type of IDH-mutant astrocytoma graded II, III, or IV. Moreover, grading IDH-mutant diffuse astrocytic tumors is no longer purely histological—it is also based on the presence of the CDKN2A/B homozygous deletion

mutation, which results in a CNS WHO grade of IV, even in the absence of microvascular proliferation or necrosis [23].

The classification also includes TERT promoter mutation, EGFR gene amplification, and combined gain of entire chromosome 7 and loss of entire chromosome 10 (+7/−10) as qualifying for the diagnosis of GBM, IDH-wildtype. As a result, GBM, IDH-wildtype should be diagnosed in the case of an IDH-wildtype diffuse and astrocytic glioma in adults if there is either microvascular proliferation, necrosis, TERT promoter mutation, EGFR gene amplification, or +7/−10 chromosome copy number changes. Pediatric patients, however, should be diagnosed for IDH-wildtype diffuse astrocytomas using different types of pediatric-type gliomas [23].

3. Pathogenesis of GBM

3.1. GBM Site in the Brain

GBM is far more commonly localized in the supratentorial compartment [24] than the subtentorial compartment, and most frequently in the frontal lobe [25]. The rarest locations of GBM are the brainstem and cerebellum (Figure 1).

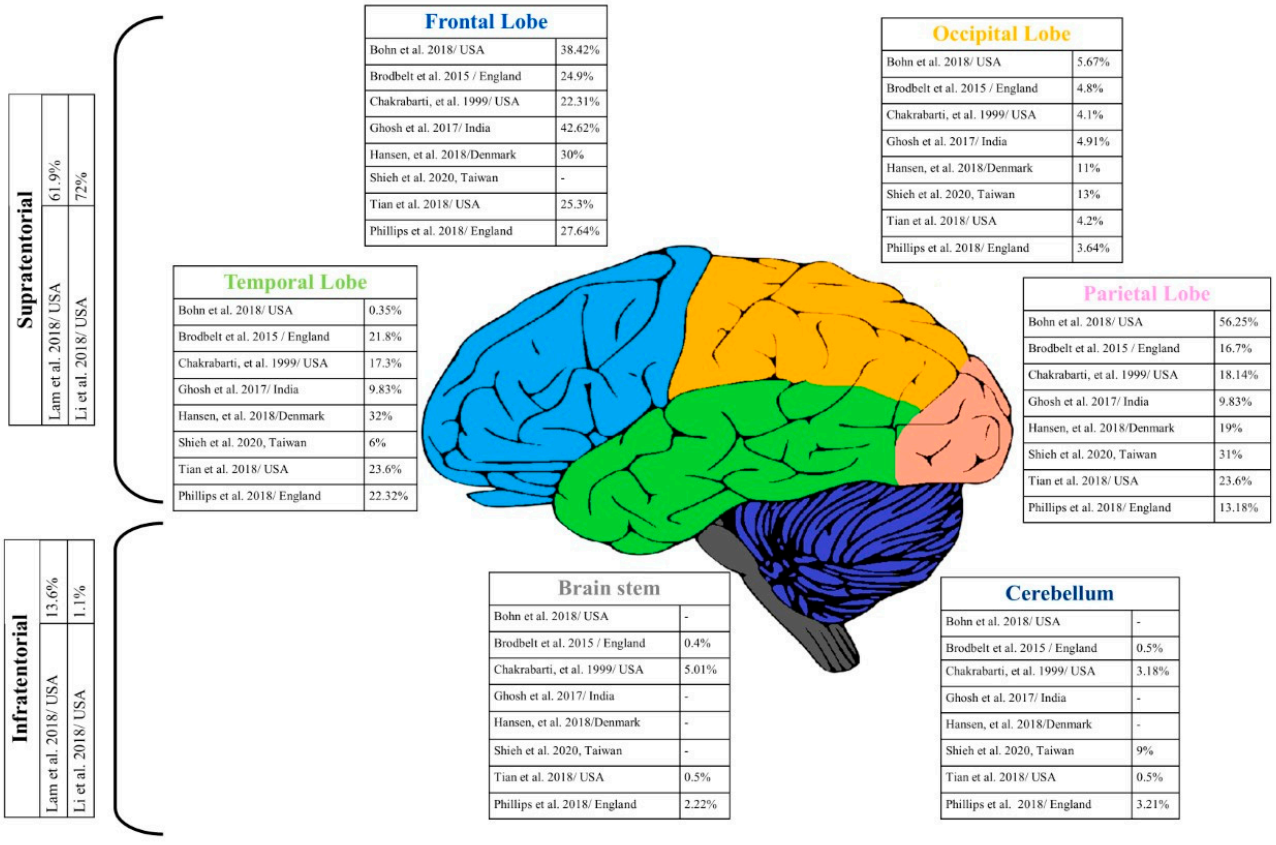


Figure 1. Summary of GBM locations in the central nervous system (%); some data also distinguished locations: Brain, NOS (not otherwise specified), Chamber NOS (not otherwise specified), Brain Overlapping Brain Damage, and Other, which are not shown in the figure; therefore, the sum of the percentages of the structures is not 100% [23,24,26–32]. own figure, no permission required for publication.

3.2. Genetic Pathogenesis

Modern advances in genomic technology have improved our understanding of the key molecular changes that trigger GBM. The molecular markers described below carry prognostic and predictive information, facilitate the differentiation of specific tumor types, and offer hope for understanding tumor progression and creating targeted therapies.

1. ***ATRX* (*a-thalassemia/mental-retardation-syndrome-X-linked*) mutation.** The *ATRX* gene located on Xq21.1 encodes a protein involved in the chromatin-rearrangement pathway, allowing histone H3.3 to be incorporated into heterochromatin [33]. *ATRX* mutations occur in approximately 57% of secondary GBM; in GBM cells, *ATRX* mutations occur more frequently in *IDH*-mutant GBM than in wildtype (71% vs. exceptions) [15] and co-occur with *IDH1* and *TP53* mutations [34,35]. *ATRX* mutations are positive prognostic factors [36,37]. In a prospective study conducted on a cohort of patients with astrocytic tumors (grade I-IV), those with a loss of ATRX expression had a better prognosis than those who retained ATRX expression and a co-occurring *IDH* mutation [38].
2. ***TERT* (*Telomerase Reverse Transcriptase*)** promoter mutation. The *TERT* gene encodes telomerase, an enzyme responsible for adding the missing 3′ end of a DNA strand during replication. The mutation of the *TERT* gene promoter results in increased telomerase activity and telomere elongation, suggesting that maintaining the presence of telomeres is essential for brain tumor formation [39,40]. The two most common mutations of the *TERT* promoter are C228T and C250T, located at base pairs 124 and 146, respectively, which encode this promoter [41]. These mutations can lead to up to a fourfold increase in *TERT* expression [39,42]. *TERT* mutations are present in up to 80% of GBM [39,43–46]. The role of *TERT* promoter mutation as a prognostic factor has not been unequivocally determined due to numerous confounding co-occurring factors (age, surgical intervention, *IDH*, *EGFR* mutations, and *MGMT* methylation status) [47]. *TERT* promoter mutations occur more frequently in *IDH*-wildtype GBM than *IDH*-mutant GBM (72% vs. 26%) [15]. Further prospective studies are needed on large cohorts of a homogeneous patient population (for example, *IDH*-wildtype and O^6*-methylguanine DNA methyltransferase* (*MGMT)* promoter-unmethylated glioma) to independently assess the prognostic impact of *TERT* promoter mutations [47].
3. ***TP53* (*Tumor protein P53*) mutation:** The *TP53* gene is located on human chromosome 17p13.1. The functional p53 protein is a homotetramer that plays a key role in the regulatory network, controlling proliferation, survival, genome integrity, and other cellular functions. The presence of *TP53* mutations is associated with the progression of GBM [48]. The inactivation of p53 correlates with increased invasiveness [49], decreased cell apoptosis [50], and increased proliferation [51]. Cell lines carrying the p53-inactivating mutation show greater resistance to DNA-damaging chemotherapeutics, such as cisplatin [50]. Although *TP53* mutations correlate with poor prognoses in other cancers [52], they have no prognostic value in GBM [51,53,54]. *TP53* mutations are more common in *IDH*-mutant GBM than *IDH*-wildtype GBM (81% vs. 27%) [15]. The gain of function (GOF) *TP53* mutation results in a new function or altered expression; in GBM, it leads to increased malignancy of cells by increasing their proliferation, migration, invasion, metastasis, drug resistance, and genome instability, and increasing survival [55–58]. Wang, Xiang et al. [59] reported that GOF mutations are associated with worse OS, and that they reduce GBM sensitivity to temozolomide by increasing *MGMT* expression [59].
4. **B-RAF V600E mutation:** B-RAF is part of the RAS-RAF-MEK-ERK MAP kinase pathway. This precisely regulated pathway is responsible for cell growth; mutations that confer the constitutive B-RAF kinase activity would result in uncontrolled cell proliferation and tumor formation. The V600E mutation involves the substitution of valine for glutamate at position 600 of the B-RAF protein, producing the permanently activated serine/threonine kinase B-RAF, which activates extracellular signal-regulated kinase 1 and 2 (ERK1/2) and other mitogen-activated protein (MAP) kinases. In the literature, the frequency of all B-RAF mutations in GBM is estimated at 2–6%. In one study [60], patients with GBM from four studies were evaluated, and 8 out of 505 (1.5%) showed the presence of the B-RAF V600E mutation. The mutation may be a convenient point of entry for effective personalized anticancer therapy with kinase

inhibitors, as evidenced by published case reports, such as the clinical response to vemurafenib (a B-RAF kinase inhibitor) in three pediatric high-grade gliomas [61].

5. **GATA4** (GATA-binding protein 4)**:** GATA4 is a transcription factor of the GATA6 family, considered a suppressor gene. In normal astrocytes, GATA4 does not affect cell growth; however, in mice with knockout *GATA4* genes and null-p53 status, the absence of GATA4 induces a transformation associated with increased proliferation, resistance to chemotherapy, and radiotherapy-induced apoptosis [62]. A series of studies conducted by Agnihotri et al. [63] showed that: (i) in 94/163 human GBM tumor cells, GATA4 expression was lost, (ii) GATA4 inhibited transformation to GBM in vitro and in vivo, and (iii) the re-expression of GATA4 in GBM cells sensitized them to telosomide, regardless of the MGMT mutation status. The role of GATA4 in response to telosomide suggests the utility of the GATA4 mutation status as a predictive biomarker, and this needs to be confirmed in further studies.
6. **FGFR1** (Fibroblast Growth Factor Receptor (1)): the FGFR family of proteins is a group of transmembrane receptors with tyrosine kinase function. The exact impact of signaling by FGFR on the pathobiological aspects of individual cancers remains unknown [64]. The strongest evidence suggests that FGFR1 contributes to poor prognosis in GBM, and signaling through this pathway is associated with increased radioresistance, invasiveness, and stemness [65–67].
7. **EGFR** (epidermal growth factor receptor): EGFR is a receptor with tyrosine kinase activity that is activated by EGF (epidermal growth factor). EGFR promotes cell proliferation by activating the MAPK and PI3K-Akt pathways [68]. The EGFR gene is located at locus 7p12 and its amplification is observed in approximately 40% of GBM cases [69]. The amplification of EGFR has been associated with poor prognoses by some authors, but the results are inconclusive [70–74]. EGFR amplification is more common in *IDH*-wildtype GBM than *IDH*-mutant GBM (35% vs. exceptional) [15]. The most common EGFR mutation is variant-III EGFR mutation (EGFRvIII), involving a deletion without a shift of the reading frame of base pair 801 extending from exon 2 to 7, and it has attracted much research interest. This mutation leads to the constitutive activation of EGFR, resulting in the activation of downstream tyrosine kinase pathways. EGFRvIII mutation occurs almost exclusively in the presence of EGFR amplification [75]. Attempts to create a vaccine targeting EGFRvIII (Rindopepimut) ended in the phase-IV clinical trial in 2016 due to a lack of improvement in OS.
8. **MGMT** (O^6-methylguanine DNA methyltransferase): the *MGMT* gene is located on chromosome 10q26 and encodes a protein responsible for DNA repair, removing an alkyl group from the O^6 position of guanine, an important DNA alkylation site. The presence of MGMT promoter methylation is a positive predictor of better OS. The authors of a study published in *NEJM* [76] suggested the usefulness of determining the *MGMT* promoter methylation status by methylation-specific PCR in order to identify patients who may benefit from including temozolomide with standard radiotherapy compared with radiotherapy alone. Temozolomide works by methylating DNA at the N^7 and O^6 atoms for guanine and N^3 for adenine. Methylation sites can be repaired by specialized enzymes, such as MGMT. In a paper published in 2012 [77], the efficacy of temozolomide was greater for a methylated *MGMT* promoter in GBM cells (i.e., reduced MGMT expression). The use of the MGMT inhibitor O^6-benzylguanine (O^6-BG) restored temozolomide (TMZ) sensitivity to TMZ-resistant cell lines LN-18 and T98G [78].
9. ***WT1* (*The Wilms tumor gene*)**: *WT1* was first identified as the gene responsible for the development of the Wilms kidney tumor that primarily affects children. The *WT1* gene is located at locus 11p13 and functions as a zinc finger-like transcription factor. Despite the initial classification of *WT1* as a suppressor gene, the overexpression of WT-1 in many cancers (breast cancer and acute leukemias) [79,80] has led to its recognition as an oncogene [81]. In a study conducted in 2004 [41], 48 out of 51 GBM samples (94%) showed positive staining for the WT-1 protein.

10. **PTEN** (Phosphatase and tensin homolog): the *PTEN* gene is a suppressor gene located on 10q23. LOH (loss of heterozygosity) or methylation mutation disrupt the pathways that use phosphatidylinositol 3-kinase (PI3K) and are found in at least 60% of GBM cases [82]. Loss of PTEN function due to mutation or loss of heterozygosity (LOH) is associated with poor prognosis of GBM. PTEN is a protein with protein phosphatase and lipid phosphatase functions, and most of the onco-suppressive properties are due to the lipid phosphatase properties [83]. The PI3K/Akt pathway is blocked by PTEN, and loss of functional PTEN impairs the regulation of cell survival, cell growth, and proliferation [84]. According to Koul [82], loss of PTEN expression is indicative of the progression of a highly malignant tumor—PTEN is present in most low-grade tumors. Brito et al. [26] reported that PTEN deletion in *IDH*-wildtype GBM is associated with better OS.

4. Survival and Prognostic Factors

4.1. Incidence

The incidence of GBM shows minor locational variability (Figure 1). The data in Table 1 show the incidence of GBM by gender; unfortunately, due to different methodologies, they cannot be directly compared.

The location of the glioblastoma multiforme is concentrated in most cases in the frontal, temporal, and parietal lobes, and, less often, it affects other structures. In the last two decades, the increase in the number of detected cases (increase in morbidity/better diagnostic techniques) has been particularly noticeable, especially in the areas of the frontal and temporal lobes, [27]. In most reports, the incidence is similar to the work of Bohn et al., 2018 [28] and Tian et al., 2018 [85]—from the most common to the least common: frontal–temporal–parietal–occipital lobe–other structures of the brain.

Table 1. Incidence rates of GBM for both genders.

Reference	Year Range	Country	Population Size (*n*)	Incidence/ 100,000/Year Male	Incidence/ 100,000/Year Female	Incidence/ 100,000/Year Male and Female	Age
Brodbelt et al., 2015 [29]	2007–2011	England	10,743	5.87 (5.73–6.02) [b]	3.54 (3.44–3.65) [b]	4.64 (4.56–4.73) [b]	all
Chakrabarti et al., 2005 [30]	1974–1999	USA	3832	2.68 (95% CI 2.56–2.80) [a]	1.67 (95% CI 1.59–1.75) [a]	2.11 (95% CI 2.0–2.17) [a]	≥20
Dho et al., 2017 [86]	2013	Republic of Korea	19	0.12 [j]	0.17 [j]	0.87 [j]	0–19
			629	0.99 [j]	0.78 [j]		all
Dobec-Meić et al., 2006 [87]	1996–2004	Croatia	63	5.1 [g]	4.6 [g]	4.8, (95% CI 3.7–6.2) [g]	≥18
Dobes et al., 2011 [88]	2000–2008	Australia	2197	-	-	3.96 (3.37–4.52) [a]	all/not specified
Fabbro-Peray et al., 2019 [6]	2008–2015	France	2053	-	-	3.3 [f]	all
Fleury et al., 1997 [89]	1983–1990	France	764	3.09 [b]	1.94 [b]	2.38 [b]	all
Fuentes-Raspall et al., 2011 [90]	1994–2005	Spain	134	-	-	1.59 [d]	all
Fuentes-Raspall et al., 2017 [91]	1994–2013	Spain	463	5.05 (4.45; 5.72) [e]	3.44 (2.97; 3.96) [e]	4.17 (95% CI 3.80; 4.57) [e]	all
Gittleman et al., 2018 [92]	2000–2014	USA	150,399			4.40 (4.38–4.42 95% CI) [a]	>20
Gousias et al., 2009 [93]	2005–2007	Northwest Greece	36	4.12 [k]	3.26 [k]	3.69 [k]	all
Hansen et al., 2018 [31]	2009–2014	Denmark	1364	6.3 [h]	3.9 [h]	-	19–89
Ho et al., 2014 [94]	1989–2010	Netherlands	9504	3.2 [b]	1.9 [b]	2.5 [b]	≥18
Jazayeri et al., 2013 [95]	2000–2009	Iran	3101	-	-	0.76 (0.70–0.83) [g]	all

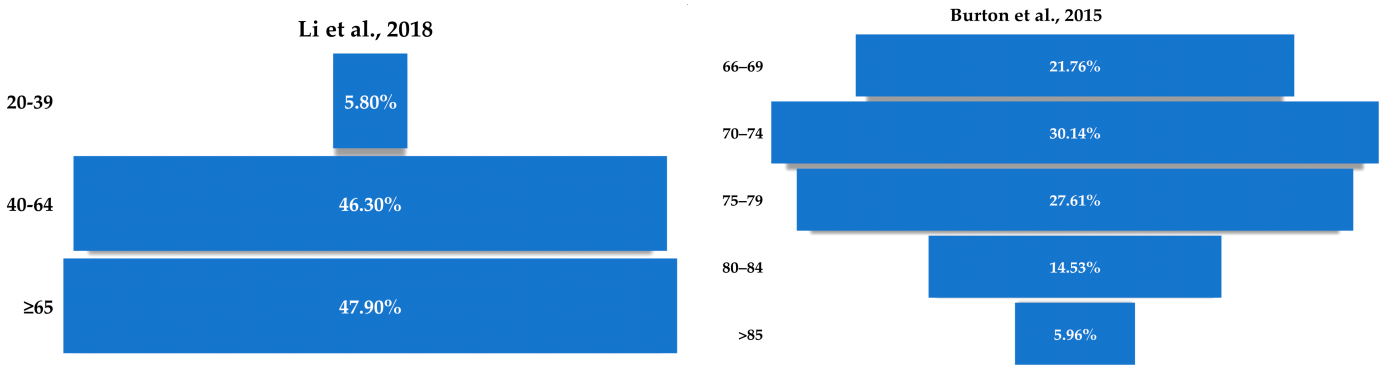
Table 1. *Cont.*

Reference	Year Range	Country	Population Size (*n*)	Incidence/ 100,000/Year Male	Incidence/ 100,000/Year Female	Incidence/ 100,000/Year Male and Female	Age
Jung et al., 2013 [96]	2010	Republic of Korea	523	0.89 [j]	0.95 [j]	0.77 [j]	all
Li et al., 2018 [32]	1973–2014	USA	28,835	-	-	4.1 [l]	>20
Larjavaara et al., 2007 [97]	2000–2002	Finland	154	-	-	2 [d]	20–69
Natukka et al., 2019 [98]	1990–2006	Finland	2284	-	-	3.8 (95% CI 3.7–4.0) [e]	all
Natukka et al., 2019 [98]	2007–2016	Finland	1776	-	-	3.5 [e]	all
Ohgaki et al., 2004 [99]	1980–1994	Switzerland	715	3.32 (CI, 2.69–4.09) [d]	2.24 (CI, 1.56–3.22) [d]	-	all
Ostrom et al., 2013 [100]	2006–2010	USA	50,872	3.97 [a]	2.53 [a]	3.19 [a]	all
Ostrom et al., 2017 [101]	2010–2014	USA	56,421	3.99 (3.95–4.03 95% CI) [a]	2.52 (2.49–2.56 95% CI) [a]	3.20 (3.17–3.23 95% CI) [a]	all
Ostrom et al., 2020 [14]	2013–2017	USA	60,056	4.03 (95% CI 3.98–4.07) [a]	2.54 (95% CI 2.50–2.57) [a]	3.23 (CI (95% 3.20–3.25) [a]	all
Schoenberg et al., 1976 [102]	1935–1964	Connecticut	1167	2.07 [i]	1.51 [i]	-	all
Walker et al., 2019 [103]	2009–2013	Canada	5830	4.91 (95% CI 4.75–5.08) [c]	3.24 (3.11; 3.37) [c]	4.06 (3.95; 4.16) [c]	all
Wanner et al., 2020 [104]	2009–2012	Georgia	72	0.59 (0.42; 0.82) [a]	0.42 (0.29; 0.59) [a]	-	all

[a] Age-adjusted using the 2000 US standard population, [b] European age-adjusted incidence, [c] Adjusted to the 2011 Canadian census age distribution, [d] Age-standardized to the world's standard population, [e] Adjusted to the 2013 European standard population, [f] Crude rate, [g] No information, [h] Age adjustment not specified, [i] All age adjustments used the direct method and the population of the United States in 1950 as the standard, [j] Adjusted to Segi's world standard population, [k] Adjusted to the Greek population, [l] Annual age-standardized incidence rates (ASRs; per 100,000 population), *n*—number of subgroup members.

4.2. Age

Age is an important factor in the development of diseases such as cancer. Many studies confirm that age significantly affects the incidence of GBM, where the vast majority of cases occur in people over 40 years of age. This was confirmed by Kai et al. [32], where, for 47.9% of the subjects, the age of GBM diagnosis was ≥65 years, and 46.3% of the subjects were between 40 and 64 years. In the study by Tian et al. [85], far more cases of GBM (55.2%) were diagnosed among respondents between 41–60 years of age (Figure 2).



(**a**) (**b**)

Figure 2. *Cont.*

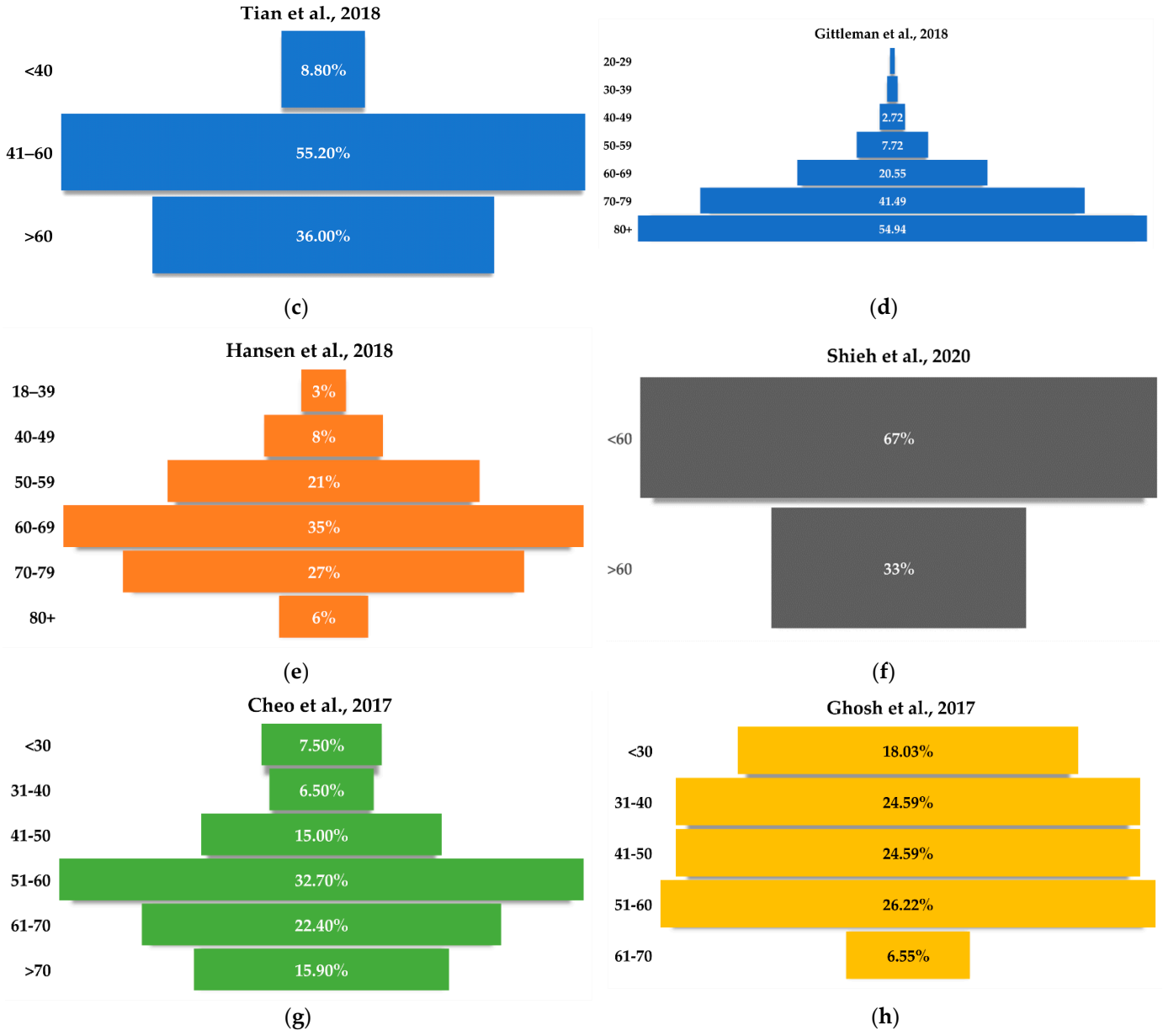


Figure 2. Age-related incidence of GBM in (**a**) [32], (**b**) [105], (**c**) [85] (**d**) [92] the USA, (**e**) Denmark [31], (**f**) Taiwan [106], (**g**) China [107], and (**h**) India [25].

Based on the Central Brain Tumor Registry of the United States (CBTRUS) reports in 2013, 2017, and 2020, it was observed that the incidence of GBM increases with age, peaking at 75–84 years and decreasing after 85 years (Figure 3). Gittleman et al. [92] showed that the incidence rate of GBM increases with age.

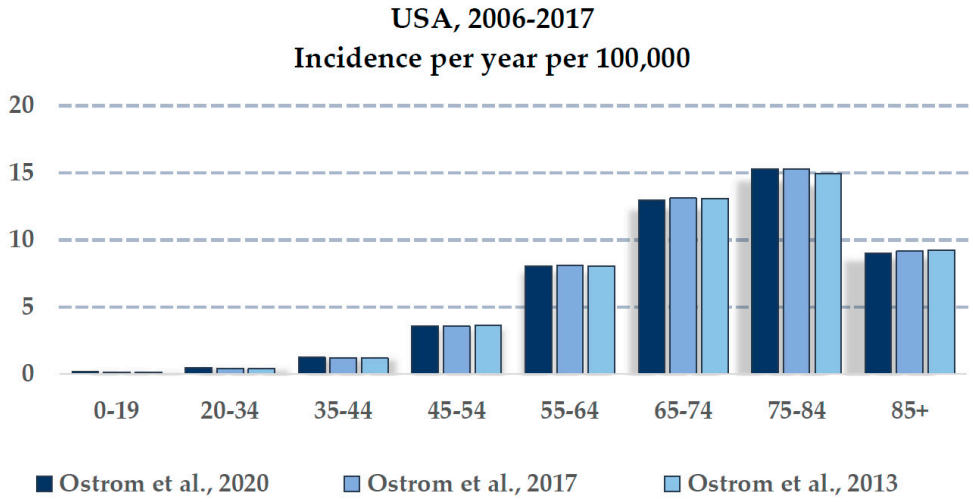


Figure 3. Age-related incidence of GBM (100,000/years) [14,100,101].

4.3. Survival

GBM is the most aggressive diffuse glioma of the astrocyte lineage and remains an incurable tumor. Table 2 presents survival data for GBM. In most cases, the median survival was less than 15 months. In the study by Lam et al. [24], the median survival was 20, with 46.9% of subjects surviving for up to 2 years after diagnosis. In contrast, Fabbro-Peray et al. [6], Gittelman et al. [92], and Ostrom et al. [101] observed that patients most often lived for up to 1 year after diagnosis.

Table 2. Survival time of GBM patients.

Reference	Year Range	Country	Population Size (*n*)	Survival Me (Months)	Survival (%).			
					1 Year	2 Years	5 Years	10 Years
Brodbelt et al., 2015 [29]	2007–2011	England	10,743	6.1	28.4	11.5	3.4	-
Cheo et al., 2017 [107]	2002–2011	China	107	15.1	-	23.5	-	-
Fabbro-Peray et al., 2019 [6]	2008–2015	France	2053	11.2	47.1	20.1	4.5	-
Fuentes-Raspall et al., 2017 [91]	1994–2013	Spain	463	-	24.0	-	3.3	-
Ghosh et al., 2017 [25]	2012–2014	India	61	8	19.15	3.27		
Gittleman et al., 2018 [92]	2000–2014	USA	33,469	-	39.5	16.9	5.4	2.7
Hansen et al., 2018 [31]	2009–2014	Denmark	1364	11.2	-	-	-	-
Lam et al., 2018 [24]	2000–2010	USA	302	20	-	46.9	-	-
Narita and Shibui, 2015 [108]	2001–2004	Japan	1489	15	-	-	10.1	-
Ostrom et al., 2017 [101]	2000–2014	USA	33,951	-	39.7	17.2	5.5	-
Yan Yuan et al., 2016 [109]	1992–2008	Canada	14,120	-	26.5	9.5	4.0	-

Me—median, *n*—number of subgroup members.

4.4. Urban/Rural Socioeconomic Status

In the CBTRUS Statistical Report [14], the incidence in urban counties was 2.1% ($p = 0.0715$) higher than that in rural counties, at 3.17 cases per 100,000 population, (95% CI = 3.14–3.2) and 3.1 per 100,000 population (95% CI = 3.04–3.17), respectively.

In a study conducted in the United States by Cote, Ostrom, et al. [110], glioma incidence was higher in counties with a higher socioeconomic status compared to counties of lower socioeconomic status. Counties of high socioeconomic status also had lower glioma mortality. The authors associated the differences in incidence and mortality with race and socioeconomic status, rather than the area of residence (urban vs. rural).

In 1976, Barker et al. [111] reported the incidence of glioma in the south of England at 3.94 per 100,000, which was lower in large urban areas. In contrast, E. V. Walker et al. [112] described lower mortality during the first 5 weeks of a GBM diagnosis in a group of people living in rural areas compared to those living in urban areas (hr: 0.86; 95% CI: 0.79 to 0.99). Higher mortality during the first 1.5 years after GBM diagnosis was also observed in the low-income group compared with the high-income group (hr: 1.15; 95% CI: 1.08 to 1.22).

5. Protective Factors

5.1. Gender and Hormones

The protective effects of female sex hormones on the development of GBM tumors, and their effect on increasing the incidence of meningiomas, are fairly well documented in the literature. Cowppli-Bony et al. [71], in a review on female sex hormones and the incidence of gliomas, observed an increased risk of developing these cancers in women with late first menstruation and late menopause, and a decreased risk in users of oral hormonal contraception and hormone replacement therapy (but the duration of use was not significant). In contrast, Michaud, Dominique S. et al. [113] and Wigertz et al. [114] reported no effect of the level of exogenous estrogen on the risk of GBM. Table 3 shows the incidence rate of GBM in both sexes. All studies presented indicate a higher incidence of GBM in men.

Table 3. Incidence rates of GBM presented for both genders.

Reference	Year Range	Country	Population Size (*n*)	M:F Ratio
Bohn et al., 2018 [28]	2010–2014	USA	3473	1.40
Brodbelt et al., 2015 [29]	2007–2011	England	10,743	1.66
Bruhn et al., 2018 [115]	2001–2012	Sweden	143	1.6
Burton et al., 2015 [105]	1997–2009	USA	3759	1.15
Chakrabarti et al., 2005 [30]	1974–1999	USA	3832	1.6
Cheo et al., 2017 [107]	2002–2011	China	107	1.55
De Witt Hamer et al., 2019 [116]	2011–2014	Netherlands	2382	1.63
Dobec-Meić et al., 2006 [87]	1996–2004	Croatia Varazdin County	63	1.12
Dobes et al., 2011 [88]	2000–2008	Australia	2197	1.6
Fabbro-Peray et al., 2019 [6]	2008–2015	France	2053	1.5
Ghosh et al., 2017 [25]	2012–2014	India	61	2.59
Gousias et al., 2009 [93]	2005–2007	Northwest Greece	36	1.25
Hansen et al., 2018 [31]	2009–2014	Denmark	1364	1.6
Helseth and Mork, 1989 [117]	1955–1984	Norway	2813	1.48
Jung et al., 2013 [96]	2010	Republic of Korea	523	1.22

Table 3. *Cont.*

Reference	Year Range	Country	Population Size (*n*)	M:F Ratio
Li et al., 2018 [32]	1973–2014	USA	28,835	1.35
Nomura et al., 2011 [118]	1995–2004	Osaka, Japan	713	1.25
Ostrom et al., 2017 [101]	2010–2014	USA	56,421	1.36
Ostrom et al., 2013 [100]	2006–2010	USA	50,872	1.57
Shieh et al., 2020 [106]	2005–2016	Taiwan	48	1.18
Tian et al., 2018 [85]	2000–2008	USA	6586	1.60
Wiedmann et al., 2017 [119]	1963–1975	Norway	3102	1.33
J.-C. Xie et al., 2018 [120]	2004–2015	USA	30,767	1.38
Zampieri et al., 1994 [121]	1986–1988	Italy	72	1.25

n—number of subgroup members, M:F ratio—Male:Female ratio.

5.2. Non-Steroidal Anti-Inflammatory Drugs and Paracetamol

The proven protective effect of acetylsalicylic acid in inflammatory bowel disease on colorectal cancer risk [122] and the potential beneficial effect on other cancers encourages the evaluation of the effect of nonsteroidal anti-inflammatory drugs (NSAIDs) on the risk of developing GBM. The potential protective effect of NSAIDs in brain tumors is likely due to the inhibitory effect on prostaglandin E_2 (PGE_2) synthesis, a prostaglandin that may play a role in direct mutagenic effects, tumor growth, invasion, metastasis, immunosuppression, and angiogenesis [123,124]. Additionally, the potential role of elevated PGE_2 levels associated with malignant brain tumors is evidenced by the decrease in the PGE_2 levels after the surgical removal of a malignant brain tumor [125].

Altinoz, Meric et al. [126] described the effects of aspirin and its metabolites on GBM cells. Gentisic acid (GA), a metabolite of acetylsalicylic acid, blocks the attachment of fibroblastic growth factor (FGF) to its receptor, and the sulfonate metabolite dobesilic acid blocks the growth of C6 GBM cell line cells in vivo.

However, the molecular rationale behind the protective effect of NSAIDs on GBM development is not unequivocally reflected in the literature. Publications vary in their results and are often inconclusive due to many potential confounding variables and due to a failure to obtain adequate numbers of GBM patients. Collecting reliable data on NSAID use is a major challenge, especially because of the cognitive impairment during GBM treatment and the natural course of this neuroproliferative disease. In addition, the high heterogeneity of GBM documented in the Cancer Genome Atlas (TCGA) [127,128] and its intricacy may influence the different responses of different tumor subtypes to NSAIDs and result in discrepancies.

Scheurer et al. [129] described a 20% reduction in GBM risk after using NSAIDs, but the confidence interval was very close to 1.00. The studied subjects with developed glioma were less likely to report regular aspirin use in the past (OR: 0.69; CI: 0.56, 0.87). A case–control study published in 2004 by Sivak-Sears et al. [130] compared San Francisco Bay Area residents with GBM (n = 236) to a matched control group (n = 401). Based on the interviews collected, the GBM group reported consuming at least 600 fewer NSAID tablets in the 10 years prior to their disease than the control group (OR = 0.53, 95% CI: 0.3–0.8). Another case–control study conducted between 2007 and 2010 with a group of 517 GBM patients and 400 participants in the control group showed an inverse relationship between NSAID use for at least 6 months and the risk of developing GBM (OR = 0.68, 95% CI 0.49–0.96) [131].

In the literature, some publications argue a lack of correlation between NSAID use and the risk of glioma or GBM. A prospective study initiated in 1995 on a group of 302,767 US residents by Daugherty et al. [132] described the occurrence of 341 cases of gliomas in that group, including 264 GBM cases. Regular aspirin use (more than twice a

week) was not associated with the risk of GBM (HR = 1.17; 95% CI, 0.83–1.64) compared with non-use. For the use of the rest of the NSAIDs, excluding aspirin, there was also no correlation with the occurrence of GBM (HR = 0.83; 95% CI, 0.56–1.20) compared with no NSAID use.

A Danish case–control study of a large group of patients (n = 2688) and a control group of n = 18,848 based on the Danish Cancer Registry and NSAID prescriptions from 2000 to 2009 also found no correlation between NSAID use and glioma risk. Bruhns et al. [133] showed no statistically significant difference in OS between patients using and not using NSAIDs in therapy (p = 0.75; 95% CI: 10.12–18.13).

5.3. Other Medications

5.3.1. Antihistamines

Scheurer et al. [129] observed an OR of 0.89 (95% CI 0.63, 1.25) for GBM in antihistamine users. In a subsequent study, Scheurer et al. [134] observed that antihistamine use was associated with a 37% increased risk of glioma (odds ratio 1.37; 95% CI: 0.87; 2.14). In contrast, a 3.56 times increase in risk was observed in patients reporting a history of asthma or allergies who used antihistamines regularly for more than 10 years.

Schlehofer et al. [135] found a 30% reduction in the risk of glioma in adults using antihistamines. Schoemaker et al. [136] also reported a slight, but non-significant, reduction in risk associated with the use of antihistamines by those reporting conjunctivitis, allergic rhinitis, and hay fever.

McCarthy et al. [137] noticed that oral antihistamine use was inversely associated with glioma risk, although the adjusted OR was not statistically significant for those with low-grade glioma.

5.3.2. Statins

A meta-analysis by Xie et al. [138] showed no lengthening of OS and PFS in statin users. In the study by Ferris et al. [131], taking statins showed a statistically significant inverse relationship between the duration of therapy and glioma risk.

Cote et al. [139] observed that associations between statin use and the risk of glioma were similar in the combined cohorts (HR = 1.30, 95% CI 0.99–1.69), and were statistically significant among men (HR = 1.58, 95% CI 1.06–2.34), but not among women (HR = 1.10, 95% CI 0.77–1.58).

A meta-analysis by Rendom et al. [140] showed that statins were potent anti-cancer drugs that suppressed glioma growth through various mechanisms in vitro. However, these effects were not statistically significant in terms of glioma incidence and survival.

5.3.3. Cannabinoids

Cannabinoids originally referred to the bioactive components of the Cannabis sativa plant, namely the psychoactive cannabinoid Δ^9-tetrahydrocannabinol (THC) and other phytocannabinoids, e.g., cannabinol, cannabidiol (CBD), and cannabigerol, or the flavor and aroma agent β-caryophyllene (BCP) [133]. Most cannabinoids bind to G protein-coupled cannabinoid receptors, CB_1 and CB_2, which act as agonists or inverse agonists. Importantly, GBM tumors express both major cannabinoid-specific receptors (CB_1 and CB_2), and the expression of these receptors has been detected in GBM cell lines, in ex vivo primary tumor cells from GBM patients, and in situ in GBM tissue biopsies. It has been observed that highly malignant gliomas, such as GBM, express high levels of CB_2, which is positively correlated with the degree of malignancy [141]. In view of this fact, it was considered that cannabinoid receptor agonists could be used as anticancer agents [142]. This was confirmed by Guzman et al. [143], who reported a reduction in cancer cell proliferation after THC administration in two of nine patients. In contrast, several in vivo studies demonstrated that cannabinoids can significantly reduce tumor volume in orthotopic and subcutaneous animal models of glioma [144].

Three major mechanisms have been observed to inhibit tumor growth:

- Apoptosis and cytotoxic autophagy;
- Mechanisms that inhibit cell proliferation;
- Anti-angiogenic mechanisms.

This was confirmed by a 2013 review by Ellert-Miklaszewska [141], in which the authors described the occurrence of apoptosis involving proteins from the Bcl-2 family in response to treatment with cannabinoids. Hernandez-Tiedra et al. [145] observed that cannabinoids alter the permeability of the membranes of autophagosomes and autolysosomes, causing the release of cathepsins in the cytoplasm, which activates cell death by apoptosis. In contrast, Massi et al. [146] reported that cannabinoids induce apoptosis through oxidative stress. In addition to cancer cell apoptosis, cannabinoids can induce cell cycle arrest and thus inhibit cancer cell proliferation [147].

5.3.4. Atopy

Lehrer et al. [148] showed, in their study taking into account 1p19q deletion, TERT overexpression, and *TP53* and *ATRX* mutations, that OS was greater in subjects with allergies (p = 0.025, HR 0.525, 95% CI 0.299–0.924). Schwartzbaum et al. also described the protective effect of asthma-associated gene polymorphisms on the risk of GBM [149].

There is an inverse relationship between atopy and the risk of developing glioma cancers.

A meta-analysis conducted in 2007 by Linos et al. [150] on 3450 patients with gliomas showed that the pooled relative risks (RRs) of glioma incidence in people with atopy compared to those without a history of atopy for allergy, asthma, and eczema were 0.61 (95% CI = 0.55 to 0.67), 0.68 (95% CI = 0.58 to 0.80), and 0.69 (95% CI = 0.58 to 0.82), respectively.

The mechanism responsible for these relationships has not been clearly established. A plausible explanation for this phenomenon is an as-yet unexplained increased sensitivity of the immune system. Allergy has been implicated in tumorigenesis, not only in gliomas, but also in pancreatic cancer. Interestingly, some IgE antibodies directed against allergens cross-react with glioma antigens [151], and some authors have reported an inverse correlation between the plasma antibody levels and glioma risk [150,151].

6. Risk Factors

6.1. Tobacco Smoking and Nitrosamines

Cigarette smoking has not been clearly linked to an increased risk of developing GBM [152,153] and glioma [152–154]. Because of the mixed findings, further attempts to establish a correlation or lack thereof are desirable, especially because cigarette smoke is a proven risk factor for the development of malignancies in certain organs. Cigarette smoke mutagens, such as tobacco-specific nitrosamines (TSNAs) and polycyclic aromatic hydrocarbons (PAHs), penetrate the blood–brain barrier [155], which may potentially affect the development of central nervous system tumors [156]. Considerable scientific evidence also points to the carcinogenic effect of TSNA in causing malignancies of the lung, pancreas, esophagus, and oral cavity. The most recent International Agency for Research on Cancer (IARC) monograph did not classify the nervous system as an organ in which carcinogenesis is caused by tobacco products [156].

Nitrosamines can originate from cigarette smoke, but also from the reaction of nitrates and nitrites used in meat products—hams, bacon, and sausages. N-nitrosodimethylamine (NDMA) is one of the most common nitrosamines in food [157–159]. NDMA is a potent carcinogen capable of inducing cancer in animal models [160]. Nitrates present in food entering the digestive system are absorbed into the blood and then secreted into the saliva. Following ingestion, they are passed into the stomach, where they are converted to nitrosamines in an acidic environment [161]. A study of patients diagnosed between 1987 and 1991 in Israel found that N-nitroso compounds were not directly linked to brain tumors [162].

In a study by Michaud et al. [163], neither the group consuming the most processed meat products nor the nitrate-exposed group had an increased risk of glioma (RR: 0.92; 95% CI: 0.48, 1.77 and RR: 1.02; 95% CI: 0.66, 1.58, respectively).

A meta-analysis by Saneei et al. [164] included data from 18 observational studies and found no association between the consumption of processed red meat and increased incidence of glioma.

6.2. Race/Ethnicity

There is a limited association between specific ethnic groups and the risk of developing GBM. Ostrom et al. [100] reported a 2.97 times higher incidence of GBM in Caucasians compared to Asians, and a 1.99 times higher incidence in Caucasians compared to African Americans.

A 2006 study by Fukushima et al. [165] compared mutations found in primary GBM in a Japanese group with mutations found in the Swiss group described by Ohgaki et al. [99]. The results of the study by Fukushima et al. [165] suggest high molecular similarity of GBM, despite the different genetic backgrounds of Asians and Caucasians (Table 4).

Table 4. Relationship between race/ethnicity and the incidence of GBM.

Reference	Year Range	Country	Population Size (*n*)	Age	Race/Ethnicity (%)				
					White	Black	Hispanic	Asian	Unknown/Other
Bohn et al., 2018 [28]	2010–2014	USA	3473	≥18	83.21	5.90	5.53	5.36	-
Burton et al., 2015 [105]	1997–2009	USA	3759	>65	93.11	3.14	-	-	3.75
Cheo et al., 2017 [107]	2002–2011	China	107	13–85	-	-	-	Chinese 76.6 Malay 9.3 Indian 7.5	6.5
Li et al., 2018 [32]	1973–2014	USA	28 835	>20	91.4	4.7	-	-	3.9
Ostrom et al., 2013 [100]	2006–2010	USA	50,872	all	91.78	5.62	6.77	2.22	0.38
Ostrom et al., 2017 [101]	2010–2014	USA	56,421	all	91.11	6.12	7.34	2.38	0.39
Ostrom et al., 2020 [14]	2013–2017	USA	60,056	all	91.47	6.21	7.87	1.87	0.45
Xie et al., 2018 [120]	2004–2015	USA	30,767	all	89.6	5.5	-	-	4.7
Reference	**Year Range**	**Country**	**Population Size (*n*)**	**Age**	**Race/Ethnicity (Incidence per 100,000)**				
					White	**Black**	**Hispanic**	**Asian**	**Unknown/Other**
Chakrabarti et al., 2005 [30]	1974–1999	USA	3832	>20	Latino 1.83 (1.65–2.01 95% CI) Non-Latino white 2.53 (2.44–2.62 95% CI)	1.45 (1.27–1.62 95% CI)	-	-	-
Ostrom et al., 2013 [100]	2006–2010	USA	50,872	all	3.45 (3.41–3.48 95% CI)	1.67 (1.60–1.73 95% CI)	2.45 (2.36–2.53 95% CI)	1.67 (1.57–1.78 95% CI)	1.48 (1.26–1.72 95% CI)
Ostrom et al., 2017 [101]	2010–2014	USA	56,421	all	3.46 (3.43–3.49 95% CI)	1.79 (1.73–1.85 95% CI)	2.42 (2.35-2.5095% CI)	1.47 (1.26–1.69 95% CI)	1.61 (1.52–1.70 95% CI)
Ostrom et al., 2020 [14]	2013–2017	USA	60,056	all	3.51 (3.45–2.54 95% CI)	1.77 (1.71–1.83 95% CI)	2.46 (2.39–2.53 95% CI)	1.18 (1.11–1.25 95% CI)	1.49 (1.30–1.69 95% CI
Shabihkhani et al., 2017 [166]	2001–2011	USA	21,184	-	5.1 (95% CI 5.0–5.3)	-	3.4 (95% CI 3.3–3.5)	-	-

n—number of subgroup members.

6.3. Ionizing Radiation

Ionizing radiation is a recognized risk factor for many cancers. Direct damage to genetic material or the generation of free radicals in the vicinity of DNA strands results in an increased incidence of mutations within the genetic material of cells. Since controlled clinical trials on the effects of radiation on carcinogenesis are not feasible for ethical reasons, case–control studies play a major role in describing this phenomenon. Ron et al. [167] already in 1988 linked doses of 1–2 Gy to an increased risk of neuronal tumors. A literature review by Bowers et al. [168] in 2013 documented an 8.1–52.3 times increased risk of

central nervous system cancer after radiotherapy to the head for a CNS tumor in childhood compared to the general population, proportional to dose.

Most studies on the relationship between computed tomography (CT) and the risk of glioma development in children have not shown an increased risk, apart from a study describing one excess brain tumor per 10,000 patients over a 10-year period after exposure to one CT scan [169].

6.4. Head Injury

Because of the described anecdotal cases of CNS tumors (not just GBM) being diagnosed after head trauma, further studies on head trauma as an etiologic factor of brain tumors have been conducted, with mixed results. Unfortunately, the available research is quite limited. Proving a causal relationship is very difficult in this case [170]. In a study on the Danish population, gliomas were not diagnosed more frequently in patients after head injury—the standardized incidence ratio (SIR) after the first year was 1.0 for glioma (CI = 0.8–1.2) compared to the general Danish population. Tumors detected during the first-year period were not considered due to the detection of incidental lesions already existing during the trauma [171]. A study conducted in 1980 showed an increased odds ratio (odds ratio = 2.0, $p = 0.01$) in women compared with the control group in the incidence of meningiomas following head trauma [172]. In contrast, a case–control study evaluating the incidence of meningiomas and gliomas after head injury documented a higher risk of meningiomas, but a lower risk of gliomas (OR = 1.2, 95% CI: 0.9–1.5 for any injury; OR = 1.1, 95% CI: 0.7–1.6) [173]. Potential problems with the study may include the use of diagnostic methods using ionizing radiation, which is a proven risk factor for cancer, and potential problems with recalling past injuries and the non-standardized assessment of their extent.

6.5. Obesity

Adipose tissue has many functions in the human body. In addition to storing nutrients in the form of fats, it has a secretory role, for example, secreting estrogens [174] and pro-inflammatory substances [175,176]. For these reasons, it may have a potential impact on the development of cancer, including GBM.

Current data suggest that low body weight (BMI < 18.5 kg/m^2) at age 21 is associated with a lower risk of developing gliomas later in life, although the results were only statistically significant in the group of women [177]. Moore et al. [178] found that patients who were obese at age 18 (BMI 30.0–34.9 kg/m^2) had nearly four times the risk of developing gliomas compared to those who had a BMI of 18.5–24.9 kg/m^2 at age 18 (RR = 3.74; 95% CI = 2.03–6.90; p trend = 0.003).

In the study by Kaplan et al. [162], increased fat and cholesterol consumption was inversely related to the incidence of glioma (high fat intake OR = 0.45, 95% Cl 0.20–1.07; high cholesterol intake: OR = 0.38, 95% Cl 0.14–1.01). Cote et al. [174] observed an inverse relationship between hyperlipidemia and glioma.

A study on a group of patients diagnosed between 1987 and 1991 in Israel found a relationship between the occurrence of gliomas and meningiomas and a protein-rich diet (OR = 1.94, 95% CI 1.03–3.63) [162]. Wiedmann et al. [119] did not observe an increased risk of glioma in overweight or obese individuals.

Seliger et al. [179] described a decrease in the risk of GBM in people with diabetes (OR = 0.69; 95% CI = 0.51–0.94). The decrease in risk was most pronounced in men with more than 5 years of disease or with poor glycemic control (HbA1c $\geq$ 8). In contrast, the effect of lower GBM risk was absent in women (OR = 0.85; 95% CI = 0.53–1.36).

6.6. Growth

Although a tall stature is associated with a higher incidence of certain cancers [180,181], the exact mechanism of this phenomenon has not been explained. It is likely that the insulin-like growth factor (IGF) and growth hormone (GH) pathways, which determine growth and final height in humans, are involved. The IGF concentrations peak at puberty and

then decline in the third decade of life [182]. More than 80% of GBM tumors overexpress insulin-like growth factor binding protein-2 (IGFBP-2), one of the biomarkers of GBM malignancy [183,184]. In less aggressive tumors, IGFBP-2 is usually undetectable and appears with tumor progression [185].

In the paper published by Moore et al. [178], the risk of developing glioma among tall people (over 190 cm) was twice as high as that among people less than 160 cm tall (multivariate relative risk [RR] = 2.12; 95% confidence interval [CI] = 1.18–3.81; p trend = 0.006). In contrast, a study by Little et al. [176] did not link adult height to the risk of developing glioma.

6.7. Metals

The International Agency for Research on Cancer (IARC) lists cadmium, cadmium compounds, chromium compounds, and nickel compounds as human carcinogens, with lead as a potential carcinogen. None of these have been found to be associated with brain tumors. The ability of some heavy metals to penetrate the blood–brain barrier and to enter through the olfactory nerve pathway [186] prompts a closer examination of their effects on the risk of GBM.

A study conducted in 1970 examining job-exposure matrix (JEM)-based exposures to individual metals did not observe an increased risk of glioma in relation to occupational exposure to chromium, nickel, or lead among 2.8 million male workers (n = 3363 cases of glioma).

Parent et al. [187] reported an increased incidence of glioma associated with occupational exposure to arsenic, mercury, and petroleum products. However, they did not report an increased OR for glioma for welders exposed to lead, cadmium, or welding fumes [187]. Lead may also induce oxidative stress and disturbances in energy metabolism, induce apoptosis, and affect certain signaling pathways [187–191]. A meta-analysis by Ahn et al. [192] reported an increased risk of malignant brain tumors associated with lead exposure (pooled OR = 1.13, 95% CI: 1.04–1.24). Rajaraman et al. [193] observed no relationship between lead exposure and glioma risk.

Bhatti et al. [191] examined the potential carcinogenicity of lead by analyzing the modification of single-nucleotide polymorphisms (SNPs) within genes functionally related to oxidative stress. The study included 494 controls, 176 GBM patients, and 134 meningioma patients who were evaluated for occupational lead exposure. *Rac family small GTPase 2* (*RAC2)* and *glutathione peroxidase 1* (*GPX1)* gene polymorphisms significantly modified the relationship between cumulative lead exposure and GBM risk.

6.8. Nutritional Factors, Chemicals, and Pesticides

Brain tissue necrosis associated with GBM invasion leads to the release of triglycerides and may be accompanied by the release of toxins co-stored in phospholipid-rich neural tissue [194].

In a 1992 study using data from the Canadian National Cancer Incidence Database and Provincial Cancer Registries, Morrison et al. [195] found a statistically significant relationship between the risk of death from GBM and increased exposure to fuel/oil emissions (test for trend p = 0.03, RR for highest-exposure quartile was 2.11, 95% confidence interval = 0.89–5.01). They further suggested inverse associations of cholesterol and fat consumption with brain tumor risk, which they described as inconsistent with other studies [162].

In a study on T98G and U138-MG GBM cells, researchers attempted to determine the cytotoxic or proliferative effects of chemical compounds. The proliferative effect occurred only for the T98G line with perfluorodecanoic acid (PFDA), perfluoroacetate sulfonate (PFOS), and testosterone. However, perfluorinated salt (ammonium perfluoroacetate) and dehydroepiandrosterone (DHEA) showed no proliferation-stimulating effect, suggesting that the proliferative effect is not mediated by androgen receptor activation. The authors concluded that exposure to certain substances released during necrolysis may affect the subsequent growth of GBM and the adoption of more aggressive forms of GBM [194].

An in vitro study subjected the U87 GBM cell line to long-term exposure to low doses of a mixture of pesticides (chlorpyrifos-ethyl, deltamethrin, metiram, and glyphosate). Exposure resulted in the development of resistance to chemotherapeutics (cisplatin, temozolomide, 5-fluorouracil, among others) and increased expression of ATP-binding cassette (ABC) proteins [196].

Kuan et al. [197] reported weak or null associations between food groups, nutrients, or dietary patterns and glioma risk. They found no trends of decreasing glioma risk with increasing intake of total fruit, citrus fruit, and fiber, and a healthy diet.

6.9. Coffee and Tea

Coffee and tea may have potential cancer-protective effects. The presence of antioxidants, such as polyphenols, caffeic acid, diterpenes (including kahweol and cafestol), and heterocyclic compounds [198–201], could explain the molecular basis for this finding. A study by Kang et al. [201] reported the inhibition of GBM cell growth in vitro after exposure to caffeine by the inhibition of inositol trisphosphate receptor subtype 3. Polyphenol (2)-epigallocatechin-3-gallate restores the expression of methylated (silenced) genes in cancer cells, including MGMT, a protein with a DNA repair function [202]. Huber et al. [203] described elevated MGMT protein levels in rat livers after exposure to Kahweol and Cafestol (diterpenes).

Studies on the effects of coffee and tea on glioma risk are inconclusive. Holick et al. [204] reported an inverse relationship between caffeine consumption and glioma risk among men, but not among women. In contrast, in a cohort of 545,771 participants, Dubrow et al. [205] found no reduction in glioma risk with increased coffee and tea consumption. However, in a full multivariate model, there was an almost statistically significant inverse relationship between the highest level of tea consumption (three cups per day) and glioma risk (HR = 0.75; 95% CI, 0.57–1.00).

In a more recent study on a British population cohort (2,201,249 person-years and 364 GBM cases), Creed et al. [206] observed an inverse relationship between tea consumption and glioma risk that was statistically significant for all gliomas, and for GBM in men. In the same year, Cote et al. [207] published a paper using data from the Nurses' Health Study (NHS), Nurses' Health Study II (NHSII), and Health Professionals Follow-Up Study (HPFS) (6,022,741 person-years; 362 cases of GBM). The authors did not observe a relationship between coffee consumption and glioma risk, but noted a borderline inverse relationship between tea consumption and glioma risk for the combined cohort of men and women (HR for >2 cups/day versus <1 cup/week 0.73, 95% CI: 0.49–1.10, p-trend = 0.05).

Michaud et al. [208] observed a statistically significant inverse relationship between coffee intake and glioma risk in a group consuming 100 mL or more of coffee or tea per day compared to a group consuming less than 100 mL of coffee or tea per day. Based on the six studies included in the meta-analysis, Malerba et al. [209] suggested no association between coffee or tea consumption and the risk of glioma, but their work had limitations due to the small number of papers analyzed.

6.10. Alcohol Use

Alcohol can cross the blood–brain barrier and, therefore, can affect glial cells; in addition, it is a recognized risk factor in multiple cancers [210]. The metabolism of alcohol (at higher concentrations in the body) produces acetaldehyde and reactive oxygen species that have toxic effects on cells; acetaldehyde has been shown to be neurocarcinogenic in animals [211]. Additionally, alcoholic beverages contain N-nitroso compounds that cause brain tumors in animals [211–213]. Despite this, the study by Qi et al. [214] based on 19 meta-analyses reported no association between glioma incidence and alcohol consumption. These observations were confirmed by a recent study by Cote et al. [215], who even indicated that low to moderate alcohol consumption may reduce the risk of glioma.

6.11. Sleep and Melatonin

Samatic et al. [216] noticed that sleep duration is not linked with the risk of glioma. Oreskovic et al. [217] reported that there are mechanisms of pro-tumor effects of sleep disorders, including phase shifts, decreased antioxidant levels, immunosuppression, metabolic changes, melatonin deficiency, cognitive impairment, or epigenetic changes. All of these changes significantly affect the poorer prognosis of patients with malignant brain tumors and are potential exacerbating factors for tumor progression. In addition, the occurrence of a brain tumor contributes to sleep disorders.

Lissoni et al. [218] evaluated the effects of melatonin co-treatment in patients with GBM undergoing radical or adjuvant radiotherapy. They observed that the patient survival percentage of the RT and melatonin group was significantly higher than that of the RT alone group (6/14 vs. 1/16 patients).

Cutando et al. [219] reported that melatonin administration reduces the incidence of malignant tumors in vivo and increases the survival time of patients with GBM treated by radiotherapy. A study by Martin et al. [220] showed that melatonin sensitizes human malignant glioma cells against TRAIL-induced cell death. Furthermore, the melatonin/TRAIL combination significantly increases apoptotic cell death compared to TRAIL alone. A study by Zheng et al. [221] confirmed the anti-glioma function of melatonin to be mediated partly by suppressing glioma stem cell (GSC) properties through EZH2-NOTCH-1 signaling.

6.12. Inflammation

Even in a healthy body, gene mutations can lead to tumorigenesis and GBM. Numerous mechanisms are in place to offset these processes so that altered cells are effectively destroyed by the immune system before tumor formation [222,223]. Even when a tumor forms, the immune system can destroy it at an early stage. A molecular mechanism that facilitates this process is inflammation [224]. Chronic inflammation, on the other hand, can facilitate tumor formation [225] by damaging DNA, resulting in mutations and tumorigenesis [225,226]. Additionally, chronic inflammation triggers mechanisms that can inhibit an otherwise robust immune system response [227] and thus inhibit the immune system from fighting newly formed cancer cells. For this reason, the factors that trigger this physiological state will increase susceptibility to cancer. Some of the best-studied inflammation-related factors involved in GMB are tumor necrosis factor α (TNFα) and interleukins 1 and 6 (IL-1 and IL-6).

Tumor necrosis factor α (TNFα) is a soluble cytokine involved in directing the systemic inflammatory response [228]. It can exert antitumor effects on glioma cells, but can also enhance tumor progression. TNFα can facilitate angiogenesis by increasing epidermal growth factor receptor (EGFR) activity [229]; it induces immune cell suppression through the activation of the NF-κB and STAT3 pathways [230], and decreases the expression of the tumor-suppressor gene PTEN in glioma [231]. Our results suggest that TNFα is involved in reduced macrophage infiltration, suggesting that TNFα plays a suppressive role by demonstrating the ability to promote tumorigenesis [232]. Since abnormal epidermal growth factor receptor EGFR signaling is widespread in GBM, EGFR inhibition seemed to be a promising therapeutic strategy. However, EGFR inhibition in GBM causes a rapid upregulation of TNFα, which in turn leads to the activation of the JNK-Axl-ERK signaling pathway involved in resistance to EGFR inhibition [233]. A previous study showed that TNFα induces the upregulation of angiogenic factors in malignant glioma cells, which plays a role in RNA stabilization [234]. This confirms that TNFα in GBM cells may play an important role in tumor progression.

Interleukin 1 (IL-1) is a potent inducer of proangiogenesis and proinvasion factors, such as VEGF, in human astrocytes and glioma cells. IGF2 induction [235,236] is strongly stimulated by IL-1 in astrocytes [237]. IL-1 is also a major inducer of astrocyte/glioma miR-155, a microRNA involved in inflammation-induced cancer formation [238]. The specific microRNA (miR-155) targets cytokine signaling suppressors, potentially leading to the overactivation of STAT3, a transcription factor important in glioma progression.

IL-1α has been implicated in cancer pathogenesis, but there is little evidence of its role in GBM. To date, its function has been shown to be both pro- and anti-tumor in various cancer types [239]. IL-1α secretion by tumor cells causes the constitutive activation of NF-κB, which results in the expression of genes involved in the cascade of metastatic processes and angiogenesis [240].

GBMs have been shown to produce large amounts of IL-1β, which plays a key role in glioma aggressiveness and survival. IL-1β is a major pro-inflammatory cytokine that triggers a number of tumorigenic processes by activating various cells to upregulate key molecules involved in oncogenic events. Elevated levels of IL-1β have been observed in cultures of GBM cell lines [241] and in samples from human GBM tumors [242]. IL-1β receptor (IL-1R) is found in GBM cells and tissues [243]. The binding of IL-1β to IL-1R activates a cascade of NF-κB and mitogen-activated protein kinase (MAPK) signaling pathways [244]. IL-1β-induced ERK activation can also have mitogenic effects on human glioma U373MG cells and significantly increase GBM cell proliferation [245]. IL-1β-dependent activation of the NF-κB, p38 MAPK, and JNK pathways in GBM cells also leads to increased expression of VEGF, which promotes angiogenesis, migration, and invasion [246]. In addition, IL-1β-mediated up-regulation of factor HIF-1 [247] is involved in molecular responses to hypoxia, which is a key component of GBM progression.

The glioma environment is subject to chronic inflammation, and IL-6 is one of the cytokines strongly associated with the chronic inflammatory phenotype often associated with GBM. Tumor-associated macrophages make up a large majority of noncancerous cells in tumors and are major producers of IL-6 [248]. Interleukin 6 (IL-6) has been shown to be a factor involved in the malignant progression of glioma [249]—it promotes regeneration, invasion, and angiogenesis. In glioma, the elevated expression of IL-6 and its receptor is associated with poor patient survival [250]. IL-6 promotes tumor survival by suppressing immune surveillance through the recruitment and stimulation of tumor-associated myeloid-derived suppressor cells and neutrophils. This paralyzes the response of surrounding type-1 helper T cells and cytolytic T cells, ultimately leading to T cell dysfunction and the inhibition of tumor cell clearance. IL-6 is specifically involved in GBM as the stimulation of brain tumor cells by IL-6 promotes three major signal transduction pathways involved in gliogenesis—(1) p42/p44-MAPK, dysregulated in approximately one-third of all cancers and strongly involved in the detection and processing of stress signals [251]; (2) PI3K/AKT, a signaling pathway associated with enhancing angiogenesis, activating the EMT transition to increase invasion, and promoting metastasis [252]; and (3) JAK-STAT3, a pathway that blocks tumor recognition by immune cells and promotes cell cycle progression and the inhibition of apoptosis [253].

6.13. Electromagnetic Radiation

With the popularization of electronic devices, such as microwave ovens and cell phones, the impact of exposure to electromagnetic waves and the risk of developing CNS tumors became a controversial topic. The impact of phones on tumor development remains inconclusive due to the mixed results from studies, the relatively short time since the prevalence of smartphones, and the numerous confounding factors in the research.

Today, people are commonly exposed to radio-frequency electromagnetic fields (RF-EMF) (30 kHz–30 GHz) through electronic devices, such as cell phones, cordless phones, radios, and Bluetooth. These devices are located in close proximity to users so that even low-power transmitters are not precluded from potential effects on health. The specific RF energy absorption rate (SAR) of the most common source, mobile telephones, is influenced by many factors, such as the design of the device, the position of the antenna in relation to the user's head, the anatomy of the user's head, how the phone is held, and the quality of the connection between the cell phone and the network station. A working group [254] in 2011 concluded that, despite the high risk of error in the available studies, the potential carcinogenic effects of RF-EMF cannot be ruled out.

A pooled analysis of Swedish case–control studies of people who had used cell phones for more than 25 years was conducted by Hardell and Carlberg [255], showing that the OR of developing glioma was 3.0 (95% CI: 1.7–5.2). In contrast, Villeneuve et al. [256] suggested that the lack of increase in glioma incidence rates with the increasing popularization of cell phones supports the lack of a causal relationship.

In a study published in 2010 [257], a group who used a cell phone at least once a week over a six-month period had a lower risk of developing glioma than the group who never used a cell phone (OR = 0.81 (95% CI: 0.70–0.94)), but the most exposed (10th decile (≥1640 h)) in terms of cumulative exposure had a 40% higher risk of developing glioma (OR = 1.40, 95% CI = 1.03–1.89). This indicates the possible presence of confounding factors, study biases, and suboptimal selection of study participants.

In studies on the effect of cellphone use on the survival of GBM patients, Olsson et al. [258] did not report any reduced OS compared to those who did not use cell phones regularly.

7. Treatment of GBM

Current treatment methods are based on a combination of surgical approaches along with radiotherapy and chemotherapy. Recent significant changes in therapeutic methods involve the addition of temozolomide (alkylating chemotherapeutics) to the treatment regimen. Currently, the determination of mutations of specific tumor cell genes has a high predictive and prognostic value and allows the development of targeted therapies.

GBM remains an incurable cancer [2]. The goal of medical management is to make a diagnosis by the biopsy of the tumor and to prolong and improve the quality of the patient's life. A definitive diagnosis is made on the basis of a mandatory histopathological examination and molecular studies [259]. Most patients are treated with multiple-modality therapy. Symptomatic treatment (symptoms occurring as a result of local pressure damage to the centers—epilepsy, neurological losses, hydrocephalus, and elevated intracranial pressure (ICP)) is an important part of treatment. Surgical treatment remains the primary focus of care and patient management [260]. Tumor shedding makes it difficult to distinguish the tumor cell mass from the surrounding healthy tissue. For this reason, despite achieving complete macroscopic resectability, local recurrence does occur. Roh et al. [261] compared gross total resection (GTR) with lobectomy including the tumor and surrounding noncancerous tissue (SupTR), but did not report statistically significant differences in the postoperative Karnofsky Scale scores in the operated patients. The median PFS was longer in the SupTR group (30.7 months (95% CI 4.3–57.1)) compared to that in the GTR group (11.5 months (95% CI 8.8–14.2)), which was significant ($p = 0.007$). However, it should be mentioned that SupTR was only performed when the tumor was located in the frontal or temporal lobe and without damage to the motor cortex. The SupTR surgical intervention probably did not damage a significant portion of healthy neurons, but removed cells whose axons would have been damaged by GTR surgery anyway.

The failure to achieve clear surgical margins is due to the presumed progression of tumor cells along neuronal fibers (neuropils) without macroscopic changes, which does not give the operator a chance to achieve microscopic resectability and cure the patient with surgery alone. Extensive resection (or SupTR) has the limitation of potentially damaging nerve centers and pathways, where dysfunction dramatically decreases the patient's quality of life and well-being. Currently, fractionated adjuvant radiotherapy is the standard of care after surgical resection [259].

Another form of GBM treatment is chemotherapy. In a paper published in 2005 in *NEJM*, Stupp et al. [261] reported greater efficacy of fractionated radiotherapy (fractionated focal irradiation in daily fractions of 2 Gy administered 5 days per week for 6 weeks, for a total of 60 Gy) combined with temozolomide administration (75 mg per square meter of body surface area per day, 7 days per week from the first to the last day of radiotherapy), followed by six cycles of adjuvant temozolomide (150 to 200 mg per square meter for 5 days during each 28-day cycle) compared with radiotherapy alone.

Furthermore, in this study, the efficacy of temozolomide was greater in the presence of a methylated *MGMT* promoter in GBM cells. Subsequent adjuvant telosomide chemotherapy combined with radiotherapy (TMZ/RT → TMZ) significantly improved median survival, and 2- and 5-year survival and is currently the standard of care for patients with GBM under 70 years of age, or 70+ in good physical condition. TMZ is administered daily for the duration of the radiotherapy and then for 5 days every 4 weeks for six cycles as maintenance (adjuvant) therapy after the completion of radiotherapy. Mutation of the MGMT gene promoter has been shown to be the strongest predictive marker, and the greatest benefit of chemotherapy appears to occur in patients with this mutation.

Li X. et al. [262] linked GBM treatment to a higher incidence of subsequent cancers. This is probably due to the use of radiotherapy, with consequential mutagenic effects and the individual's predisposition to develop tumors. The consequences of anticancer treatment also relate to the side effects of temozolomide. In rare cases, TMZ may result in the development of myelodysplastic syndrome, acute myeloid leukemia, and even acute lymphoblastic leukemia [263].

Currently, there is no screening tool or test to detect GBM before the onset of clinical symptoms. The gold standard for imaging studies showing GBM is MRI [264].

8. Conclusions

This review presents the pathogenesis of GBM, including the key molecular changes that trigger GBM. In addition, the risk factors for GBM, as well as factors with protective effects, were analyzed. Unfortunately, due to significant differences in the methodology of epidemiological reports, it proved impossible to compare some data, e.g., compare GBM incidence in various regions of the world. It would, therefore, be valuable to establish an international cancer registry to reliably document key data on GBM.

Analysis of the data collected in this article confirms that GBM is in the higher grade of primary brain tumors and is far more common in men. Moreover, the risk of being diagnosed with glioma increases with age, while median survival remains low, despite medical advances. Despite the alarming growing trend in the incidence of GBM, it is still difficult to directly determine the causes of its occurrence, which is why further research into the etiology and treatment of GBM tumors should continue. In addition, it is difficult to clearly determine what impact the use of stimulants, certain drugs (e.g., NSAIDs), or the use of cell phones has on the development of glioma. It is also worth noting that today's treatments cannot cure GBM patients, but only slightly extend their total life span.

9. Limitation

Although GBM is the most common and aggressive primary brain tumor, in the past several decades, we have seen little progress in finding better treatment options for patients diagnosed with this cancer. The complexity of the disease, the heterogeneity or highly invasive potential of GBM tumors, and the fact that, until recently, GBM research received less funding compared to other tumor types have contributed to little advancement in the treatment of this disease. Due to the high complexity of GBM, it is important to conduct studies with high reproducibility. Unfortunately, available epidemiological reports or studies on the risk of GBM are characterized by significant differences in results, which is probably due to differences in intra-population sex ratios, insufficient sample size relative to population size, and different inclusion and exclusion criteria. Because of these important differences, there is significant difficulty in comparing studies. Our review covers most of the areas of GBM epidemiology and indicates that the existing literature on GBM has its strengths and weaknesses. We have established significant challenges in the epidemiology of GBM, including short patient survival, cognitive changes in patients that hinder the collection of data, and low incidence that makes it difficult to carry out a prospective study. All these factors significantly affect the possibility of understanding the epidemiology of GBM. Because of the specificity of this disease, it seems important to

extend research in GBM epidemiology, e.g., by analyzing a broader range of risk factors or biomarker-specific morbidity.

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Review

CC Chemokines in a Tumor: A Review of Pro-Cancer and Anti-Cancer Properties of Receptors CCR5, CCR6, CCR7, CCR8, CCR9, and CCR10 Ligands

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Abstract: CC chemokines (or β-chemokines) are 28 chemotactic cytokines with an N-terminal CC domain that play an important role in immune system cells, such as $CD4^+$ and $CD8^+$ lymphocytes, dendritic cells, eosinophils, macrophages, monocytes, and NK cells, as well in neoplasia. In this review, we discuss human CC motif chemokine ligands: CCL1, CCL3, CCL4, CCL5, CCL18, CCL19, CCL20, CCL21, CCL25, CCL27, and CCL28 (CC motif chemokine receptor CCR5, CCR6, CCR7, CCR8, CCR9, and CCR10 ligands). We present their functioning in human physiology and in neoplasia, including their role in the proliferation, apoptosis resistance, drug resistance, migration, and invasion of cancer cells. We discuss the significance of chemokine receptors in organ-specific metastasis, as well as the influence of each chemokine on the recruitment of various cells to the tumor niche, such as cancer-associated fibroblasts (CAF), Kupffer cells, myeloid-derived suppressor cells (MDSC), osteoclasts, tumor-associated macrophages (TAM), tumor-infiltrating lymphocytes (TIL), and regulatory T cells (T_{reg}). Finally, we show how the effect of the chemokines on vascular endothelial cells and lymphatic endothelial cells leads to angiogenesis and lymphangiogenesis.

Keywords: chemokine; CC chemokine; cancer; tumor; organ-specific metastasis; angiogenesis; lymphangiogenesis; tumor microenvironment

1. Introduction: The Dual Properties of Chemokines Are Key for Understanding of the Tumor Microenvironment during Therapy

The CC (β) subfamily of chemokines is a group of chemotactic cytokines known as CC motif chemokine ligands (CCL)1–28. Their shared characteristic is the N-terminal CC domain and digits in their symbols depend on the order of discovery [1,2]. The actual number of CC chemokines is 27, as CCL9 and CCL10 denote the same chemokine. All these chemokines are ligands for 10 receptors—CC motif chemokine receptors (CCR)1–10. Just like the rest of chemokines, CC chemokines are crucial for the functioning of the immune system cells [2]. However, apart from their anti-cancer properties, they also show some pro-cancer characteristics and thus play an important role in neoplasia.

Below, we have listed the properties of selected chemokines in the tumor. These properties can be divided into anti- and pro-cancer. The former are associated mainly with the recruitment of anti-cancer tumor-infiltrating lymphocytes (TIL), which destroy cancer cells [3]. In turn, the pro-cancer properties

of chemokines are related to recruiting cells supporting tumor development [4], as well as increasing or causing the proliferation [5], migration, and invasion of cancer cells [6]. Very often, if not always, a given chemokine shows both pro- and anti-cancer properties, something we wished to emphasize in this paper. Therefore, the increased expression of a single chemokine is not always a clear indicator in establishing a patient prognosis in all cancers. The table below shows where the increased expression of a given chemokine improves the prognosis for one type of cancer or worsens the prognosis for patients with other cancers [7,8] (Tables 1 and 2). There is no single CC chemokine that would worsen or improve the prognosis in all types of cancers.

Table 1. Influence of increased expression of individual CC chemokines discussed in this review on the prognosis of patients with various cancers according to "The Human Protein Atlas". (https://www.proteinatlas.org/) [7,8].

Type of Cancer	Receptor										
	CCL1	**CCL3**	**CCL4**	**CCL5**	**CCL18**	**CCL19**	**CCL20**	**CCL21**	**CCL25**	**CCL27**	**CCL28**
Glioma	N/A	–	–	↓	↓	–	↓$p = 0.082$	N/A	–	N/A	↓
Thyroid cancer	N/A	↑	–	↑	–	–	–	↓	↑$p = 0.066$	N/A	–
Lung cancer	↓$p = 0.058$	↓$p = 0.089$	–	↑	–	–	↓	↓	–	N/A	↓
Colorectal cancer	N/A	–	↑	↑$p = 0.086$	↓$p = 0.057$	↑	–	↓$p = 0.099$	–	N/A	↑
Head and neck cancer	↑	–	↑$p = 0.070$	↑	↑	↑	↓	↑	↑	N/A	↑
Stomach cancer	–	–	–	↑	–	↓$p = 0.080$	–	↓	↓$p = 0.064$	N/A	–
Liver cancer	N/A	–	↑$p = 0.91$	↑$p = 0.087$	–	↑	↓	↑	↑	N/A	↓
Pancreatic cancer	N/A	↑$p = 0.072$	↑$p = 0.086$	↓	↓	↑$p = 0.083$	↓	↑	–	N/A	↓
Renal cancer	N/A	↓	↓	↓	↑	↓	↓	↓	↓	N/A	–
Urothelial cancer	N/A	↑	↑	↑	↓	–	–	↓$p = 0.089$	–	N/A	↑$p = 0.065$
Prostate cancer	N/A	–	–	–	↓	–	–	–	–	N/A	–
Testis cancer	–	↓$p = 0.093$	–	↓$p = 0.075$	–	↓	–	–	–	N/A	–
Breast cancer	N/A	–	↑$p = 0.060$	↑	↑$p = 0.089$	↑	–	↑	↑	N/A	↑
Cervical cancer	↑$p = 0.065$	–	↑	↑	↑	↑	↓	↑	↑	N/A	–
Endometrial cancer	N/A	↑	↑	↑	↑$p = 0.096$	–	↑	↑$p = 0.055$	–	N/A	↓
Ovarian cancer	–	–	–	↑	↑	↑	–	↑	↑	N/A	↓
Melanoma	–	↑	↑	↑	–	↑	–	↓$p = 0.081$	–	N/A	↓

↑ blue background—better prognosis with higher expression of a given chemokine in a tumor; ↓ red background—worse prognosis with higher expression of a given chemokine in a tumor; ——no correlation with higher expression of a given chemokine in a tumor.

Table 2. Effects of increased expression of individual CC chemokine receptors discussed in this review on the prognosis of patients with various cancers according to "The Human Protein Atlas" (https://www.proteinatlas.org/) [7,8].

Type of Cancer	Receptor					
	CCR5	**CCR6**	**CCR7**	**CCR8**	**CCR9**	**CCR10**
Glioma	↓	↑$p = 0.076$	↓	↓	↑	↓$p = 0.061$
Thyroid cancer	↑	–	–	–	–	↑
Lung cancer	↑	↑	↑	–	↑	–
Colorectal cancer	↑	↑	↑	↑	↑$p = 0.056$	↓$p = 0.057$
Head and neck cancer	↑	↑	↑	↑	↑	↑
Stomach cancer	↑$p = 0.083$	↓	–	↑$p = 0.080$	–	↓
Liver cancer	↑	–	↑	–	–	↓
Pancreatic cancer	–	↑	↑	↑$p = 0.074$	↓$p = 0.081$	↑
Renal cancer	↓	↓	↓	↓	↑	↓
Urothelial cancer	↑	–	↑$p = 0.079$	–	↑	–
Prostate cancer	↑$p = 0.053$	↑	–	–	–	↓
Testis cancer	↓	↓$p = 0.080$	↓	↓	–	–
Breast cancer	↑	↑	↑	–	↑	↑
Cervical cancer	↑	↑	↑	↑	–	↑
Endometrial cancer	↑	–	↑	–	–	↑
Ovarian cancer	↑$p = 0.062$	–	↑	↑	–	–
Melanoma	↑$p = 0.077$	–	↑	↑	–	↓$p = 0.095$

↑ blue background—better prognosis with higher expression of a given chemokine in a tumor; ↓ red background—worse prognosis with higher expression of a given chemokine in a tumor; ——no correlation with higher expression of a given chemokine in a tumor.

Another important premise of this review is the intratumor heterogeneity. A tumor is not a homogenous environment and consists of areas with different properties. The most significant is the area affected by chronic hypoxia [9], characterized by the accumulation of tumor-associated macrophages (TAM) [10–13], regulatory T cells (T_{reg}) [14–16], and myeloid-derived suppressor cells (MDSC) [17,18]. The functions of these recruited pro-cancer cells in this microenvironment are enhanced by chronic hypoxia [11,19–21] and cancer acidification [22], which increases the resistance of cancer cells to anticancer therapy and the action of the immune system [20,22–24]. In such hypoxic areas, chemokines show only pro-cancer properties, despite their aforementioned dual nature. However, during the effective anti-cancer response of the immune system, the same chemokines will exhibit anti-cancer properties [3] (Figure 1).

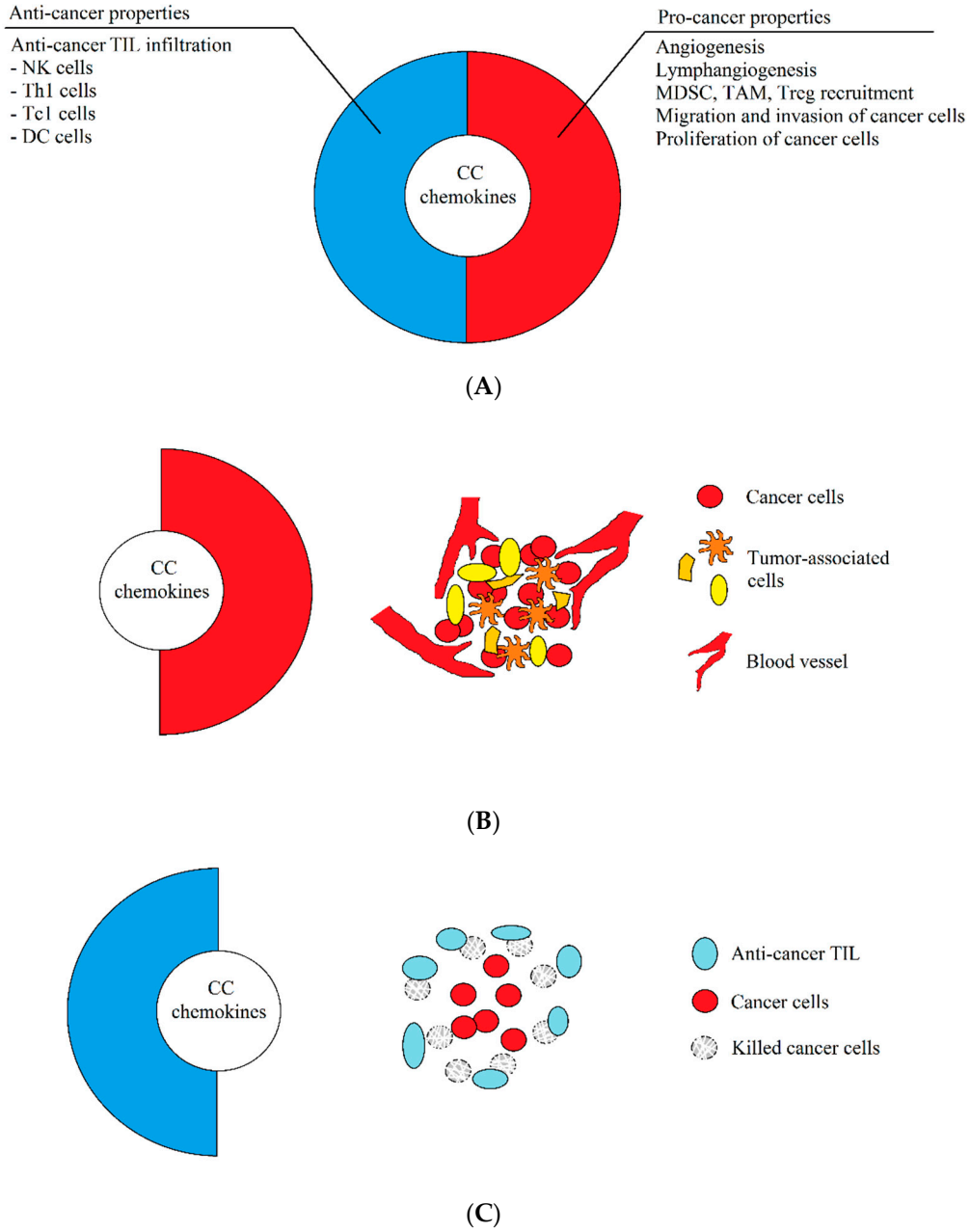

Figure 1. The dual properties of CC chemokines. (**A**) Most, if not all, the chemokines described in this paper have both pro- and anti-cancer properties. The anti-cancer properties consist of the recruitment of anti-cancer tumor-infiltrating lymphocytes (TIL), which infiltrates the tumor and destroys tumor cells. The pro-cancer properties of chemokines, on the other hand, consist in causing angiogenesis and lymphangiogenesis, recruitment of pro-cancer cells supporting the development of the tumor, and the stimulation of proliferation, the induction of migration, and the invasion of cancer cells. (**B**) In a growing tumor, CC chemokines have enhanced pro-cancer properties, while anti-cancer properties are suppressed. As a result, these chemokines participate in the development of a tumor by causing angiogenesis, migration of tumor cells, and recruitment of cells supporting the development of a tumor, which results in the progress of cancer. (**C**) During immunotherapy or an effective anticancer response of the immune system, the same CC chemokines show enhanced anti-cancer properties, which result in the infiltration of a tumor by anti-cancer TIL, which destroy tumor cells. The immune system fights with the tumor, which often leads to recovery.

Knowledge of the anti-cancer and pro-cancer properties of individual chemokines allows a prediction of the consequences to then improve the effectiveness of anti-cancer therapies. One example is radiotherapy, which leads to an increased expression of certain chemokines, e.g., CCL2 and CCL5, resulting in the recruitment of TAM and T_{reg} [25–27]. This has a pro-cancer effect and nullifies the therapeutic benefits of radiotherapy. On the other hand, the same chemokines have anti-cancer properties, because they infiltrate the tumor with anti-cancer TILs [3,28]. For this reason, radiotherapy may be more effective if used prior to immunotherapy [29]. Another application of knowledge presented in this paper is the use of gene therapy to enhance the expression of a given chemokine followed by immunotherapy [30–33] or chemotherapy [34]. As in the previous case, the increased expression of a chemokine may enhance the effectiveness of immunotherapy.

The role of chemokines in cancer has been the subject matter of a considerable number of papers. PubMed (https://pubmed.ncbi.nlm.nih.gov/) includes almost 22 thousand articles containing the words "chemokine" and "cancer" (title + abstract + keywords). However, the sheer number of papers makes it difficult to write a comprehensive and meaningful review. One solution is to focus on literature reviews that deal with individual chemokines, although one problem is that due to the fast advances in science they quickly become out-of-date. Another problem is the low attention paid in literature to less important chemokines and the emphasis on the best known chemokines. That is why, in this review, we address the significance of all human chemokines in cancer. Due to the considerable amount of data, we decided to discuss in this paper only CC chemokines that have been assigned to receptors: CCR5, CCR6, CCR7, CCR8, CCR9, and CCR10 (CCL1, CCL3, CCL4, CCL5, CCL18, CCL19, CCL20, CCL21, CCL25, CCL27, and CCL28) (Tables 3 and 4).

Table 3. Nomenclature for CC chemokines discussed in this review, including cells recruited into the tumor niche.

Chemokine	Alternative Name of the Chemokine	Receptor	Effect on Recruiting Cells to a Tumor Niche	Effect on Tumor Vascularization	Organ-Specific Metastasis
CCL1	I-309	CCR8	TAM, T_{reg}	Angiogenesis	Lymph node
CCL3	MIP-1α	CCR1, CCR5	CAF, MDSC, T_{reg}, TIL, Kupffer cells	Angiogenesis	
CCL4	MIP-1β	(CCR1), CCR5	CAF, MDSC, T_{reg}, TIL	Angiogenesis, lymphangiogenesis	
CCL5	RANTES	CCR1, CCR3, CCR5	MDSC, TAM, Th17, TIL, T_{reg}	Angiogenesis, lymphangiogenesis	
CCL18	PARC, MIP-4	PITPNM3, CCR8	T_{reg}	Angiogenesis	
CCL19	ELC	CCR7	TIL, T_{reg}	Angiogenesis, lymphangiogenesis	Lymph node
CCL20	LARC	CCR6	T_{reg}, Th17	Angiogenesis	Liver
CCL21	SLC	CCR7	TIL, T_{reg}	Angiogenesis, lymphangiogenesis	Lymph node
CCL25	TECK	CCR9			Gastrointestinal tract
CCL27	ESkine	CCR10	TIL, Th22	Lymphangiogenesis	Skin
CCL28	MEC	CCR3, CCR10	TIL, T_{reg}, cancer-associated stellate cells	Angiogenesis, lymphangiogenesis	

CAF—cancer-associated fibroblasts; MDSC—myeloid-derived suppressor cells; PITPNM3—phosphatidylinositol transfer protein 3; TAM—tumor-associated macrophages; Th17—T helper 17; TIL—anti-cancer tumor-infiltrating lymphocytes; T_{reg}—regulatory T cells.

Table 4. Receptors for CC chemokines described in this review and their respective ligands and functions in a tumor.

Receptor	Ligands	Effect on Recruiting Cells to a Tumor Niche	Effect on Tumor Vascularization	Organ-Specific Metastasis
CCR5	CCL3, CCL4, CCL5, CCL7, CCL11, CCL14, CCL16	CAF, TIL, MDSC, TAM, T_{reg}	Increased VEGF expression, which leads to angiogenesis	
CCR6	CCL20	TAM, Th17, T_{reg}	Angiogenesis	Liver
CCR7	CCL19, CCL21	TIL, T_{reg}	Increased expression of VEGF-A, VEGF-C, and VEGF-D, which leads to angiogenesis and lymphangiogenesis	Lymph node
CCR8	CCL1, CCL16, CCL18	TAM, T_{reg}	Angiogenesis	Lymph node
CCR9	CCL25			Gastrointestinal tract
CCR10	CCL27, CCL28	TIL, T_{reg}	Lymphangiogenesis	Skin

CAF—cancer-associated fibroblasts; MDSC—myeloid-derived suppressor cells; TAM—tumor-associated macrophages; Th17—T helper 17; TIL—anti-cancer tumor-infiltrating lymphocytes; T_{reg}—regulatory T cells; VEGF—vascular endothelial growth factor.

2. CCR5 Ligands

2.1. CCL5

Chemokine CCL5 (also known as regulated on activation, normally T cell expressed and secreted (RANTES)) is a ligand for receptors CCR5 [35,36], CCR3 [37–39], and CCR1 [1,2,40]. After secretion of this chemokine outside the cell, two amino acids are truncated from the N-terminus by CD26/dipeptidyl peptidase IV (DPPIV), resulting in an increased affinity to CCR5 and reduced affinity to CCR1 and CCR3 [41]. CCL5 is also a ligand for G-protein-coupled receptor 75 (GPR75), whose expression occurs on brain nerve cells [42,43]. The effect of CCL5 is mitigated by the atypical chemokine receptor 2 (ACKR2)/D6, which reduces the level of this chemokine [44–46].

Pro-inflammatory cytokines elevate CCL5 expression, which triggers the accumulation of various immune cells at inflammatory sites [47], e.g., contributing to asthma by CCR3–mediated chemotaxis of eosinophils [47]. The CCL5→CCR5 axis also induces the accumulation of and increases the cytotoxic properties of anti-cancer TIL in a tumor [28,30,48–54]. CCL5 is responsible for the infiltration of a tumor by NK cells [48,49,52,53], conventional type 1 dendritic cells [54], T helper cell type 1 (Th1) [28], and type 1 cytotoxic cells (Tc1) [22]. For this reason, some postulate that cancer therapies should combine immunotherapy with increasing the expression of CCL5 in the tumor to enhance the infiltration of the tumor by various immune cells [30,49,50]. On the other hand, the genome of Kaposi's sarcoma-associated herpesvirus 8 contains viral macrophage-inflammatory protein-II (vMIP-II) [55–57]. Cells infected by this virus secrete vMIP-II, which acts as a CCR5 antagonist and therefore reduces the infiltration of the tumor by anti-cancer TIL. vMIP-II also induces the recruitment of Th2-type cells via CCR3 and CCR8, which decreases the activity of cytotoxic lymphocytes [58,59].

On the other hand, CCL5 has also several pro-cancer properties (Figure 2). Its expression is elevated in breast cancer [60], glioblastoma multiforme [61], and hepatocellular carcinoma [62]. In a tumor, CCL5 expression is found in cancer cells [63,64], cancer-associated fibroblasts (CAF) [65], mesenchymal stem cells (MSC) [66], MDSC [67], TAM [68–71], and TIL [72]. CCL5 expression also occurs in lymphatic endothelial cells, which is crucial for the formation of the metastatic niche [73].

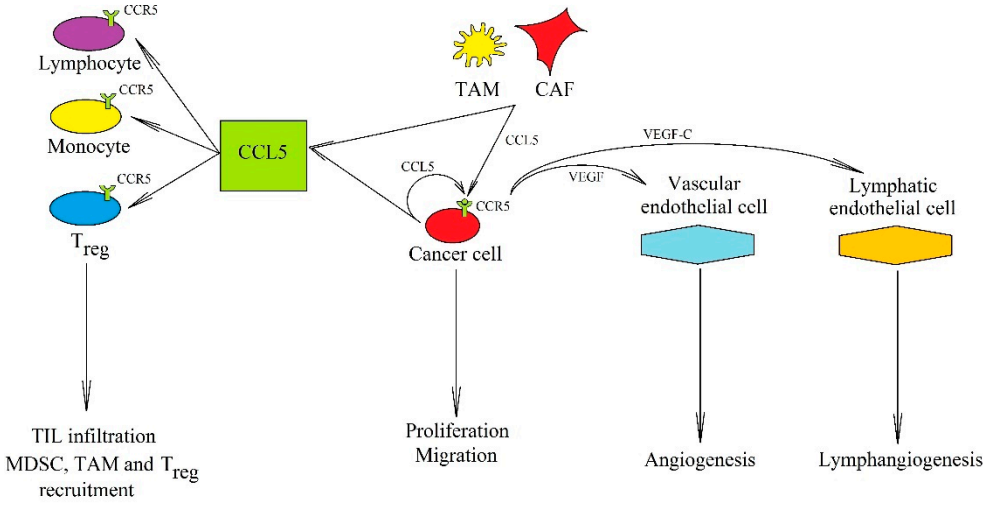

Figure 2. The significance of the CCL5→CCR5 axis in cancer processes. In a tumor, CCL5 is secreted by tumor cells and also by TAM and CAF. Its anti-cancer properties consist of inducing cytotoxic TIL infiltration that increases the anticancer response of the immune system. Its pro-cancer effect is associated with (i) recruitment of cells participating in cancer immune evasion: MDSC, TAM, and T_{reg}, (ii) induction of proliferation, migration, and invasion of cancer cells, and (iii) causing an increased production of VEGF, which leads to angiogenesis and lymphangiogenesis.

CCL5 acts on cancer cells by increasing their proliferation [62,64,74,75]. By activating the Wnt/β-catenin→signal transducer and activator of transcription 3 (STAT3) signaling pathways, it induces the self-renewal of prostate cancer stem cells [70]. Similar to other chemokines, CCL5 causes migration, invasion, and epithelial-to-mesenchymal transition (EMT) of cancer cells via its receptor [62,64,76,77], which is related to the activation of hedgehog [78], Wnt/β-catenin [70,79], and the Akt/PKB→nuclear factor κB (NF-κB) pathways [80–82]. The induction of migration by CCL5 is associated with increased secretion of matrix metalloproteinases (MMPs) by cancer cells [80,83,84]. In addition, CCL5 increases apoptosis resistance and drug resistance through the activation of Akt/PKB→NF-κB and STAT3 pathways [72,85–87]. It also causes an increase in programmed death-ligand 1 (PD-L1) expression on cancer cells, which protects them against cytotoxic lymphocytes [71].

CCL5 not only affects cancer cells but also tumor-associated cells. It recruits MDSC [88,89], MSC [90], TAM [77,91,92], and T-helper cells 17 (Th17) [93] into the tumor niche. Via CCR5, it participates in recruiting T_{reg} and elevating its activity in the tumor niche [67,94–96]. CCL5 is secreted by diffuse large B cell lymphoma, which causes the recruitment of monocytes that increase the proliferation of those cancer cells [97]. In human papillomavirus (HPV) 16, E7 protein increases the expression of CCL5 [98]. This chemokine is responsible for recruiting mast cells and through these cell enhances the growth of a tumor.

CCL5 participates in angiogenesis, which is associated with an increase in vascular endothelial growth factor (VEGF) expression in cancer cells and vascular endothelial cells via the activation of CCR1 and CCR5 [99–102]. CCL5 may cause differentiation of cancer stem cells into endothelial cells as observed in the ovarian cancer model [103]. This chemokine is also induced by the Epstein–Barr virus (EBV) (human herpesvirus 4) in nasopharyngeal carcinoma cells, which leads to angiogenesis [104]. It may also indirectly cause lymphangiogenesis by increasing VEGF-C expression, as observed in chondrosarcoma cells [105].

2.2. CCL3 and CCL4

CCL3 (also known as macrophage inflammatory protein-1α, i.e., MIP-1α) and CCL4 (also known as macrophage inflammatory protein 1β, i.e., MIP-1β) are pro-inflammatory chemokines. In humans,

there are also additional copy number variations (CNV) of *CCL3* and *CCL4* genes, known as *CCL3L* and *CCL4L*, which are produced by duplicating the genes of the corresponding chemokines [106,107]. The products of these additional genes have the same amino acid sequence as CCL3 and CCL4 and the same properties.

CCL3 is a ligand for receptors CCR1 and CCR5 [35,36], while CCL4 interacts with CCR5 [35,36], and with low affinity with CCR1 [40]. CCL3 and CCL4 are not ligands for CCR3 [38,39,108], but Combadiere et al. show that CCL3 and CCL4 are potent agonists for CCR3 [37]. Due to their anti-cancer properties, we decided to include them in the section on CCR5 ligands. Both these CC chemokines are important in the onset of the immune response [109]. They induce the recruitment of dendritic cells, neutrophils, monocytes, macrophages, NK cells, and T cells to inflammatory sites [109]. CCL3 is responsible for the correct function of $CD8^+$ T cells and CCL4 acts on $CD4^+$ T cells [110–112]. The increase in the number of immune cells, induced by CCL3 and CCL4, makes these chemokines a potentially important element of cancer immunotherapy [113–115].

In a tumor, an active immune response results in the production of CCL3 and CCL4 by B cells [116] and basophils [117]. The produced chemokines act as chemoattractants for anti-cancer TIL with CCR1 and CCR5 [115,118–121]. However, in a tumor both CCL3 and CCL4 may be cleaved by cathepsin D [122] and chemokine decoy receptor ACKR2/D6 [2,123], which suppresses the anti-cancer effect of these two CC chemokines.

On the other hand, CCL3 and CCL4 also have pro-cancer effects. Their expression has been found in tumor-associated and cancer cells. In a tumor, they are produced by TAM [69,124–127], MDSC [68], and MSC [66], which promotes tumor growth. The expression of CCL4 may also occur in tumor-associated neutrophils (TAN) [128]. CCL3 may be expressed in a cancer cell [127]. The immune response reduces the expression of CCL3 in cancer cells via the activation of β-catenin [129]. Both CCL3 and CCL4 are also expressed in chronic myeloid leukemia and multiple myeloma [130–133]. CCL3 and CCL4 lead to the inhibition of normal osteoblast function and an increase in osteoclast activity and thus to bone destruction [130,134–136]. In addition, the production of CCL3 by acute myeloid leukemia cells causes severe anemia by inhibiting erythropoiesis [137]. In bone marrow, hypoxia induces an increase in CCR1 expression on multiple myeloma cells [138]. The activation of this receptor by CCL3 affects the function of CXC motif chemokine receptor 4 (CXCR4), which lead to the egress of those cells to the blood. The elevated level of CCL3 in serum is associated with a worse prognosis for patients with diffuse large B cell lymphoma [139], extranodal NK/T-cell lymphoma [140], and multiple myeloma [141].

Both chemokines can cause T_{reg} recruitment via CCR1 and CCR5. CCL3 causes the recruitment of T_{reg} to the leukemic hematopoietic microenvironment, which promotes the development of acute myeloid leukemia [142]. CCL4 also induces the recruitment of T_{reg} to the tumor niche, as shown in melanoma [67]. Both CCL3 and CCL4 cause the recruitment of MDSC into the tumor niche via CCR5 [143,144].

There are no reports on the recruitment of TAM by CCL3 or CCL4; they support the anticancer functions of monocytes [145], so they will not recruit TAM but will be more responsible for the infiltration of anti-cancer M1 macrophages. In a hepatocellular carcinoma model, CCL3—via CCR1—causes the recruitment of Kupffer cells, resident macrophages in the liver [146]. In addition, CCL3 [147] and CCL4 [148] cause the recruitment of CAF via CCR5, which is of great importance for the functioning of a tumor and the initial stages of metastatic niche formation in bones.

Although CCL3 and CCL4 have an anti-cancer effect by causing cytotoxic TIL infiltration to the tumor, they can support the development of the tumor if they act directly on a cancer cell. For example, CCL3 increases cancer cell proliferation [144]. In multiple myeloma, this chemokine causes drug resistance by activating the PI3K→Akt/PKB→mTOR and extracellular signal-regulated kinase (ERK) mitogen-activated protein kinase (MAPK) pathways [133]. In acute lymphoblastic leukemia, anticancer drugs increase the expression of CCL3 and CCL4, which leads to drug resistance of these cells [149].

CCL3 causes migration and invasion of cancer cells via CCR5 [124,127,144]. This is related, among other things, to the activation of PI3K→Akt/PKB and ERK MAPK pathways [127]. Both CCL3 and CCL4 may also participate in metastasis. CCL3 is secreted by the tumor, which causes an increase in CCL2, CCL7, and CCL8 expression in the lungs and brain [150], which facilitates the metastasis to these organs. After getting to the blood, a cancer cell is encased in platelets, which protects it against NK cells [151]. Platelets also secrete many factors, including CCL3, which support the cancer cell [152]. After reaching another organ, CCL3 participates in the formation of the metastatic niche. The cancer cell recruits macrophages through the CCL2→CCR2 axis [153]; these macrophages then secrete CCL3, which causes the retention of these cells in the metastatic niche in an autocrine manner via CCR1. In bones, on the other hand, there is an increase in the expression of CCL3 in bone-marrow-derived monocytes caused by the secretion of epidermal growth factor (EGF) by cancer cells. This causes the differentiation of these monocytes into osteoclasts [154], which leads to bone remodeling around the forming metastases.

CCL3 and CCL4 also indirectly cause angiogenesis. CCL3 [127,155] and CCL4 [156] cause increased expression of VEGF in a cancer cell, a growth factor causing angiogenesis. CCL4 also increases the expression of VEGF-C in a cancer cell, which leads to lymphangiogenesis and lymph node metastasis [157].

3. CCR6 and CCL20

CCL20 (also known as liver activation regulated chemokine (LARC) or macrophage inflammatory protein-3α (MIP-3α) or Exodus-1) is a proinflammatory chemokine; a ligand for CCR6 [1,2,158,159]. This makes it significant for the correct function of dendritic cells, T cells, and B cells—cells with expression of this receptor [159]. CCL20 is produced by Th17 cells and is thus responsible for the function of these lymphocytes [160]. In addition, CCL20 also plays an important role in neoplastic processes (Figure 3).

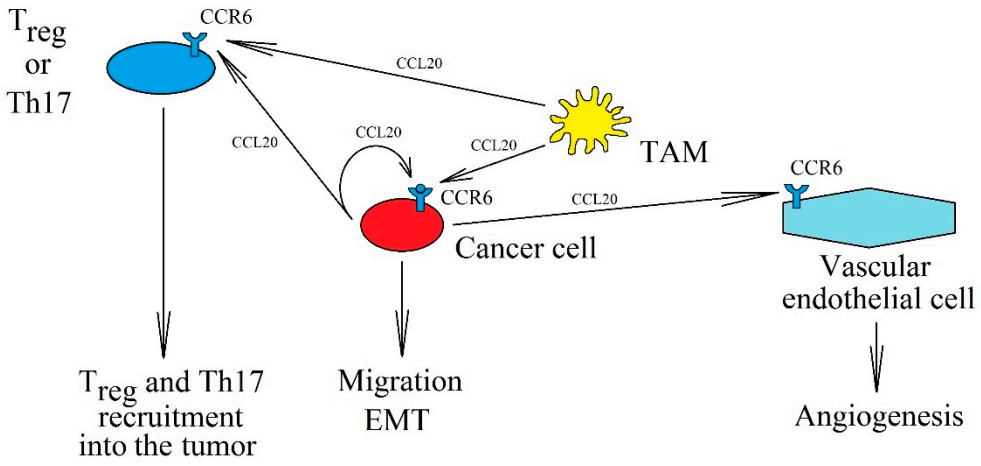

Figure 3. The significance of the CCL20→CCR6 axis in cancer processes. CCL20 is expressed in tumor cells and TAM, which can activate CCR6 on cancer cells in an autocrine manner, causing their migration and EMT. In addition, increased concentration of CCL20 in the cancer microenvironment recruits T_{reg} and Th17 into the tumor niche and causes angiogenesis via CCR6.

CCL20 expression is elevated in many cancers, such as breast cancer [60], hepatocellular carcinoma [161], and pancreatic cancer [162,163]. While its expression occurs in cancer cells [63,162], TAM are also a significant source of this chemokine in the tumor [162,164,165]. CCR6 expression has also been shown on tumor cells. Thus, in some cancers, the cells can stimulate their own proliferation and migration in an autocrine manner via CCL20→CCR6 [63,166–168]. At the same time, CCL20

directly causes angiogenesis by activating CCR6 on vascular endothelial cells [169]. CCR6 activation may also increase the expression of VEGF in cancer cells, which contributes to angiogenesis [170].

The main primary function of CCL20 in a tumor is to recruit T_{reg} and Th17 for the tumor niche [171–174]. This leads to tumor immune evasion. The CCL20 also recruits TAMs [175] through CCR6. This chemokine is also responsible for recruiting dendritic cells, which increases the anticancer response of the immune system [176]. However, it seems that the impact of cancer immune evasion is stronger than this process.

In addition to recruiting cells to a tumor niche, CCL20 increases cancer cell proliferation [166,177], causes cancer cell migration and invasion [167,177–179], and induces EMT of cancer cells [165,180,181]. It also participates in organ-specific patterns of metastasis—high expression of CCL20 in the liver results in the metastasis of tumor cells with high expression of CCR6 to this organ [182–184]. CCR6 is also important in lung metastasis in breast cancer patients [185] and adrenal metastasis in lung cancer patients [186]. However, in the adrenal gland and lungs, CCL20 expression is low [187]. This indicates that this chemokine can only participate in the induction of cell migration into the bloodstream. The expression of CCR6 on B-cell non-Hodgkin's lymphomas leads to the localization of those cells at mucosal sites [188]. The CCL20→CCR6 axis is also significant in osteolytic bone lesions caused by multiple myeloma [189].

4. CCR7, CCL19, and CCL21

The most important physiological functions of CCL19 (also known as EBI1 ligand chemokine (ELC) or macrophage inflammatory protein-3 (MIP-3) or Exodus-3) and CCL21 (also known as secondary lymphoid tissue chemokine (SLC) or 6Ckine or Exodus-2) include the homing of T cells to a lymph node [190–194]. This process is dependent on the receptor of these chemokines: CCR7 [2,190]. Therefore, the increased expression of these chemokines in a tumor has an anti-cancer effect via cytotoxic TIL [195–198].

However, this axis may also have pro-cancer properties if CCR7 is located on a cancer cell. Hypoxia [199–201] and prostaglandin E_2 (PGE_2) [202,203] increases the expression of CCR7 on a cancer cell (Figure 4). CCR7 enhances proliferation [204–206] and stemness of cancer cells [207–209]. The activation of CCR7 increases angiogenesis by activating NF-κB and thus increases VEGF-A expression in esophageal squamous carcinoma cells [210]. In contrast, in colorectal cancer cells, CCL19 inhibits angiogenesis by increasing the expression of miR-206, which inhibits the ERK MAPK→HIF-1→VEGF-A pathway [211]. Activation of CCR7 also increases the expression of VEGF-C and VEGF-D in non-small-cell lung cancer cells [210], head and neck cancer cells [212], and esophageal squamous carcinoma cells [213]. These are growth factors responsible for lymphangiogenesis. The activation of CCR7 on a cancer cell causes EMT and migration of cancer cells [199,200,204–206,209]. In blood and lymphatic vessels CCR7 on cancer cells prevents anoikis [205,214]. In lymphangiogenesis, cancer cells penetrate lymphatic vessels where a cancer cell with CCR7 expression is kept in a lymph node [215–221], which is related to the high expression of a ligands for this receptor [190–192,194] in these peripheral lymphoid organs. In addition, some leukemias, such as B cell chronic lymphocytic leukemia but not multiple myeloma cells, show CCR7 expression [222]. This leads to the homing of those cells to secondary lymphoid tissues. CCR7 is also significant in the dissemination of non-Hodgkin's lymphoma [223]. In leukemias, the expression of CCR7 is a significant prognostic factor. Higher expression is associated with worse prognosis for patients with diffuse large B-cell lymphoma [224]. Metastasis to lymph nodes is not only associated with the described mechanism but also with the high expression of CCL1 in a lymph node combined with the expression of CCR8 on a cancer cell [225]. This process is crucial for the metastasis of malignant melanoma to a lymph node. CCR7 expression has also been associated with skin metastasis in patients with breast cancer [185].

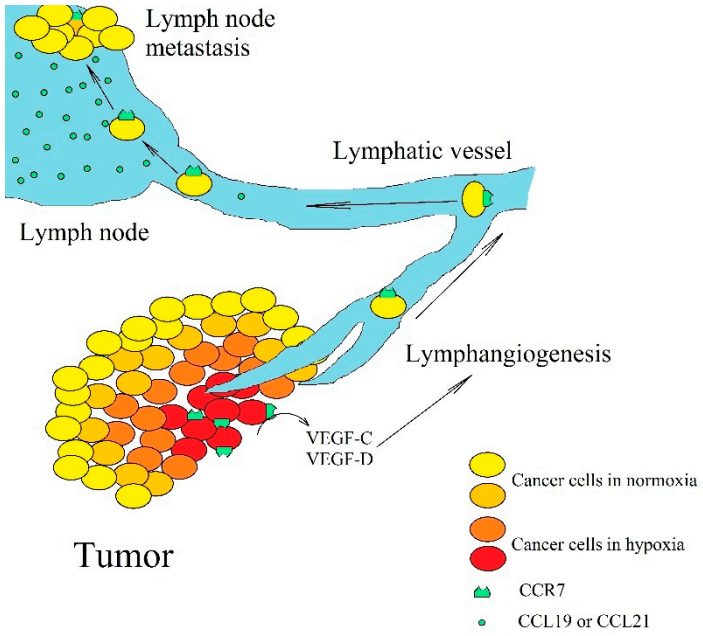

Figure 4. The significance of CCR7 expression in lymph node metastasis. Hypoxia increases CCR7 expression on cancer cells, which leads to an increase in VEGF-C and VEGF-D expression, which in turn, leads to lymphangiogenesis. Then, cancer cells enter lymphatic vessels where they migrate to lymph nodes. As lymph nodes have a high expression of CCL19 and CCL21, cancer cells with CCR7 expression become trapped and form metastasis there.

In addition to the effect on a cancer cell, the described axis CCL19/CCL21→CCR7 also affects the cellular composition of a tumor. Increased expression of CCL19 and CCL21 in a tumor results in anti-cancer TIL infiltration and an improved prognosis for many tumor patients [195–198]. Cancer immune evasion mechanisms reduce the expression of these chemokines in a cancer cell [129]. Nevertheless, if the tumor microenvironment has favorable conditions for tumor development, then CCL19 and CCL21 may participate in T_{reg} recruitment to the tumor niche [226,227]. T_{reg} are cells involved in cancer immune evasion and so their recruitment to a tumor niche inhibits the correct anti-cancer response of the immune system.

5. CCR8

5.1. CCL1

Chemokine CCL1(also known as I-309) was first identified as a cytokine secreted by activated human T lymphocytes [228]. CCL1 is a ligand for just one receptor, CCR8 [1,2,229], and is considered a Th2-related cytokine due to the expression of the CCR8 receptor on Th2 cells, but not on Th1 cells [230]. Therefore, this chemokine plays an important role in the pathogenesis of asthma [231]. The CCL1→CCR8 axis also plays an important role in the homing of lymphocytes to the healthy skin and therefore plays an important role in the physiology of this tissue [232,233].

Elevated expression of CCL1 in a cancer cell occurs in leukemia caused by viruses, e.g., human T cell leukemia virus type 1 (HTLV-1) [234,235]. Properties similar to this chemokine are also shown by viral macrophage inflammatory protein-I (vMIP-I) (homolog to CCL1)—a viral protein expressed in tumor cells transformed by the herpes virus [236]. Another example is Kaposi sarcoma-related human herpes virus-8 [237,238]. In addition, encoded in by the genome of that virus, vMIP-II leads to the recruitment of Th2-type cells via CCR3 and CCR8, which reduces the activity of cytotoxic lymphocytes [58]. In breast cancer, however, the expression of CCL1 is no different than in healthy tissue [60]. In hepatoma cells, it is absent or very low [239]. In gliomas, on the other hand, it is even lower than in the brain tissue [61]. In hepatocellular carcinomas, the expression of CCL1 is elevated

in tumor stroma and peritumoral tissue [240]. In solid tumors, CCL1 is produced by CAF [241,242], TAM [243], CCR8$^-$CD11b$^+$ myeloid cells [244], and T_{reg} [245]. The expression of this chemokine has also been demonstrated in breast cancer stem cells [246], bladder cancer tumors [244,247], and renal cell carcinomas [244].

In cancer, CCL1 has an antiapoptotic activity and induces chemoresistance to anticancer drugs due to the activation of the ERK MAPK cascade via CCR8 (Figure 5) [234,236,242,248,249]. This has a special significance in apoptosis resistance adult T-cell leukemia [234] and murine T cell lymphomas [248]. CCL1 also stimulates proliferation [247] and causes the migration of bladder cancer cells [241]. At the same time, due to the expression of CCL1 in lymph nodes, this chemokine participates in metastasis to these peripheral lymphoid organ through the CCR8 receptor on cancer cells that have entered lymphatic vessels [225]. This process is important in metastasis to lymph nodes in malignant melanoma, which often leads to increased CCR8 expression in the cancer cells of this tumor [225]. However, the CCL1→CCR8 axis is not the only molecular route of metastasis to lymph nodes. In this process, CCL19 and CCL21 acting on CCR7 are also significant [215–221].

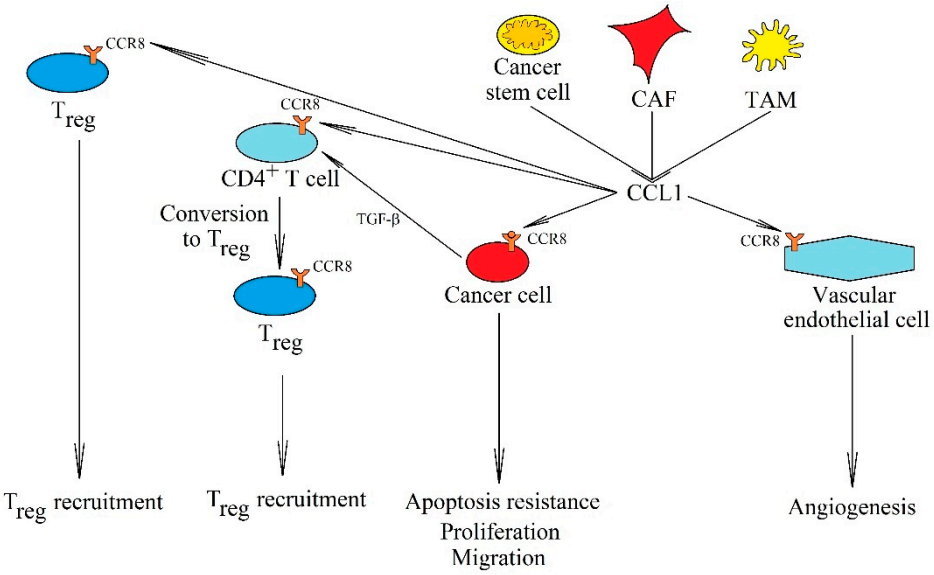

Figure 5. The significance of the CCL1→CCR8 axis in cancer processes. CCL1 is secreted into the cancer microenvironment by cancer stem cells, CAF and TAM. This chemokine has many primary tumor functions. By activating the CCR8 receptor on cancer cells, it causes their proliferation, apoptosis resistance, and migration. It also causes angiogenesis by activating its CCR8 receptor on endothelial cells. Another important function is the recruitment of T_{reg} into the tumor niche and causing the conversion of CD4$^+$ T cells into T_{reg}.

In addition to the effect on a cancer cell, CCL1 causes angiogenesis on vascular endothelial cells via CCR8 [238,250]. It is involved in the recruitment of CCR8$^-$CD11b$^+$ myeloid cells [244] and T_{reg} [246,251,252] to a tumor niche. However, CCL1 can also participate in the conversion of CD4$^+$ T cells into T_{reg} [245], in a process dependent on transforming growth factor-β (TGF-β), which increases the expression of CCL1 in CD4$^+$ T cells. This is followed by an autocrine conversion of this cell to T_{reg}, which also involves CCL1. In addition, CCL1 supports the immunosuppressive function of T_{reg} in a tumor niche [245], which is crucial for the interaction of cancer stem cells and CAF with T_{reg} [241,242,246]. Finally, CCL1 increases the expression of interleukin 6 (IL-6) in MDSC, which acts as a proinflammatory in the cancer microenvironment [244].

5.2. CCL18

CCL18 (also known as pulmonary and activation regulated chemokine (PARC), alternative macrophage activation-associated C-C chemokine-1 (AMAC-1), dendritic cell-derived C-C chemokine 1 (DC-CK1), and macrophage inflammatory protein 4 (MIP-4)) is produced by dendritic cells, especially in germinal centers of regional lymph nodes. This chemokine then causes the chemoattraction of naïve T cells to these cells [253–255]. This leads to the initiation of an immunological response. CCL18 also acts as an anti-inflammatory chemokine, a marker for the macrophage M2 subset [256].

In a tumor, CCL18 is mainly produced by TAM (Figure 6) [69,257,258]. This chemokine causes cancer cell migration and EMT by activating its receptors, phosphatidylinositol transfer protein 3 (PITPNM3) and CCR8 [2,259–263]. CCL18 also increases the expression of cancer stem cell markers [264], is involved in angiogenesis by acting on vascular endothelial cells via PITPNM3 [265], and affects non-cancer cells in the tumor niche. Acting as an immunosuppressive cytokine, it causes polarization of macrophages to phenotype M2 [256] and recruits naïve $CD4^+$ T cells into the tumor niche via its receptor PITPNM3 [266], which then differentiate into T_{reg} cells responsible for tumor immune evasion. CCL18 recruits immature dendritic cells to the tumor niche [267]. In addition, this chemokine participates in the differentiation of immature dendritic cells into tumor-associated dendritic cells (TADC) [268,269]. Other functions of CCL18 in the tumor include participation in the intercellular communication dependent on extracellular vesicles [270]. This chemokine binds to glycosaminoglycans on extracellular vesicles, which allows them to be retained on cells with an expression of CCR8, a receptor for CCL18. CCL18 also increases the proliferation of cancer cells, but this effect depends on the type of tumor. For example, CCL18 decreases the proliferation of acute lymphocytic leukemia B cells [271] and cutaneous T-cell lymphoma (CTCL) [272] in a process dependent on GPR30 that affects the activity of CXCR4 [271]. Increased expression of CCL18 in lesional skin and serum of patients with CTCL [273] and in patients with diffuse large B cell lymphoma [274] is associated with a worse prognosis. In addition, CCL18 decreases the proliferation of non-small-cell lung cancer cells [67] and increases the proliferation of glioma cells [275] and oral squamous cell carcinoma cells [276].

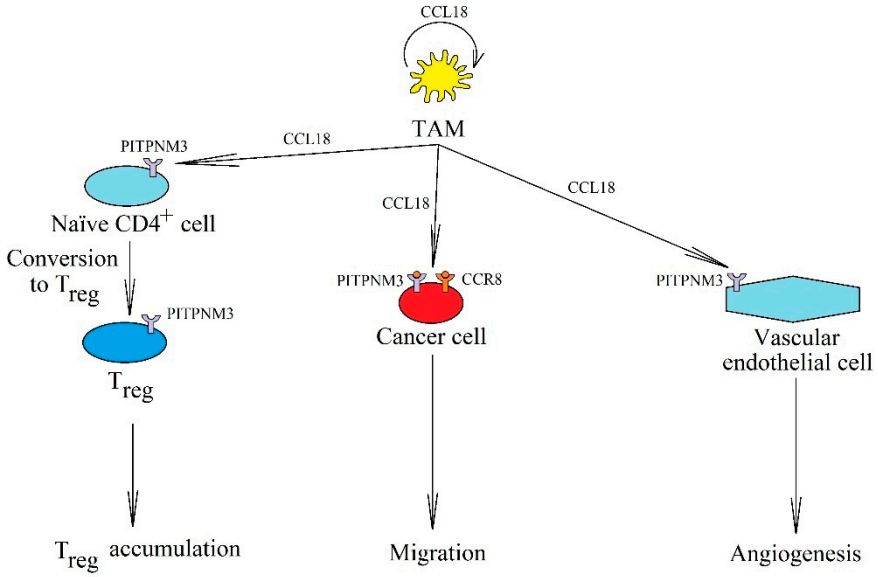

Figure 6. The significance of CCL18 in cancer processes. In a tumor, it is produced and secreted by TAM, where it acts on these cells in an autocrine manner. It also causes cancer cell migration and invasion via receptors PITPNM3 and CCR8. CCL18 is important in angiogenesis by acting on PITPNM3 on endothelial cells. It also causes the recruitment of naïve $CD4^+$ T cells into the tumor niche.

6. CCR9 and CCL25

CCL25 (also known as thymus-expressed chemokine (TECK)) is a ligand for just one receptor: CCR9 [1,2,277,278]. This chemokine is important for the correct function of the thymus [279,280]. Due to the expression of CCL25 in the gastrointestinal tract, lymphocyte homing to the tissues of this system is one of the main functions of the CCL25→CCR9 axis [281–283]. This is of great importance in the immunological functions of the intestinal and gastric mucosa. However, this function of CCL25 results in intestinal metastasis of cancer cells with CCR9 expression [284,285]. The CCL25→CCR9 axis is also significant in the homing of diffuse large B-cell lymphoma and follicular lymphoma to the gastrointestinal tract [286].

In a tumor, CCL25 is produced by cancer cells, such as breast cancer cells [287] and pancreatic cancer cells [288], and also by cancer-related cells, e.g., pancreatic stellate cells in pancreatic cancer [289]. CCL25 participates in the migration and invasion of cancer cells by causing an increase in metalloproteinase expression [290–292]. It also causes EMT of cancer cells [287]. However, in colon cancer, CCR9 activation inhibits cancer cell migration [293]. The activation of CCR9 is also associated with apoptosis resistance and drug resistance, which is related to Akt/PKB→PI3K pathway activation [294–296]. Additionally, the CCL25→CCR9 axis stimulates tumor cell proliferation [288].

There are no data on the influence of CCL25 on the recruitment of cells cooperating in the development of a tumor or its participation in angiogenesis [297]. However, this chemokine may cause infiltration of the tumor by cytotoxic TIL exhibiting CCR9 expression, which has an anticancer effect [298]. There are also indications that CCL25 causes lymphangiogenesis because activation of the CCL25→CCR9 axis increases the expression of VEGF-C and VEGF-D on the non-small-cell lung cancer cells [292]. CCL25 can also recruit MDSC into a tumor niche, as indicated in a study by Sun et al. on endometriosis [299].

7. CCR10, CCL28, and CCL27

CCL28 (also known as mucosae-associated epithelial chemokine (MEC)) is a chemokine essential for normal mucosal immune function [300,301]. It activates two receptors: CCR3 [302] and CCR10 [303]. Another chemokine that activates CCR10 is CCL27 (also known as ESkine) [2,304,305], and for this reason, its effects are similar to those of CCL28. CCL27 causes $CD3^+$ and $CD4^+$ lymphocyte migration [306]. CCL28 and CCL27 cause B cell and T cell migration via CCR10, especially IgA plasma blasts [300,301,307]. CCL27 is produced by keratinocytes and therefore acts mainly in the skin [301], whereas CCL28 is produced in mucosal tissues [301]. For this reason, these chemokines are important in the homing of immune system cells to mucosal and epithelial tissues and thus participate in immunological reactions against microorganisms. CCL28 also causes eosinophil migration via CCR3 [302], which is associated with the development of allergies.

CCL27 also has an intracellular function. The CCL27 gene, through alternative splicing, creates—in addition to CCL27—PESKY protein [306,308,309], a nuclear protein with expression in the eye, brain, and testes. This protein alters the expression of genes associated with the actin cytoskeleton, which leads to cell migration and changes in cell morphology [308,310]. CCL27 also contains a nuclear location sequence—after internalization of the CCR10 receptor with this chemokine, CCL27 is transported to the cell nucleus where it has a similar function to PESKY [308].

Expression of CCL28 is down-regulated in breast cancer [311], colon cancer [312], and multiforme glioblastoma [61]. In endometrial cancer [313], basal cell carcinoma [314], and squamous cell carcinoma [314], the expression of CCL27 is reduced. This suggests that these chemokines have an anticancer effect, at least in the early stages of tumor development. This is confirmed by studies on the survival of patients with different types of breast cancer [315]. In patients with luminal-like breast cancer, elevated concentrations of CCL28 in the tumor improve prognosis, in contrast to triple-negative breast cancer [315]. In turn, elevated CCL27 expression improves the prognosis in patients with cutaneous malignant melanoma [316]. CCL28 and CCL27 participate in the anticancer response of the immune system, causing an infiltration of the tumor by anticancer NK cells, which leads to

improved prognosis with an increased expression of these chemokines in the tumor [313,317–319]. For this reason, gene therapies that increase the expression of these two chemokines have an anticancer effect [317,318,320].

However, numerous in vitro studies show that CCL28 and CCL27 support tumor development (Figure 7). For this reason, some researchers suggest that CCL28 can act locally, especially in the hypoxic regions of the tumor [14,16,17,321,322]. CCL28 and CCL27 stimulate proliferation and have anti-apoptotic effects on cancer cells [323–325]. In vitro experiments also show that CCL28 reduces migration and EMT in oral squamous cell carcinoma [326], but the effect on migration varies among other types of cancer. In hepatocellular carcinoma, CCL28 increases the migration of these cells [327]. The same activation of CCR10 by both the ligands causes the migration of breast cancer cells [325,328] and glioblastoma multiforme cells [324]. After entering the bloodstream, a cancer cell is trapped in organs and tissues, which have a high expression of chemokines for the receptors on the cancer cell. Due to the production of CCL28 and CCL27 in the skin, the expression of CCR10 on a cancer cell increases the probability of skin metastasis [329].

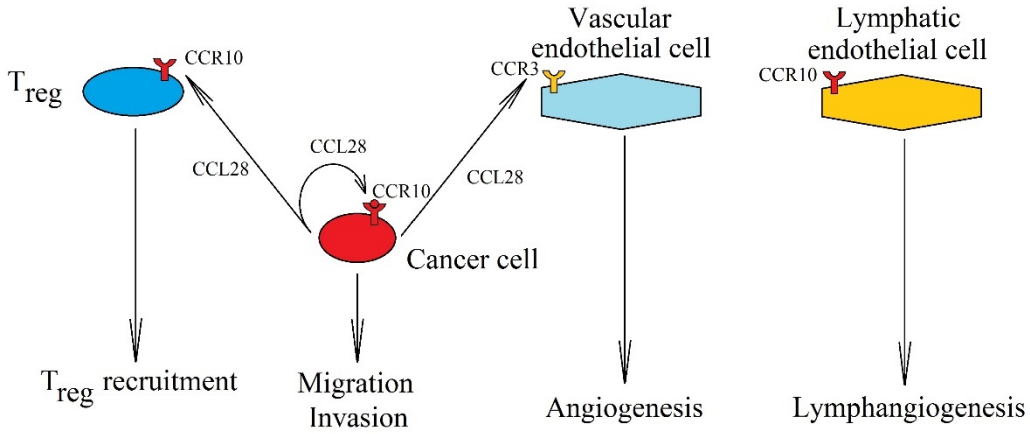

Figure 7. Significance of CCL28 expression increase in cancer processes. Expression of CCL28 in a cancer cell causes the migration and invasion of cancer cells via CCR10. CCL28 is also involved in T_{reg} recruitment into the tumor niche by interacting with receptor CCR10 on these cells. It also causes vascularization of the tumor, causing angiogenesis by activating CCR3 on endothelial cells and lymphangiogenesis by activating CCR10 on lymphatic endothelial cells.

In addition to its effect on cancer cells, CCL28 also acts on non-cancer cells in a tumor niche. In particular, it participates in the recruitment of T_{reg} [14,16], and in pancreatic ductal adenocarcinoma, in the recruitment of cancer-associated stellate cells [330] caused by the expression of CCR10 on these cells. The CCL27→CCR10 axis has also been shown to be involved in the recruitment of Th22 to malignant ascites; even though no effect of an increased number of these cells on patient prognosis has been demonstrated [331], Th22 may have a pro-cancer effect, as shown in a study on colorectal cancer [332]. In turn, CCL28 causes angiogenesis by activating CCR3 on vascular endothelial cells [322]. Both chemokines, CCL28 and CCL27, cause lymphangiogenesis by activating CCR10 on lymphatic endothelial cells [333].

8. Further Direction in the Research on the Role of CC Chemokines in Cancer Processes

The role of individual chemokines in neoplastic processes is very well known. They cause the recruitment of various cells into the cancer niche and cause cancer cell migration and invasion. However, little is known about the interactions between cells in a tumor, particularly the direct or indirect influence of cancer cells on non-cancer cells and the interactions between non-cancer cells. Especially interesting are cancer-cell-induced changes in the expression of chemokines produced by cancer-associated cells, e.g., by TAM [68–71,257,258], MDSC [67], and CAF [65]. In a tumor, cancer cells

are not isolated but interact with cancer-associated cells, and that is why these cells (e.g., TAM, TIL, MDSC, CAF) should be investigated more often to discover new mechanisms in a tumor. Unfortunately, there are few research tools that can be used to show the interactions between cancer-associated cells and the actual cancer cells. The most notable are the co-culture of cancer cells and cancer-associated cells [334] and the use of a conditioned medium from cancer cells to culture non-cancer cells [335,336]. The understanding of those interactions could help foster new therapeutic approaches if the discovered mechanisms are universal in one type of tumor or even common for all neoplastic diseases.

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Abbreviations

CAF	Cancer-associated fibroblasts
CCL	CC motif chemokine ligand
CCR	CC motif chemokine receptor
EMT	Epithelial-to-mesenchymal transition
ERK	Extracellular signal-regulated kinase
MAPK	Mitogen-activated protein kinase
MDSC	Myeloid-derived suppressor cells
NF-κB	Nuclear factor κB
NK	Natural killers
TAM	Tumor-associated macrophages
Th17	T-helper cells 17
TIL	Tumor-infiltrating lymphocytes
T_{reg}	Regulatory T cells
VEGF	Vascular endothelial growth factor

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CCL18 Expression Is Higher in a Glioblastoma Multiforme Tumor than in the Peritumoral Area and Causes the Migration of Tumor Cells Sensitized by Hypoxia

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Abstract: Glioblastoma multiforme (GBM) is a brain tumor with a very poor prognosis. For this reason, researchers worldwide study the impact of the tumor microenvironment in GBM, such as the effect of chemokines. In the present study, we focus on the role of the chemokine CCL18 and its receptors in the GBM tumor. We measured the expression of CCL18, CCR8 and PITPNM3 in the GMB tumor from patients (16 men and 12 women) using quantitative real-time polymerase chain reaction. To investigate the effect of CCL18 on the proliferation and migration of GBM cells, experiments were performed using U-87 MG cells. The results showed that CCL18 expression was higher in the GBM tumor than in the peritumoral area. The women had a decreased expression of PITPNM3 receptor in the GBM tumor, while in the men a lower expression of CCR8 was observed. The hypoxia-mimetic agent, cobalt chloride ($CoCl_2$), increased the expression of CCL18 and PITPNM3 and thereby sensitized U-87 MG cells to CCL18, which did not affect the proliferation of U-87 MG cells but increased the migration of the test cells. The results indicate that GBM cells migrate from hypoxic areas, which may be important in understanding the mechanisms of tumorigenesis.

Keywords: CCL18; PITPN3; CCR8; hypoxia; glioblastoma multiforme; tumor-associated macrophage; brain tumor

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1. Introduction

Glioblastoma multiforme (GBM) is the most severe of brain tumors at grade IV [1]. Compared to other cancers, while its incidence is low at approximately 3.2 per 100,000 population [2], and despite the use of surgery, radiotherapy or chemotherapy, GBM patients show a very short median survival prognosis following diagnosis and treatment of only about 12 months [3,4] and a 5-year survival rate of less than 5% [2,5]. For this reason, GBM is being intensively studied to better understand the tumorigenesis-related mechanisms of this cancer, to help develop more effective therapeutic approaches.

One of the current key directions in the study of tumorigenic processes in GBM tumors is to understand the communication between tumor cells, and between a tumor cell and tumor-associated cells, where chemokines play an important role. The chemokine group of approximately 50 chemotactic cytokines [6] plays significant roles in the migration, invasion and metastasis of tumor cells, as well as in the angiogenesis and recruitment of tumor-associated cells into the tumor niche [7].

The expression of some chemokines is elevated in cancer tumors, with GBM showing elevated expressions of 17 chemokines in the tumor compared to healthy brain tissue [8]. The greatest increase in expression (at 70 times more, relative to controls) was noted for CC motif chemokine ligand 18 (CCL18) [8], from the β-chemokine sub-family. In cancer tumors, CCL18 is mainly produced and secreted into the tumor microenvironment by tumor-associated macrophages (TAM) [9–14] and in much smaller amounts by GBM cancer cells [11], as shown in non-small cell lung cancer cells [12] and melanoma cells [15].

The best-researched CCL18 receptor is phosphatidylinositol transfer membrane-associated protein 3 (PITPNM3)/PYK2 N-terminal domain interacting receptor 1 (Nir1) [16–20]. Activation of this receptor causes tumor cell migration in many cancers. Activation of PITPN3 by CCL18 on endothelial cells also leads to angiogenesis [21]. No less a significant receptor for CCL18 in cancer processes is CC motif chemokine receptor 8 (CCR8). In bladder cancer, activation of CCR8 by CCL18 causes the migration and invasion of tumor cells, as well as increased expression of vascular endothelial growth factor (VEGF)-C, which then causes lymphangiogenesis [22]. The CCL18→CCR8 axis has previously been shown to be significant in other cancer processes as well. CCL18 is involved in the availability of extracellular vesicles [23]. However, the significance of CCR8 in the pro-tumorigenic effects of CCL18 has not yet been sufficiently investigated.

Another important direction of cancer research is to understand the impact of hypoxia on cancer processes [24,25]. Hypoxia is an oxygen deficiency characteristic of certain areas of a cancerous tumor. The cellular response to hypoxia is associated with activation of the hypoxia-inducible factor (HIF)-1 and HIF-2 pathways [26]. Oxygen deficiency decreases the activity of the oxygen-dependent enzymes prolyl hydroxylase (PHD) [27,28] and factor-inhibiting HIF (FIH) [29,30]. As a consequence, HIF-1 and HIF-2 subunits are not proteolytically degraded. This accumulation in the cell is followed by the production of HIF-1 and HIF-2 and an increase in hypoxia-dependent gene expression. One of the model genes induced by HIF-1 (and thus by hypoxia) is vascular endothelial growth factor (VEGF) [31–34]. Hypoxia also alters gene expression independently of HIFs. This is associated with the reduced activity of the oxygen-dependent enzymes ten-eleven translocation (TET) and Jumonji C family (JmjC), which respectively lead to DNA and histone methylation [35].

The effect of CCL18 has been fairly well established in various cancer models. In comparison, very little research has been devoted to the expression of CCL18 receptors, in particular PITPNM3. Therefore, the aim of the present study was to investigate the contributions of CCL18 and the receptors CCR8 and PITPNM3 to tumorigenic processes in GBM. In particular, we focused on the importance of hypoxia and patient gender in the studied mechanisms of tumorigenic processes.

2. Results

2.1. CCL18 Expression in GBM Tumors Is Elevated Relative to the Peritumoral Area

CCL18 expression in the enhancing tumor region and the tumor core was more than seven times higher than in the peritumoral area (Figure 1). These differences were statistically significant (enhancing tumor region vs. peritumoral area, $p = 0.044$; tumor core vs. peritumoral area, $p = 0.018$). The expression of the PITPNM3 and CCR8 receptors did not differ in enhancing tumor region and the tumor core vs. peritumoral area ($p > 0.05$).

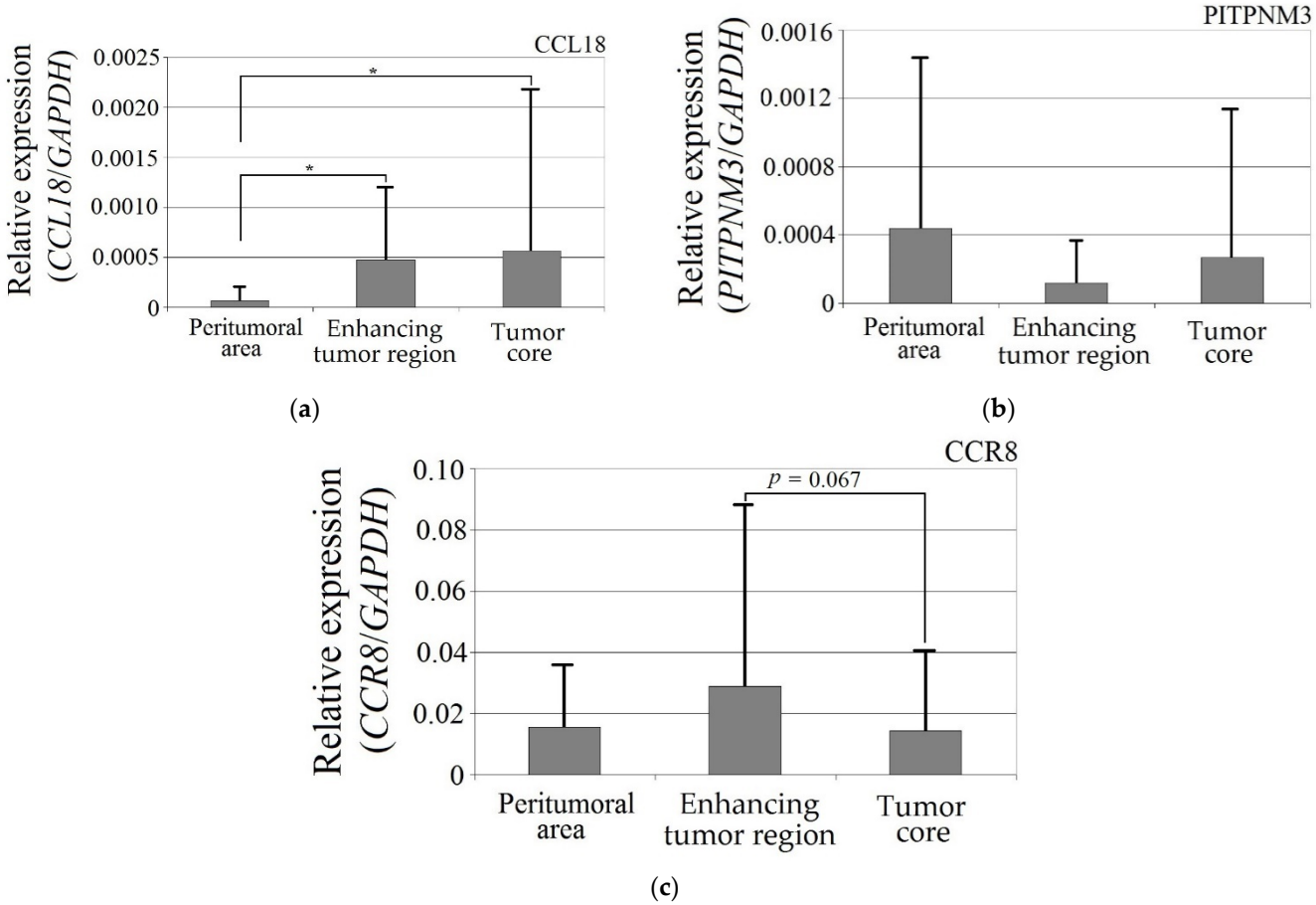

Figure 1. Expression of (**a**) CCL18, (**b**) PITPNM3 and (**c**) CCR8 in GBM tumor and peritumoral area. * $p = 0.05$.

CCL18 expression in GBM tumors was statistically significantly higher in women ($p < 0.05$) in both the enhancing tumor region and the tumor core relative to the peritumoral area, but otherwise did not differ between the genders (Figure 2).

Expression of PITPNM3 in women was lower in the tumor core relative to both the enhancing tumor region ($p = 0.049$) and peritumoral area ($p = 0.049$). Expression of PITPNM3 in the tumor core in the women was also lower than in the men ($p = 0.0011$), but not statistically significantly ($p = 0.062$) in the enhancing tumor region in the women.

CCR8 expression in the peritumoral area and enhancing tumor region did not differ between genders. CCR8 expression in the tumor core in men was lower than in the peritumoral area ($p = 0.0033$) and lower in the tumor core compared to the women ($p = 0.0077$).

2.2. Hypoxia Increases the Expression of CCL18 and the Receptor for This Chemokine: PITPNM3 in U-87 MG Cells

The hypoxia-mimetic agent $CoCl_2$ increased the expression of CCL18 ($p < 0.0001$), PITPNM3 ($p < 0.0001$) and VEGF expression ($p = 0.002$) in U-87 MG cells (Figure 3). There was no effect of cobalt chloride ($CoCl_2$) on CCR8 expression ($p > 0.05$). Nutrient deficiency did not affect the expression of CCL18 or its receptors, PITPNM3 and CCR8 ($p > 0.05$), but did decrease VEGF expression ($p = 0.0007$) in U-87 MG cells.

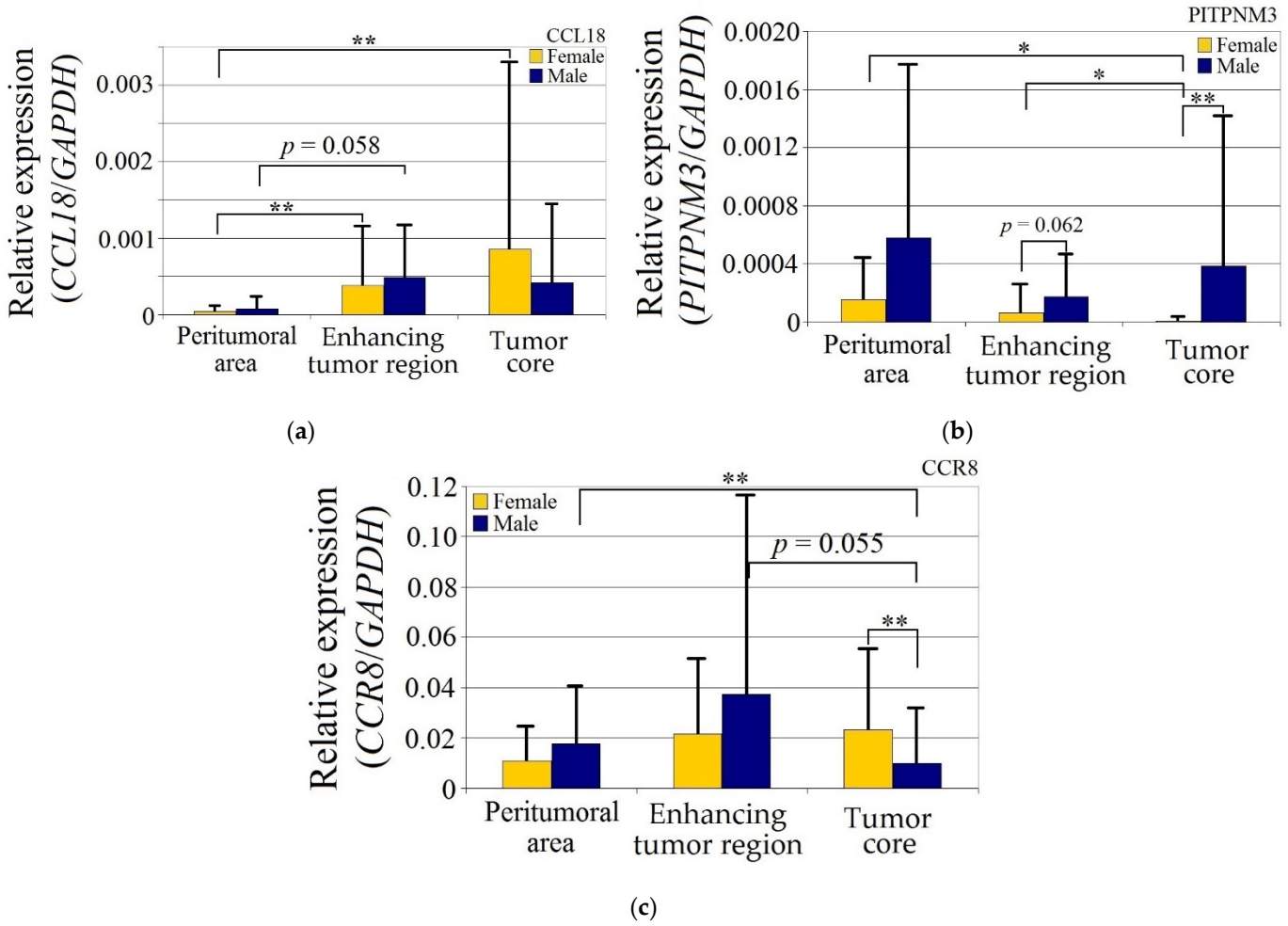

Figure 2. Expression of (**a**) CCL18, (**b**) PITPNM3 and (**c**) CCR8 in GBM tumor and peritumoral area relative to patient gender. * $p = 0.05$, ** $p = 0.005$.

2.3. CCL18 Does Not Affect U-87 MG Cell Proliferation

CCL18 had no effect on U-87 MG cell proliferation at concentrations ranging from 10 ng/mL to 50 ng/mL ($p > 0.05$) (Figure 4).

2.4. CCL18 Induces Migration of U-87 MG Cells. This Effect Is Enhanced by Preincubation with $CoCl_2$

CCL18 increased U-87 MG cell migration at concentrations ranging from 10 ng/mL to 50 ng/mL ($p < 0.01$) (Figures 5 and 6). At a concentration of 10 ng/mL, CCL18 increased cell migration by 36%, while at a concentration of 50 ng/mL it increased expression three-fold. This effect was synergistic with preincubation of U-87 MG cells with $CoCl_2$ where it increased the number of migrating cells four-fold. U-87 MG line cells showed migration to CCL18, which was further stimulated by the hypoxia-mimetic agent $CoCl_2$.

2.5. Macrophages and CCL18 Expression Are Co-Localized with Each Other in the GBM Tumor

In tumor-transformed brain tissue, there were areas with many macrophages (Figure 7, red arrows) that were metabolically active to synthetize and liberate CCL18 into ECM of glioblastoma (Figure 7, green arrows) within the region where hypoxia and malnutrition occurred.

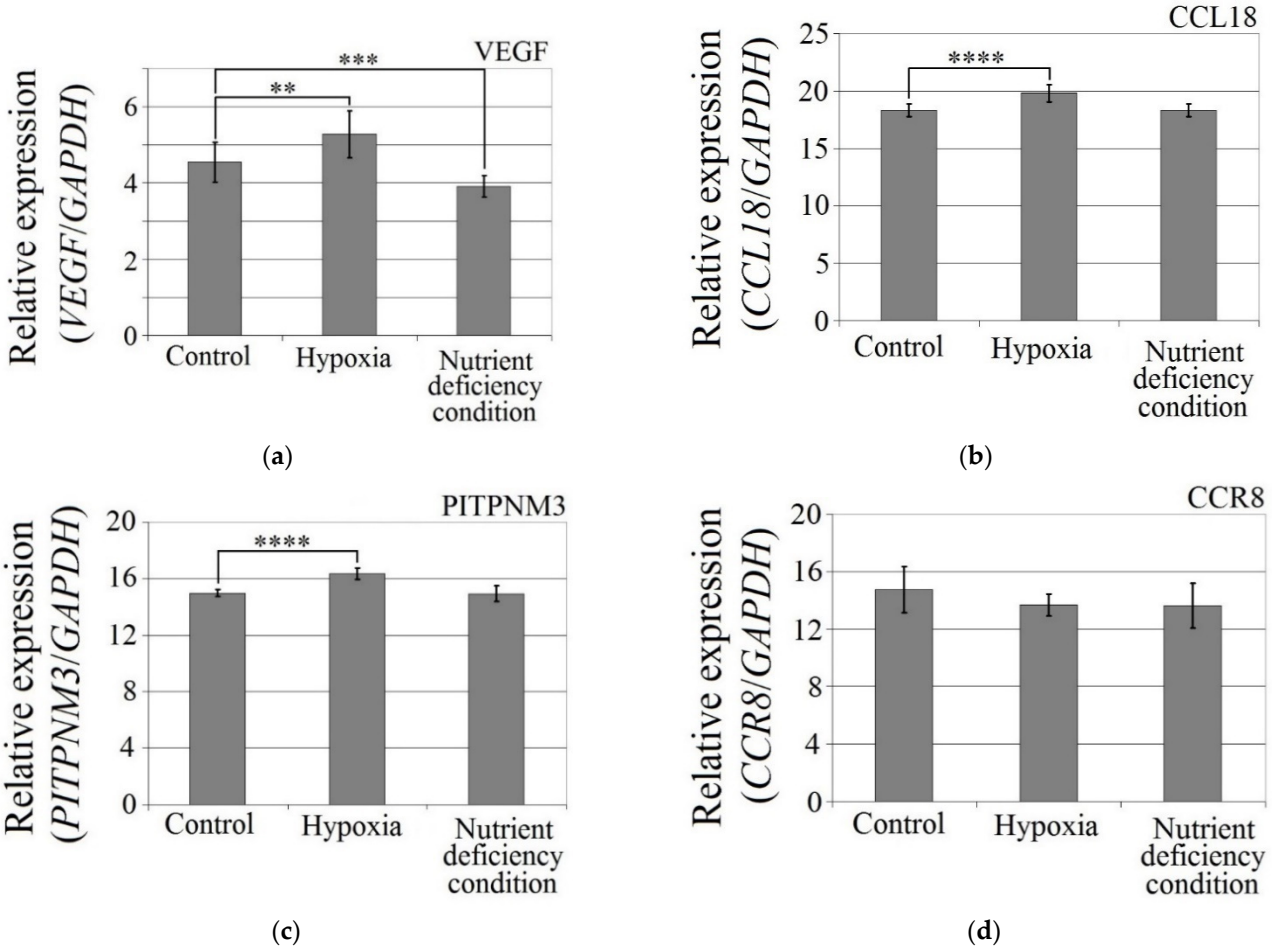

Figure 3. Effects of the hypoxia-mimetic agent $CoCl_2$ and nutrient deficiency on the expressions of (**a**) VEGF, (**b**) CCL18, (**c**) PITPNM3 and (**d**) CCR8 in U-87 MG cells. ** $p = 0.005$, *** $p = 0.001$, **** $p = 0.0001$.

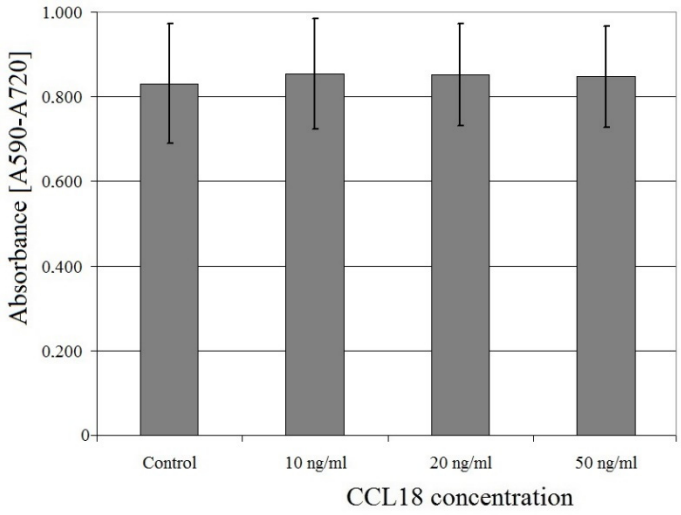

Figure 4. Effect of CCL18 on U-87 MG cell proliferation.

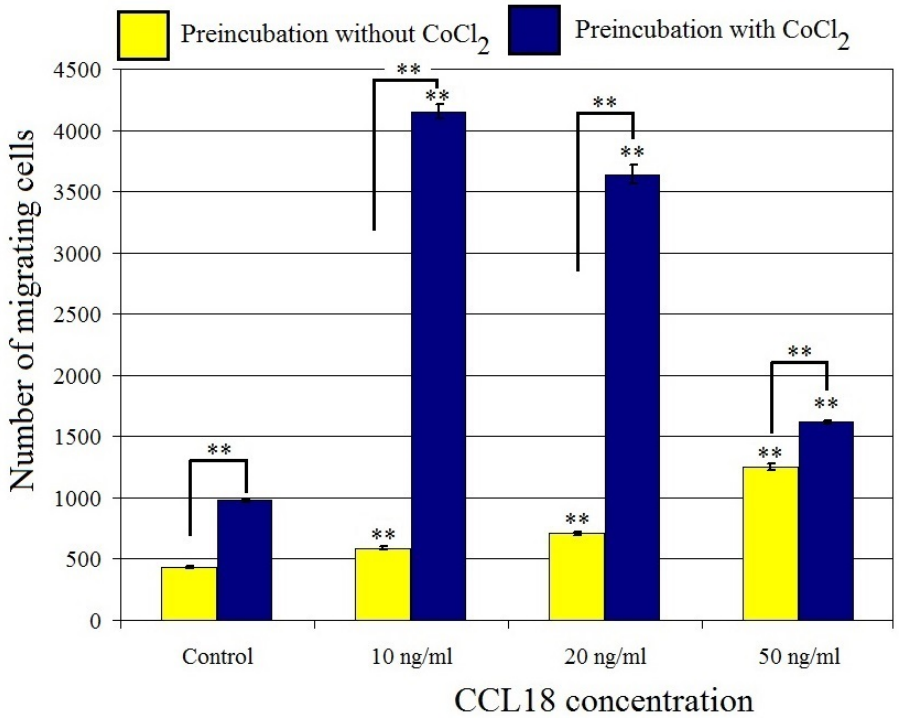

Figure 5. Effect of CCL18 on the migration of U-87 MG cells. ** $p = 0.005$.

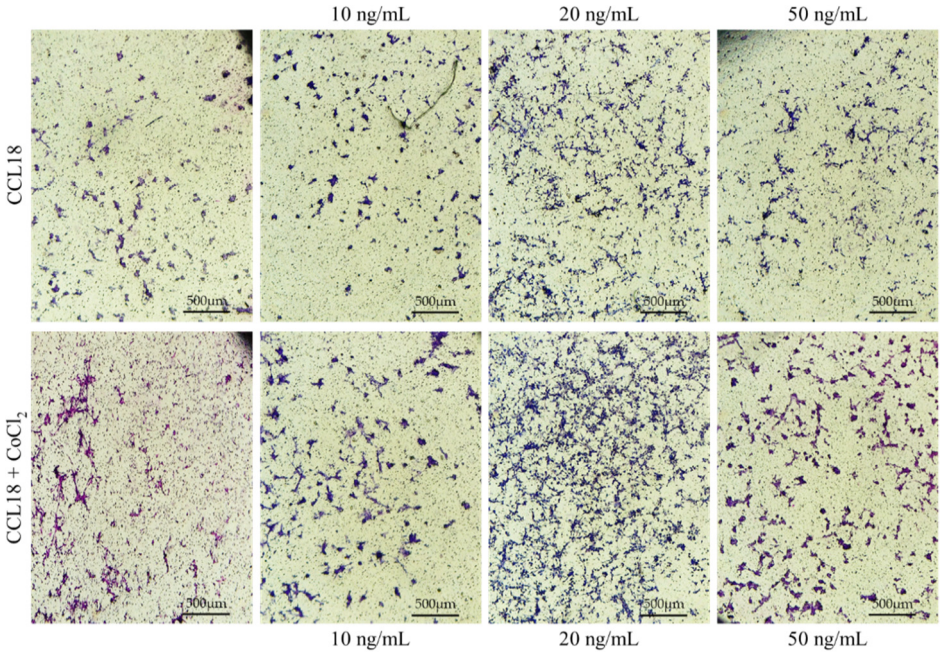

Figure 6. Representative micrographs showing the migration assay. U-87 MG cells cultured in $CoCl_2$ hypoxia mimic agent (lower panel) showed a stronger response to CCL18 compared to cells grown under normal conditions (upper panel). CCL18 concentrations were 10 ng/mL, 20 ng/mL and 50 ng/mL. Control medium contained PBS. Pictures were captured with a Leica DM5000B, Wetzlar, Germany, objective magnification ×4, scale bar 500 µm.

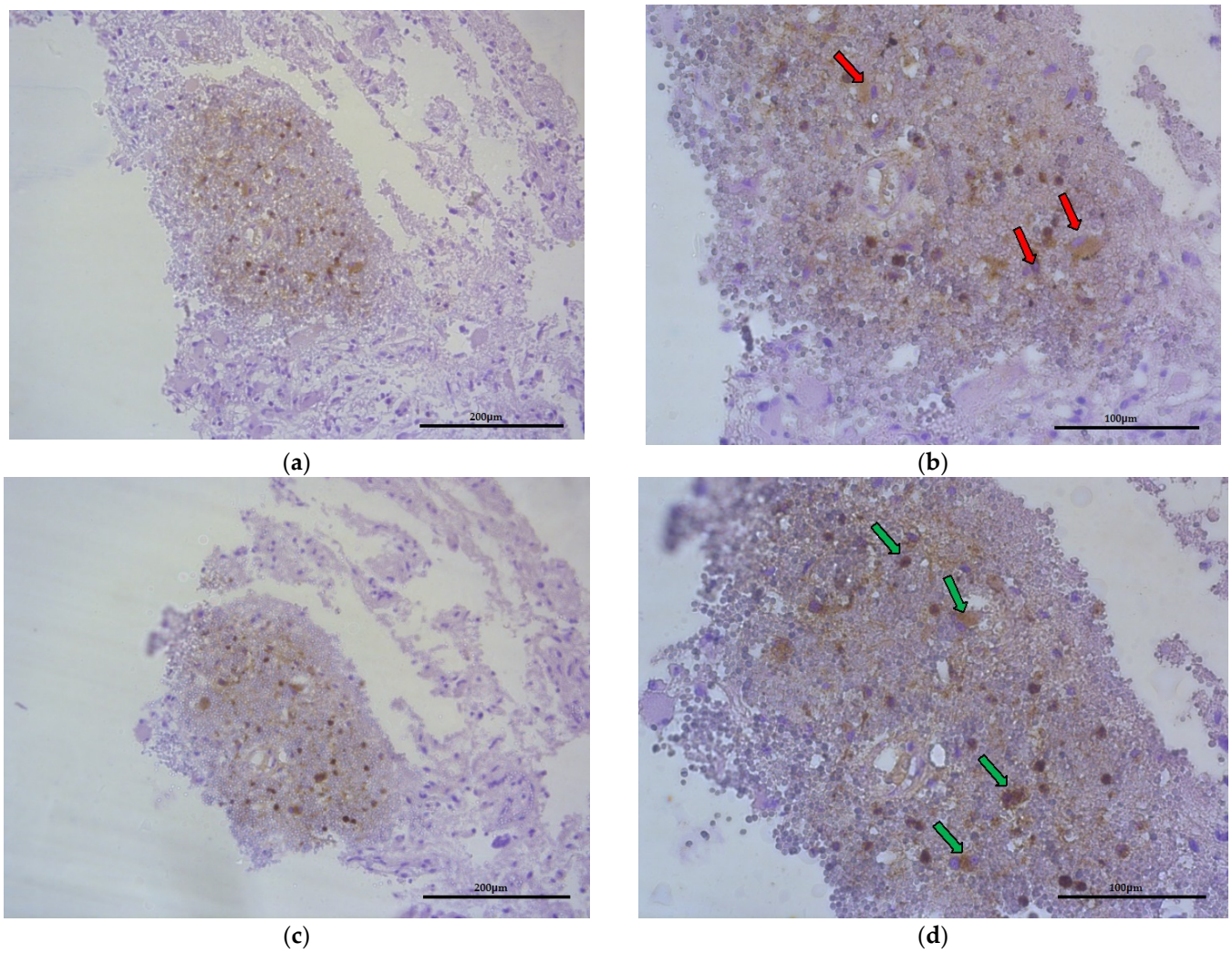

Figure 7. Representative microphotography showing the same region of tumor (obj. magnification a, b × 20, scale bar 200 µm; a, b × 40, scale bar 100 µm). (**a**,**b**) Immunoexpression of CD68 (marker of macrophages), red arrows indicate this cells. (**c**,**d**) Immunoexpression of CCL18, green arrows show that macrophages produce and liberate CCL18 into ECM of glioblastoma.

3. Discussion

3.1. In Vivo Expression of the Studied Genes

In our study, we demonstrated that the expression of the chemokine CCL18 is elevated in GBM tumors. These results are consistent with literature data, where the expression of CCL18 in GBM tumor can be 70 times higher [8] to 100 times higher [36] than in post-mortem brain biopsies. CCL18 plays an important role in tumorigenesis in GBM, as shown by the survival data of patients with this cancer which is inversely correlated with CCL18 levels in the tumor [23]. Additionally, data from the Human Protein Atlas (Available online: http://www.proteinatlas.org, accessed on 22 August 2021) [37–39] show that patients with high CCL18 expression in glioma tumors have a much worse prognosis.

Our study is the first to analyze the expression of the receptor for the CCL18 chemokine, i.e., PITPNM3 in GBM. Therefore, it cannot be directly compared to the literature data. We showed that the expression of PITPNM3 did not change in GBM tumors in all studied patients. However, when taking gender into account, it did decrease in women compared to the peritumoral area. The influence of the PITPNM3 receptor on tumorigenic processes has been best studied in breast cancer models, which showed an elevated expression of this receptor [16,40], as well as non-small cell lung cancer [20] and oral squamous cell

carcinoma [41]. It has been reported that this receptor may play an important role in the development of some cancers. Data included from the Human Protein Atlas (Available online: http://www.proteinatlas.org, accessed on 22 August 2021) [37,38] indicate that higher expression of PITPNM3 in a tumor is associated with a worse prognosis for breast cancer patients [42]. The same regularity can be observed for glioma [43]. Hence, PITPNM3 appears to play a key role in GBM development. Our results may explain the higher incidence and mortality of male patients with GBM [44,45], although there is a lack of literature data on the influence of gender on PITPNM3 expression.

In our study, CCR8 expression did not change relative to peritumoral area. However, there was some trend of decreased CCR8 expression in the necrotic core relative to peritumoral area. CCR8 expression decreased in the necrotic core in male patients. Previous literature has shown that CCR8 expression in GBM tumors is either unchanged [8] or increased [23]. CCR8 is important in the development of GBM because, as previously shown, patients with increased CCR8 expression in the tumor show a worse prognosis [23]. These results are in line with data presented in the Human Protein Atlas (Available online: http://www.proteinatlas.org, accessed on 22 August 2021) [37,38,46].

3.2. Effects of Hypoxia, Oxidative Stress and Nutrient Deficiency Condition on CCL18 Expression and Function

In our model, hypoxia increased the expression of VEGF. This shows that the experiment was properly performed because VEGF is a model gene that is inducible by hypoxia and by $CoCl_2$ [32,34,47]. In our study, nutrient deficiency induced the downregulation of VEGF expression, which is consistent with the results of Sarkar et al. (2020) who showed a reduction in VEGF expression in MG-63 osteosarcoma cells in response to reduced fetal bovine serum (FBS) concentration [48]. GBM tumors are also characterized by a decrease in nutrient availability in hypoxic zones. Both nutrient deficiency and hypoxia affect VEGF expression in GBM cells in hypoxia-affected zones.

In our study, hypoxic conditions increased CCL18 expression in U-87 MG cells. However, the observed effect may depend on the research model used. In non-neoplastic cells, hypoxia reduces CCL18 expression, as shown in immature dendritic cells [49,50], monocytes [51] and macrophages [52]. This is associated with a decrease in JMJD3 activity and consequently an increase in histone methylation in the *CCL18* gene promoter. As the CCL18 gene promoter does not contain a hypoxia-response element (HRE), it is therefore not induced by HIF-1 or HIF-2 [50]. In contrast, in lung adenocarcinoma cells, hypoxia reduces CCL18 expression, but the observed effect was not statistically significant [53].

Our results show that the expression of PITPNM3 is higher under hypoxia, which is consistent with the literature data. PITPNM3 is a gene that undergoes increased expression under hypoxia. This is confirmed by studies on PC-3 prostate cancer cells and SK-OV-3 ovarian cancer cells [33].

In our study, hypoxia did not alter CCR8 expression in U-87 MG cells. Currently, there is no data available on the effect of hypoxia on the expression of the CCR8 receptor, which supports the results obtained in whole transcriptome analysis using microarrays using three cell lines, in which no effect of hypoxia on CCR8 expression was demonstrated [33].

3.3. Effect of CCL18 on U-87 MG Cell Proliferation

In the present study, CCL18 had no effect on U-87 MG cell proliferation. These results are partially consistent with the literature data. As shown, CCL18 did not affect the proliferation of BxPC-3 and PANC-1 pancreatic ductal adenocarcinoma cells [54], MGC-803 gastric cancer cells and GES-1 gastric epithelial cells [55]. Nevertheless, the effect of CCL18 seems to depend on the research model. For example, CCL18 has been shown to increase the proliferation of U-251 GBM cells [36], MDA-MB-231 and MCF-7 breast cancer cells [56]. It is likely that the effect of CCL18 on proliferation depends on the expression of receptors and other proteins significant in the induction of proliferation by this chemokine.

3.4. Effect of CCL18 on U-87 MG Cell Migration

In our study, CCL18 induced the migration of U-87 MG cells. These results support literature data showing that CCL18 causes the migration and invasion of breast cancer cells [16], bladder cancer cells [22], hepatocellular carcinoma cells [19], non-small cell lung cancer cells [20] and prostate cancer cells [17]. Our study demonstrated for the first time that hypoxic conditions increase cancer cell migration. Our results have significant implications for understanding cancer tumor function, particularly in GBM, where hypoxia-affected zones which form during tumor development induce cancer cell migration.

4. Materials and Methods

4.1. Patient Samples

The material used in the present study was obtained from patients under surgery for brain tumors who had been diagnosed by neuroimaging (magnetic resonance imaging (MRI) or computed tomography (CT)) at the Department of Neurosurgery and Pediatric Neurosurgery of the Pomeranian Medical University in Szczecin, Poland. The present project and archiving of material were initiated in 2014 by the Department of Biochemistry and the Department of Neurosurgery and Pediatric Neurosurgery of the Pomeranian Medical University in Szczecin and concerned the engagement of purinergic receptors in GBM progression. The project was accepted by the local bioethical commission (KB-0012/96/14) and the study was conducted in accordance with the Declaration of Helsinki.

Tissue samples from tumors were collected during surgery from 28 patients (16 males and 12 females) diagnosed with a central nervous system (CNS) tumor and GBM (Table 1 and Figure 8). All patient brain tumor samples were analyzed in 6 replicates. Patients presented with symptoms resulting from increased intracranial pressure: dizziness andnausea, and those resulting from local tumor growth: sensory and motor disorders as well as disorders of higher nervous functions (Table 2).

Table 1. Statistical characteristics of the study group.

	N	Mean	Standard Deviation	Median	Minimum	Maximum	First Quartile	Third Quartile	InterQuartile Range
Age at surgery	24	60.7	12.5	64	36	81	54	68.5	14.5
Weight	24	84	19	89	55	130	67.5	95	27.5
Height	23	172	12	172	147	196	163	182	19
BMI	23	28.7	4.8	27.9	21.5	38.9	24.7	31.9	7.2
Physical activity	21	3.05	1.16	3	1	4	3	4	1
Limitation of physical activity caused by the tumor	21	2.10	0.83	2	1	3	1	3	2
Limitation of cognitive abilities	21	2.19	0.87	2	1	3	1	3	2

N, number of patients included in the analysis. Physical activity, limitation of physical activity and limitation of cognitive abilities were calculated based on the levels indicated in the questionnaires. Physical activity: everyday, 4; a few times a week, 3; rarely, 2; almost never, 1. Limitation of physical activity: none, 1; partial, 2; considerable, 3. Limitation of cognitive abilities: none, 1; partial, 2; considerable, 3.

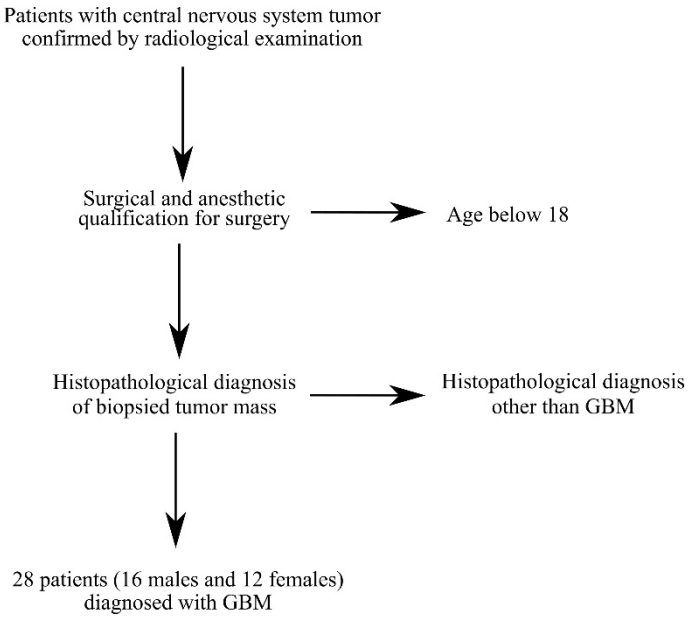

Figure 8. Criteria for selecting patients for the study. Patients were selected based on CNS tumor diagnosis, GBM diagnosis and age over 18 years.

Table 2. Statistical characteristics of the study group.

	N	Mean	Standard Deviation	Median	Minimum	Maximum	First Quartile	Third Quartile	InterQuartile Range
					Men				
Age at surgery	14	60.6	11.9	62	41	81	57.5	67	9.5
Weight	14	93.8	15.4	93	73	130	82.5	97.6	15.1
Height	14	178	8.6	178	163	196	172	184	12
BMI	14	29.5	4.2	28.0	24.7	38.9	27.0	31.7	4.6
Physical activity	12	3.17	1.19	4	1	4	2.75	4	1.25
Limitation of physical activity caused by the tumor	12	2.33	0.78	2.5	1	3	2	3	1
Limitation of cognitive abilities	12	2.5	0.67	3	1	3	2	3	1
					Women				
Age at surgery	12	60.8	14.1	66	36	79	53.5	70.2	16.8
Weight	12	70.3 **	15	63.5	55	95	59	83.5	24.5
Height	11	162 **	8	160	147	173	158	168	10
BMI	11	27.4 *	5.7	25.4	21.5	36.2	22.2	31.9	9.7
Physical activity	11	2.89 **	1.17	3	1	4	3	4	1
Limitation of physical activity caused by the tumor	11	1.78 **	0.83	2	1	3	1	2	1
Limitation of cognitive abilities	11	1.78 **	0.97	1	1	3	1	3	2

N, number of patients included in the analysis. Physical activity, limitation of physical activity and limitation of cognitive abilities were calculated based on the levels indicated in the questionnaires. Physical activity: everyday, 4; a few times a week, 3; rarely, 2; almost never, 1. Limitation of physical activity: none, 1; partial, 2; considerable, 3. Limitation of cognitive abilities: none, 1; partial, 2; considerable, 3. The difference between men and women statistically was significant at ** $p < 0.001$; * $p < 0.05$.

Each patient was recommended for neurosurgery following radiological diagnosis of a CNS tumor. After qualifying for surgery, patients underwent a standard anesthetic procedure (general anesthesia with endotracheal intubation). During the neuronavigation procedure, craniotomy and tumor resection were performed according to the classical method (bone removal and dura incision, tumor visualization, resection, biopsy for histopathological and molecular examination, closure of the dura, bone restoration in some patients, subcutaneous tissue and skin closure in some patients). The range of resection was determined by the extent of the tumor and its topography.

Clinical radiological morphology made it possible to distinguish three tumor zones commonly found in the literature and in clinical practice: the non-enhancing tumor core

(TC) (usually located in the central part of the tumor), the enhancing tumor region (ET) (surrounding the tumor core) and the peritumoral area (PA) (a buffer zone between the tumor and healthy tissue, with individual foci of infiltration) (Figure 9) [57,58]. We considered the peritumoral area as the experimental control; as previously shown, this is a suitable control for GBM-related experiments [59].

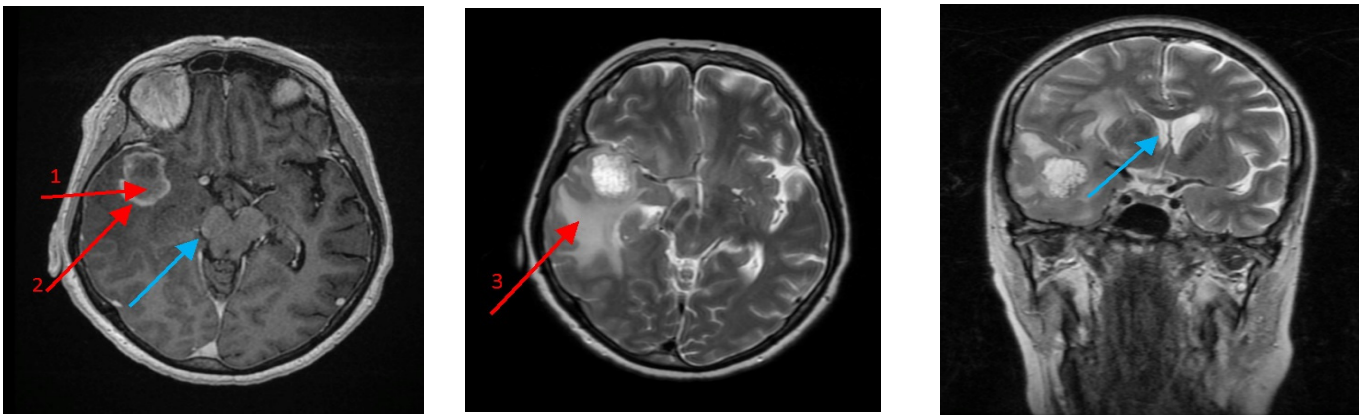

Figure 9. Magnetic resonance imaging (MRI) scan of the brain with the glioblastoma multiforme (GBM) tumor. A 64-year-old female with no antecedent medical history developed a headache, malaise and weight loss prior to hospital admission. On the brain MRI exam, a tumor was revealed in the right temporal lobe of 3–5 cm diameter with a **necrotic tumor core (1)** covered by a marginal **growing tumor area (2)** more dense in Clarisan, i.e., (**a**) On the T2 protocol, a tumor was seen as surrounded with an abundant oedematic area—**peritumoral area (3)**, (**b**) and caused a right brain peduncle compression (**a**) (blue arrow), as well as a lateral ventricle shift, (**c**) (blue arrow). The biopsy revealed GBM, which was then followed by craniotomy and the tumor was removed in gross totally by neuronavigation assistance.

The use of neuronavigation helped in mapping the tumor and determining the topographies of the zones. The results of MRI scans at both 1.5 T and 3 T were entered into the control station of the neuronavigation device and used during the operation to determine the position of surgical instruments in relation to cancer tissue with the assistance of a video camera. The camera monitored the surgical movement in relation to the radiological image to a precision of 2–3 mm. This allowed a safe and reliable resection in places where the image of the operating microscope was uncertain and the macroscopic tumor boundaries were difficult to distinguish. Neuronavigation during biopsy and craniotomy allowed material to be extracted from the three separate zones. Each sample was subjected to histopathological examination to confirm the criteria of a grade IV brain tumor defined by WHO: *IDH* mutation, 1p19q codeletion and *MGMT* gene promoter methylation.

4.2. Cell Culture and Treatment

Human brain cells (glioblastoma astrocytoma, U-87 MG cell line) from the European Collection of Authenticated Cell Cultures (ECACC) were cultured in EMEM medium (Sigma-Aldrich, Poznań, Poland) supplemented with 10% (*v*/*v*) heat-inactivated fetal bovine serum (FBS; Gibco Limited, Poznań, Poland), 2 mM L-glutamine, 1 mM sodium pyruvate (Sigma-Aldrich, Poznań, Poland), 1% non-essential amino acids (Sigma-Aldrich, Poznań, Poland), 100 U/mL penicillin (Gibco Limited, Poznań, Poland) and 100 µg/mL streptomycin (Gibco Limited, Poznań, Poland), at 37 °C in a humidified atmosphere of 95% air and 5% CO_2. The U-87 MG cells were seeded in 6-well plates at a density of 20,000 cells/cm^2 in full medium. After 72 h of incubation (70–80% confluence), the cells were washed three times with pre-warmed phosphate buffer saline (PBS) solution (37 °C). Next, the cells were cultured for 24 h under three different conditions (control, nutrient

deficient and necrotic). The control cells were suspended in a full medium, the starved cells were grown in a medium with a low concentration of L-glutamine (0.2 mM) and without sodium pyruvate (volume supplemented with PBS). For the induction of necrotic conditions, cells were incubated in a medium supplemented with 200 µM H_2O_2. After 24 h of incubation the U-87 MG cells were trypsinized (0.25% trypsin-EDTA solution, Sigma-Aldrich, Poznań, Poland) from the plate. After centrifuging (25 °C, 300 G, 5 min), the supernatant was discarded, the pellet rinsed with PBS and centrifuged again for RNA analysis.

In vitro studies were performed to analyze the influence of hypoxia and nutrient deficiency condition on the gene expression of *CCL18, CCR8* and *PITPNM3*. All cell culture studies were analyzed in 6 replicates in each of the study groups.

Characteristic of GBM is the presence of structures called pseudopalisades in the tumor [60–62]. These structures exhibit hypoxia and nutrient deficiency associated with the long distance or blocked lumen of blood vessels running through the pseudopalisade. To analyze the effect of conditions in pseudopalisades on GBM cells, we examined the effect of hypoxia and nutrient deficiency on the expression of *CCL18, CCR8* and *PITPNM3* genes. For this purpose, the U-87 MG cells were treated with cobalt chloride ($CoCl_2$) (200 mM, Poznan, Poland), a hypoxia-mimetic agent widely used in hypoxia experiments [63,64]. In order to demonstrate properly performed hypoxia-mimetic conditions, the effect of $CoCl_2$ on *VEGF* expression was investigated. *VEGF* is a model gene whose expression is upregulated under hypoxia, such as in incubation with $CoCl_2$ [32,34,47].

In addition, to better reflect the conditions in which GBM cells live, we studied the effect of nutritional deficiency on the expression of *CCL18*, *CCR8* and *PITPNM3* in U-87 MG cells. These cells grew in a medium with a low concentration of L-glutamine (0.2 mM) and without sodium pyruvate. However, they were still exposed to 1.0 g/L (5.5 mM) of glucose in the medium. Under these conditions, the concentration of substances such as mineral salts, vitamins, other amino acids or growth factors did not change.

The aforementioned experimental conditions reflect nutritional deficiency conditions. GBM cells have two sources of energy and building blocks: glucose and glutamine [65,66]. GBM cells convert glucose to pyruvate, which is then metabolized in the Crebs cycle or is converted to lactate. The reduction in pyruvate decreases the availability of glucose already metabolized. GBM cells also need a second component for their metabolism—glutamine [67,68], a source of carbon for proliferating GBM cells. A significant decrease in the concentration of glutamine is part of a nutritional deficiency condition.

4.3. Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Quantitative analysis of the mRNA expression of *CCL18, CCR8* and *PITPNM3* genes was performed by two-step reverse transcription PCR (RT-PCR). Total RNA was extracted from 50–100 mg tissue samples using an RNeasy Lipid Tissue Mini Kit (Qiagen, Hilden, Germany) and, for the in vitro study, from 300,000 cells using an RNeasy Mini Kit. cDNA was prepared from 1 µg of total cellular RNA in 20 µL of reaction volume using a FirstStrand cDNA synthesis kit and oligo-dT primers (Fermentas, Waltham, MA, USA). Quantitative assessment of mRNA levels was performed using an ABI 7500Fast real-time RT-PCR analyzer with Power SYBR Green PCR Master Mix reagent (Applied Biosystems, Waltham, MA, USA). Real-time conditions were 95 °C (15 s), 40 cycles at 95 °C (15 s), and 60 °C (1 min). According to melting point analysis, only one PCR product was amplified under these conditions. Each sample was analyzed in two technical replicates, and mean Ct values were used for further analysis. The relative quantity of a target, normalized to the levels of endogenous controls, *glyceraldehyde-3-phosphate dehydrogenase (GAPDH)*, was calculated as the fold difference (2^{dCt}) and further processed using statistical analysis. Data were presented as tumor tissue absolute expression.

The *GAPDH* reference gene was selected because it is considered a suitable control in research on the expression of various genes in GBM [69]. The following primer pairs were used:

(5′-TCA TGG GTG TGA ACC ATG AGA A-3′ and 5′-GGC ATG GAC TGT GGT CAT GAG-3′) for *GAPDH*,

(5′-CTCTGCTGCCTCGTCTATACCT-3′ and 5′-CTTGGTTAGGAGGATGACACCT-3′) for *CCL18*,

(5′-GTGTGACAACAGTGACCGACT-3′ and 5′-CTTCTTGCAGACCACAAGGAC-3′) for *CCR8*,

(5′-TCGCTTGTCTCACCTGAAC-3′ and 5′-CAGGAACTCTCTGTAGACCTGG-3′) for *PITPNM3*.

4.4. Proliferation

U-87 MG cells were seeded in a 96-well plate at a density of 20,000 cells/cm^2 in a complete medium. After 24 h of incubation, the medium was drawn off and the cells were washed with warm PBS. Then, full medium with either 10 ng/mL, 20 ng/mL or 50 ng/mL of CCL18 (SigmaAldrich, Poznań, Poland) was added to the wells. For each given concentration of CCL18 under study, 8 replicates were performed. The control for each concentration was a sample (8 replicates) with an appropriate amount of PBS added so that the final concentrations of the medium components in the compared samples were equivalent to each other. Cells were incubated in the provided medium for 48 h. Then 20 µL of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) (5 µg/µL, SigmaAldrich, Poznań, Poland) was added to each well and incubated for 2 h in an incubator. In the next step, the culture medium was gently and thoroughly removed and 150 µL of dimethyl sulfoxide (DMSO) (SigmaAldrich, Poznań, Poland) was added to each well and incubated in the dark for 10 min. The absorbance of each well was measured using a microplate reader (EZ Read 2000, Biochrom, Poland) at 590 nm.

4.5. Measuring Cell Confluence Using ImageJ

Micrographs (Leica DM5000B, Wetzlar, Germany, magnification ×4) of chambers from migration assay stained with hematoxyline were analyzed with the use of ImageJ Fiji software (Johannes Schindelin, Albert Cardona, Mark Longair, Benjamin Schmid, and others, https://imagej.net/software/fiji/downloads, version 1.2, accessed on 20 September 2021). First, the downloaded image was opened in Fiji software and color deconvolution was performed (Image > Color > Color Deconvolution), and the hematoxyline option was selected. Next, the threshold was selected (Image > Adjust > Treshold); the minimum threshold value was set at zero and the maximum threshold value was adjusted so that the background signal was removed, without removing the true signal from the hematoxyline stained cells. The percentage of area covered by the cells was measured (Analyze > Measure). Three independent analyses (with a slightly changed maximum threshold) were performed and the results were averaged.

4.6. Migration

U-87 MG cells were seeded in two 6-well plates at a density of 20,000 cells/cm^2 and cultured for 48 h under different conditions. One was cultured in a complete medium with $CoCl_2$ (200 mM, SigmaAldrich, Poznań, Poland) and the other without $CoCl_2$. The medium was then extracted and the cells washed twice with warm phosphate buffer saline (PBS) solution. Full medium with CCL18 (SigmaAldrich, Poznań, Poland) at concentrations of 10 ng/mL, 20 ng/mL and 50 ng/mL, and medium with PBS was added to 5 wells of both plates so that the final concentration of medium components corresponded to the lowest in the other wells. Cells with a complete medium supplemented with CCL18 or PBS were incubated for 16 h. In the next step, the medium was extracted and the cells were washed twice with warm PBS, 1 mL of trypsin-EDTA solution (0.25%) was added to each well and incubated for 4 min until the cells detached. Complete medium was then added and centrifuged (25 °C, 300 G, 5 min). The resulting cell pellets were placed in a medium without FBS but with 1% bovine serum albumin (BSA) (SigmaAldrich, Poznań, Poland). Cells were counted for each well, and the corresponding volume of cell mixtures along

with the corresponding volume of medium without FBS but with 1% BSA was added to the upper chamber of Nunc™ Polycarbonate Cell Culture Inserts in multi-well plates (8.0 UM PC, Life Technologies, Warsaw, Poland) obtaining 1×10^5 cells suspended in 300 µL of medium without FBS but with 1% bovine serum albumin (BSA). 750 µL of medium with 20% FBS was added to the lower chamber. The cells were incubated for 8 h. In a further step, a procedure was performed to fix the cells that migrated through the membrane. For this purpose, Polycarbonate Cell Culture Inserts were washed twice with PBS, then cells were fixed with formaldehyde solution (4%, buffered, pH 6.9) (SigmaAldrich, Poznań, Poland) for 3 min at 25 °C. The Polycarbonate Cell Culture Inserts were washed twice with PBS. In all, 100% methanol (Honeywell, Warsaw, Poland) was added to both chambers and incubated for 20 min at 25 °C. The Polycarbonate Cell Culture Inserts were again washed twice with PBS. The cells were then stained with hematoxylin (SigmaAldrich, Poznan, Poland) for 2 min at 25 °C, the chamber was rinsed twice with PBS, and then the top layer of cells from the Polycarbonate Cell Culture Inserts was wiped off leaving only the cells that had migrated the membrane. To obtain the results of the experiment, the Polycarbonate Cell Culture Inserts were placed on primary slides and the number of cells in each sample was counted using a microscope (Leica DMi1, KAWA.SKA, Poznań, Poland).

4.7. Immunohistochemistry

The dissected glioblastoma were fixed in 10% formalin for at least 24 h and then washed with absolute ethanol (3 times over 3 h), absolute ethanol with xylene (1:1) (twice over 1 h) and xylene (3 times over 20 min). Then, after 3 h of saturation of the tissues in liquid paraffin, the samples were embedded in paraffin blocks. Using a microtome (Microm HM340E), 3–5 µm serial sections were obtained and placed on polysine microscope slides (Thermo Scientific, Altrincham, UK; cat. no. J2800AMNZ). The sections of the glioblastoma were deparaffinized in xylene and rehydrated in decreasing concentrations of ethanol, and then used for IHC reaction.

In order to expose the epitopes to IHC procedure, the deparaffinized and rehydrated sections were boiled twice in Target Retrieval Solution (DacoCytomation, Carpinteria, CA, USA, S2367, S2369) in a microwave oven (700 W twice for 5 min). Once cooled and washed with PBS, the endogenous peroxidase was blocked using a 3% solution of perhydrol in methanol, and then the slides were incubated over night at 4 °C with primary antibodies against: CD68 (Abcam, Cambridge, UK, EPR20545, final dilution 1:5000), CCL18 (also now as MIP-4 from Santa Cruz Biotechnology, Dallas, TX, USA, sc-374438; final dilution 1:250). Antibodies were diluted in antibody diluent with background-reducing components (Dako, Santa Clara, CA, USA, S3022). To visualize the antigen–antibody complex, a Dako LSAB + System-HRP was used (DakoCytomation, K0679), based on the reaction of avidin–biotin–horseradish peroxidase with DAB as a chromogen, according to the staining procedure instructions included. Sections were washed in distilled H_2O and counterstained with hematoxylin. For a negative control, specimens were processed in the absence of a primary antibodies. Positive staining was determined microscopically (Leica DM5000B, Wetzlar, Germany) by visual identification of brown pigmentation.

4.8. Statistical Methods

The expressions of the desaturase genes were calculated in relation to the expression of *GAPDH*. The relative expression values for the three zones (tumor core, enhancing tumor region and peritumoral area) in each patient and their ratios were calculated, e.g., enhancing tumor region/tumor core as a ratio of relative expression in the enhancing tumor region to the relative expression in the tumor core. The distribution of expression values significantly differed from a normal distribution (Shapiro–Wilk test), and therefore statistical analysis was based on nonparametric tests: Mann–Whitney U tests for comparisons between groups of patients, Wilcoxon signed-rank tests for comparisons between the zones of the tumor and in vitro studies. Spearman rank correlation coefficient was used for analysis of correlations between the expressions of the tested genes in the three zones. The median and quartile

values were given as descriptive statistics in tables and graphs. The Kruskal–Wallis ANOVA test was used to test the demographic and basic characteristic of the study group regarding sex. The statistical significance threshold was $p < 0.05$. Calculations were performed using Statistica 13 software.

5. Conclusions and Significance of the Results Obtained

Macrophages accumulate in regions of the GBM affected by hypoxia [70]. The specific conditions in these regions polarize these cells toward M2 macrophages [71]. Macrophages with such polarization show increased expression of CCL18 [72,73]. CCL18 is even considered a kind of M2 marker of macrophages, and hence its first name: alternative macrophage activation-associated CC chemokine 1 (AMAC-1) [74]. Hypoxia causes increased expression of PITPNM3, a receptor for CCL18 in cancer cells [33], which was confirmed in the present study. The increase in CCL18 expression in the tumor cell environment and the increase in PITPNM3 receptor expression in the tumor cell simultaneously stimulate the migration of tumor cells. This mechanism contributes to GBM tumor dissemination and worsens the prognosis for patients with GBM. Experimental data also indicate that the survival of patients with a high expression of PITPNM3 and/or CCL18 have a worse prognosis than those with a lower expression of these genes [39,43], as shown by data from the Human Protein Atlas (Available online: http://www.proteinatlas.org, accessed on 22 August 2021) [37,38].

6. Limitations of the Study

This study showed that macrophages secrete CCL18, which increases the migration of GBM cells sensitized by hypoxia. It also demonstrated gender differences in the expression of receptors for CCL18. Nevertheless, it was associated with certain limitations. First, this study considered patients from a population from a relatively small region that is relatively genetically homogeneous, so the obtained results may differ from a similar study conducted elsewhere. For this reason, the experiment should be replicated in other countries to see whether its results were specifically related to the studied population or GBM in general. Second, the mechanism by which hypoxia sensitizes GBM cells to CCL18 should be investigated in greater detail. Our results indicate that hypoxia increases the expression of PITPNM3, the receptor for CCL18, which points to a potential mechanism in which hypoxia sensitizes GBM cells to CCL18 by increasing PITPNM3 expression. However, we did not focus on the importance of the PITPNM3 receptor in this process, but only on the potential role of CCL18 in GBM tumorigenesis. For this reason, future research should investigate the importance of PITPNM3 in GBM cell migration under hypoxia. Particularly useful could be experiments using siRNA or shRNA targeting PITPNM3 and CCR8 receptors.

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Institutional Review Board Statement: The study was conducted in accordance with the Declaration of Helsinki, and approved by the local bioethical commission (KB-0012/96/14).

Informed Consent Statement: Informed consent was obtained from all subjects involved in the study.

Data Availability Statement: The data presented in this study are available on request from the corresponding author.

Conflicts of Interest: The authors declare no conflict of interest.

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ANEKS

OŚWIADCZENIA WSPÓŁAUTORÓW

Szczecin, dnia 10.01.2023 r.

OŚWIADCZENIE

Dotyczące pracy

Korbecki Jan, **Grochans Szymon**, Gutowska Izabela, Barczak Katarzyna, Baranowska-Bosiacka Irena: *CC chemokines in a tumor: A review of pro-cancer and anti-cancer properties of receptors CCR5, CCR6, CCR7, CCR8, CCR9, and CCR10 ligands*. International Journal of Molecular Sciences 2020 Oct 15;21(20):7619. doi: 10.3390/ijms21207619.

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Szymon Grochans

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Barczak Katarzyna
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Podpis

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podpis

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Grochans Szymon, Korbecki Jan, Simińska Donata, Żwierełło Wojciech, **Rzeszotek Sylwia**, Kolasa Agnieszka, Kojder Klaudyna, Tarnowski Maciej, Chlubek Dariusz, Baranowska-Bosiacka Irena: *CCL18 expression is higher in a glioblastoma multiforme tumor than in the peritumoral area and causes the migration of tumor cells sensitized by hypoxia.* International Journal of Molecular Sciences 2022 Aug 1;23(15):8536. doi: 10.3390/ijms23158536

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Rzeszotek Sylwia
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podpis

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OŚWIADCZENIE

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Grochans Szymon, Korbecki Jan, Simińska Donata, Żwierełło Wojciech, Rzeszotek Sylwia, **Kolasa Agnieszka**, Kojder Klaudyna, Tarnowski Maciej, Chlubek Dariusz, Baranowska-Bosiacka Irena: *CCL18 expression is higher in a glioblastoma multiforme tumor than in the peritumoral area and causes the migration of tumor cells sensitized by hypoxia.* International Journal of Molecular Sciences 2022 Aug 1;23(15):8536. doi: 10.3390/ijms23158536

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Dotyczące pracy

Grochans Szymon, Korbecki Jan, Simińska Donata, Żwierełło Wojciech, Rzeszotek Sylwia, Kolasa Agnieszka, **Kojder Klaudyna**, Tarnowski Maciej, Chlubek Dariusz, Baranowska-Bosiacka Irena: *CCL18 expression is higher in a glioblastoma multiforme tumor than in the peritumoral area and causes the migration of tumor cells sensitized by hypoxia.* International Journal of Molecular Sciences 2022 Aug 1;23(15):8536. doi: 10.3390/ijms23158536

Ja niżej podpisany/a **Kojder Klaudyna**, oświadczam, iż mój wkład w przygotowanie pracy polegał na przeglądzie piśmiennictwa.

Jednocześnie wyrażam zgodę, aby powyższa praca stanowiła części rozprawy doktorskiej lek. Szymona Grochansa.

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Grochans Szymon, Cybulska Anna Maria, Simińska Donata, Korbecki Jan, Kojder Klaudyna, Chlubek Dariusz, Baranowska-Bosiacka Irena : *Epidemiology of glioblastoma multiforme - literature review.* Cancers: 2022 May 13;14(10):2412.doi: 10.3390/cancers14102412.

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Szymon Grochans

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.....Cybulska.....

podpis

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Simińska Donata

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podpis

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podpis

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Ja niżej podpisany/a **Kojder Klaudyna**, oświadczam, iż mój wkład w przygotowanie pracy polegał na przeglądzie piśmiennictwa.

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Klaudyna Kojder

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podpis

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Grochans Szymon, Cybulska Anna Maria, **Simińska Donata**, Korbecki Jan, Kojder Klaudyna, **Chlubek Dariusz**, Baranowska-Bosiacka Irena.: *Epidemiology of glioblastoma multiforme - literature review.* Cancers: 2022 May 13;14(10):2412.doi: 10.3390/cancers14102412.

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