Aus dem Lehrstuhl für Neurologie
Prof. Dr. Ralf Linker
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der Universität Regensburg

Analysis of CCL2/MCP-1 and TNF-α in Serum of Patients with Amyotrophic Lateral Sclerosis Treated with G-CSF and their Relevance as Potential Biomarkers

Inaugural - Dissertation
zur Erlangung des Doktorgrades der Medizin

der
Fakultät für Medizin
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vorgelegt von
Katharina Adriana Prietzel

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Abstract in German

Zielsetzung: Ziel dieser Arbeit ist es, die Entwicklung der Serumspiegel der immunmodulatorischen Zytokine CCL2 und TNF-α während der Progression der amyotrophen Lateralsklerose (ALS) unter G-CSF-Behandlung zu untersuchen und zu bewerten, ob diese Zytokine als Biomarker für den Krankheitsverlauf der ALS-Erkrankung unter G-CSF-Behandlung dienen können. Mit Hilfe valider Biomarker könnte die diagnostische Sensitivität und Spezifität verbessert, die Diagnose der ALS möglicherweise früher gestellt und die individuelle Prognose besser abgeschätzt werden. Dies könnte eine an den individuellen Krankheitsverlauf angepasste, optimierte Therapie ermöglichen.

Material und Methoden: Bei 36 ALS Patienten, die nach individualisierten Therapieregimen mit subkutanem G-CSF behandelt wurden, wird untersucht, ob sich die Patientengruppen der Kurz- und der Langzeitüberlebenden in Bezug auf ihre CCL2- und TNF-α-Ausgangsserumspiegel vor Beginn der G-CSF Behandlung unterscheiden. Außerdem wird die Entwicklung der CCL2- und TNF-α-Serumspiegel während des Krankheitsverlaufs unter der G-CSF Therapie analysiert (zum einen als absolute Werte, zum anderen als relative Werte bezogen auf den Basiswert vor Behandlung). Zusätzlich wird untersucht, ob der CCL2- oder der TNF-α-Basisserumspiegel als methodische Validierung des klinisch gewählten Cut-off-Zeitpunkts als Grenzwert zwischen den kurz- und langzeitüberlebenden Patienten verwendet werden können.

Ergebnisse: Hinsichtlich der Untersuchung auf Unterschiede der Zytokin-Ausgangsserumspiegel zwischen Kurz- und Lanzeitüberlebenden zeigt sich für CCL2, dass sich die Mediane der Ausgangsserumspiegel der Gruppen signifikant unterscheiden und dass der Median der CCL2-Ausgangsserumspiegel der Kurzzeitüberlebenden signifikant höher ist als der der Langzeitüberlebenden. Für TNF-α zeigt sich kein signifikanter Unterschied der Mediane der Ausgangsserumspiegel der beiden Gruppen.

Hinsichtlich der Untersuchung auf Unterschiede zwischen den Kurz- und den Langzeitüberlebenden unter der Behandlung zeigt sich für CCL2, dass der Median der absoluten CCL2 Serumspiegel der Kurzzeitüberlebenden höher ist als der Median der absoluten CCL2 Serumspiegel der Langzeitüberlebenden. Der Median der relativen CCL2 Serumspiegel der Kurzzeitüberlebenden hingegen ist im Durchschnitt niedriger als der der Langzeitüberlebenden. Für TNF-α zeigt sich erneut, dass sich die Mediane

der absoluten und relativen TNF-α Serumspiegel der Kurzzeitüberlebenden nicht signifikant von denen der Langzeitüberlebenden unterscheiden.

Ob sich unter der Behandlung insgesamt signifikante Veränderungen der Zytokinserumspiegel einstellen, wurde untersucht, indem die Mediane der Zytokinserumspiegel nach Behandlungsbeginn mit den Medianen vor Behandlungsbeginn verglichen wurden. Für CCL2 zeigt sich, dass der Median der Zytokinserumspiegel nach Behandlungsbeginn signifikant niedriger ist als der Median der Ausgangsserumspiegel. Für TNF-α zeigt sich, dass der Median nach Behandlungsbeginn signifikant höher ist.

Ob sich nach Behandlungsbeginn unter dem Einfluss wiederholter G-CSF Applikationen kontinuierliche und dementsprechend im zeitlichen Verlauf zunehmende signifikante Veränderungenen der Zytokin-Serumspeigel einstellen, wurde untersucht, indem die Steigungen der einfachen linearen Regressionen der Serumspiegel analysiert wurden. Für CCL2 zeigt sich, dass die absoluten Serumspiegel der Kurzzeitüberlebenden vor den G-CSF-Gaben im Behandlungsverlauf signifikant sinken. Für TNF-α zeigt sich, dass die absoluten TNF-α-Serumspiegel der Langzeitüberlebenden nach den G-CSF-Gaben und unabhängig vom Status der G-CSF-Behandlung unter dem Einfluss der G-CSF-Therapie signifikant sinken. Es zeigt sich außerdem, dass die relativen TNF-α-Serumspiegel der Langzeitüberlebenden vor den G-CSF-Gaben unter dem Einfluss der G-CSF-Therapie signifikant steigen, während die relativen TNF-α-Serumspiegel der Langzeitüberlebenden nach den G-CSF-Gaben signifikant sinken.

Ob sich der Ausgangsserumspiegel zur methodischen Validierung des gewählten Grenzwerts von 30 Monaten nach Behandlungsbeginn zur Gruppenzurodnung eignet, wurde untersucht, indem die Höhe des P-Werts als Funktion des Unterschieds der Ausgangsserumspiegel zwischen Kurz- und Langzeitüberlebenden zu den unterschiedlichen möglichen Grenzwerten analysiert wurde. Für CCL2 zeigt sich, dass die Patienten am besten anhand ihres Ausgangsserumspiegels als Kurz- oder Langzeitüberlebende klassifiziert werden können, wenn der Grenzwert zur Gruppenzuordnung zwischen 29 und 31 Monaten nach Behandlungsbeginn liegt. Für TNF-α lassen sich keine signifikanten Unterschiede hinsichtlich des TNF-α Ausgangsserumspiegels zwischen Kurz- und Langzeitüberlebenden feststellen.

Fazit: Im Bezug auf CCL2 weisen die Ergebnisse dieser Arbeit darauf hin, dass der absolute CCL2-Basisserumspiegel ein geeigneter Biomarker mit prädiktivem Wert für

das kurz- und langfristige Überleben unter G-CSF-Behandlung ist, und dass höhere absolute CCL2- Basisserumspiegel mit einem schwereren Krankheitsverlauf bei ALS-Patienten (auch) unter G-CSF Therapie einhergehen. Der abslute CCL2-Basisserumspiegel ist zudem zur methodischen Validierung des in der vorliegenden Arbeit gewählten Grenzwertes zur Unterscheidung der Patienten in Kurz- und Langzeitüberlebende geeignet. Es lässt sich außerdem ableiten, dass der absolute CCL2-Serumspiegel während der Behandlung ein geeigneter Biomarker mit prädikativem Wert für die Schwere des Verlaufs und die Überlebensdauer bei ALS Patienten unter G-CSF-Behandlung ist, und dass höhere absolute CCL2-Serumspiegel bei Patienten unter G-CSF-Therapie mit einem kürzeren Überleben assoziiert sind. Zudem stützen die Ergebnisse die Annahme, dass die G-CSF Behandlung den CCL2 Serumspiegel bei ALS Patienten senkt, da die zentralen Tendenzen der absoluten und relativen CCL2 Follow-up-Werte trotz fortschreitender Erkrankung mit zunehmender Neuroinflammation im Behandlungsverlauf signifikant niedriger sind als die der Ausgangsserumspiegel.

Was TNF-α betrifft, kann aus den Ergebnissen dieser Arbeit gefolgert werden, dass der absolute TNF-α-Basisserumspiegel nicht mit der Überlebensdauer von Patienten unter G-CSF-Therapie assoziiert ist und sich nicht als prognostischer Parameter für die Schwere des Krankheitsverlaufs und die Überlebensdauer eignet. Auch zur methodischen Validierung des in dieser Arbeit gewählten Grenzwertes zur Unterscheidung der Patienten in Kurz- und Langzeitüberlebende ist der absolute TNF-α-Basisserumspiegel nicht geeignet. Es lässt sich außerdem ableiten, dass der absolute TNF-α-Serumspiegel während der Behandlung kein geeigneter Biomarker für die Schwere des Erkrankungsverlaufs oder die Überlebensdauer bei ALS Patienten unter G-CSF-Behandlung ist. Die Ergebnisse stützen die Hypothese, dass der TNF-α-Serumspiegel bei ALS Patienten auch unter G-CSF-Therapie ein geeigneter Biomarker für das Ausmaß der Neuroinflammation ist, da die zentralen Tendenzen der absoluten und relativen TNF-α Follow-up-Werte im Rahmen der fortschreitenden Erkrankung unter der G-CSF Behandlung signifikant höher sind als die der entsprechenden Ausgangsserumspiegel. Theoretisch könnte dies aber auch darauf hinweisen, dass die G-CSF-Behandlung die TNF-α Serumspiegel langfristig erhöht und der antiinflammatorische Effekt von G-CSF bei ALS Patienten nicht durch eine Senkung des TNF-α-Serumspiegels zustande kommt.

List of Abbreviations

6-OHDA	6-hydroxydopamine
Aβ	amyloid beta
AD	Alzheimer's disease
ALS	amyotrophic lateral sclerosis
ALSFRS	Amyotrophic Lateral Sclerosis Functional Rating Scale
ALSFRS-R	Revised Amyotrophic Lateral Sclerosis Functional Rating Scale
AP-1	Activator protein 1
APP	amyloid precursor protein
APP/PS1 mice	double transgenic mice expressing a chimeric mouse/human amyloid precursor protein (Mo/HuAPP695swe) and a mutant human presenilin 1 (PS1-dE9)
Arg1	arginase 1
AUC	area under the curve
BBB	blood-brain barrier
BMC	bone marrow-derived cells
BMEC	brain microvascular endothelial cells
C9orf72	chromosome 9 open reading frame 72
CCL2	chemokine (C-C motif) ligand 2, also known as monocyte chemoattractant protein 1 (MCP1), respectively JE, the murine analog
CCL2-	chemokine (C-C motif) ligand 2 deficient
CCL7	chemokine (C-C motif) ligand 7, also known as monocyte chemoattractant protein 3 (MCP3)
CCL8	chemokine (C-C motif) ligand 8, also known as monocyte chemoattractant protein 2 (MCP2)
CCL12	chemokine (C-C motif) ligand 9, also known as monocyte chemoattractant protein 5 (MCP5)
CCL13	chemokine (C-C motif) ligand 13, also known as monocyte chemoattractant protein 4 (MCP4)
CCR2	C-C chemokine receptor type 2, also known as cluster of differentiation 192 (CD192)
CCR2-	C-C chemokine receptor type 2 deficient
CCR2ko	C-C chemokine receptor type 2 knock-out

CD	Cluster of Differentiation
CNS	central nervous system
COX2	cyclooxygenase-2, also known as Prostaglandin-endoperoxide synthase 2 (PTGS2)
CSF	cerebrospinal fluid
CX3CL1	chemokine (C-X3-C motif) ligand 1
CX3CR1	CX3C motif chemokine receptor 1, also known as fractalkine receptor or G-protein coupled receptor 13 (GPR13)
CXCR2	CXC motif chemokine receptor 2, also known as Interleukin 8 receptor (IL8RB)
DN-TNF	dominant-negative TNF inhibitor, a class of biologics that selectively inhibits soluble tumor necrosis factor-alpha
Dx	diagnosis
EAE	experimental autoimmune encephalomyelitis
FADD	Fas-associated protein with death domain
fALS	familial ALS
Fc region	fragment crystallizable region, the tail region of an antibody that interacts with cell surface receptors
FIZZ1	Found in Inflammatory Zone 1, also known as Resistin-like Molecule (RELM) alpha
FUS/TLS	RNA-binding protein FUS/TLS (FUsed in Sarcoma/Translocated in LipoSarcoma)
G93A	missense mutation G93A (SOD1-G93A transgenic mice express a G93A mutant form of human SOD1 and are the most frequently used animal model to study ALS)
GAG	glycosaminoglycan
G-CSF	Granulocyte-Colony Stimulating Factor, also known as colony-stimulating factor 3 (CSF 3)
GM-CSF	Granulocyte-macrophage colony-stimulating factor, also known as colony-stimulating factor 2 (CSF2)
G protein	guanine nucleotide-binding protein
GTP	guanosine-5'-triphosphate
HBMvECs	Human brain microvascular endothelial cells
hCMEC/D3	human Cortical Microvessels Endothelial Cells/D3, also known as Human blood-brain barrier cell line, Human cerebral endothelial

	cell line, CMEC/D3 cell Line, a patented cell line that is a model of human blood-brain barrier function
HSC	hematopoietic stem cells
huGFAP	human glial fibrillary acidic protein
IFN-γ	Interferon-γ
IgG	Immunoglobulin G
IL	interleukin
IL-1RA	interleukin-1 receptor antagonist protein
IL-4Rα	interleukin 4 receptor alpha
lenti-DN-TNF	a lentiviral vector encoding a dominant-negative TNF
LPS	Lipopolysaccharide
M1	classically activated macrophage
M2	alternatively activated macrophages
Matplotlib	plotting library for the Python programming language and its numerical mathematics extension NumPy
MBEC4	mouse brain capillary endothelial cells, a transformed cell line
M-CSF	macrophage colony-stimulating factor, also known as colony-stimulating factor 1 (CSF1)
MHC	major histocompatibility complex
MMSE	Mini-Mental-State-Examination
MPTP	1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine, used as an animal model for Parkinson's disease
MS	multiple sclerosis
mSOD1	mutant Cu2+/Zn2+ superoxide dismutase
mTNF-α	membrane-bound tumor necrosis factor-alpha
NADPH	Nicotinamide adenine dinucleotide phosphate
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
NO	nitric oxide
NO_2^-	nitrogen dioxide
NumPy	Numerical Python
Pandas	Python Data Analysis Library
PBMC	peripheral blood mononuclear cells

PD	Parkinson's disease
PEG	polyethylene glycol
PET	positron emission tomography
RIP	receptor-interacting protein
ROS	reactive oxygen species
sALS	sporadic ALS
SciPy	Scientific Python
SD	standard deviation
SOCS3	Suppressor of cytokine signaling 3
SOD1	Cu2+/Zn2+ superoxide dismutase
sTNF-α	soluble tumor necrosis factor-alpha
sTNFR1	soluble Tumor necrosis factor receptor 1
sTNFR2	soluble Tumor necrosis factor receptor 2
T1 cells	T helper type 1 cells, also known as Th1 cells
T2 cells	T helper type 2 cells, also known as Th2 cells
tg	transgenic
TNF-α	tumor necrosis factor-alpha
TNFR1	tumor necrosis factor receptor 1, also known as tumor necrosis factor receptor superfamily member 1A (TNFRSF1A) or as Cluster of Differentiation 120a (CD120a)
TNFR2	tumor necrosis factor receptor 2, also known as tumor necrosis factor receptor superfamily member 1B (TNFRSF1B) or as Cluster of Differentiation 120b (CD120b)
TRAF	tumor necrosis factor receptor-associated factor
Tx	treatment
VE-cadherin	vascular endothelial cadherin, also known as Cadherin 5, type 2 or Cluster of Differentiation 144 (CD144)
WT	wild type
Ym1	also known as chitinase-like protein 3 (Chil3)

1 Introduction

1.1 Inflammation of the Central Nervous System (CNS): Common Feature of Pathologies in Neurodegenerative Diseases

It is becoming increasingly evident that neuroinflammation is a crucial aspect of most neurodegenerative diseases and a key determinant of the disease process. Its development depends on a complex interplay of various cells of innate and adaptive immunity, interacting in a non-cell autonomous, well-orchestrated dialogue.

Practically every neurological impairment entails an inflammatory reaction leading to proliferation and alteration of the phenotype of glial cells, a phenomenon termed ‘‘reactive gliosis’’. An acute insult, like trauma, hypoxia, and stroke, impairs neuronal functioning and results in neuroinflammation, as microglia get activated, acquire a phagocytic phenotype, and produce inflammatory cytokines and chemokines. By reducing further tissue injury and promoting the repair process, acute neuroinflammation is in general primarily beneficial to the CNS. Although acute neurodegenerative pathologies may temporarily cause nitrosative and oxidative stress, they are only short-lived and usually not deleterious to neuronal health.

Contrary, chronic neuroinflammation is prolonged and often self-sustaining and persists for a long time after an initial lesion or injury. The permanent inflammation is associated with a continuous increase in neurotoxic substances and pro-inflammatory mediators such as cytokines and chemokines, produced by proliferating glial cells and by infiltrating peripheral monocytes and other leukocytes. These secreted factors are strongly detrimental to the neuronal cells in the long term. Neurodegenerative CNS disorders including amyotrophic lateral sclerosis (ALS), but also other diseases like multiple sclerosis (MS), Alzheimer's disease (AD), and Parkinson's disease (PD), are associated with chronic neuroinflammation and elevated levels of several cytokines. Thus, the duration of the inflammatory response as well as the nature of the microglial activation may determine the protective or harmful consequences of an inflammatory reaction in the CNS (1, 2).

The role of the immune system during the progression of ALS is highly complex, however, prominent neuroinflammation and the associated cellular responses of the resident glia and infiltrating immune cells are pathological hallmarks of the disease.

1.2 Amyotrophic lateral sclerosis

ALS, also known as Lou Gehrig's disease, is the most common adult-onset motor neuron disease. It is a devastating progressive neurodegenerative disease, characterized by the selective loss of motor neurons at all levels of the motor system: motor cortex, brainstem, and spinal cord. ALS is a heterogeneous disorder with various underlying gene mutations and a relatively great pathophysiological and phenotypic variability (3). Depending on the site of disease onset, early-stage symptoms include proximal or distal limb muscle weakness, palsy, dysarthria, or dysphagia. This initial stage is followed by upper motor neuron signs like hyperreflexia, spasticity, cloni, Hoffmann, and Babinski signs. As the disease progresses, muscle atrophy, fasciculations, and paralysis occur due to the degeneration of lower motor neurons and the corresponding muscles. Further symptoms such as cognitive impairments and/or behavioral dysfunctions arise in about 50% of ALS patients (4). Up to half of the individuals present with mild changes in behavior and cognition, and 15% experience symptoms of frontotemporal dementia. ALS is typically fatal within 3–5 years of symptom onset, in most cases due to final respiratory insufficiency. So far, there is no satisfactory treatment for ALS that will stop or even significantly delay its inexorable progression. The glutamate antagonist riluzole and the antioxidant edaravone are currently the only drugs approved and recommended for ALS by the FDA. However, nothing but the small benefits of a slightly increased life expectancy (in weeks) with decelerated disease progression and prolonged disease duration can be achieved by these treatments.

More than 90% of ALS incidences are believed to be sporadic; only about five to ten percent of patients have a family history of ALS. Except for a few instances, the majority of familial ALS (fALS) mutations are dominant. Familial cases are associated with a wide range of genetic mutations, among them the most investigated and best characterized form of fALS, caused by a mutant gene encoding Cu2+/Zn2+ superoxide dismutase (SOD1) (5). This mutation accounts for approximately 20% of people with fALS and leads to motor neuron degeneration due to a toxic gain of function of this enzyme. Five percent of patients with the diagnosis of sporadic ALS (sALS) also have SOD1 mutations. Other fALS-causing mutations include the well-known mutation in the gene for TAR binding protein 43 (6), mutations in the FUS/TLS gene (7), and in the C9orf72 gene (8), among a great variety of other underlying mutant genes. The onset of ALS most frequently occurs in the fifth or sixth decade of life. The juvenile form of

ALS, defined by a disease onset before the 25th year of life, is less common and is often characterized by a comparatively slow disease progression (9). Apart from these genetic predispositions identified to date, there are also several non-genetic risk factors for ALS concerning lifestyle, occupation, and environment (10).

Even though the exact mechanisms of motor neuron degeneration in ALS remain to be further elucidated, potential molecular pathways involve mitochondrial dysfunction (11, 12) and likely include abnormalities in protein aggregation and disorganization of intermediate filaments (13, 14), as well as glutamate-mediated excitotoxicity (15) and deficiencies in intracellular calcium homeostasis (16–18). The pathogenesis of motor neuron degeneration in ALS involves excitotoxicity due to a highly augmented calcium influx caused by abnormal activation of glutamate receptors (19). Injury and loss of motor neurons and associated astrocytic gliosis are the pathological hallmarks of ALS. Furthermore, so-called Bunina bodies, small eosinophilic intracellular inclusions, consisting of cystatin C and transferrin among other components, can be found in degenerating lower motor neurons and in motor neurons of different cranial nerve nuclei and might be pathognomonic for ALS (20).

Numerous studies have been conducted to gain deeper insights into the pathomechanisms of ALS. For this purpose, a broad range of in vitro and in vivo models of the disease have been developed. The best studied in vivo model of ALS is the genetically engineered SOD1 mouse, which overexpresses the missense mutation described above and shows age-dependent degeneration of motor neurons accompanied by limb weakness. Contrary to several other models of neurodegenerative diseases, the SOD1 mouse model effectively reproduces neuronal cell death in a manner that resembles the profile of cell death seen in the human disease and thus provides a valuable model to study the interplay of neurodegeneration and the immune system. (21)

1.3 The Double Role of Immunity in ALS

The relevance of immune-mediated mechanisms in the pathogenesis of ALS is widely accepted and neuroinflammation is a prominent feature at sites of motor neuron degeneration. It implies infiltration of T cells and monocytes, astrogliosis, and microglial activation. Innate as well as adaptive immunity actively affect disease progression in ALS patients and in in vivo disease models. Many findings have established the

hypothesis that the immune response can be separated into two different phases: a more protective Type 2 response followed by a more toxic Type 1 response (22, 23).

At the earliest stage of ALS disease, during the so-called presymptomatic or stable phase, the immune response to signals from stressed or dying neurons involves a protective cytokine environment in an attempt to prevent further neuronal injury and support repair processes. This phase is called the T2 stage of the immune response. It includes M2-like microglia (discussed below) and regulatory T cells. It is beneficial by promoting the clearing of debris, tissue repair, synaptic pruning, and release of neurotrophic factors. During the T2 stage microglial cells produce the chemokine CCL2 (further discussed below) to attract monocytes. Due to an altered cytokine milieu, regulatory T cells, which slow down disease progression (24–26), and Th2 effector cells (CD4-positive), which are neuroprotective in ALS (27–29), accumulate in the CNS. These two cell types maintain an anti-inflammatory environment by expression of IL-4, TGF-β, and IL-10 (24, 25).

As the pathgophysiologic process and thus ALS deteriorates and the disease burden increases, a shift to a detrimental immune response takes place. It significantly aggravates the disease condition, entailing a rapidly changing cytokine profile, M1-like microglia, and T1 cells. Levels of inflammatory markers continuously rise during the disease course (30). Pro-inflammatory cytokines of the T1 phase induce astrocytic dysfunction, enhance motor neuron injury, and activate glial cells. The activated glial cells recruit peripheral monocytes as well as T cells to the injured CNS, resulting in exacerbation of the disease. In the T1 phase high amounts of inflammatory cytokines such as TNF-α (further discussed below), IFN-γ, and IL-6 attract T1 cell types or promote their differentiation in ALS mouse models (22, 29) and similarly in ALS patients. Microglia get polarized towards an M1-like phenotype, cytotoxic CD8 T cells are recruited to the brain and spinal cord and naive CD4 T cells develop into T1 effector cells (22, 23). Large numbers of monocytes are still attracted, and in this later phase of the disease, they are induced to become macrophages or M1 dendritic cells (22, 23).

Importantly, however, the transformation from T2 to T1 immune response, as described above, happens incrementally, partly incomplete, and takes several months in mice and likely months until years in human patients. This shift observed in ALS takes place in reversed order to the typical immune response after an infection or trauma, in

which there is an aggressive T1 phase followed by a T2 phase inducing debris removal and supporting repair mechanisms. One possible hypothesis is that the T2 to T1 shift happens due to the gradual polarization of immune cells in the spinal cord and brain. In this context, one glial cell type, the microglia (further discussed below), deserves particular attention, as its phenotypical and functional changes seem to play a pivotal role in this transition during the disease course of ALS.

Altered cytokine profiles have an important influence on the microglial transition from neuroprotective to neurotoxic immune cells and accordingly on the disease progression of ALS. The particular role of two cytokines, known for their relevance during the human immune response, namely CCL2, and TNF-α, will be further discussed in the following and will be investigated in this work.

In ALS, several pathophysiological processes can be targeted to alter and slow down disease progression. However, so far no truly satisfactory drug has yet been found to treat this lethal disease. One promising candidate for decelerating disease progression is the neurotrophic immunomodulatory glycoprotein G-CSF (further discussed below). Many researchers have already investigated the effects of G-CSF in neurodegenerative diseases such as AD, PD, and ALS and several studies have demonstrated the anti-inflammatory properties of G-CSF.

1.4 Question and Objective

This work aims to investigate whether the immunomodulatory cytokines CCL2 and TNF-α have a relevant influence on the progression of ALS in patients under G-CSF treatment and whether they could act as biomarkers for the disease course of ALS under G-CSF treatment to improve diagnostic specificity and sensitivity, to potentially accelerate the diagnosis of ALS, and to better assess individual prognosis.

In the present work, ALS patients were treated with repeated subcutaneous G-CSF injections, applied either in specific intervals or continuously on single days, in an attempt to modify and slow disease progression. Changes in CCL2 and TNF-α serum levels were analyzed with increasing disease progression. It was examined if the cytokine serum levels or the respective changes in cytokine serum levels correlate with the patients' survival duration or G-CSF treatment status. Based on these investigations, the possible influence of CCL2 and TNF-α on the progression of ALS and their potential as biomarkers in patients under G-CSF treatment is analyzed and discussed.

2 Theoretical Background

2.1 Primary Immune Defense of the Brain: The Microglia

2.1.1 Activation and Functions, M1-/M2-like Reaction

Microglia, which account for 10-20% of the glial cells, are a unique population of resident macrophages of the brain and spinal cord. They play a vital role in the initiation and development of neuroinflammation. Within the parenchyma of the CNS, their distribution varies, being more abundant in the gray than in the white matter. The morphology and the density of microglia are region-specific (31), and there is a range of different microglial subpopulations with distinct features (32, 33). Generally, microglial cells found in meningeal and perivascular areas are more macrophage-like whereas parenchymal microglia display a highly ramified phenotype (34). All microglial cells originate from precursor cells of the myeloid lineage which invade the CNS tissue during embryonic development and can display a great variety of different functions (35–37). The phenotypic appearance and the functions of microglial cells differ depending on their state of activation. The activation state, in turn, is influenced by the surrounding physiological milieu. Different subsets of microglia exert both neuroprotective and neurotoxic functions (38). Already during early development, immunologically active microglia colonize the CNS and are involved in its organization and formation. In the normal adult brain microglial cells are in a resting state, which is maintained by signals arising from surrounding neuronal and glial cells.

In their quiescent state the microglia show a downregulated phenotype: As the local cells of the innate immune defense, these ramified, highly mobile cells of stellate morphology use their fine, ceaselessly palpating, extending, and retracting sinuous processes to continuously sample and monitor their immediate microenvironment. The quiescent microglia produce different secreted factors that can act both in an autocrine and paracrine manner and are regulated by soluble factors as well as directly by cellular interactions (39, 40). They are in close contact with their neighboring cells. Receptor-ligand interaction with the neighboring cells is of great importance in keeping or rapidly changing their state of activation. Neurons produce CD200 and fractaline, which stop microglia from becoming neurotoxic. Under healthy conditions in the normal brain, microglia get inhibitory stimulation via the fractaline receptor CX3CR1 which binds chemokine CX3CL1 that is continuously released, predominantly by healthy

neurons. Microglial cells display functional plasticity in response to activation. In vivo, CX3CR1 deficiency dysregulates microglial functions and leads to cell-autonomous microglial neurotoxicity (41, 42). If the repressive signaling of CX3CL1 is removed under pathologic conditions, microglia transform into active phagocytic amoeboid-like cells with altered surface receptor expression and changed expression of cytokines and growth factors. These changes can happen in response to either systemic inflammation (43) or local CNS damage including disruption of the blood-brain barrier, changes in neurotransmitter levels or loss of neuronal stimuli, neuronal injury, cell death, or any other disturbance of neuronal homeostasis (39).

The former concept that microglia steadily proceed from resting to activated states in a linear process, varying merely by degree of activation is now replaced by the notion that microglia engage different modes of progression from a downregulated state to cells holding various and specific activation-associated effector functions in diverse disease conditions. Their response is dictated by the type of stimulation, the range of different involved receptors, and the prior state of the microglia, depending on the way the morphology is already changed depending on previous stimulation. Following a distinct primary stimulus, including circulating cytokines like TNF-α, IFNγ, M-CSF, or GM-CSF, the resting microglia become primed. This results in an altered morphology with thickened soma and shorter and fewer processes. They are increasing the surveillance of their microenvironment and present antigens via MHC class II molecules. As soon as the primed microglia are exposed to secondary stimuli, for instance, TNF-α, IL-1, or IL-6, they attain a state of maximal activation and release different inflammatory mediators (44, 45). This implies that the microglial response to a given stimulus is strongly dependent on a prior activation event or ‘‘priming’’ by a previous stimulus.

Although some research questions this assumption (46, 47), it is widely accepted that in several neurodegenerative diseases, microglial activation states can be subclassified into classically activated microglia (M1-like) that initiate a pro-inflammatory response, and into alternatively activated microglia (M2-like) which are more of a protective type. Quite similar to macrophage activation programs, which can be distinguished by the associated release of cytokines, arginine metabolism, and antigenicity (48), the microglial activation state seems to be determined by the local cytokine milieu and by the activating stimulus (40, 48). Also, after completion of CNS development, microglia are still highly mobile under certain circumstances as in CNS damage or disease. To

reach their target sites during neuroinflammatory processes they have to migrate through dense extracellular matrix. It is therefore obvious and could be proven that classically and alternatively activated microglia differ in morphology, cytoskeleton, migration and invasion capacity, and use of enzymes (49).

M1-like microglia polarize to the M1 phenotype upon exposure to pro-inflammatory cytokines, such as IL-1β, IL-6, IFN- γ, and TNF-α, and cellular and bacterial debris. Other known inducers of M1-like microglia are the TLR4 agonist LPS and IFN-γ. To eliminate the foreign pathogen and facilitate an adaptive immune response, M1-like microglia produce nitric oxide (NO), potent reactive oxygen species (ROS), COX2, and CX3CL. They enhance the release of pro-inflammatory cytokines like TNF-α, IL-1β, and IL-6 and increase the expression of redox molecules and antigen-presenting molecules. Their secretion of neurotrophic factors is decreased. (50–53)

Contrary to this, M2-like microglia block pro-inflammatory responses and contribute to the resolution of inflammation by secretion of anti-inflammatory cytokines (54), neurotrophic factors, including brain-derived neurotrophic factor and glial cell line-derived neurotrophic factor (33), IGF-1, IL-4 and IL-10, one of the most potent autocrine inhibitors of pro-inflammatory cytokine production. Whereas usually T1 signaling is associated with phagocytosis of pathogens, M2-like microglia of the T2 response perform clearance of apoptotic cells and cellular debris. Due to their specific gene expression profile, three distinct M2-like microglial phenotypes can be distinguished. M2a microglia are referred to as the type I of alternatively activated microglia that facilitate the resolution of neuroinflammation. Microglia of the M2a-like phenotype result from stimulation with IL-4 or IL-13, important immunosuppressive mediators of the CNS, and produce increased levels of arginase-1, FIZZ1, and Ym1 (24, 55). M2b microglia are considered to be type II alternatively activated microglia and are associated with increased phagocytic and immunomodulatory activity. Microglia of the M2b-like phenotype are induced by immune complexes and ligands of the Toll-like receptors. Markers useful to identify M2b-immunomodulatory microglia include increased IL-1RA and SOCS3 (56). Microglia of the M2c-like phenotype, also referred to as the Type III of alternatively activated microglia or as the acquired deactivation phenotype, develop due to stimulation by the anti-inflammatory cytokines IL-10 or TGF-β and show an upregulated expression of CCR2 and scavenger receptors (57) as well as increased levels of IL-10 and TGF-β and enhanced IL-4Rα, Arg1, SOCS3 and CD206 expression

(56). They are associated with anti-inflammatory actions, tissue repair, and clearance of myelin debris.

Restrictively, it must be mentioned that although microglia can be polarized into distinct activation states, the specific functions of these states are still a debated issue. Due to the advances in the understanding of the different states of activated microglia, the debate focuses increasingly on their relative contributions and functions in neuroinflammatory diseases. This has led to increasing interest in translational human studies. The transfer of the in vitro results with mostly murine cells to human M1- or M2-like cells is not without limitations. Since several commonly used markers, such as Arg1 and Ym1, are not expressed in human myeloid cells, the ability to identify distinct human microglial phenotypes is somewhat restricted. However, other markers appear to be consistent in human myeloid cells, so there is strong evidence that human microglia have a similar spectrum of activation states with the respective functions, defined by the environmental milieu in normal and disease conditions (for review see (58)).

2.1.2 Microglia in ALS

One of the major topics of current research about microglia is their dichotomy between neuroprotective and neurotoxic responses and the dual role they play in the complex process of neurodegeneration (33, 59). It has been shown that microglia influence the different pathomechanisms underlying neuronal damage in many CNS diseases such as Alzheimer’s disease, Parkinson’s disease, multiple sclerosis, and other neurodegenerative pathologies and may strongly contribute to disease onset, progression, and extent. Non-cell-autonomous processes and microglia-mediated cytotoxicity also play a crucial role in the pathogenesis of ALS in the development of motor neuron degeneration. Microgliosis at sites of neuronal injury is a neuropathological hallmark of ALS. Different microglia alterations precede disease onset and happen during the early symptomatic stage (60). Microglial activation is widespread during the disease course (61, 62), and has been shown to correspond with neuronal degeneration in in vivo models of ALS (30).

Different studies using transgenic mice carrying the mutant SOD1 (mSOD1) have evidenced that expression of mSOD1 in motor neurons alone is not sufficient to cause ALS and suggest that the involvement of non-neuronal cells is required. In these studies, the expression of mSOD1 in motoneurons alone did not cause significant motor

neuron death. Remarkably, motor neurons without mSOD1 gene mutation showed signs of ALS pathology when surrounded by mSOD1 expressing cells. Moreover, the non-neuronal cells that were free of mSOD1 slowed down degeneration and significantly prolonged the survival of mutant SOD1-expressing motor neurons (63, 64). Some authors therefore suggest the possibility that microglia might be the cells in which mutant SOD1 acts to initiate fALS in the transgenic mouse models (64). Also, based on the analysis of several other studies, microglia are considered a contributor to motor neuron injury in ALS (65).

Predominantly based on in vitro studies microglial responses to an inflammatory stimulus in murine models of ALS were investigated. Some of these studies aimed to examine the potential effect of mSOD1 on microglial cells. In one study, primary cultured microglia from mSOD1-transgenic mice were compared to microglia from non-transgenic litter mates (66). Lipopolysaccharide (LPS), a well-known activator of microglia, was used as inflammatory stimulus. The authors reported that mSOD1 expression increased the production of TNF-α and attenuated IL-6-release by LPS-activated adult microglia. In contrast, neonatal microglia showed no difference between wild-type and mSOD1 cells in terms of cytokine expression under both unstimulated and LPS-stimulated conditions. Accordingly, the authors proposed the possibility of an increased cytotoxic potential of adult mSOD1 microglia, which only becomes apparent after microglial activation (66). In another in vitro study, microglia isolated from the mutant human SOD1 G93A transgenic mouse model of ALS were compared to SOD1 wildtype microglia and to microglia from non-transgenic littermate controls (67). To detect the activation state of quiescent microglia and the extent of activation by the inflammatory stimulus LPS, the morphological properties of SOD1 G93A and SOD1 wildtype microglia were compared to microglia from respective non-transgenic littermate controls in this study. It was reported that SOD1 G93A microglia showed altered morphological properties and had an increased response to lipopolysaccharide (LPS) used as an inflammatory stimulus. The authors suggested that SOD1G93A microglia cause a neurotoxic microenvironment due to elevated inflammatory cytokine and ROS expression, with attenuated phagocytic activity (67). Taken together, these results indicate that depending on their activation state, microglia may develop cytotoxic and neurodegenerative potential in ALS by increased expression of inflammatory cytokines.

It has not yet been fully proven that these in vitro findings about microglial responses apply equally in vivo. However, in numerous in vivo models of ALS in mice, it was shown that the use of healthy microglia instead of mutant SOD1 microglia had little effect on disease onset and early disease phase, but delayed later disease progression and slowed motor neuron loss (68). Moreover, lowering microglial mutant SOD1 expression in mSOD1 transgenic mice by intraventricular injection of a selective toxin against the monocyte/macrophage system and subsequent replacement of mSOD1 microglia by bone marrow transplantation of wild-type microglia prolonged survival in mice significantly and slowed down the disease (69). One study examined the role of donor-derived mSOD1 microglia in PU.1 knockout transgenic mice that are unable to develop myeloid and lymphoid cells and therefore lack macrophages, neutrophils, T and B cells, and microglia (70). Interestingly, this study found that bone marrow transplantation of donor-derived mSOD1-expressing microglia did not induce ALS-like symptoms typical of mSOD1-tg mice. Interestingly however, in double-transgenic mice with PU.1 knockout and mSOD1 expression, transplantation of wild-type donor-derived microglia slowed motor neuron loss and prolonged survival when compared to mice receiving mSOD1-expressing cells (70). These observations suggest that expression of mSOD1 alone in microglia does not appear to be sufficient for the development of ALS-like symptoms in mice, but that mSOD1 microglia play a critical role in survival duration when other cell types in the animals also carry the SOD1 mutation. These findings are consistent with earlier in vivo animal studies reporting that expression of mSOD1 in motoneurons alone is not sufficient to induce or accelerate motoneuron disease in mice (64) and expression of mSOD1 in non-neuronal cells contributes to motor neuron degeneration and disease progression (63).

There is strong evidence that activated microglia hold a trophic role at the early disease stage (40). However, microglia seem to lose their surveillance potential and switch to a neurotoxic phenotype as the disease progresses. Decreased neuroprotective activity of mSOD1 microglia was found in a resting state (71) and at the disease end-stage (72). There are conflicting opinions about eventual microglial polarization during the disease course of ALS. Although there are also opposing views, based on recent reports which rather support the idea of unique neuron disease-specific microglial neurodegenerative characteristics instead of a typical M1-like or M2-like microglial phenotype (72), it is generally assumed and commonly described in in vitro studies with

mSOD murine cells as models of ALS that morphologically activated microglia lose their prior M2-like anti-inflammatory phenotype after the early phase of the disease and acquire a pro-inflammatory M1-like phenotype during ALS disease progression and end-stage (for review see (21, 56)).

Although these studies with in vivo mouse models of ALS using mSOD murine cells indicate that microglial activation is a fundamental point of convergence of stimuli that induce or accelerate neuronal degeneration, it is important to distinguish between murine and human microglia as well as between animal models of ALS, in which clinical progression is relatively predictable because of genetic homogeneity, and human disease, where heterogeneity of disease is the rule. The fact that transgenic mouse experiments provide only limited insights into the heterogeneity of the disease and the variable rates of progression in ALS patients needs to be considered and kept in mind when debating how inflammation might affect the human CNS during ALS. It is important to take into account that the cells might react differently to the inflammatory state in vivo in ALS patients. Unfortunately, therefore the leap to human ALS disease is not completely without problems, due to the lack of in vivo studies with murine models of ALS or translational studies with in vitro or in vivo human cells of individuals with ALS. Still, altered cytokine profiles and altered microglial properties appear to similarly potentiate the inflammatory response in human ALS. It is well established that changes in the immune response in ALS, including microglial transformation during disease progression, are mediated by the complex interplay of disease-specific changes in the levels of cytokines and chemokines, two of particular interest being CCL2 and TNF-α.

2.2 The Chemokine CCL2

2.2.1 General Definition of Chemokines

Chemokines are a family of structurally related cytokines; small homologous chemoattractant low-molecular-weight signal proteins, primarily known for their capability to induce migration and activation of leukocytes (73). They can be divided into four different subfamilies according to function, gene localization, and structure (74). All share the structure of four cysteine residues that differ in their relative position. On this basis, they can be subclassified by the number of amino acids separating the two most NH2-terminal located cysteine residues (C, CC, CXC, and CX3C) (75). The beta chemokine subfamily (CC subfamily), distinguished by the absence of amino acid between the two

conserved cysteines in adjacent positions at the amino-terminal end of the molecule, is one of the two major chemokine families. Members of the CC-subfamily mainly act on eosinophils, T lymphocytes, monocytes, and basophils.

Chemokines can be expressed by various cell types and are involved in a great diversity of processes and physiological functions, including development, cell proliferation and growth, leukocyte trafficking, and T cell polarization of the immune response (74, 76). These functions are all mediated by ligand interaction with different low- and high-affinity receptors on the surface of distinct target cells (77). Generally speaking, chemokines can be divided into two groups: inducible chemokines which are especially expressed after physiological stress, and constitutive chemokines, which are necessary for basal leukocyte migration and other functions. Contrary to cytokines, chemokines are highly specific in their effect and act on particular leukocyte subpopulations and dendritic cells without pleiotropic effects (78).

It was first proposed that chemokines could play a vital role in various CNS diseases because of their highly selective stimulation of the migration of specific leukocyte subsets to the intrathecal space during different pathologic processes. It was shown in vitro and in vivo that parenchymal CNS cells belong to the major producers of chemokines (74). In the CNS, chemokines regulate homeostasis and influence the differentiation and proliferation of neuronal and glial cells as well as axonal guidance and cytokine production. They attract and activate immune cells, as they contribute to the infiltration of bloodborne monocytes through the blood-brain barrier and to the recruitment of resident astrocytes and microglia. Chemokines moreover influence glial reactivity (79) and act on neurons by affecting cell survival and neuronal activity (80). Thus, they are of great importance in the inflammatory response in most CNS pathologies (81).

2.2.2 The MCP Family

The beta chemokine subfamily contains the important group of the monocyte chemoattractant proteins. It consists of five structural similar molecules (82), classified as CCL2 (MCP1), CCL8 (MCP2), CCL7 (MCP3), CCL13 (MCP4), and CCL12 (MCP5) (83) which share about two-thirds of sequence homology (82). There is some redundancy between the different MCPs since they show overlapping ligand-receptor interaction. One MCP can stimulate several receptors, and an individual receptor can bind

more than a single MCP, but still, they all differ in chemoattractant potency and act on distinct leukocyte target populations.

2.2.3 General Aspects of CCL2 Biology: Structure and Biological Function of CCL2 and its Receptor CCR2

CCL2, also known as monocyte chemoattractant protein-1 (MCP-1) (respectively JE, the murine analog), is located on chromosome 17 (chr.17, q11.2), consists of 76 amino acids (82), and has a molecular weight of approximately 13-15 kDa, depending on levels of glycosylation. By mutational analysis, it could be revealed that the biological activity of CCL2 depends on two specific regions of the primary structure. The first domain comprises the sequence from Thr-10 to Tyr-13, whereas the second important segment is formed by residues 34 and 35 (84). Apart from this, it has also been reported that the integrity of the NH2-terminal residues 1-6 is critical for CCL2 function, since their deletion results in a loss of activity (85). Noteworthy, some N-terminus deletion mutants appear to function as CCL2 antagonists and have been shown to inhibit MCP-1 activity in vitro and in vivo (86, 87). The NH2-terminal residues 7-10 are involved in receptor desensitization (85). The secondary structure of CCL2, which forms symmetrical homodimers in solution is composed of two helical regions and four regions of β-sheet that consist of residues 9–11 (β0), 27–31 (β1), 40–45 (β2), and 51–54 (β3) (88). The quaternary structure of CCL2 shows similarities to the chemokines RANTES and MIP-1β in many respects. (for detailed review of the structure and function of CCL2 see also (89))

Numerous cell types have been found to express CCL2, including monocytic, mesangial, epithelial, endothelial, and smooth muscle cells as well as microglia, astrocytes, and fibroblasts (90–95). However, macrophages and monocytes appear to be the main source of CCL2 production. CCL2 was the first characterized human CC chemokine (96, 97), and since then various studies have demonstrated its powerful chemoattractant effects and its ability to induce transmigration and activation of monocytes in vitro and in vivo. Predominantly cells of the myeloid lineage like monocytes/macrophages and microglia, but also T cells, B cells, and dendritic cells migrate to sites of inflammation according to a concentration gradient of CCL2 (78, 98–103). Moreover, CCL2 and its main receptor CCR2 also play a significant role in T1- or T2-polarized immune responses (104–108). Thus the chemokine can be regarded as an important actor at the intersection of adaptive and innate immunity.

CCL2 signaling happens mostly by interaction with its primary receptor CCR2, but also CCR2-independent signaling pathways have been reported (109), among others the cell surface receptor CCR4 (110). The main receptor of CCL2, CCR2 is a member of the so-called G protein-coupled receptor superfamily, seven-transmembrane domain molecules that mediate intracellular signaling through heterotrimeric GTP-binding proteins (83). CCR2 interacts with multiple ligands and can for example be stimulated also by at least four other pro-inflammatory chemokines of the MCP family in vitro (CCL7, CCL8, CCL12, and CCL13) (111) apart from its most powerful activator CCL2. However, in vivo, CCL2 is commonly estimated to be the only functionally relevant ligand of CCR2, with the CCL2/CCR2 signaling network being of great importance in a broad range of physiologic and pathologic processes. In contrast to rodents, which express only one CCR2 isoform, humans have two distinct isoforms of the CCR2 receptor, CCR2A and CCR2B (112), with alternatively spliced carboxyterminal tails. CCR2A is expressed by mononuclear cells and vascular smooth muscle cells, whereas CCR2B is the dominantly expressed form, accounting for about 90% of total CCR2, and is prevalent in monocytes and activated natural killer cells (113). Both isoforms use different signaling pathways (114), a finding that indicates that the human CCL2-CCR2 axis might be regulated already at receptor levels (115). Notably, CCR2 signaling can induce both pro- and anti-inflammatory responses. Its pro-inflammatory function depends on receptor expression by T cells and antigen-presenting cells, while its anti-inflammatory role is related to regulatory T cells (89).

Apart from interaction with its G protein-coupled receptor CCR2, secreted CCL2 also binds glycosaminoglycan (GAG) chains of cell surface proteoglycans and soluble GAGs (116). This mechanism is estimated to increase the local chemokine concentration at the production site and thereby enables the formation of a haptotactic and chemotactic chemokine gradient which functions as a directional signal for migrating cells. Additionally, this GAG binding mechanism is reported to induce dimerization/oligomerization of CCL2 (117, 118). There are contradictory theories, about whether this dimerization can be regarded as indispensable for the in vivo function of CCL2 (118), or whether CCL2 functions rather as a monomer (for review see (115)). Yet, it is generally assumed that the receptor CCR2 binds dimerized CCL2 and is thereupon internalized and removed from the cell membrane (119), a process which might contribute to the regulation of extracellular CCL2 levels, acting as a self-limiting feedback mechanism

(120–122). Receptor activation by CCL2 sets off several different intracellular transduction pathways such as signaling via downstream targets like mitogen-activated protein kinases (123, 124), phosphatidylinositol-3-OH kinase and protein kinase C (125), implying a broad spectrum of heterogeneous cellular responses.

2.2.4 CCL2/CCR2 Expression in the Brain

CCR2 expression in the CNS is limited to specific cell types. Cells of the immune system, namely dendritic cells, T cells, and monocytes (82) have been observed to constitutively express this receptor. Apart from this, CCR2 expression has been shown in human fetal astrocytes (126) and human neurons (127), as well as in microglia (128), astrocytes, and cholinergic and dopaminergic neurons (80, 129) in the rat brain.

Contrary to the restricted expression of its receptor, the chemokine CCL2 can be secreted by a large number of different cell types. It is expressed constitutively in the brain but is also involved in diverse CNS pathologies as a critical mediator of neuroinflammation (78).

Under physiologic conditions, CCL2 is detectable in comparatively low concentrations in astrocytes (90, 130) and neuronal cells (131) as well as in microglial and endothelial cells at a minimal level. In some neuroanatomical regions, CCL2 is found in arginine vasopressin magnocellular neurons, as well as in melanin-concentrating hormone-expressing neurons (131). Furthermore, CCL2 can be co-localized with classical neurotransmitters, since it is present in cholinergic and dopaminergic neurons in specific areas of the CNS (131). The neuronal expression in the healthy brain raises the possibility that CCL2 could be an important modulator of neuronal functioning and might be involved in neuroendocrine regulation.

In numerous neurodegenerative diseases, CCL2 is of high relevance as a mediator of inflammatory processes in the CNS. Under conditions of pathological neuroinflammation, astrocytes and microglia represent the most important sources of upregulated CCL2 production but also epithelial cells as well as infiltrating macrophages and, to a lesser extent, lymphocytes contribute to the highly elevated levels of CCL2 expression (132). This indicates that cell recruitment and induction of migration into the brain parenchyma are regulated and maintained at least partly in an autocrine manner. Increases in microglial or astrocytic CCL2 production have been reported in acute CNS pathologies such as cerebral ischemia (133–135) and traumatic brain injury (132, 136,

137), as well as in chronic neurodegenerative disorders like multiple sclerosis (138–140), Alzheimer's disease (141), ALS (62, 142) and several others.

2.2.5 CCL2 and CCR2: Role in the Development of T1 versus T2 Immunity

It is commonly known and has often been documented that the presence of CCL2 exerts a significant influence on the T1/T2 immune response polarization. CCL2 is primarily considered to be a pro-inflammatory mediator of the T1 phase of the inflammatory response. However, several studies also link CCL2 signaling to the T2 phase of the immune response. Still, the role of CCL2 in the T2 immune response might be less pronounced compared to its role in the T1 immune response. Interestingly, the nature of the immune response appears to vary by context and specific immune response, possibly also influenced in part by whether the chemokine binds to its major receptor or interacts with other receptors to trigger different cellular responses via CCR2-independent signaling pathways. Thus, the issue of CCL2-mediated T1 versus T2 polarization requires a differentiated approach.

Several authors described that expression or in vitro administration of CCL2 correlates with the initiation of a T2-type immune response (108, 143, 144). Apart from this, it was repeatedly reported that CCL2 stimulates T cell-dependent IL-4 production which is characteristic of the T2 phase of immunity, and CCL2 neutralization results in decreased IL-4 levels (145–148).

Contrary to its ligand, several studies using CCR2 knockout mice indicated that the receptor promotes the induction of a T1-type response. Animals deficient for CCR2 showed reduced levels of interferon γ (IFN γ), probably due to impairments in the recruitment of antigen-presenting cells (149). Moreover, they had significant defects in the production of other cytokines typical for the T1 immune response (121, 150, 151). The absence of CCR2 led to deficits in the initiation of an efficient T1 response and resulted in a shift to a T2 response instead (152).

The cause of these remarkable disparities in the mediation of immunity types between CCL2 and its major receptor is yet unknown, but it seems possible that CCL2 signaling might happen via another receptor in case of CCR2 deficiency.

Several findings query the general validity of the strict CCL2/CCR2 dichotomy regarding the presumed opposing T polarization activity of ligand and receptor. One study

associated CCL2 deficiency with an enhanced T2 immune response and contrarily a reduced T1 response (107). Other authors described that the production of IFN γ observed in CCR2 deficient cells compared to CCR2 positive cells varied, depending on different time points of measurement (153). At an early stage, CCR2-/- cells secreted considerably fewer amounts of IFN γ than CCR2 +/+ cells, whereas a second measurement revealed a similar production in both cell types. At a later stage, levels of secreted IFN γ were even higher in CCR2-deficient mice than in CCR2-positive animals (153). These observations suggest a delay instead of an impairment in the induction of T1 immune response in the absence of CCR2. Another group observed that CCL2 neutralization as well as CCR2 deficiency in mice led to decreased leukocytic IFN-γ production and elevated secretion of the cytokines IL-4 and IL5, characteristic of a T1 to T2 shift of the immune response (154). In this study, antigen-specific IFN-γ-producing cells were detected in CCL2-neutralized animals but not in CCR2-deficient mice. Upon stimulation, cells derived from CCR2-negative animals produced significantly reduced amounts of T1 response-associated cytokines in comparison to CCL2-neutralized or WT mice, probably due to impaired trafficking of antigen-presenting cells. The observation that CCL2 neutralization in CCR2 deficient mice did not affect the development of the immune response indicates that the T2 polarization was not due to the comparatively high concentrations of CCL2 in these mice (154). An in vivo study using differently immunized mice, resulting in either a T1 or a T2 immune response (depending on the type of immunization), reported that overexpression of CCL2 at the site of immunization during different stages of T1- and T2-type granulomatous responses led to led to inconsistent effects (155). In this study, CCL2 overexpression only partly altered cytokine expression, and alterations of the cytokine profiles were shown to depend on the respective phase of the immune response.

Accordingly, overall, it seems quite conceivable that the T1 or T2-inducing activity of CCL2 and its receptor is influenced or probably even determined by additional factors such as the inflammatory milieu, the type of antigen, the localization of inflammation, the timing of CCL2 production, or the stage and type of immune response.

2.2.6 The CCL2-CCR2 Axis in CNS Pathologies with Neuroinflammation

2.2.6.1 CCL2 and Blood-Brain Barrier Permeability

The blood-brain barrier (BBB) consists of brain microvascular endothelial cells (BMECs) with tight junctions, astrocytes, and pericytes. Together with the endothelial and the parenchymal basal laminae, these cells build the "neurovascular unit" and separate the fluid spaces of the systemic compartment and of the CNS. Its main function is to enable neuronal functioning by maintaining homeostasis in the brain due to control of the highly selective transport processes between CNS parenchyma and circulation. BBB compromise is an important hallmark of many neurodegenerative disorders and BBB breakdown is often associated with disease progression. Recent findings suggest that the occurrence of BBB perturbation may contribute to the onset of AD and ALS as a causative factor. CCL2 upregulation can be found in many CNS disorders with BBB injury, and accumulating evidence indicates that CCL2 might affect the integrity of the BBB, leading to increased permeability and thereby enhancing disease progression (119, 156). Under in vitro conditions, cells in astrocyte/BMEC co-cultures treated with CCL2 have been reported to show redistribution of several tight junction proteins (157, 158). Furthermore, BMECs exposed to CCL2 appear to undergo considerable alterations in their actin cytoskeleton and increase the formation of stress fibers (159). Notably, CCR2 deficiency markedly reduces the effect of CCL2 on the BBB in vitro and in vivo. The morphological changes of BMECs described above, which critically impair the barrier function in the in vitro BBB model also seem to lead to hyperpermeability of the BBB in vivo. They have not been observed in the absence of CCR2 in cultured endothelial cells (157, 158), nor in mice lacking this receptor (157). Moreover, apart from its direct effects on cells of the BBB, CCL2 also induces disruption of the BBB indirectly by the infiltration of peripheral immune cells. It has been shown that BBB opening correlates significantly with CCL2-mediated leukocyte recruitment and that depletion of peripheral monocytes before CCL2 treatment considerably reduces BBB permeability in mice (157).

But even though many studies have been conducted in recent years to elucidate the modulatory effects of CCL2 on the BBB properties (for detailed review see (115)), the specific interactions and exact molecular mechanisms remain to be further explored.

2.2.6.2 Consequences of CCL2 Overexpression in the Brain

Under normal conditions, CCL2 expression in the CNS happens at consistently low levels. However, genetically engineered mice overexpressing this chemokine provide the opportunity to investigate the consequences of highly elevated CCL2 levels in the brain. There are several transgenic mouse models of overexpression, varying in terms of different promotors controlling CCL2 expression (160–164).

Using the myelin basic protein gene promotor, which is present in cells intrinsic to the CNS, allows for determining the effects of CCL2 overexpression selectively in the brain and thymus without an increase in other organs. Taking advantage of this fact, previous studies have demonstrated that transgenic mice show augmented migration of mononuclear cells into the brain, confirming CCL2 as a primarily monocytic chemoattractant in vivo (160, 162). This elevated infiltration was further enhanced by LPS administration, indicating that the chemoattractant properties of CCL2 can be amplified by exposure to other inflammatory stimuli (160). A subsequent study produced similar results since CCL2 overexpressing mice were showing a pronounced mononuclear cell infiltration. Moreover, an increased sensitivity and a higher lethality in response to systemic LPS injection could be observed in these mice (162). Additionally, it was reported that cerebral ischemia led to remarkable increases in infarction size when comparing transgenic CCL2 overexpressing mice to wild-type controls. The ischemic damage in the CCL2 overexpressing mice was related to the perivascular accumulation of invading macrophages and neutrophils (162).

Other studies use mice overexpressing CCL2 under the control of the human glial fibrillary acid protein promoter (huGFAP-CCL2hi tg mice) (161, 164). One of these studies reported that although no destruction of neuronal tissue was detected, the transgenic mice developed neurological impairments termed delayed encephalopathy and exhibited perivascular leukocyte infiltration and blood-brain barrier disruption (164). Furthermore, the study found that microglia in huGFAP-CCL2hi tg mice exhibited morphological signs of activation but could not acquire an amoeboid phenotype. These observations indicate that CCL2 overexpression causes an abnormal morphologic transformation and may change the process of microglial activation. In line with these findings are the results of a subsequent study by the same group. In this study, administration of Pertussis toxin plus Freud's complete adjuvant in tg mice with high levels of astrocytic CCL2 overexpression caused serious symptoms of a rapid-onset,

transient encephalopathy accompanied by perivascular leukocyte infiltrates (161). Noteworthily, mice overexpressing CCL2 without the inflammatory stimulus of Pertussis toxin and Freud's complete adjuvant injection failed to develop signs of neurological impairment in this study.

Taken together all these findings support the hypothesis that CCL2 overexpression in the CNS leads to a highly exaggerated immune response to inflammatory stimuli, often resulting in neurological damage.

2.2.6.3 CCL2 in other Neurodegenerative Diseases

It is known that CCL2 plays a significant role in various neurodegenerative diseases. A multitude of in vitro and in vivo studies have been conducted, confirming the potential of this chemokine as a possible biomarker in brain tissue, cerebrospinal fluid (CSF), and blood within the realm of neurodegeneration.

As for MS, CCL2 was reported in both active and chronic MS autopsy tissue (139, 140, 165) and also the experimental autoimmune encephalomyelitis (EAE) animal model of MS provides evidence for a major role of CCL2 in the disease process. In murine EAE, there is a significant predominantly astrocytic upregulation of CCL2 mRNA expression during the acute and relapsing phase of the disease with a correlation between the extent of CNS inflammation and disease severity (166–169) and also infiltrating neutrophils, macrophages, and CD8 T cells express CCL2 during EAE(170). A study performed with CCL2 knockout mice found that animals deficient for CCL2 proved to be relatively resistant to EAE, likely due to diminished local macrophage recruitment into the CNS mice (107). Other studies analyzed the consequences of CCR2 deficiency in EAE mice and confirmed a non-redundant role of CCR2-mediated signaling in EAE. Contrary to wild-type mice, most CCR2-deficient animals did not display clinical disease symptoms (105, 106) and did not develop histological signs of sustained inflammation or axonal demyelination (105). In human MS, expression of CCL2 and CCR2 has been reported in three compartments, namely the brain, cerebrospinal fluid (CSF), and blood (138, 139, 171–176). Studies using autopsy tissue from MS patients demonstrated an upregulation of CCL2 in the brain parenchyma (139, 140, 165, 176). Remarkably, in contrast to the elevated expression reported in lesions in the CNS parenchyma, numerous authors measured consistently decreased CCL2 protein levels in the cerebrospinal fluid (CSF) of MS patients in comparison to healthy controls or to

patients suffering from other neuroinflammatory or noninflammatory neurological diseases (171, 173, 177–181). Contrary to the coherent findings about CCL2 levels in MS CSF, the results of studies on CCL2 serum levels due to chemokine production by peripheral blood mononuclear cells (PBMC) in MS patients are strongly divergent. Whereas some studies reported a decrease in serum CCL2 levels in MS patients (177, 182), others observed elevated CCL2 levels in serum (183) or in supernatants of unstimulated PBMCs from MS patients (184) and again others found no significant difference in serum CCL2 levels between MS patients and control subjects (185) and reported no evidence of systemic dysregulation of CCL2 in MS (186).

In the context of AD it has frequently been proposed that a change in CCL2 expression and possibly even function could be a prognostic marker for the development of AD, and that CCL2 levels might reflect the severity or progression rate of the disease. CCL2 first came into scientific focus in association with the pathogenesis of AD when it was found that CCL2 expression is enhanced in reactive microglia and mature senile plaques in autopsy tissue samples derived from AD patients (141). Only shortly after, the upregulation of CCL2 was also demonstrated in brain microvessels (187), neurons, and astrocytes (188) of AD patients. Different research groups investigated the potential impact of CCL2 on the production or clearance of Aβ, as well as the reciprocal influence of Aβ on CCL2 expression in the brain. Several in vitro tests revealed that incubation with Aβ stimulates the synthesis of significant amounts of CCL2 in peripheral monocytes (189, 190), microglia (190), and astrocytoma cells (191) as well as in primary cultured astrocytes (192, 193), oligodendrocytes (193), and human brain endothelial cells (194). In in vivo studies transgenic AD mice showed significant upregulation of CCL2 correlating with increased microglial and macrophage numbers in the entorhinal cortex (195). Other scientific approaches investigated whether CCL2 has an impact on Aβ accumulation. An in vivo study using transgenic AD mice overexpressing CCL2 (APP/CCL2 mice) found that APP/CCL2 mice showed elevated numbers of recruited mononuclear phagocytic cells, but also accelerated and enhanced Aβ aggregation in the cortex and hippocampus (163). Other authors observed early memory deficits and impairments of synaptic transmission in APP/CCL2 mice and reported that CCL2 did not reduce Aβ degradation but promoted microglial Aβ uptake and intracellular oligomerization and increased Aβ deposition (196). The results of another study, using an in vitro as well as an in vivo approach indicated that CCL2 and

APP both stimulate mononuclear phagocytic cell-mediated Aβ clearance synergistically (197). In transgenic CCL2 deficient AD mice (APP/PS1/CCL2- mice) it was observed that CCL2 deficiency caused an increase in both soluble Aβ oligomers and Aβ aggregates compared to non-deficient APP/PS1 mice and that microglial cell accumulation around plaques and microglial Aβ phagocytosis were decreased and neurogenesis was reduced in CCL2-deficient animals (198). The upregulation of CCL2 and a possible association with the accumulation of Aβ and phosphorylated tau protein were also investigated in human AD patients, where a positive correlation was observed between CCL2 CSF concentrations and CSF Aβ levels as well as between CCL2 CSF values and CSF phosphorylated tau levels (199). One aspect of major clinical interest is the question of whether CCL2 may be used as an indicator of the severity or progression rate of AD and various studies have measured CCL2 levels in CSF and serum/plasma samples to evaluate the potential role of the chemokine as a biomarker for AD with heterogeneous results. It was repeatedly reported that CCL2 CSF levels were increased in AD patients (199, 200) and also a recent meta-analysis found that CCL2 CSF levels were significantly elevated in AD patients (201). As for analyses of serum or plasma CCL2 levels in AD, some authors reported that plasma CCL2 levels were significantly higher and CCR2 expression was decreased on CD14+ monocytes in AD patients (202). Another study examined serum CCL2 levels in different patient subgroups and reported elevated serum CCL2 concentrations in subjects with MCI and subjects with mild to moderate AD, but not in patients with severe AD as compared to controls (203). Two other studies detected lower baseline CCL2 concentrations in PBMC of subjects with AD than in healthy individuals (204, 205).

2.2.6.4 CCL2 in ALS

The interrelations between neuroinflammation and ALS pathogenesis are solidly established. However, what remains to be further explored is how specific immune pathways and proteins affect the disease. Consistent with the observations in other neurodegenerative conditions, recent discoveries suggest a significant involvement of the chemokine CCL2 in the pathogenesis and disease progression of ALS.

2.2.6.4.1 CCL2 in Models of ALS in Vitro and in Vivo

To investigate the association between CCL2 expression and ALS pathology, different studies have been conducted, utilizing the mutant human SOD1 mouse model of ALS.

Results of an in vitro study with cultured microglia derived from mSOD1 transgenic mice allow the conclusion that microglia are an important source of the increased CCL2 levels found in ALS patients and in the ALS mouse model since CCL2 production was found to be more than 3 fold higher in mSOD1 microglia compared to SOD1 wild type microglia under activating conditions (67). By examining mSOD1 murine spinal cord tissue, it was demonstrated that CCL2 mRNA and immunoreactivity were upregulated in mSOD1 neuronal and glial cells already at early stages of the disease, preceding any signs of microglial activation or manifest symptoms of the disease (62). Spinal cord tissue CCL2 mRNA levels were considerably elevated in mSOD1 mice at all ages in this study. Other authors compared the effects of CCL2 on astrocytic proliferation rate, examining primary cultured astrocytes from mSOD1 mice and non-transgenic littermates (95). In this study, administration of recombinant murine CCL2 significantly and dose-dependently stimulated proliferative activity in cells of ALS mice whereas no increase was found in control cultures. The same study also analyzed CCL2 and CCR2 mRNA expression in murine lumbar spinal cords. Levels were quantitatively compared between tissue samples of mSOD1 mice and controls and additionally between presymptomatic, onset, and postsymptomatic animals. An age-dependent CCL2 upregulation was detected in the ALS mice, but not in the spinal cord of non-transgenic littermates. At all disease stages, CCL2 mRNA concentrations were markedly higher in mSOD1 animals compared to age-matched controls. CCR2 mRNA levels were significantly increased in presymptomatic and onset mSOD1 mice than in postsymptomatic animals or age-matched non-transgenic mice. Whereas CCL2 immunoreactivity was primarily observed in spinal cord motor neurons, staining for CCR2 was mainly detected in astrocytes of ALS mice (95). Another group of authors reported that specific splenic monocytes (Ly6Chi monocytes) expressed a M1-like macrophage phenotype, correlating with a significant upregulation of CCR2, before disease onset and at the terminal stage of the disease in a mouse model of ALS (206). This upregulation of CCR2 was paralleled by an increased CCL2 production in spinal cord microglia at disease onset.

2.2.6.4.2 CCL2 in Human ALS

A broad variety of different studies evaluated CCL2 levels in human ALS disease by examining CNS tissue, CSF, serum, and plasma of ALS patients. CCL2 mRNA upregulation and augmented CCL2 protein levels have been observed in glial cells and

macrophages in spinal cord tissue samples of ALS patients compared to samples of control subjects with other neurological diseases (62), and a substantial increase of CCL2 values in comparison to controls was also found in the CSF (62, 142, 201, 207–215) and the serum or plasma of ALS patients (142, 209, 210, 214–216) as compared to controls, although blood levels were usually less elevated than CSF levels. However, a recent meta-analysis study did not find any significant differences in CCL2 blood levels between ALS patients and controls (217).

Concerning the association between CCL2 levels in CSF and disease duration, progression rate, or severity, one study reported a trend towards higher CSF CCL2 values in ALS patients with a shorter time between the appearance of first symptoms and final diagnosis (207). In line with this another study found that higher CCL2 CSF levels were associated with a faster disease progression (209). In addition, other authors observed that CCL2 CSF levels in ALS patients were negatively correlated with the ALSFRS-R score, suggesting that higher CCL2-CSF levels are associated with a more severe disease state (211). In contrast to these studies, another report found a positive correlation between CCL2 concentrations in the CSF of ALS patients and scores on the Norris Total Scale, a scale used to assess ALS disease status, with high scores indicating a mild disease state (215). Accordingly, this study reported that lower CCL2 CSF levels were associated with a more severe disease state. No association between CCL2 CSF levels and disease duration was found in this study. In another report, no association was found between CCL2 levels in CSF and disease duration or disease severity (212).

As for CCL2 blood levels, a negative correlation between CCL2 concentrations and ALSFRS-R scores of ALS patients and a positive correlation with disease progression rate was reported, suggesting that higher CCL2 levels in serum and CSF are associated with worse disease severity and faster disease progression (209). In line with this are the results of another study that found that plasma levels of CCL2 correlated positively with symptom durations in ALS indicating higher levels of circulating CCL2 being present at a more severe disease state (218).

2.3 The Cytokine TNF-α

2.3.1 General Aspects of TNF-α Biology: Structure and Biological Function of TNF-α and its Receptors

The human TNF-α gene is located on chromosome 6 between 6p21.1 and 6p21.3, within the human leukocyte antigen class III region. TNF-α is synthesized as a 233-amino acid-long 26 kDa polypeptide precursor, arranged in stable homotrimers (219). The nonglycosylated type II transmembrane protein is cleaved into a soluble 51 kDa homotrimer by the metalloprotease TACE (TNF-α converting enzyme)/ADAM17 (220). TNF-α is a soluble trimer and consists of three protomers with a molecular weight of 17 kDa. Each subunit is 185 amino acids long and is composed of two anti-parallel beta-sheets (221). Both the membrane-bound (mTNF-α) as well as the soluble form (sTNF-α) are biologically active and can interact with the two receptors TNFR1 (p55/CD120a) and TNFR2 (p75/CD120b), which mediate complex signaling cascades described in more detail below.

TNF-α is an important pro-inflammatory cytokine inducing pleiotropic responses in a broad variety of cells. Since its versatile autocrine and paracrine effects are often cell type dependent and determined by specific intracellular transduction pathways, TNF-α is implicated in a broad range of physiological and pathological processes. These processes comprise gene induction, cellular proliferation, and differentiation, but also inflammation, immunity as well as apoptosis, and necrosis (221–224). Generally, the cytokine's principal role is in the regulation of immune cells. As a critical mediator of the immune response, TNF-α initiates and amplifies inflammatory processes, for instance by recruiting leukocytes to sites of inflammation. Its effects on the immune response are modulated by the general context and stage of an inflammatory reaction. An abnormal or excessive TNF-α production is involved in a great number of human diseases. TNF-α is a member of the large TNF ligand family and is mainly produced by activated macrophages or monocytes, but to a lesser extent also by a variety of other cells. In the CNS, it can be expressed by microglia, astrocytes, and neurons, but it is presumed that during neuroinflammation microglia are the cytokines' main source.

2.3.2 Receptors and Molecular Mechanisms of TNF-α Signaling

TNFR1 (tumor necrosis factor receptor 1), located on chromosome 12, and TNFR2 (tumor necrosis factor receptor 2), located on chromosome 1, belong to the TNF

receptor superfamily, whose 27 members bind TNFs via an extracellular cysteine-rich domain. TNF receptors comprise one to six cysteine-rich repeats in their extracellular domain (221–223); TNFR1 and TNFR2 both contain four cysteine-rich repeats in their extracellular domain. The majority of the receptors are type I transmembrane proteins with elongated shapes due to a scaffold of disulfide bridges (223), whose tips fit in the grooves between each two of the three subunits of the trimeric TNF-α ligand. After ligand-induced oligomerization, their downstream signaling usually depends on different cytoplasmic adaptor proteins (222, 223). The two receptors differ not only in structure but also in binding affinity to their ligands, tissue expression, and activation of signaling pathways, causing disparate and sometimes opposing effects. Constitutive TNFR1 expression at low levels is found in all nucleated cells, whereas TNFR2 expression can be detected predominantly in cells of the immune system (221). Both receptors are found on neurons throughout the brain. TNFR1 can be fully activated by both the soluble and the membrane-bound form of TNF-α and appears to be the primary mediator of sTNF-α, whereas TNFR2 is more strongly activated by the transmembrane form (225). Most knowledge about TNF-α signaling was obtained and deduced from studying TNFR1, thus the functional relevance of TNFR2 may probably be underrated. The extracellular domains of both receptors can be proteolytically cleaved, releasing soluble truncated receptor fragments that appear to regulate levels of TNF-α by its neutralization due to ligand binding (226). The intracellular receptor domains are structurally different between TNFR1 and TNFR2. Both receptors contain a domain interacting with the adaptor protein TRAF (TNF receptor-associated factor), but contrary to the indirect recruitment of TRAF by TNFR1, the receptor TNFR2 recruits TRAF directly. Thus, TRAF plays an essential regulatory role in the transduction pathways of both receptors, and its recruitment is the first step in the TNF-α mediated activation of gene expression. Apart from TRAF, other cytoplasmic proteins, such as TRADD (tumor necrosis factor receptor type 1-associated death domain protein), RIP (receptor interacting protein), and FADD (Fas-associated protein with death domain) are of importance in the TNFR1 signaling pathway. Unlike TNFR2, TNFR1 contains a death domain that can interact with the death domains of other intracellular proteins and thereby induces apoptosis via caspase activation. FADD is required for the activation of caspase-8 which in turn mediates apoptosis either in a caspase-3 dependent or a caspase-3 independent way. No directly induced apoptotic effects were found for TNFR2. Nonetheless, an indirect role in TNF-α-associated cytotoxicity was reported

for TNFR2 via the potentiation of TNFR1-mediated cytotoxicity (227). However, it should be noted that - although of great importance in many physiological and pathological conditions - TNF-α-mediated cell death plays a minor role in comparison to the cytokine's multiple and important other effects in the inflammatory process. Furthermore, its apoptotic activity is sometimes attenuated or even outweighed by the anti-apoptotic effects of TNFR1-dependent NF-κB activation. Apart from the induction of apoptosis, TNFR1 downstream signaling can result in the activation of the anti-apoptotic NF-κB pathway that promotes cell survival and proliferation. Other important signaling pathways are the extracellular signal-regulated kinases pathway, which regulates cellular processes such as cell proliferation and differentiation, the stress-related c-Jun N-terminal kinase pathway, which is also associated with cell proliferation and differentiation and involves activation of the nuclear transcription factor AP-1. Furthermore, TNFR1 is associated with the p38 mitogen-activated protein kinase kinase pathway, which also controls cell proliferation, and differentiation and regulates apoptosis, the Acidic Sphingomyelinase pathway, which has a strategic role in lipid metabolism and cellular stress response, and the Neutral Sphingomyelinase pathway, which has a major regulatory role in ceramide-dependent cell growth and apoptosis. Extensive crosstalk happens not only between these various and partly conflicting TNFR1-associated pathways but also between TNFR1 and TNFR2-mediated signal transduction cascades. (for detailed review of TNF-α and TNF-α-mediated signaling pathways see also (221–224, 228))

2.3.3 TNF-α Signaling in CNS Pathologies with Neuroinflammation

2.3.3.1 TNF-α and Blood-Brain Barrier Permeability

Consistent with its deleterious role in the process of neurodegeneration, elevated TNF-α levels have been strongly linked to dysfunction and breakdown of the BBB. Several laboratories have investigated the cytokines' effects on endothelial permeability utilizing different in vitro models of the BBB with similar results. Using the immortalized human brain microvascular endothelial cell line hCMEC/D3, one group described a TNF-α-induced increase of endothelial permeability as well as a redistribution of zonula occludens-1 and VE-cadherin. Higher TNF-α concentrations led to caspase activation and apoptotic cell death (229). Another team, also employing the hCMEC/D3 cell line, investigated the expression of the barrier constituting adherens junction and tight

junction transmembrane proteins occludin, claudin-5, and VE-cadherin. Protein levels of occludin and claudin-5 were significantly reduced following TNF-α administration, and a downregulation of claudin-5 and VE-cadherin mRNA gene expression was observed (230). Other researchers, utilizing human brain microvascular endothelial cells (HBMvECs) to analyze the effects of TNF-α on endothelial monolayer permeability, similarly found the protein expression of the interendothelial junction proteins VE-cadherin, occludin and claudin-5 dose- and time-dependently decreased in response to TNF-α treatment (231). They attributed the dose-dependent increase in monolayer permeability to TNF-α-induced NADPH oxidase activation. Another in vitro study with HBMvECs demonstrated that the TNF-α/NF-κB signaling pathway has a key role in methamphetamine-induced endothelial dysfunction (232). In this study, blocking of TNF-α prevented the rise of methamphetamine-induced endothelial permeability. These findings were supported by the results of another laboratory examining the cellular responses to TNF-α in primary cultured mouse brain microvascular endothelial cells (233). The authors of this study reported the involvement of NF-κB activation in TNF-α-induced downregulation of claudin-5 promoter activity which resulted in a partial loss of claudin-5 protein and mRNA expression. In an in vitro co-culture system with microglia and mouse brain capillary endothelial cells (MBEC4), it was demonstrated that TNF-α release by microglia due to LPS administration led to marked hyperpermeability of the MBEC4 cell layer (234). Blocking of TNF-α with a neutralizing antibody was reported to inhibit this effect, indicating that the cytokine is implicated in BBB disruption. In line with these results are the findings of a study that compared MPTP-treated wildtype mice and TNF-α knockout animals in terms of BBB leakage (235). This study found that TNF-α deficiency resulted in reduced numbers of activated microglia and a significantly attenuated dysfunction of the BBB. Furthermore, TNF-α was demonstrated to be involved in the increase of BBB permeability in mice during sepsis (236), acute liver failure (237), and radiotherapy (238). All these studies provide important evidence that the cytokine TNF-α considerably affects the integrity of the BBB under various conditions and thereby contributes to the induction of pathological processes in the brain.

2.3.3.2 TNF-α in other Neurodegenerative Diseases

The pivotal role of TNF-α in neurodegenerative diseases and its potential utility as a biomarker have been well established by extensive in vitro and in vivo investigations.

Recent research supports the idea that cytokine-mediated neurotoxicity and inflammation-associated oxidative stress promote the progression of PD and highlights the relevance of TNF-α in the pathogenic mechanisms. To model nigral neurodegeneration, in vivo studies use animals treated with the dopaminergic neurotoxins 6-OHDA (6-hydroxydopamine) (239) or MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) (240). In rats receiving injections of 6-ODH TNF-α levels were increased in the nigrostriatal dopaminergic regions of the injection side (241) and retrograde nigral degeneration was markedly attenuated by neutralization of sTNF-α with an engineered dominant-negative TNF-α inhibitor (242). Inhibition of sTNF-α by injection of a lentiviral vector encoding a dominant-negative TNF (lenti-DN-TNF) into the rat substantia nigra significantly decreased dopaminergic neuronal degeneration resulting from concomitant 6-OHDA injection and behavioral deficits were markedly attenuated (243). Also the delayed administration of lenti-DN-TNF two weeks after 6-OHDA injection prevented further loss of nigral dopaminergic neurons and reduced microglial activation (244). Another study utilizing an antisense oligodeoxyribonucleotide for TNF-α inhibition found that early TNF-α inhibition had neurotoxic effects, whereas late inhibition was neuroprotective (245). Also after injection of MPTP into mice a significant upregulation of TNF-α mRNA is observed in the murine substantia nigra (246–248). In MPTP-treated monkeys significantly elevated TNF-α plasma levels were detected one year after injection and animals with moderate PD symptoms exhibited lower cytokine concentrations than more severely impaired conspecifics (249). However, in MPTP-injected mice with genetic ablation of the TNF-α gene TNF-α knockout partially reduced MPTP-associated neurotoxicity (248). Moreover, it was found that MPTP mediated toxicity was not attenuated in transgenic mice deficient for one individual receptor, either TNFR1 or TNFR2, but double knockout of both TNFR-α receptors was demonstrated to result in complete protection (250, 251). In human PD augmented concentrations of TNF-α were repeatedly measured in brain tissue (252, 253), CSF (253), and serum (254–257) and a significant association between higher plasma or serum TNF-α levels in PD patients and poorer cognitive function and an advanced disease stage was observed (255–260).

Many studies also corroborate the significance of TNF-α in the pathomechanisms involved in the progression of AD. One in vitro study reported a protective role of TNF-α regarding Aβ-induced neurotoxicity in cultures of hippocampal rat neurons, since

pretreatment with TNF-α attenuated neuronal death in cells exposed to Aβ (261). Other authors reported that TNF-α treatment increased Aβ production in cultures of cortical neurons and astrocytes derived from AD mice (262). Interestingly, co-administration of TNF-α and IFNγ, but not TNF-α administration alone, decreased Aβ degradation in cultures of WT microglia in this study. Other in vitro research suggests that TNFR1-mediated signaling pathways are crucial for Aβ-induced neurodegeneration since Aβ-induced neuronal apoptosis was significantly higher in primary hippocampal neuron cultures overexpressing TNFR1, whereas neurons derived from TNFR1 knockout mice were hardly affected by Aβ-mediated neurotoxicity even at high amyloid dosages (263). In vivo animal experiments provide further evidence for the notion of TNF-α as an important driver of AD development and progression. Upregulated TNF-α mRNA expression was repeatedly reported in the cortical tissue of genetically engineered AD mice (195, 264). Moreover, several laboratories analyzed the consequences of TNF-α inhibition/knockouts in animal models of AD and came to interesting results. In AD mice, blocking TNF-α resulted in significantly reduced pre-plaque Aβ-associated pathology (265) and attenuation of TNF-α signaling or genetic TNF-α knockout led to markedly reduced behavioral abnormalities although TNF-α deficiency resulted in increased hippocampal Aβ deposition (266). Also, intracerebroventricular TNF-α signaling inhibition by different means was reported to decrease tau phosphorylation as well as Aβ plaque formation (267), ameliorate AD-associated recognition memory deficits (268) and reduce Aβ-induced cognitive impairments (269) in AD mice and intraperitoneal TNF-α signaling inhibition was reported to prevent memory deficits and synaptic loss in AD mice and monkeys (270). Not only ligand neutralization or ablation but also the inactivation of the TNF-α receptors by inhibition or genetic deletion of TNFR1 and TNFR2 is a valuable approach to study the role of the TNF-α system in AD. Knockout of TNFR1 in AD mice resulted in attenuated Aβ deposition (271) reduced Aβ-associated cognitive and synaptic dysfunction (269, 271), whereas TNFR2 gene deletion led to increased AD-like pathology with elevated Aβ concentrations and enhanced microglial activation and amyloid plaque formation (272).Contrary, mice deficient for both TNFR1 and TNFR2 showed significantly exacerbated Aβ- and tau-associated pathology as well as decreased microglial Aβ phagocytosis activity (273). The first indication for the involvement of TNF-α in human AD pathology was the observation of elevated microglial TNF-α expression in autopsy brains (274) and of increased TNF-α concentrations in the CSF (275) of AD patients. Regarding serum TNF-α levels, the data are

inconsistent. One study observed lower TNF-α serum values in AD patients and a negative correlation between TNF-α serum concentrations and the extent of cognitive impairment in AD patients (276), other authors found no differences in TNF-α serum levels between AD patients and healthy controls (275, 277–280), and again other authors reported elevated TNF-α serum levels in AD patients (281–285). In line with the findings of the latter studies is a report that describes significantly higher TNF-α serum concentrations in patients with severe AD than in individuals with mild to moderate disease (286) and a publication of a negative correlation between TNF-α serum levels and cognitive performance (279). Similarly, another study found that high TNF-α serum baseline levels had prognostic significance for the severity of disease progression and were associated with a 4-fold increase in the rate of cognitive decline (287). Inhibition of TNF-α by weekly perispinal extrathecal injections of the TNF-α neutralizing ligand-binding recombinant fusion protein Etanercept led to significant cognitive improvements in patients with mild to severe AD in a small pilot study (288). A subsequent case report by the same authors described rapid improvement of mental functions in a patient with late-onset AD after the same anti- TNF-α-tratment (289). However, a recent randomized double-blind phase 2 clinical trial, which investigated the effects of weekly subcutaneous Etanercept injections in AD patients found no statistical differences in cognitive, behavioral, or global functions when compared to placebo (290).

2.3.3.3 TNF-α in ALS

Since numerous studies point to a relevant involvement of TNF-α in the pathogenesis of neurodegenerative diseases, it is reasonable to investigate a potential activation of the TNF system in the context of ALS.

2.3.3.3.1 TNF-α in in Vitro Models of ALS

The results of multiple in vitro studies utilizing mouse models of ALS support the hypothesis of TNF-α involvement in the pathogenesis of ALS. In one study the interaction of TNF-α, IL-6, and IFNγ proteins was modeled in vitro using Walker EOC-20 murine microglia, a microglial cell line used in neuroscience research, with nitrite (NO_2^-) efflux as a quantitative index of cell response in vitro (291). In this study, TNFα was found to cause significant NO_2^- production, whereas IL-6 had a lesser effect and neither IFNγ nor IL1β had any effect when applied singly. The TNF-α mediated effect was even more enhanced in the presence of the pro-inflammatory cytokines IFN γ and IL-6,

which potently amplified NO_2^- generation (291). This finding suggests that already comparatively small increases in the concentrations of synergistically acting cytokines induce a disproportionately severe stimulation of microglia. This strong activation results in deleterious effects on the already degenerating CNS.

In another in vitro study primary cultured astrocytes derived from presymptomatic mSOD1 mouse neonates were found to express increased basal amounts of TNF-α mRNA (292). Additionally, these astrocytes showed significantly higher elevations of TNF-α mRNA levels upon stimulation with IFNγ or TNFα than non-transgenic cells. This indicates that the expression of mutant human SOD1 stimulates astrocytes to adopt a neuroinflammatory state already at a very early disease stage. The expression of TNF-α death receptor-associated components was also upregulated in transgenic cells (292).

2.3.3.3.2 TNF-α in Animal Models of ALS

Several research groups determined TNF-α levels in the spinal cord of transgenic mice and reported an upregulation of TNF-α mRNA and protein expression occurring already before signs of disease onset like motor neuron death at presymptomatic stages (293–295). Enhanced expression was also found in end-stage disease (293, 295) and levels were observed to rise from asymptomatic to terminal stage in one study (293). The increase of microglial TNF-α secretion in mSOD1 mice was demonstrated to be dependent on the enhanced generation of ROS and to be regulated at a posttranscriptional level via proteolytic procession of the membrane-bound TNF-α form by several ROS-sensitive enzymes including TACE (296). Elevated expression of TNF-α mRNA and protein was found in mSOD1 transgenic animals in vivo (291). Another study similarly detected the upregulation of proteins associated with the TNF-α system in the spinal cord of SOD1 mice. TNFR1 and FADD expression was found to be increased at 80 days and was even further elevated at 120 days in transgenic animals (297). Other authors reported that inhibition of TNF-α by oral administration of the TNF-α antagonist nordihydroguaiaretic acid, which shows antioxidant and anti-inflammatory properties in vitro and in animal models, slowed down motor dysfunction in mSOD1 transgenic mice. Total lifespan and life expectancy after the start of treatment were prolonged significantly in comparison to untreated animals (298). Another study investigated the effects of intraperitoneal injections of IgG derived from sALS patients in mice. This treatment resulted in only subclinical signs of motor neuron disease, but

interestingly, a significant upregulation of TNF-α expression was observed in the murine spinal cord (299).

2.3.3.3.3 TNF-α in Human ALS

In human ALS, elevated TNF-α levels were found in the CSF (201, 211) and the blood (217, 300–305).

Regarding a possible association between circulating TNF-α levels and survival, disease severity, or rate of disease progression, the literature is inconsistent. There are on one hand studies that didn´t find a correlation between TNF-α plasma concentrations and ALSFRS-R scores (303) and also no association between serum TNF-α levels and disease stage, disease duration, or clinical disease parameters in general (300). On the other hand, some publications reported an association between higher circulating TNF-α values and disease duration (301) and a negative correlation between TNF-α plasma levels and survival in one subgroup of the analyzed ALS patients (306).

Moreover, increased peripheral TNF-α levels were reported to be accompanied by increased circulating concentrations of its receptors sTNFR1 (217, 300) and sTNFR2 (300) but no correlation between one of these levels and disease duration or disease severity was found in these studies.

Taken together, all these findings strongly support the idea of TNF-α being a central actor in the disease course of ALS.

2.4 G-CSF

2.4.1 Biological Function and Pharmaceutical Variants of G-CSF

The growth factor Granulocyte colony-stimulating factor (G-CSF), also known as colony-stimulating factor 3 (CSF 3) is a cytokine and hormone with neuroprotective, neurotrophic, anti-apoptotic, antioxidant, and immunomodulating properties. The glycoprotein has various biological functions related to white blood cells, the hematopoietic system, and neurons and can overcome the BBB (307, 308). G-CSF is produced by different tissues, including endothelial cells, macrophages, and some other immune cells. It regulates and increases the proliferation, differentiation, function, and survival of neutrophil precursor cells and mature neutrophils. G-CSF also stimulates hematopoietic

stem cell release from the bone marrow into the bloodstream. Moreover, G-CSF has neuroprotective potential. Its receptor is expressed by neurons in the brain and spinal cord and, as a neurotrophic factor, it acts as an antagonist to apoptosis, induces neurogenesis, and increases neuroplasticity.

There are two pharmaceutical analogs of the natural glycoprotein. The pharmaceutical variants are called filgrastim, sold under the brand name Neupogen among others, and lenograstim, sold under the brand name Granocyte. Filgrastim and polyethylene glycol (PEG)-filgrastim are two commercially available forms of recombinant human G-CSF. Filgrastim is synthesized in an E. coli expression system. Its structure is slightly different from the structure of naturally occurring G-CSF. Due to its comparatively short half-life, daily injections are necessary, whereas PEG-filgrastim has a much longer half-life, reducing the need for daily injections. In contrast, lenograstim is synthesized in Chinese hamster ovary cells. Due to its synthesis in a mammalian cell expression system, lenograstim is indistinguishable from the natural human G-CSF form. Until now, no clinical or therapeutic consequences of the differences between filgrastim and lenograstim have been reported.

2.4.2 G-CSF: Neurogenesis and Neuroprotection in Neurodegenerative Diseases

Different studies suggest an important role of G-CSF in neurogenesis (for review see (309, 310)). In a study examining G-CSF knock-out mice, hippocampal neurogenesis was strongly reduced, and the mice showed deficits in the development of motor skills and memory formation (311). The G-CSF receptor is expressed in various brain regions and it is upregulated in response to neuronal insults. This indicates an autocrine neuroprotective signaling mechanism of G-CSF by reducing or inhibiting inflammation and apoptosis and stimulating neurogenesis. Because of its neuroregenerative potential G-CSF appears to be a promising target to reduce neurodegeneration in different neurological diseases including ALS.

2.4.2.1 G-CSF in other Neurodegenerative Diseases

Different findings point towards a possible beneficial role of G-CSF treatment as a neuroprotective agent in the therapy of neurodegenerative diseases.

Studies on murine AD models indicate that G-GSF acts as a neurotrophic factor in AD. As early as 2007, an in vivo study using two different AD mouse models (an acute and

a chronic AD model) found that subcutaneous G-CSF injections improved neurological behavior and restored cognitive and memory abilities accompanied by an accumulation of hematopoietic stem cells (HSC) and by local neurogenesis surrounding the Aβ aggregates (312). Another in vivo study using an AD mouse model, conducted in 2009, showed that G-CSF administration reduced systemic inflammation by suppressing the production or function of major plasma pro-inflammatory cytokines, increased overall microglial activity, decreased Aβ deposition, and improved cognitive performance (313). In a study utilizing AD transgenic mice, carried out in 2011 treatment with subcutaneous G-CSF injections in combination with injections of stem cell factor was reported to result in a long-term decrease of β-amyloid deposition, augmented circulating bone marrow cells, a higher level of bone marrow-derived microglia and to the co-localization of β-amyloid deposits and bone marrow-derived microglial cells (314). In 2013 it was reported that in another in vivo study with AD rats, subcutaneous G-CSF-administration was reported to lead to improved neurobehavioral, memory and motor functions as well as to increased numbers of progenitor cells (315).

Also in the context of PD, G-CSF received attention during the last years due to its neurotrophic effects. In 2006, a study examining the neuroprotective properties of G-CSF in vitro and in vivo in a model of PD reported that G-CSF application led to considerable protection against dopaminergic cell death in vitro and that subcutaneous G-CSF treatment rescued dopaminergic neurons in the substantia nigra from cell death in mice (316). In 2018 another study using another mouse model of PD found that treatment with G-CSF markedly improved working memory and motor function and restored the nigrostriatal dopamine level (317). In 2020, other authors reported that co-injection of G-CSF and bone marrow-derived stem cells had synergistic effects in increasing the expression of antioxidant enzymes and decreasing the activity of pro-inflammatory cytokines in PD rats (318). In addition to the preclinical findings, in 2017 four patients with early-stage PD were treated with G-CSF injections for 5 days and 6 courses over one year in a phase I trial pilot study and showed a slower nigrostriatal degeneration in comparison to prior reports of PD (319).

2.4.2.2 G-CSF in ALS

Regarding ALS a variety of pathophysiological processes must be addressed to modify the disease course. Therefore, a growth factor appears to be a reasonable therapeutic agent, since it addresses multiple pathways of pathophysiology in parallel, and

according to multiple studies, treatment with G-CSF seems to be a promising possibility to counteract ALS pathology and decelerate disease progression. Different studies and clinical trials have been conducted to investigate the feasibility, safety, and efficacy of G-CSF administration in animal models of ALS as well as in human disease (for review see (309, 310, 320)), and some studies suggest that G-CSF treatment has neuroprotective effects in ALS and may delay disease progression.

2.4.2.2.1 G-CSF in Models of ALS in Vitro and in Vivo

The effects of G-CSF were first examined in cultured motoneuronal cells and the SOD1(G93A) tg mouse model of ALS in 2008. In this study, G-CSF was applied by continuous subcutaneous injection and by CNS-targeted transgenic overexpression. In the motoneuron cell line, G-CSF reduced caspase-3 activation and apoptotic cell death. In the transgenic mice, G-CSF slowed down disease progression by decreasing muscle denervation atrophy, increasing motor neuron survival, improving motor functions, decelerating the onset of motor impairment, and extended overall survival time (321). A study in 2010 showed that in mSOD1 tg mice, the decrease in the number of neurons and large myelinated axons could be reduced by repeated daily subcutaneous G-CSF administration and that the recruitment of microglia was increased by the G-CSF treatment (322). Similarly, another animal study, conducted in 2011, examined the potential effect of long-term subcutaneous G-CSF administration in mSOD1 tg mice and observed an attenuated inflammatory reaction and a decelerated disease progression (323). The authors reported that long-term G-CSF treatment from presymptomatic stage until end stage of the disease decreased the levels of pro-inflammatory cytokines, reduced microgliosis and astrogliosis, and prolonged the animals' overall survival. Interestingly, some authors reported a sex-specific effect of subcutaneous G-CSF treatment, as the protective effect of G-CSF in survival, motor function, and synaptic dysfunction was observed only in male mSOD mice, whereas the treatment was not beneficial in female mSOD mice (324).

In contrast to animal studies in which G-CSF was administered subcutaneously, one group examined the effects of intraspinal viral G-CSF delivery compared to intramuscular viral G-CSF delivery in SOD-1 (G93A) mice. The authors demonstrated that, compared to intramuscular injection, intraspinal G-CSF administration increased the efficacy of G-CSF treatment in the ALS mouse model by slowing disease progression and prolonging overall survival (325). Additionally, an in vitro study in 2015 investigated

the effects of G-CSF treatment on isolated lumbar motoneurons from SOD1 G93A mice compared to non-transgenic littermates regarding the gene expression profiles and reported that G-CSF administration restored the transcriptomic deregulations of SOD1G93A motoneurons (326).

Overall, a favorable effect of G-CSF on the course of the disease and survival can be derived from several in vitro and in vivo models of ALS. Even if the exact mechanisms are still only partially understood, these results appeared sufficiently promising to allow G-CSF to be increasingly used and investigated in ALS patients over time.

2.4.2.2.2 G-CSF in Human ALS

2.4.2.2.2.1 Pilot Studies

One of the first reports of G-CSF treatment in ALS patients was published in 2001. In this trial, three ALS patients were treated with G-CSF for stem cell mobilization. G-CSF was administered only once for stem cell mobilization, and although no improvement in disease was observed thereafter, none of the patients experienced disease deterioration related to the G-CSF application (327). In another pilot study conducted in 2008, eight patients with a diagnosis of probable or definite ALS received subcutaneous daily G-CSF injections for 5 to 6 days at a dose of 300-600 µg, depending on the patient's body weight for the collection and reinfusion of stem cells. Again, no adverse events were reported during clinical or autopsy examinations (328). Only shortly thereafter, in 2009 and 2012, a group of authors conducted two similar studies in which patients with confirmed ALS received subcutaneous G-CSF injections at a dose of 300 µg for three consecutive days to mobilize bone marrow-derived cells (BMC) followed by autologous stem cell transplantation into the frontal motor cortex. The first study included ten patients whereas the second study included sixty-seven patients. In both reports treatment with G-CSF was well tolerated, without serious adverse events, and increased absolute monocyte and lymphocyte counts (329, 330). Although these early studies did not establish a beneficial role for G-CSF treatment itself in ALS, they paved the way for conducting larger studies with an optimized G-CSF regimen by demonstrating the feasibility, tolerability, and safety of the therapy in ALS.

2.4.2.2.2.2 Observation of Beneficial Effects

In 2009, a study was conducted with thirteen patients with the diagnosis of probable or definite ALS who received subcutaneous G-CSF administration of 2 µg/kg once a day

for five days within an open trial setting. The observation period was three months before to six months after the injections. The study reported that G-CSF treatment significantly reduced the decreases in ALSFRS (ALS Functional Rating Scale) and CMAP (compound muscle action potential) amplitude during the first three months after administration, compared to the declines measured before G-CSF administration (331). As for the decline in ALSFRS, the change in the decline was even greater in the second three months after G-SCF injections. In addition, the percentage of CD34+ cells was increased (331). The results implied the efficient mobilization of bone marrow-derived cells and provided the first important indication of the possible beneficial effects of G-CSF on the rate of motor decline and the speed of disease progression. In another double-blind pilot study from 2010 thirty-nine patients with definite or probable ALS received G-CSF or placebo treatment in intervals of three months over one year. In this study, G-CSF application didn´t statistically significantly slow down disease progression, but a trend of disease deceleration was observed, and again, no major adverse effects were found (332). Yet, it should be mentioned that the described trends and statistically significant effects of G-CSF on the disease course of ALS can only be derived to a limited extent due to the small size of the patient cohorts in these studies.

2.4.2.2.2.3 Lack of Observation of Beneficial Effects

However, not all studies showed improvements in terms of disease progression. In an open-label multicenter study, also from 2010, twenty-four ALS patients received subcutaneous G-CSF treatment at a dose of 5 μg/kg twice daily for 4 consecutive days every 3 months for a total of four cycles. From the third day of G-CSF injections, mannitol was additionally administered intravenously four times a day for consecutive 5 days to increase the permeability of the BBB. While this study did not find a decelerating effect on disease progression, interestingly it did find a significant reduction in CSF CCL2 levels and, in line with prior trials, reported only transient and no serious side effects (333). In 2011, a double-blind, placebo-controlled, randomized trial was conducted with a total of ten patients with confirmed ALS. The patients were either treated with placebo injections or with subcutaneous administration of G-CSF in a dose of 10 μg/kg for the first 10 days and from day 20 to 25. Although this study reported no significant differences in terms of brain volumetry and clinical examination, it was again reported that G-CSF treatment was well tolerated (334). In 2015 a large randomized,

double-blind, placebo-controlled study investigated the tolerability, safety, and efficacy of subcutaneous G-CSF treatment in forty patients with probable or definite ALS and also failed to detect a beneficial effect of G-CSF on the disease course. The participants received either subcutaneous G-CSF administration with a dose of 5 µg/kg or placebo injections once for five consecutive days. The authors observed no significant differences between the two groups in clinical tests and nerve conduction velocity studies (335). Remarkably, the study reported that the decline in ALSFRS-R scores was greater in female patients with G-CSF treatment than in females in the placebo group. Therefore, the authors concluded the G-CSF treatment protocol used in this study did not seem to be a promising treatment option and might even promote disease progression in female patients. Interestingly, however, there was a trend towards a positive correlation between CCL2 CSF baseline levels and change in ALSFRS-R scores in both groups, indicating more rapid disease progression in patients with higher CCL2 CSF baseline levels (335).

2.4.2.2.2.4 Effects of Long-Term Treatment

Since data on the effect of long-term G-CSF treatment by repeated injections in patients with normal bone marrow function are limited, in 2010 a study was conducted with 26 patients with the diagnosis of definite, probable, or probable laboratory-supported ALS, who received repeated courses of subcutaneously administered G-CSF at a dose of 5 µg/kg every 12 h for 4 consecutive days, repeated in 3-month intervals for a total of four courses. Apart from two severe but only transient side effects, namely a temporal prolactin level increase in a patient with a history of prolactinoma, and a deep vein thrombosis in another patient, only minor and transitory side effects like bone pain, headache, and asthenia were observed and no treatment-related worsening of the disease course was reported (336). In addition, the authors found that repeated G-CSF administration resulted in the repeated mobilization of BMC and that most of the mobilized BMC co-expressed stem cell markers. In 2014 another group conducted a study including six patients with definite or probable ALS who received repeated monthly subcutaneous G-CSF administration on five consecutive days every four weeks. The initial dose of G-CSF was 5 µg/kg per day as a single injection and the maximum dose was 10 µg/kg as two injections. Hematologic parameters in particular were monitored during the observation period of up to three years. G-CSF treatment resulted in significantly increased white blood cell count and decreased platelet count,

as well as significant mobilization of CD34+ hematopoietic stem cells and constant mobilization of colony-forming cells (337). In this study, mild adverse events such as muscle and bone pain or headache were observed, but no serious treatment-associated adverse events and, in particular, no evidence of the development of hematopoietic malignancies were reported. In 2018, a previous publication preceding this work evaluated the safety, tolerability, and feasibility of long-term treatment with G-CSF in 36 patients with definite or probable ALS, who also represent the patient cohort of the present work and whose CCL2 and TNF-α levels are more extensively studied and analyzed in the present work. G-CSF was dosed by individual treatment regimens further outlined below. Long-term G-CSF administration with individually adapted treatment regimens was well tolerated and without major side effects in this study (338). Moreover, consistent with the results of the other studies outlined above, G-CSF treatment was shown to result in significant mobilization of hematopoietic stem cells into the peripheral blood. Furthermore, higher mobilization capacity correlated with longer overall survival, and sustained G-SCF administration resulted in permanent changes in serum cytokine concentrations (338). Another report by the same group of authors described individualized treatment effects of G-CSF administration in these 36 ALS patients using survival modeling and bioinformatics. The authors stated that they were able to retrospectively identify responders to G-CSF therapy already at an early disease stage (339). Not in all 36 investigated ALS patients, but only in this small cohort of G-CSF responders, G-CSF therapy was reported to have significant positive effects on the course of the disease and the duration of survival, whereas these effects could not be observed in non-responders. A strong individual response was reported to correlate with younger age, a higher HSC mobilization, a preserved pattern of fractional anisotropy, and a less aggressive inflammatory cytokine plasma profile. Interestingly, significantly increased serum levels of CCL2 were observed in nonresponding patients three, six, and nine months after initiation of G-CSF treatment (339).

3 Material and Methods

3.1 Patients, Treatment, and Data Recording

The data evaluated in this study were collected from 36 patients with definite or probable sporadic ALS according to the revised El Escorial criteria (see Supplements for detailed demographic data), which were treated with repeated subcutaneous injections

of G-CSF individually between January 2010 and July 2017 after written informed consent.
The individual treatments were reviewed and approved by the ethics committee of the University of Regensburg. The ethics committee of the University of Regensburg approved a retrospective analysis (ethics approval: 15-101-0106 and 14-101-0011).
Since this study was not conducted as a prospective clinical trial, the application of formal exclusion criteria was deemed inappropriate. Nonetheless, individuals with existing or prior neurological conditions apart from ALS, as well as participants engaged in any form of interventional study, were not provided with this treatment option.
All patients were Caucasian. Twenty-five patients were male, and 11 were female. The age range at the start of the treatment was between 26 and 77 years with a mean age of 52 years. Apart from the G-CSF treatment, all patients received standard care and were treated with riluzole 100 mg/day (339).
The long-term G-CSF treatment was provided monthly. G-CSF doses were individually set upon treatment start and individually adjusted over time with the general aim of achieving sufficient hematopoietic stem cell mobilization to peripheral blood (mean 464 Mio IU/month, range 90-2160 Mio IU/month) over a median of 13.7 months (range from 2.7 to 73.8 months) (338). Monthly safety monitoring was carried out by observation of functional loss, assessment of survival, and collection of serum retention samples, and cerebral DTI-based MRI scans were performed at baseline and over time (339).
ALS patients were examined at the outpatient clinic up to twice a month with an assessment of clinical status and further laboratory diagnostic analyses. Clinical examinations included the monitoring of ALS progression by the revised ALS functional rating scale (ALSFRS-R). Blood serum analyses included blood cell count, measurements of concentrations of multiple cytokines, and estimation of bone marrow function.

Hematopoietic stem cells were assessed in peripheral blood, and blood samples were stored for later evaluation of cytokines and chemokines. Thus, hematological parameters were examined at the same time points as cytokine levels. CD34+ and CD34+CD38- hematopoietic stem and progenitor cells were analyzed in peripheral blood by flow cytometry.

For cytokine assays peripheral blood serum samples of 25 µl were used and analyses were performed in duplicates. Cytokine serum levels were assessed by multiplex electrochemiluminescence. Cytokines were analyzed by determination of the area under

the curve (AUC approach) and the AUC for each biomarker was calculated with the R-package flux (339).

The duration of G-CSF treatment and the mean monthly G-CSF dose (specified in Mio IU/month) were individualized, and G-CSF application regimes were adapted over time. G-CSF was mainly given either in specific intervals or continuously on single days.

The different therapy regimes were as follows: A: filgrastim application once or twice a day over 5 consecutive days a month; B: as in “A” with repetition of the treatment interval once a month (i.e., 2 × 5 days); C: as in “B” with additional filgrastim application every second day between the treatment intervals; D: three days of filgrastim application a month; E: filgrastim application once or twice a day on a single day a week, F: filgrastim application every second day, and G: different combinations of filgrastim applications on single days a week (339).

The average monthly dose of filgrastim for each patient is given in the demographics in the Supplements.

Due to suspected adverse reactions (an episode of heat sensation, dyspnea, and lightheadedness), G-CSF treatment was terminated in one patient because drug-related intolerance or mild allergic reaction could not be excluded. This patient (P21) was temporarily switched to PEGylated G-CSF (Pegfilgrastim 6 mg) from the 46th to 53rd month of treatment, later the treatment was ended without further adverse reactions. Antibodies against filgrastim were not detected (339).

3.2 Data Processing and Data Analysis

Since many cytokines are already under investigation as potential biomarkers for disease progression and survival and since TNF-α and CCL2 seem to play an important role in various neurodegenerative diseases, levels of TNF-α and CCL2 before and after G-CSF injections were examined to analyze potential treatment effects. To transfer the changes in the absolute cytokine levels of the individual patients into inter-individually comparable values, the absolute cytokine levels were related to the respective individual baseline cytokine level, measured before the first G-CSF application. Therefore, the absolute cytokine levels of each patient were divided by the respective baseline level. This way, relative and interindividually comparable cytokine values were

obtained and enabled a comparison of the variations in cytokine levels among the different individuals. Initially, cytokine levels after G-CSF administration were measured for all patients in addition to baseline cytokine levels. Whereas some patients continued to receive long-term cytokine level measurements before and after G-CSF administration, others, depending on their therapy regimen, received cytokine level measurements only before or after G-CSF administration. This fact, as well as the use of different therapy regimens with different durations and doses of G-CSF administration and the fact that the patients did not appear for the measurements at predefined and always constant times, made the analyses more difficult, as this reduced the interindividual comparability of the measurements.
Survival was defined as the time between treatment start and patient death, regardless of the cause of death. The range of the recorded survival duration after treatment initiation was between 2.9 and 77.6 months. Patients were divided into two groups, namely long-term and short-term survivors, according to their survival duration. An earlier publication, investigating the same patient cohort, identified a response to G-CSF treatment in some patients as clearly relevant to their survival (339). In another previous publication with the same patient cohort, the cut-off for the classification of patients into short-term survivors and long-term survivors was set at 30 months after diagnosis (338). In contrast to this, in the present work, the cut-off was set at 30 months after the start of G-CSF treatment. This cut-off was chosen based on the assumption that treatment effects might be a relevant factor in some of the investigated patients, as reported earlier (339). This different cut-off resulted in a larger divergence between the group sizes of short-term and long-term survivors and in a different group composition in comparison to the prior publication (338) but was better suited to reflect the assumed response of some patients to G-CSF application. Thus, short-term survivors were defined as patients with a survival of less than 30 months, and long-term survivors were defined as patients with a survival of more than 30 months after the start of treatment with G-CSF.

The recording of the patient's survival was not or could not be carried out completely. Survival duration was not recorded in six patients. Three of these six patients were recorded sufficiently long enough for inclusion in the long-term survivor group. The other three patients could not be included in the recording of the survival duration and therefore could not be assigned to the short or long-term survivor group, since their

survival duration and thus their group assignment remained unclear. Four patients were still alive at the end of the study, but at that time had not lived long enough to be assigned to the long-term survivor group, so it was not possible to determine whether these patients would have belonged to the short- or long-term survivor group. Two patients died by suicide before living long enough to be classified as long-term survivors. Therefore, it was also not possible to determine whether these patients belonged to the group of short- or long-term survivors. These two patients could also not be included in the recording of the survival duration and could also not be assigned to the short or long-term survivor group. Thus, in twelve of the patients, survival duration could not be fully assessed and nine patients had to be excluded from group assignment because they had no valid data for their respective short- or long-term survival due to a lack of recording of the time of death or death by suicide before the chosen cut-off duration between short- and long-term survivors. Accordingly, the calculation of a mean survival time was not reasonably possible for these patients.

The collected data were digitized and processed using Microsoft Excel spreadsheets.

The Wilcoxon rank sum test is used to compare two independent samples. It was used to examine whether the short-term survivor group and the long-term survivor group differ significantly with respect to the central tendencies of the baseline CCL2 levels and TNF-α levels at the selected cut-off value, as shown in Figure 1. One patient (patient 28) had no recorded CCL2 and TNF-α baseline levels and could therefore not be included in this analysis. The Wilcoxon rank sum test was also used to investigate, whether the short-term survivor group and the long-term survivor group differ significantly in terms of the central tendencies of the cytokine levels during the course of treatment. Since the repeated cytokine measurements in the same patients are dependent variables, first the mean of the cytokine measurements during the observation period was calculated individually for each patient. Then, the medians of the individual patients‘ averages in the short-term survivor group and in the long-term survivor group were calculated and tested for significant differences using the Wilcoxon rank sum test.

The Wilcoxon signed-rank test is used to compare two dependent samples. It was used to examine whether the cytokine serum levels before G-CSF administrations differ significantly from the cytokine serum levels after G-CSF administrations as repeated measurements in the same patients are dependent variables. First, the means of the

cytokine measurements during the observation period, excluding baseline measurements, were calculated individually for each patient. Then the medians of the individual patients' averages, excluding baseline measurements, and the medians of the baseline measurements were calculated and tested for significant differences using the Wilcoxon signed-rank test.

The simple linear regression was calculated and plotted, as shown in Figures 2-5, to model the relationship between cytokine serum levels and survival duration. The cytokine serum levels of the short- and long-term survivor groups were divided into subgroups based on the patients' G-CSF treatment status at the respective measurement (before or after G-CSF administration). The Wald test was used to analyze whether the slope of the linear regression of the respective groups differs significantly from zero and, accordingly, whether there is a significant change in serum cytokine levels over time. An interaction term was included in the linear regression model to determine whether the slopes of short-term survivors and long-term survivors show statistically significant differences.

The change in the P value of the difference between short- and long-term survivors with respect to the cytokine baseline values of the groups was analyzed, as shown in Figure 6. For this purpose, patients were divided into two groups (short- and long-term survivors) based on their respective survival time in months after treatment initiation according to the Wilcoxon rank sum test. In this way, it was investigated whether baseline serum levels are a potential tool for methodological validation of the clinically chosen cut-off time point for classification as short- and long-term survival.

The level of statistical significance of all analyses was defined by $p \leq 0.05$ (two-tailed P value).

The programming language Python was used for data analysis. Python's scientific computing libraries NumPy (Numerical Python), SciPy (Scientific Python), and Pandas (Python Data Analysis Library) were used to perform statistical calculations and analyses (340–342). The data visualization and graphical plotting library Matplotlib was used for graph creation (343).

4 Results

In the following, it is investigated whether short-term survivors and long-term survivors show differences in the cytokine serum levels of CCL2 and TNF-α. The aim is to assess the potential of these two cytokines as biomarkers. Study participants are categorized into two groups based on their survival outcomes. Some investigations also consider the status of G-CSF treatment, distinguishing measurements taken before or after G-CSF administrations.

The initial investigation explores potential differences in baseline levels of CCL2 and TNF-α serum concentrations between the short-term survivor and long-term survivor groups. To visualize the distribution of individual baseline serum levels, boxplots are created for both groups.

Subsequently, we analyze the trajectory of cytokine serum levels during G-CSF therapy for both groups. Measurements of cytokine serum levels are associated with short-term and long-term survivor groups and plotted against time. The mean of individual patient cytokine serum level values is calculated, and it is explored whether the medians of average cytokine serum levels significantly differ between the groups. Furthermore, linear regression is applied to the individual values, investigating the slope of the linear regression to detect any consistent change in cytokine serum levels during treatment that could indicate a continuous treatment effect. Additionally, the average follow-up values for individual patients are computed, and it is examined whether the median of these values significantly differs from the median of baseline values, suggesting a non-continuous treatment effect. Potential group differences in cytokine serum levels under G-CSF therapy are investigated by comparing the behavior of cytokine serum levels during treatment.

A detailed tabular overview and comparison of most of the results presented below (cf. chapters 4.2 - 4.5) on the differences in the medians of cytokine serum levels of short- and long-time survivors, the differences between the medians of follow-up cytokine serum levels and the medians of baseline cytokine levels, and the change in the slopes of the simple linear regressions of the cytokine serum levels can be found in the Supplements (cf. chapters 7.2 – 7.4).

4.1 Absolute CCL2 and TNF-α Baseline Serum Levels of the Short- and Long-Term Survivor Group

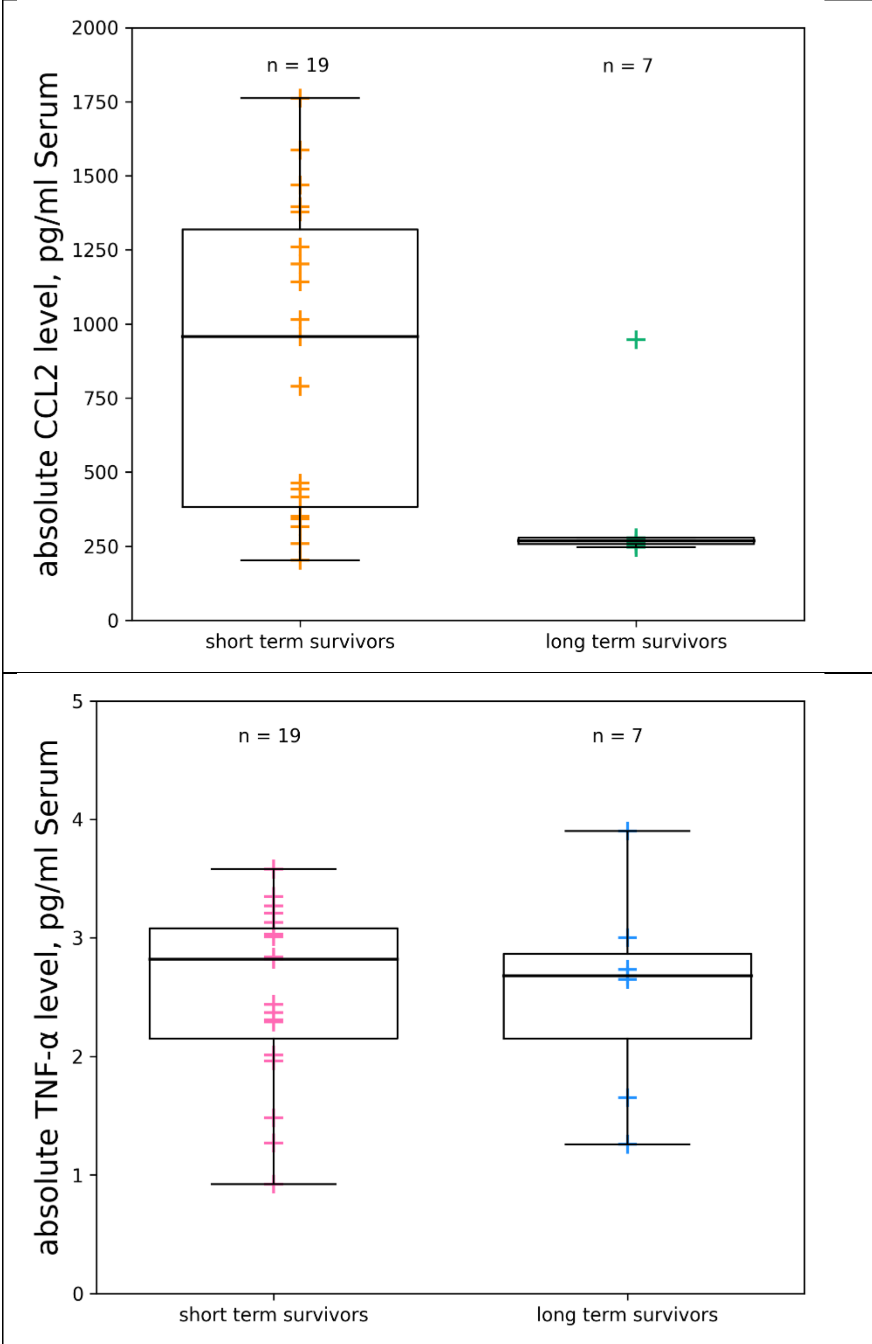

Figure 1: Boxplots of absolute baseline CCL2 and TNF-α levels of short-term and long-term survivor group, measured before the start of G-CSF injections at a threshold for grouping into short-term and long-term survivors 30 months after initiation of G-CSF treatment.

Top graph: Orange crosses represent absolute CCL2 baseline levels of short-term survivors in pg/ml Serum (left boxplot), and green crosses represent absolute CCL2 baseline levels of long-term survivors in pg/ml Serum (right boxplot), respectively. Boxes cover 25th to 75th percentiles. Whiskers cover the range between minimum and maximum values. The horizontal black solid line in the box represents the median of absolute CCL2 baseline levels of the short-term and long-term survivor group respectively.

Bottom graph: Pink crosses represent absolute TNF-α baseline levels of short-term survivors in pg/ml Serum (left boxplot), and blue crosses represent absolute TNF-α baseline levels of long-term survivors in pg/ml Serum (right boxplot), respectively. Boxes cover 25th to 75th percentiles. Whiskers cover the range between minimum and maximum values. The horizontal black solid line in the box represents the median of absolute TNF-α baseline levels of the short-term and long-term survivor group respectively.

4.1.1 Absolute CCL2 Baseline Serum Levels

The median of the absolute CCL2 baseline levels of the short-term survivors is 956.79 pg/ml Serum and 50% of all CCL2 baseline values of the short-term survivors are in the range of 382.90 pg/ml Serum to 1318.58 pg/ml Serum. The median of the absolute CCL2 baseline levels of the long-term survivors is 267.49 pg/ml Serum and 50% of all CCL2 baseline values of the long-term survivors are in the range of 256.36 pg/ml Serum to 278.08 pg/ml Serum.

The Wilcoxon rank-sum test was used to compare the two independent groups of short-term survivors and long-term survivors. At the clinically chosen cut-off of 30 months of survival duration after the start of G-CSF treatment, the median of CCL2 baseline levels of short-term survivors is significantly higher than the median of CCL2 baseline levels of long-term survivors ($p = 0.0036$).

4.1.2 Absolute TNF-α Baseline Serum Levels

The median of the absolute TNF-α baseline levels of the short-term survivors is 2.82 pg/ml Serum and 50% of all TNF-α baseline values of the short-term survivors are in the range of 2.15 pg/ml Serum to 3.08 pg/ml Serum. The median of the absolute TNF-α baseline levels of the long-term survivors is 2.68 pg/ml Serum and 50% of all TNF-α baseline values of the long-term survivors are in the range of 2.15 pg/ml Serum to 2.87 pg/ml Serum.

The Wilcoxon rank-sum test was used to compare the two independent groups of short-term survivors and long-term survivors. At the clinically chosen cut-off of 30 months of survival duration after the start of G-CSF treatment, the medians of TNF-α baseline levels of short-term survivors and long-term survivors are not significantly different ($p = 0.8214$).

4.2 Absolute CCL2 and TNF-α Serum Levels Considering the G-CSF Treatment Status

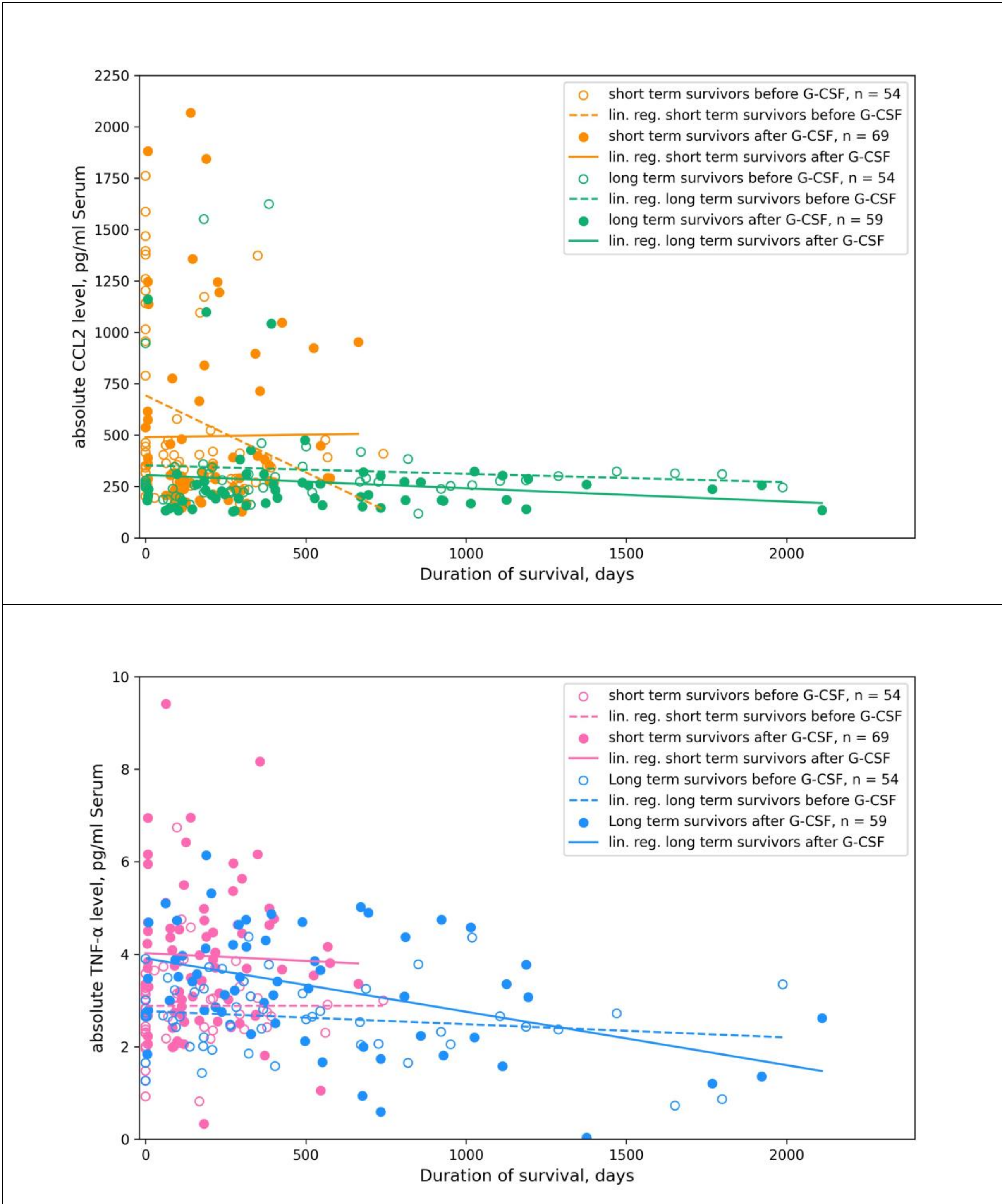

Figure 2: Absolute CCL2 levels [pg/ml Serum] and TNF-α levels [pg/ml Serum] of short-term and long-term survivors considering the G-CSF treatment status.
Top graph: Orange colour represents CCL2 levels of short term survivors, green colour represents CCL2 levels of long term survivors. Empty circles represent absolute CCL2 levels measured before G-CSF injection, filled circles represent absolute CCL2 levels measured after G-CSF injection. The dashed lines represent the linear regressions of absolute CCL2 levels before G-CSF administration over time. The solid lines represent the linear regressions of absolute CCL2 levels after G-CSF administration over time.
Bottom graph: Pink colour represents TNF-α levels of short term survivors, blue colour represents TNF-α levels of long term survivors. Empty circles represent absolute TNF-α levels measured before G-CSF injection, filled circles represent absolute TNF-α levels measured after G-CSF injection. The dashed lines represent the linear regressions of absolute TNF-α levels before G-CSF administration over time. The solid lines represent the linear regressions of absolute TNF-α levels after G-CSF administration over time.

4.2.1 Absolute CCL2 Serum Levels Considering the G-CSF Treatment Status

To compare the central tendencies of absolute CCL2 serum levels of short-term survivors and long-term survivors, the mean of the absolute CCL2 serum levels during the observation period was calculated individually for each patient. Then, the median of the individual patients' absolute CCL2 serum level averages of the short-term survivor group and the long-term survivor group before and after G-CSF administrations were calculated and tested for significant differences using the Wilcoxon rank sum test.

The absolute median serum levels of short-term survivors measured before and after G-CSF administrations (before: median = 780.69 pg/ml serum, SD = 412.68 pg/ml; after: median = 327.34 pg/ml serum, SD = 297.85 pg/ml) were compared to the respective median serum levels of long-term survivors (before: median = 249.23 pg/ml serum, SD = 239.94 pg/ml; after: median = 218.39 pg/ml serum, SD = 204.96 pg/ml). The medians of serum levels of short-term survivors are significantly higher than the medians of long-term survivors, both when measured before (p = 0.0063) and after G-CSF (p = 0.0476).

To investigate if there is a gradual and continuous change in absolute CCL2 serum levels over time the simple linear regressions of absolute CCL2 serum levels before and after G-CSF administrations were calculated and plotted. The slope of the simple linear regression of absolute CCL2 serum levels of short-term survivors measured before G-CSF administrations of is significantly negative (slope = -0.747927, p = 0.0294). The slope of absolute CCL2 levels of short-term survivors after G-CSF administrations (slope = 0.024259, p = 0.9425), the slope of absolute CCL2 levels of long-term survivors before G-CSF administrations (slope = -0.040825, p = 0.5958) and the slope of long-term survivors after G-CSF administrations (slope = -0.064545, p = 0.2617) do not deviate significantly from zero.

4.2.2 Absolute TNF-α Serum Levels Considering the G-CSF Treatment Status

To compare the central tendencies of absolute TNF-α serum levels of short-term survivors and long-term survivors, the mean of the absolute TNF-α serum levels during the observation period was calculated individually for each patient. Then, the median of the individual patients‘ absolute TNF-α serum level averages of the short-term survivor group and the long-term survivor group before and after G-CSF administrations were calculated and tested for significant differences using the Wilcoxon rank sum test.

The absolute median serum levels of short-term survivors measured before and after G-CSF administrations (before: median = 3.00 pg/ml serum, SD = 0.65 pg/ml; after: median = 3.91 pg/ml serum, SD = 1.61 pg/ml) were compared to the respective median serum levels of long-term survivors (before: median = 2.81 pg/ml serum, SD = 0.39 pg/ml; after: median = 3.37 pg/ml serum, SD = 0.83 pg/ml). The median serum levels of short-term survivors do not significantly differ from the median serum levels of long-term survivors, both when measured before ($p = 0.6456$) and after G-CSF ($p = 0.3058$).

To investigate if there is a gradual and continuous change in absolute TNF-α serum levels over time the simple linear regression of absolute TNF-α serum levels before and after G-CSF administrations were calculated and plotted. The slope of the simple linear regression of absolute TNF-α serum levels of short-term survivors measured before G-CSF administrations (slope = 0,000003, $p = 0.9975$), the slope of short-term survivors after G-CSF administrations (slope = -0.000335, $p = 0.7835$) and the slope of long-term survivors before G-CSF administrations (slope = -0.000285, $p = 0.1979$) do not deviate significantly from zero. The slope of the simple linear regression of absolute TNF-α serum levels of long-term survivors after G-CSF administrations is significantly negative (slope = -0.001155, $p = 0.0007$).

4.3 Absolute CCL2 and TNF-α Serum Levels Without Considering the G-CSF Treatment Status

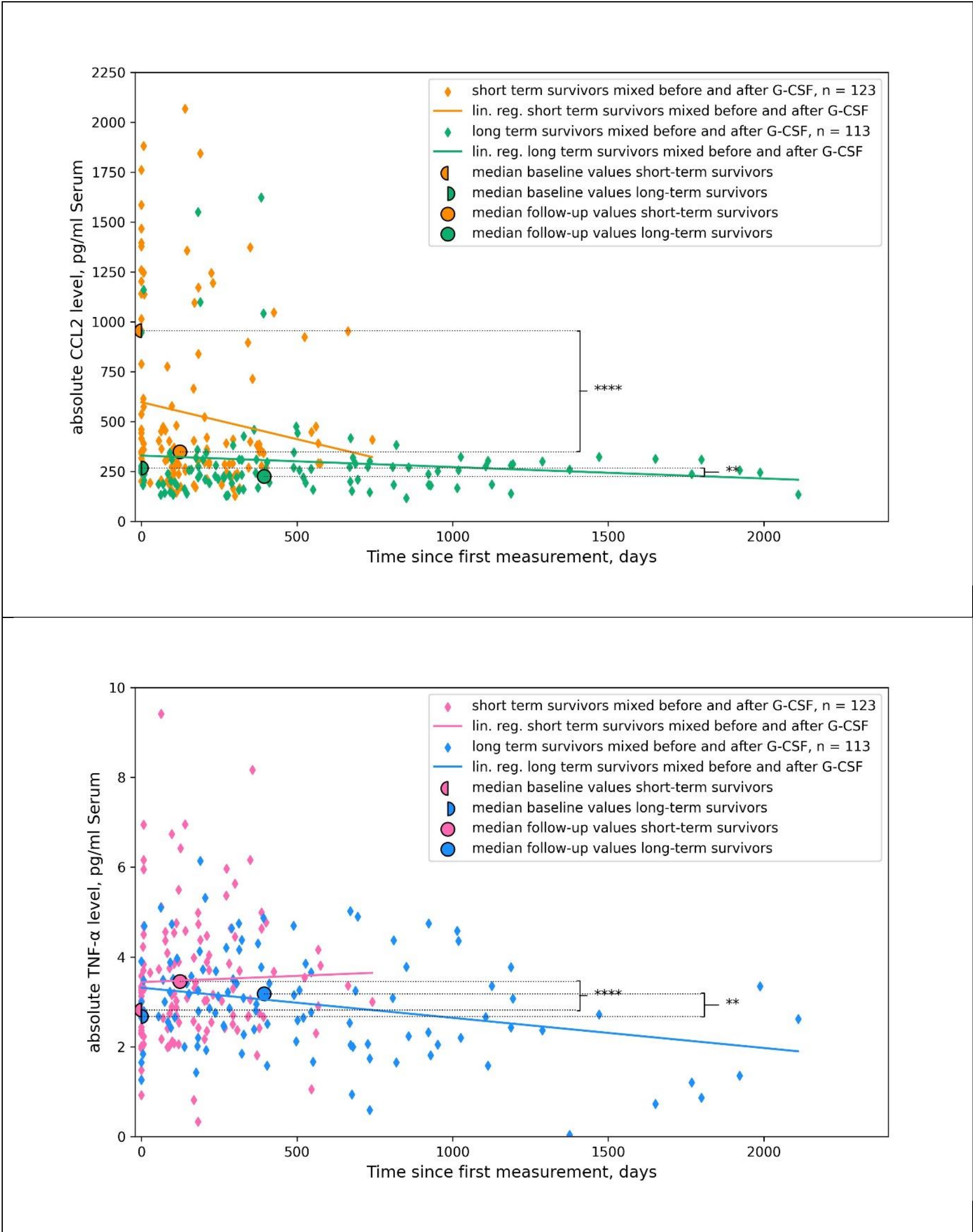

Figure 3: Absolute CCL2 levels [pg/ml Serum] and absolute TNF-α levels [pg/ml Serum] of short-term and long-term survivors, considering the G-CSF treatment status.
Top graph: Orange colour represents CCL2 levels of short term survivors, green colour represents CCL2 levels of long term survivors. Diamonds represent the individually measured absolute CCL2 levels (before and after G-CSF treatment). The lines represents the linear regression of absolute CCL2 levels over time. The semicircles represent the medians of absolute CCL2 baseline levels. The circles represent the medians of absolute CCL2 follow-up levels

(before and after G-CSF treatment). The asterisks represent the degree of significance by which the medians of the absolute CCL2 baseline values differ from the medians of the absolute CCL2 follow-up values (*: $p \leq 0.05$, **: $p \leq 0.01$, ***: $p \leq 0.001$, ****: $p \leq 0.0001$).
Bottom graph: Pink colour represents TNF-α levels of short term survivors, blue colour represents TNF-α levels of long term survivors. Diamonds represent the individually measured absolute TNF-α levels (before and after G-CSF treatment). The lines represents the linear regression of absolute TNF-α levels over time. The semicircles represent the medians of absolute TNF-α baseline levels. The circles represent the medians of absolute TNF-α follow-up levels (before and after G-CSF treatment). The asterisks represent the degree of significance by which the medians of the absolute TNF-α baseline values differ from the medians of the absolute TNF-α follow-up values (*: $p \leq 0.05$, **: $p \leq 0.01$, ***: $p \leq 0.001$, ****: $p \leq 0.0001$).

4.3.1 Absolute CCL2 Serum Levels Without Considering the G-CSF Treatment Status

To compare the central tendencies of absolute CCL2 serum levels of short-term survivors and long-term survivors, the mean of the absolute CCL2 serum levels during the observation period was calculated individually for each patient. Then, the median of the individual patients' absolute CCL2 serum level averages of the short-term survivor group and the long-term survivor group were calculated and tested for significant differences using the Wilcoxon rank sum test.

The median serum level of short-term survivors (without considering the G-CSF treatment status) (median = 522.74 pg/ml serum, *SD* = 298.82 pg/ml) was compared to the respective median serum level of long-term survivors (median = 227.23 pg/ml serum, *SD* = 222.89 pg/ml) (not shown in the graph). The median of absolute CCL2 serum levels of short-term survivors is significantly higher than the median of long-term survivors ($p = 0.0033$) (not shown in the graph).

To compare baseline absolute CCL2 serum levels to follow-up levels, the mean of the absolute CCL2 serum levels during the observation period, excluding baseline measurements, was calculated individually for each patient. Then, the median of the individual patients' averages, excluding baseline measurements, and the median of the baseline measurements were calculated and tested for significant differences using the Wilcoxon signed-rank test.

The median of absolute follow-up CCL2 serum levels of short-term survivors (median = 349.69 pg/ml serum, *SD* = 249.92 pg/ml serum) is significantly lower than the median of the respective baseline levels (median = 956.79 pg/ml serum, *SD* = 502.64 pg/ml serum); ($p < 0.0001$).

The median of absolute follow-up CCL2 serum levels of long-term survivors (median = 225.31 pg/ml serum, *SD* = 220.94 pg/ml serum) is significantly lower than the median

of the respective baseline values (median = 267.49 pg/ml serum, *SD* = 239.25 pg/ml serum); (*p* = 0.0234).

To investigate if there is a gradual and continuous change in absolute CCL2 serum levels over time, the simple linear regression of absolute CCL2 serum levels of short-terms survivors and the simple linear regression of long-term survivors were calculated and plotted. The slope of the simple linear regression of absolute CCL2 serum levels of short-term survivors (slope = -0.370768, *p* = 0.1196) and the respective slope of long-term survivors (slope = -0.057368, *p* = 0.2258) do not deviate significantly from zero. They also do not differ significantly from each other (*p* =0.129).

4.3.2 Absolute TNF-α Serum Levels Without Considering the G-CSF Treatment Status

To compare the central tendencies of absolute TNF-α serum levels of short-term survivors and long-term survivors, the mean of the absolute TNF-α serum levels during the observation period was calculated individually for each patient. Then, the median of the individual patients' absolute TNF-α serum level averages of the short-term survivor group and the long-term survivor group were calculated and tested for significant differences using the Wilcoxon rank sum test.

The median serum level of short-term survivors (without considering the G-CSF treatment status) (median = 3.25 pg/ml serum, *SD* = 0.89 pg/ml) was compared to the respective median serum level of long-term survivors (median = 3.14 pg/ml serum, *SD* = 0.66 pg/ml) (not shown in the graph). The median of absolute TNF-α serum levels of short-term survivors is not significantly different from the median of long-term survivors (*p* = 0.4980) (not shown in the graph).

To compare baseline absolute TNF-α serum levels to follow-up levels, the mean of the absolute TNF-α serum levels during the observation period, excluding baseline measurements, was calculated individually for each patient. Then, the median of the individual patients' averages, excluding baseline measurements, and the median of the baseline measurements were calculated and tested for significant differences using the Wilcoxon signed-rank test.

The median of absolute follow-up TNF-α serum levels of short-term survivors (median = 3.46 pg/ml serum, SD = 1.18 pg/ml serum) is significantly higher than the median of

the respective baseline levels (median = 2.82 pg/ml serum, *SD* = 0.72 pg/ml serum); ($p = 0.0004$).

The median of absolute follow-up TNF-α serum levels of long-term survivors (median = 3.18 pg/ml serum, *SD* = 0.68 pg/ml serum) is significantly higher than the median of the respective baseline levels (median = 2.68 pg/ml serum, *SD* = 0.81 pg/ml serum); ($p = 0.0391$).

To investigate if there is a gradual and continuous change in absolute TNF-α serum levels over time, the simple linear regression of absolute TNF-α serum levels of short-term survivors and the simple linear regression of long-term survivors were calculated and plotted. The slope of the simple linear regression of absolute TNF-α serum levels of short-term survivors does not deviate significantly from zero (slope = 0.000278, $p = 0.7288$), whereas the slope of the simple linear regression of absolute TNF-α serum levels of long-term survivors is significantly negative (slope: -0.000672, $p = 0.0018$). The slope of the simple linear regression of absolute TNF-α serum levels of short-term survivors and the slope of the simple linear regression of absolute TNF-α serum levels of long-term survivors do not differ significantly from each other ($p = 0.2072$).

4.4 Relative CCL2 and TNF-α Serum Levels Considering the G-CSF Treatment Status

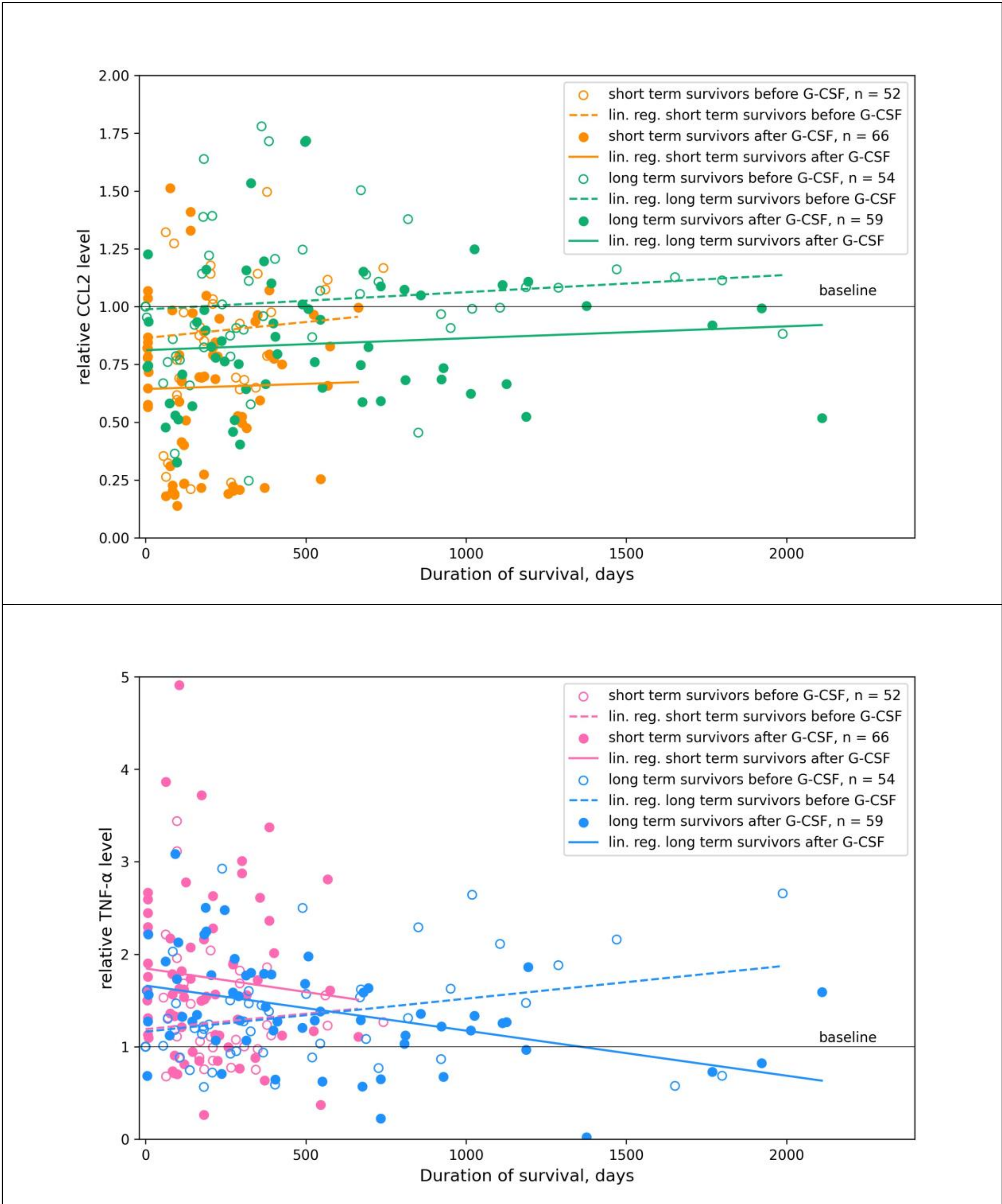

Figure 4: Relative CCL2 levels and relative TNF-α levels of short-term and long-term survivors considering the G-CSF treatment status.

Top graph: Orange colour represents CCL2 levels of short term survivors, green colour represents CCL2 levels of long term survivors. Empty circles represent relative CCL2 levels measured before G-CSF injection, filled circles represent relative CCL2 levels measured after G-CSF injection. The dashed lines represent the linear regressions of relative CCL2 levels before G-CSF administration over time. The solid lines represent the linear regressions of relative CCL2 levels after G-CSF administration over time.

Bottom graph: Pink colour represents TNF-α levels of short term survivors, blue colour represents TNF-α levels of long term survivors. Empty circles represent relative TNF-α levels measured before G-CSF injection, filled circles represent relative TNF-α levels measured after G-CSF injection. The dashed lines represent the linear regressions of relative TNF-α levels before G-CSF administration over time. The solid lines represent the linear regressions of relative TNF-α levels after G-CSF administration over time.

4.4.1 Relative CCL2 Serum Levels Considering the G-CSF Treatment Status

Relative cytokine levels of CCL2 and TNF-α were calculated by relating individual serum values over time to the respective baseline value of each patient, as detailed in the Materials and Methods section, to obtain interindividual comparable values.

To compare the central tendencies of relative CCL2 serum levels of short-term survivors and long-term survivors, the mean of the relative CCL2 serum levels during the observation period was calculated individually for each patient. Then, the median of the individual patients' relative CCL2 serum level averages of the short-term survivor group and the long-term survivor group before and after G-CSF administrations were calculated and tested for significant differences using the Wilcoxon rank sum test.

The relative median serum levels of short-term survivors measured before G-CSF administrations (including baseline levels) and after G-CSF administrations (before: median = 0.98, SD = 0.15; after: median = 0.66, SD = 0.24) were compared to the respective median serum levels of long-term survivors (before: median = 0.99, SD = 0.10; after: median = 0.82, SD = 0.11). The relative median serum level of short-term survivors measured before G-CSF administrations is not significantly different from the respective median of long-term survivors ($p = 0.3339$), whereas the relative median serum level of short-term survivors measured after G-CSF administrations is significantly lower than the respective median of long-term survivors ($p = 0.0116$).

To investigate if there is a gradual and continuous change in relative CCL2 serum levels over time the simple linear regression of relative CCL2 serum levels before and the simple linear regression of relative CCL2 serum levels after G-CSF administrations were calculated and plotted. The slope of the simple linear regression of relative CCL2 serum levels of short-term survivors under G-CSF therapy measured before administrations of G-CSF (slope = 0.000139, $p = 0.5480$), the slope of the simple linear regression of relative CCL2 serum levels of short-term survivors under G-CSF therapy measured after administrations of G-CSF (slope = 0.000045, $p = 0.8615$), the slope of the simple linear regression of relative CCL2 serum levels of long-term survivors under G-CSF therapy measured before administrations of G-CSF (slope = 0.000074, $p = 0.3896$) and the slope of the simple linear regression of relative CCL2 serum levels of long-term change under G-CSF therapy measured after administrations of G-CSF (slope = 0.000052, $p = 0.4984$) do all not deviate significantly from zero.

4.4.2 Relative TNF-α Serum Levels Considering the G-CSF Treatment Status

To compare the central tendencies of relative TNF-α serum levels of short-term survivors and long-term survivors, the mean of the relative TNF-α serum levels during the observation period was calculated individually for each patient. Then, the median of the individual patients' relative TNF-α serum level averages of the short-term survivor group and the long-term survivor group before and after G-CSF administrations were calculated and tested for significant differences using the Wilcoxon rank sum test.

The relative median serum levels of short-term survivors measured before G-CSF administrations (including baseline levels) and after G-CSF administrations (before: median = 1.00, SD = 0.28; after: median = 1.57, SD = 0.84) were compared to the respective median serum levels of long-term survivors (before: median = 1.06, SD = 0.33); after: median = 1.43, SD = 0.33). The medians of serum levels of short-term survivors are not significantly different from the medians of long-term survivors, both when measured before ($p = 0.9550$) and after G-CSF ($p = 0.3566$).

To investigate if there is a gradual and continuous change in relative TNF-α serum levels over time the simple linear regression of relative TNF-α serum levels before and the simple linear regression of relative TNF-α serum levels after G-CSF administrations were calculated and plotted. The slopes of the simple linear regressions of relative TNF-α serum levels of short-term survivors measured before G-CSF administrations (slope = 0.000334, $p = 0.4505$) and of relative TNF-α serum levels of short-term survivors measured after G-CSF administrations do not deviate significantly from zero (slope = -0.000509, $p = 0.4524$). The slope of the simple linear regression of relative TNF-α serum levels of long-term survivors measured before administrations of G-CSF is significantly positive (slope = 0.000357, $p = 0.0193$), whereas the slope of the simple linear regression of relative TNF-α serum levels of long-term survivors measured after administrations of G-CSF is significantly negative (slope = -0.000487, $p = 0.0014$).

4.5 Relative CCL2 and TNF-α Serum Levels Without Considering the G-CSF Treatment Status

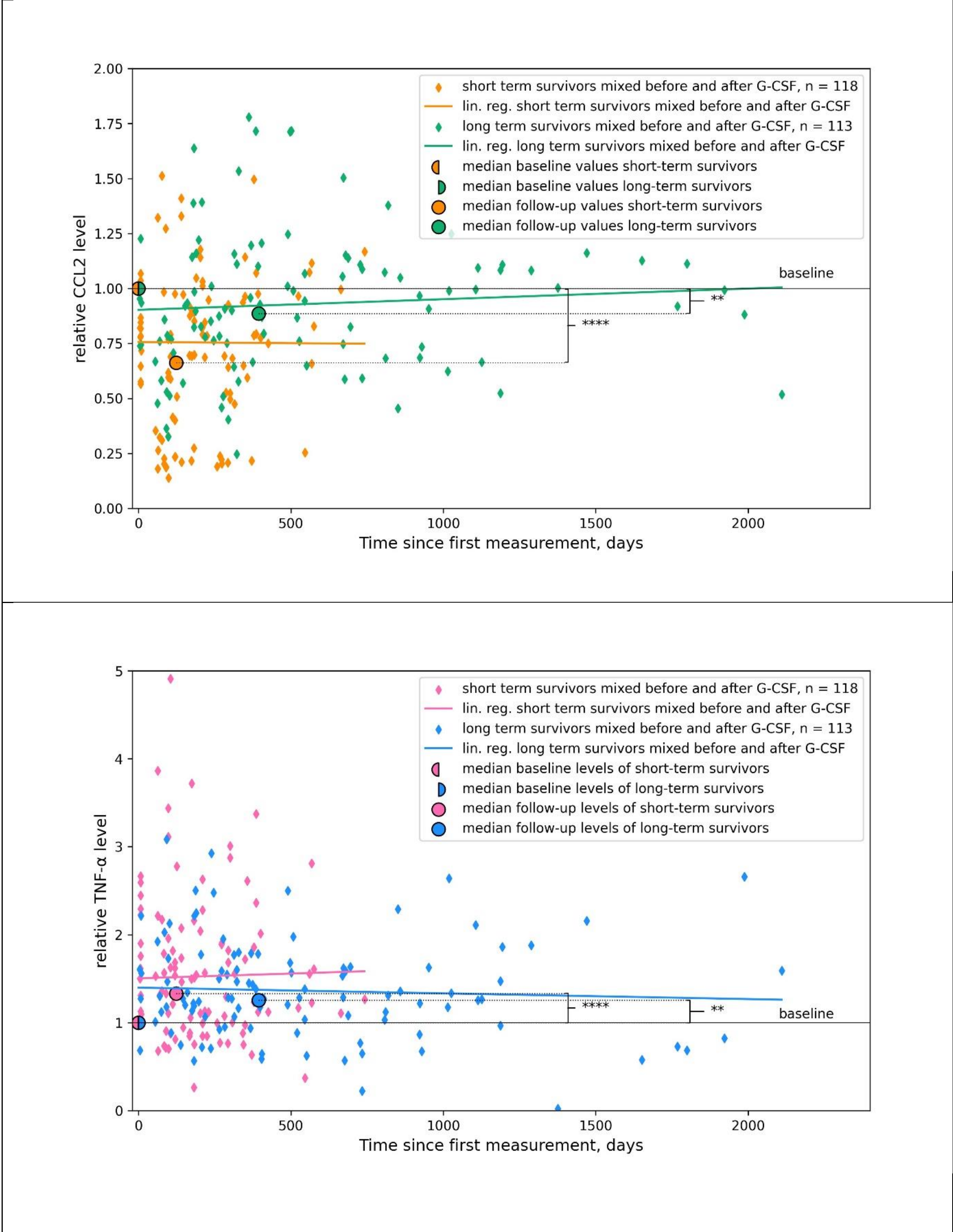

Figure 5: Relative CCL2 levels and relative TNF-α levels of short-term and long-term survivors, without considering the G-CSF treatment status.

Top graph: Orange colour represents CCL2 levels of short term survivors, green colour represents CCL2 levels of long term survivors. Diamonds represent the individually measured relative CCL2 levels (before and after G-CSF treatment). The lines represents the linear regression of relative CCL2 levels over time. The semicircles represent the medians of relative CCL2 baseline levels. The circles represent the medians of relative CCL2 follow-up levels

(before and after G-CSF treatment). The asterisks represent the degree of significance by which the medians of the relative CCL2 baseline values differ from the medians of the relative CCL2 follow-up values (*: p ≤ 0.05, **: p ≤ 0.01, ***: p ≤ 0.001, ****: p ≤ 0.0001).
Bottom graph: Pink colour represents TNF-α levels of short term survivors, blue colour represents TNF-α levels of long term survivors. Diamonds represent the individually measured relative TNF-α levels (before and after G-CSF treatment). The lines represents the linear regression of relative TNF-α levels over time. The semicircles represent the medians of relative TNF-α baseline levels. The circles represent the medians of relative TNF-α follow-up levels (before and after G-CSF treatment). The asterisks represent the degree of significance by which the medians of the relative TNF-α baseline values differ from the medians of the relative TNF-α follow-up values (*: p ≤ 0.05, **: p ≤ 0.01, ***: p ≤ 0.001, ****: p ≤ 0.0001).

4.5.1 Relative CCL2 Serum Levels Without Considering the G-CSF Treatment Status

To compare the central tendencies of relative CCL2 serum levels of short-term survivors and long-term survivors, the mean of the relative CCL2 serum levels during the observation period was calculated individually for each patient. Then, the median of the individual patients' relative CCL2 serum level averages of the short-term survivor group and the long-term survivor group were calculated and tested for significant differences using the Wilcoxon rank sum test.

The median serum level of short-term survivors (without considering the G-CSF treatment status) (median = 0.74, $SD = 0.18$) was compared to the respective median serum level of long-term survivors (median = 0.89, $SD = 0.10$) (not shown in the graph). The median of relative CCL2 serum levels of short-term survivors is significantly lower than the median of long-term survivors ($p = 0.0103$) (not shown in the graph).

To compare baseline relative CCL2 serum levels to follow-up levels, the mean of the relative CCL2 serum levels during the observation period, excluding baseline measurements, was calculated individually for each patient. Then, the median of the individual patients' averages, excluding baseline measurements, and the median of the baseline measurements were calculated and tested for significant differences using the Wilcoxon signed-rank test.

The median of relative follow-up CCL2 serum values of short-term survivors (median = 0.66, $SD = 0.25$) is significantly lower than the median of the respective baseline levels (median 1.00, $SD = 0.00$); ($p = 0.0002$).

The median of relative CCL2 serum values of long-term survivors (median = 0.89, $SD = 0.10$) is significantly lower than the median of the respective baseline levels (median = 1.00, $SD = 0.0$); ($p = 0.039$).

To investigate if there is a gradual and continuous change in relative CCL2 serum levels over time, the simple linear regression of relative CCL2 serum levels of short-

terms survivors and the simple linear regression of long-term survivors were calculated and plotted. The slope of the simple linear regression of relative CCL2 serum levels of short-term survivors (slope = -0.000011, $p = 0.9538$) and the respective slope of long-term survivors (slope = 0.000048, $p = 0.4167$) do not deviate significantly from zero. They also do not differ significantly from each other ($p = 0.752$).

4.5.2 Relative TNF-α Serum Levels Without Considering the G-CSF Treatment Status

To compare the central tendencies of relative TNF-α serum levels of short-term survivors and long-term survivors, the mean of the relative TNF-α serum levels during the observation period was calculated individually for each patient. Then, the median of the individual patients' relative TNF-α serum level averages of the short-term survivor group and the long-term survivor group were calculated and tested for significant differences using the Wilcoxon rank sum test.

The median serum level of short-term survivors (without considering the G-CSF treatment status) (median = 1.29, *SD* = 0.50) was compared to the respective median serum level of long-term survivors (median = 1.24, *SD* = 0.29) (not shown in the graph). The median of relative TNF-α serum levels of short-term survivors is not significantly different from the median of long-term survivors ($p = 0.6512$) (not shown in the graph).

To compare baseline relative TNF-α serum levels to follow-up levels, the mean of the relative TNF-α serum levels during the observation period, excluding baseline measurements, was calculated individually for each patient. Then, the median of the individual patients' averages, excluding baseline measurements, and the median of the baseline measurements were calculated and tested for significant differences using the Wilcoxon signed-rank test.

The median of relative follow-up TNF-α serum values of short-term survivors (median = 1.33, *SD* = 0.62) is significantly higher than the median of the respective baseline levels (median = 1.00, *SD* = 0.00); ($p = 0.0003$).

The median of relative follow-up TNF-α serum values of long-term survivors (median = 1.25, SD = 0.30) is significantly higher than the median of the respective baseline levels (median = 1.00, SD = 0.00); (p = 0.0391).

To investigate if there is a gradual and continuous change in relative TNF-α serum levels over time, the simple linear regression of relative TNF-α serum levels of short-

term survivors and the simple linear regression of long-term survivors were calculated and plotted. The slope of the simple linear regression of relative TNF-α serum levels of short-term survivors (slope = 0.000108, $p = 0.8033$) and the slope of relative TNF-α serum levels of long-term survivors (slope = -0.000065, $p = 0.5554$) do not deviate significantly from zero. They also do not differ significantly from each other ($p = 0.6956$).

4.6 Analysis of the P Value of the Difference in CCL2 Baseline Serum Levels and of the Difference in TNF-α Baseline Serum Levels in Relation to the Respective Cut-Off Time Point for Group Classification

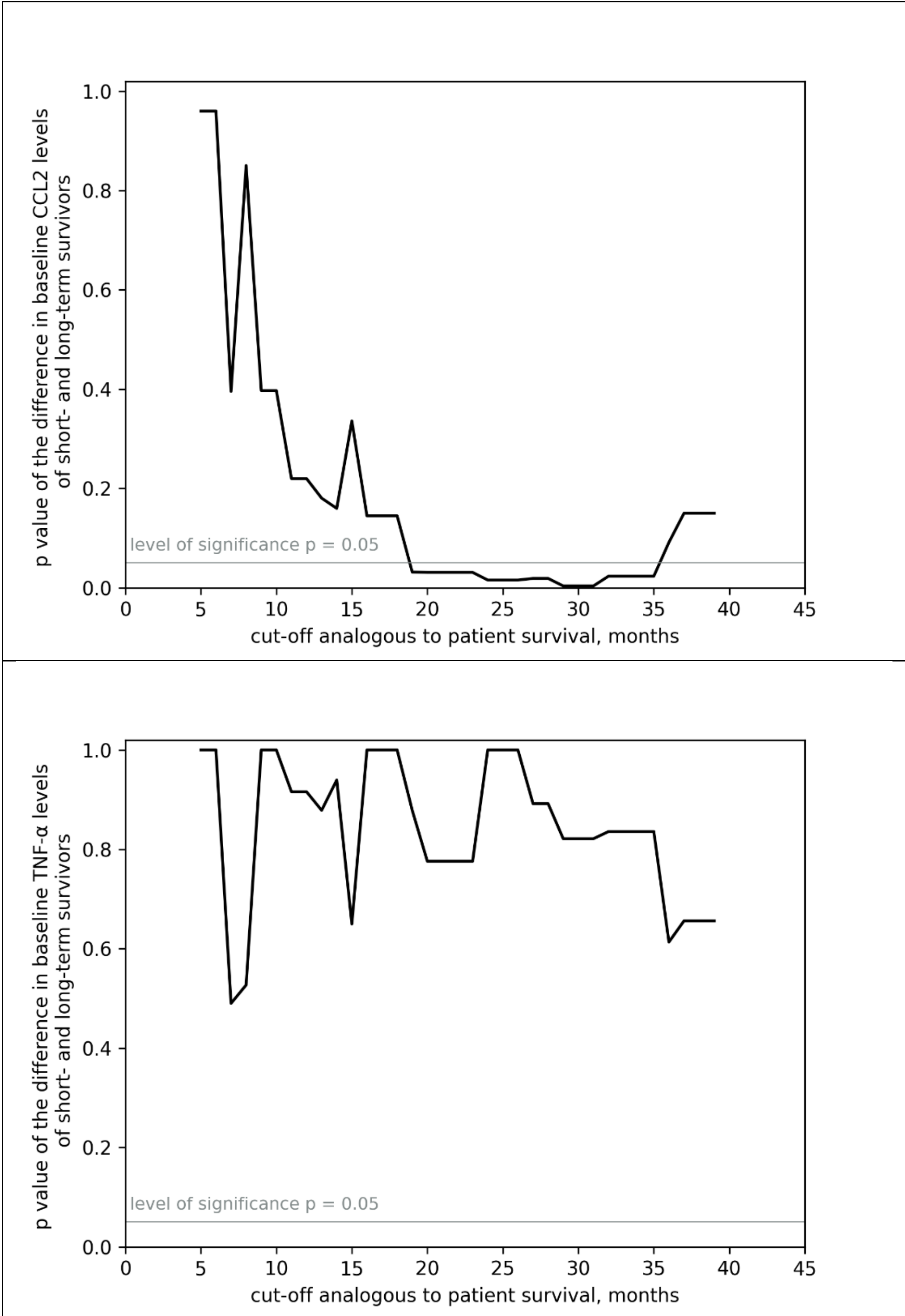

Figure 6: Change of the P value of the difference between short and long-term survivor groups regarding the groups' CCL2 baseline levels and TNF-α baseline levels when subdivided into two groups based on the respective survival period in months after treatment start according to the Wilcoxon rank sum test.

Top graph: The black solid line represents the change of the P value of the difference between short- and long-term survivor group regarding the CCL2 baseline levels for different survival durations (measured in months after treatment begin) according to the Wilcoxon rank sum test

Bottom graph: The black solid line represents the change of the P value of the difference between short- and long-term survivor group regarding the TNF-α baseline levels for different survival durations (measured in months after treatment begin) according to the Wilcoxon rank sum test

4.6.1 The CCL2 Baseline Serum Level as a Potential Biomarker for Methodological Validation of the Chosen Cut-Off

Looking at the P value of the difference in CCL2 baseline serum levels, it turns out that patients can best be classified as short- and long-term survivors if the cut-off value is between 29 and 31 months after the start of therapy and the CCL2 baseline serum level acts as a suitable biomarker for methodological validation of the clinically chosen cut-off. When regarding the medians of the CCL2 baseline levels, the P value is minimal at a threshold between 29 and 31 months survival duration after treatment start (p = 0.0026) as a cut-off between the long and the short survivor group. When subdivided into groups at a threshold of 30 months survival duration after treatment start, short-term survivors and long-term survivors differ highly significantly in the medians of the CCL2 baseline levels between the short- and long-term survivor group.

4.6.2 The TNF-α Baseline Serum Level as a Potential Biomarker for Methodological Validation of the Chosen Cut-Off

Looking at the P value of the difference in TNF-α baseline serum levels, no significant specific changes can be observed so correspondingly the TNF-α does not help as a biomarker to methodologically validate the clinically selected cut-off at 30 months after the start of therapy. When regarding the medians of the TNF-α baseline serum levels, the P value is not minimal at the threshold of 30 months survival duration after treatment start as cut off between short- and long-term survivor group (p = 0.8214). It is minimal but not statistically significant at a threshold of 7 months survival duration after treatment start (p = 0.4900).

5 Discussion

5.1 Discussion of the Applied Calculations and Analyses

Before the detailed discussion of the results, it should first be explained and discussed for what purpose the individual calculations and analyses of serum cytokine levels were performed and which analyses are particularly suitable for which investigations, so that the results can be interpreted with appropriate care. It is important to be clear for which analyses it is more appropriate to consider absolute cytokine levels and for which studies it is more appropriate to consider relative cytokine levels and to understand the difference between analyzing the change in slope of the simple linear regression of cytokine serum levels and comparing relative cytokine serum levels after treatment initiation to baseline cytokine levels.

5.1.1 Advantages and Disadvantages of Absolute Cytokine Levels

Measurement of absolute cytokine levels, quantified in pg/ml, offers direct insights into the concentration of these signaling molecules within the biological system. This approach permits a clear understanding of the magnitude of cytokine presence or absence, facilitating assessments of their potential clinical relevance. Absolute cytokine levels can efficiently elucidate pronounced changes that may occur during treatment interventions. However, the reliance on absolute values may expose the analysis to undue influences from outliers or extreme values, which can distort the interpretation of overall trends. Moreover, the inherent interindividual variability in baseline levels might obscure subtle but consistent changes over time, particularly when attempting to ascertain treatment effects. Consequently, the reliance on absolute cytokine levels necessitates careful consideration of these limitations to draw accurate inferences from the data.

5.1.2 Advantages and Disadvantages of Relative Cytokine Levels

In contrast, the assessment of relative cytokine levels, derived by normalizing follow-up measurements to baseline values, presents distinct advantages in the analysis of treatment effects. By mitigating the impact of interindividual variability, relative values provide an avenue for comparing proportional changes across individuals, thereby offering improved interindividual comparability. Such normalization can accentuate changes attributable to treatment interventions, particularly when dealing with data

characterized by substantial variability. The proportional nature of relative values can potentially unveil consistent trends that might otherwise be overshadowed by the inherent heterogeneity in baseline cytokine levels. However, the normalization process to a constant baseline value, often set at 1, might obscure the clinical significance of changed baseline values and their potential role in influencing follow-up trends. Consequently, while relative cytokine levels offer a robust means of assessing proportional changes, the interpretational context must acknowledge the implications of baseline normalization on clinical understanding.

It is important to recognize that the choice between utilizing absolute or relative cytokine levels hinges upon the research question's context, the specific characteristics of the dataset, and the desired sensitivity in detecting treatment effects. Each approach brings unique strengths and limitations that require careful consideration to ensure an accurate interpretation of the findings.

5.1.3 Advantages and Disadvantages of the Simple Linear Regression

Linear regression analysis stands as a pivotal method in deciphering relationships between variables, in this case, cytokine serum levels across distinct temporal points. It offers the capability to discern gradual and continuous changes over time, thereby presenting insights into patterns of cytokine fluctuations throughout treatment. Yet, linear regression may be limited in capturing abrupt or non-linear changes or one-time shifts in cytokine levels post-treatment initiation. It underscores continuous trends, which could potentially overlook significant variations occurring sporadically. Such continuous changes in cytokine serum levels may, but need not, occur due to a treatment effect.

5.1.4 Advantages and Disadvantages of Comparing Follow-Up Cytokine Levels With Baseline Levels

Comparing follow-up cytokine levels with baseline values offers a straightforward avenue for assessing an overall change in cytokine serum levels during treatment after treatment start. Such an overall change in cytokine serum levels may, but need not, occur due to a treatment effect. Calculating mean differences between post-treatment and pre-treatment cytokine levels provides a preliminary indicator of the overall direction of change. This method's simplicity and accessibility are noteworthy advantages. Nonetheless, a limitation of this approach is that it lacks the sophistication to account

for individual patient variability or the irregular time intervals between measurements. It neglects the temporal dimension and may fail to detect nuanced shifts in cytokine levels that evolve distinctly over time. Furthermore, it might inadvertently conflate sporadic, one-time changes with continuous, evolving trends, potentially leading to premature conclusions.

In summary, each approach is characterized by distinct strengths and weaknesses, with linear regression accentuating continuous patterns and sudden shifts, respectively. Comparing follow-up cytokine levels with baseline values offers an accessible gateway to preliminary insights, albeit at the expense of temporal sensitivity and nuanced variability.

5.2 Changes in CCL2 Serum Levels in ALS Patients Receiving G-CSF Treatment, the Possible Impact of CCL2 on Disease Progression, and its Potential as a Biomarker in ALS

5.2.1 Evaluation of the Results Regarding Baseline CCL2 Levels in Short- and Long-Term Survivors

Since the medians of CCL2 baseline serum levels of the short-term and the long-term survivor groups differ significantly and since the median CCL2 baseline serum level of the short-term survivor group is significantly higher than the median of the absolute CCL2 baseline serum value of the long-term survivor group (Figure 1), it can be hypothesized that a low CCL2 baseline serum value during therapy with G-CSF is conducive to a milder course of the disease and prolonged survival, at least in some patients. It can also be speculated that patients who show a beneficial response to G-CSF therapy and benefit from G-CSF administration are characterized by a lower CCL2 baseline serum value. Furthermore, it can be deduced that the probability is relatively high that patients under G-CSF therapy with a CCL2 baseline serum value between 250-300pg/ml have a relatively high likelihood of long-term survival of over 30 months after G-CSF treatment initiation, especially when compared to patients with higher CCL2 baseline serum levels. Overall, the results suggest that the CCL2 baseline serum level is a prognostically valuable parameter for the survival duration of ALS patients under G-CSF therapy. The finding that high baseline CCL2 serum levels are associated with faster disease progression in ALS patients under G-CSF treatment fits well with the literature data. A previous study that examined CCL2 levels under G-CSF

treatment in CSF, not in serum as this work, reported a trend towards an association between high CCL2 baseline CSF levels and a more rapid disease progression (335).

5.2.2 Evaluation of the Results Regarding Absolute CCL2 Serum Levels During Treatment

5.2.2.1 Comparison of Median Absolute CCL2 Serum Levels of Short- and Long-Term Survivors

It is found that the medians of absolute CCL2 serum levels of short-term survivors are significantly higher than the medians of absolute CCL2 serum levels of long-term survivors, both when comparing absolute CCL2 serum values of short- and long-term surviving patients before G-CSF administration (Figure 2) as well as when comparing absolute CCL2 serum concentrations of patients after G-CSF administration (Figure 2), and also when comparing absolute CCL2 serum concentrations of short- and long-term survivors without considering the G-CSF treatment status (Figure 3). The finding of higher CCL2 levels in short-term survivors fits the assumption that high CCL2 levels in ALS patients under G-CSF therapy are prognostically unfavorable and associated with shorter survival and a faster, more severe disease progression. This supports the hypothesis that CCL2 is a suitable biomarker for the severity of the disease course of ALS under G-CSF therapy and that high CCL2 serum levels in ALS patients under G-CSF treatment indicate a more severe and rapid disease progression. This is in line with earlier research on CCL2 serum levels in ALS patients (albeit lacking G-CSF treatment). One study reported a negative correlation between CCL2 concentrations and ALSFRS-R scores, alongside a positive correlation between CCL2 concentrations and disease progression rate. This suggests that elevated CCL2 serum levels are linked to increased disease severity and faster progression (209). Correspondingly, another study observed a positive correlation between CCL2 plasma levels and symptom durations in ALS, indicating higher levels of circulating CCL2 in more advanced disease stages (218).

5.2.2.2 Analysis of the Slope of the Simple Linear Regression of Absolute CCL2 Serum Levels in Short- and Long-Term Survivors and Comparison of the Medians of Absolute CCL2 Follow-Up Serum Levels and Absolute CCL2 Baseline Levels in Short- and Long-Term Survivors

A significantly negative slope of the simple linear regression of absolute CCL2 serum levels of short-term survivors measured after the start of G-CSF treatment and before the individual G-CSF administrations in the course of treatment is observed (Figure 2). This could indicate a continuous treatment effect of G-CSF in the short-term survivors, which decreases CCL2 serum levels measured before G-CSF administrations in the short-term survivors.

This assumption fits with the known neuroprotective and anti-inflammatory properties of G-CSF (330, 331). It suggests that there is not only a one-time decrease in CCL2 serum levels after the start of G-CSF therapy in the CCL2 serum levels of short-term survivors measured before G-CSF administrations, but a continuous significant decrease in CCL2 serum levels during the course of the treatment. Of course, this continuous decline in cytokine serum levels could also occur independently of the G-CSF administrations, due to other factors that have not been analyzed. The analysis only indicates a possible treatment effect. But if one attributes this change in cytokine serum levels to G-CSF administration, the change suggests that G-CSF administration appears to have a primarily long-term and not short-term decreasing effect on absolute CCL2 serum levels of short-term survivors. The observation that the decrease in the slope of cytokine levels is found for CCL2 serum levels in short-term survivors measured before G-CSF administrations, but not for CCL2 serum levels measured directly after G-CSF administrations, leads to the assumption that the G-CSF treatment effect continues to affect and lower CCL2 serum levels even until the measurement directly before the next G-CSF administration.

Provided that G-CSF has anti-inflammatory properties and that G-CSF treatment has a slowing effect on disease progression, at least in a certain proportion of responders (339), one might also expect a statistically significant decrease in the slope of the simple linear regression of absolute CCL2 serum levels of short-term survivors directly after (and not only before) G-CSF administration and for the slope of the simple linear regression of long-term survivors before and after G-CSF administration. In particular considering an earlier study, which found a decrease in CCL2 serum levels in patients

responding to G-CSF (339), a continuous favorable effect of G-CSF and therefore an even stronger statistically significant decrease in absolute CCL2 serum levels could be expected especially in the well-responding and, accordingly, longer-surviving patients. A possible reason for the lack of an observed effect of G-CSF administration on the absolute CCL2 serum levels of the long-term survivors compared to the observed significant effect in the short-term survivors could also be that regarding CCL2 serum levels short-term survivors respond even more strongly to G-CSF treatment than long-term survivors, but the response in short-term survivors is nevertheless not strong enough to provide the patients with a long-term survival. Another potential explanation for the lack of a significant change in the slope of the simple linear regression in the group of short-term survivors after G-CSG administration and the group of long-term survivors could be that the size of the observed patient cohort might be too small for a statistically significant result.

In line with this is the finding that the central tendencies of absolute CCL2 follow-up serum levels are significantly lower under G-CSF treatment than the respective absolute CCL2 baseline serum levels in both short-term and long-term survivors (Figure 3). It can be concluded that absolute CCL2 follow-up serum levels decrease during G-CSF therapy compared to absolute CCL2 baseline values measured before the start of G-CSF applications, probably due to a treatment effect. However, as mentioned previously, the examination of absolute CCL2 serum levels is generally more suitable for assessing the prognostic value as a biomarker, whereas the examination of relative CCL2 serum levels is more suitable for uncovering potential treatment effects.

5.2.3 Evaluation of the Results Regarding Relative CCL2 Serum Levels During Treatment

5.2.3.1 Comparison of Median Relative CCL2 Serum Levels of Short- and Long-Term Survivors

The finding that the central tendencies of relative CCL2 serum levels are overall significantly lower in short-term survivors than in long-term survivors (Figure 4, Figure 5) (except for the comparison of the medians of relative CCL2 serum levels before G-CSF administrations, which didn´t show a significant group difference) seems at first sight contradictory to the results regarding absolute CCL2 levels, where the medians of CCL2 serum levels are significantly higher in short-term survivors. But in fact, the

finding of overall lower relative CCL2 levels in short-term survivors is obvious and quite conclusive. Baseline CCL2 values in short-term survivors are on average higher than baseline CCL2 values in long-term survivors and relative serum levels measured in the further course of treatment are always related to the baseline. Thus, despite higher absolute serum CCL2 levels in short-term survivors, relative CCL2 levels after baseline measurements are overall lower in short-term survivors than in long-term survivors. Accordingly, it can be concluded that regarding conclusions on the prognostic value of serum cytokine levels in ALS, it is more useful to look at absolute levels to compare the short- and long-term survivor groups.

5.2.3.2 Analysis of the Slope of the Simple Linear Regression of Relative CCL2 Serum Levels in Short- and Long-Term Survivors and Comparison of the Median of Relative CCL2 Follow-Up Serum Levels and the Relative CCL2 Baseline in Short- and Long-Term Survivors

As opposed to the results concerning absolute CCL2 serum levels, there is no significant deviation from zero of the slopes of the simple linear regressions in the context of relative CCL2 serum levels and thus no continuous significant changes in relative CCL2 serum levels in the time course during repeated G-CSF applications after the start of G-CSF treatment are observed for the different groups (Figure 4, Figure 5). However, due to the relative CCL2 serum levels appearing to be more suitable for detecting a potential treatment effect, it is conceivable that the administration of G-CSF therapy does not lead to a continuous and gradual treatment impact. The negative slope of the simple linear regression of absolute CCL2 serum levels in short-term survivors before individual G-CSF administrations, which is not detectable for relative CCL2 serum levels, could potentially be attributed to the influence of outliers which have the potential to distort the interpretation of overall trends.

Interestingly, however, similar to absolute CCL2 serum concentrations, the central tendencies of relative CCL2 follow-up serum levels under G-CSF treatment are significantly lower than the respective baseline CCL2 serum levels in both short-term and long-term survivors (Figure 5). It follows that relative CCL2 serum levels decrease under G-CSF therapy when compared to baseline levels, measured before the start of G-CSF applications. In addition, it can be observed that relative CCL2 serum levels of short-term survivors decrease more under G-CSF therapy than the relative serum levels of long-term survivors, whose serum levels, however, are already lower anyway.

Again, the analysis only indicates a possible treatment effect, the change in cytokine serum levels is not necessarily due to G-CSF treatment. If one attributes this change in cytokine serum levels to G-CSF administration, the change suggests that the short-term survivors appear to respond on average even more strongly to G-CSF administration than the long-term survivors in terms of their CCL2 serum levels. This is consistent with the hypothesis made previously, when considering absolute serum CCL2 levels, that short-term survivors might be even more responsive to the G-CSF treatment in terms of CCL2 serum levels than long-term survivors, but their response is still not strong enough to achieve long-term survival so there is nevertheless a rapid disease progression. The finding that the slope of the simple linear regression in the relative CCL2 serum levels of the different groups is zero but a decrease in relative CCL2 serum levels can be found in comparison to baseline values suggests the existence of a general treatment effect from the start of G-CSF administration, but the absence of a gradual and continuous treatment effect that increases with repeated G-CSF administrations.

The finding that G-CSF treatment lowers CCL2 serum levels in the long term overall fits well with previous findings from another research group regarding the effect of long-term G-CSF treatment on CCL2 CSF levels in ALS patients. Similar to the present work, in which a significant reduction in CCL2 serum levels was observed with G-CSF treatment, a significant reduction in CCL2 CSF levels with G-CSF treatment was reported in the previous study (333).

5.2.4 Summary Evaluation of the CCL2 Serum Level as a Biomarker in ALS With Special Reference to Literature Data Regarding Other Neurodegenerative Diseases

In general, several studies support the hypothesis of CCL2 serum levels being a useful diagnostic biomarker in neurodegeneration and the results of this study are in line with prior findings in the context of ALS. As already outlined in more detail before (see also section 2.2.6.4.), studies investigating microglia and astrocytes derived from mSOD1 mice or from ALS patients in vitro revealed an upregulation of CCL2 when compared to wild-type cells or cells of healthy control patients (62, 67, 95). As for ALS disease in humans, significantly elevated CCL2 levels were found in the CSF (62, 142, 207–211, 201, 212, 213) and also in the serum or plasma of ALS patients (142, 214–216, 209, 210) as compared to controls, although blood levels were usually less elevated than CSF levels. For the sake of completeness, however, it should also be added

restrictively that several recent studies (207, 208, 210, 344) and a metanalysis from 2017 (217) did not find any significant differences in CCL2 serum or plasma levels between patients suffering from ALS compared to control subjects.

As for CCL2 CSF levels in ALS, a trend towards a positive correlation between CCL2 levels in CSF and a shorter time between the onset of first symptoms and final diagnosis (207) and a significant positive correlation between CCL2 levels in CFS and more severe ALS disease status (215) have been reported. Moreover, it has been reported that CCL2 CSF levels were significantly higher in fast-progressing patients than in slow-progressing patients, assessed by change of the ALSFRS-R scores (210), and levels of CCL2 in the CSF were reported to negatively correlate with the ALSFRS-R scores of ALS patients and positively correlate with the disease progression rate (209). Similarly, another group observed a negative correlation between CCL2 CSF values and ALSFRS-R score (211). These observations indicate that higher CCL2 CSF levels are associated with a more severe disease state and suggest a faster disease progression in patients with higher CCL2 levels in CSF. Of course, these findings can’t be applied without restriction to the expected behavior of CCL2 serum levels but still, they support the general assumption that CCL2 is a suitable biomarker for ALS disease and that an upregulation of CCL2 is associated with worse disease, what is consistent with the results of the present work.

As for CCL2 blood values in ALS, it was reported that CCL2 plasma concentrations correlated positively with disease duration, meaning higher CCL2 plasma levels were observed with disease progression (218), and additionally, a negative correlation was found between CCL2 serum values and the ALSFRS-R scores contrary to a positive correlation of CCL2 serum levels and disease progression rate (209). The author is not aware of any previous studies finding a significant positive correlation between CCL2 baseline levels in serum and a faster disease progression in ALS patients, as is the case with CCL2 serum levels in the present work, but in summary, these literature data regarding peripheral CCL2 levels in ALS patients during the disease course are very much in line with the results of the present work and support the idea that higher CCL2 levels are in general prognostically unfavorable since they indicate that higher CCL2 values are associated with a faster disease progression and a more severe disease stage. However, it should be mentioned that some studies found no correlation

between the severity of ALS disease and CCL2 CSF values (212) or CCL2 serum levels (210).

Literature data on CCL2 levels in different tissues in other neurodegenerative diseases provide evidence for the suitability of CCL2 levels as a biomarker for disease severity and progression in neurodegeneration. In light of the data available to date, the validity of the results of this work will now be evaluated. By comparing the literature data with the results of this work, it may also be possible to draw further conclusions about the potential of CCL2 serum levels as a biomarker in ALS. Therefore, the knowledge from the literature research is briefly summarized again (see also section 2.2.6.).

Similar to what has been reported by other authors for ALS, increased concentrations of CCL2 in the brain have been found in MS (140, 139, 165, 176). Interestingly however, in contrast to reports of increased CCL2 CSF levels in ALS patients, it has been repeatedly observed that CCL2 CSF levels are decreased in MS (171, 173, 177–181). The findings on CCL2 serum or plasma levels in MS are strongly divergent since some researchers did not observe differences in CCL2 serum levels between MS patients and controls (185) and found no evidence of a systemic dysregulation of CCL2 (186), whereas others reported reduced CCL2 serum levels (177, 182) and again others reported elevated CCL2 serum levels (183). It can be concluded that the findings on CCL2 levels in MS do not reliably support inferences regarding the potential utility of CCL2 serum levels as a biomarker in ALS.

As for AD, similar to ALS and MS, an upregulation of CCL2 expression in the brain (141, 187, 188) and increased CCL2 levels in the CSF (199–201) were observed. Higher CCL2 CSF concentrations were reported to correlate with a faster disease progression, indicated by an accelerated loss of cognitive function and a sharper decline in MMSE scores (345, 346), a greater extent of cerebral atrophy in the relevant brain regions (345), a faster conversion from MCI to AD (346) and increased levels of phosphorylated tau protein and Aβ in the CSF (199). As for CCL2 serum or plasma levelsin AD, CCL2 levels were observed to be significantly higher in AD patients (202) and in subjects with MCI and mild to moderate AD (203). Morevover, a progressive decline in CCL2 serum levels was found in patients who converted from MCI to AD, while patients with stable MCI showed no significant change (203). Interestingly, some studies found no link between CCL2 serum levels and the stage of AD (200, 202), whereas others

revealed a positive correlation (203), and again others reported a negative correlation with MMSE scores (347). The observation of elevated CCL2 serum levels in AD patients with shorter survival duration (203) aligns with the discovery of higher baseline CCL2 levels in ALS patients with shorter survival in the present work. The results of this work, along with existing literature on CCL2 behavior in ALS, suggest a notable similarity in CCL2 dynamics between ALS and AD. Overall, considering CCL2 as a potential biomarker in neurodegenerative diseases, more parallels emerge between ALS and AD than between ALS and MS.

In conclusion, it can be stated that CCL2 levels in neurodegenerative diseases are definitely affected by the associated pathological processes and change accordingly, but, despite some similarities, they do not necessarily evolve in the same way in different tissues (CNS tissue, CSF, and serum or plasma), due to the different neurodetrimental processes of different neurodegenerative diseases. Most findings from literature suggest that ALS is accompanied by elevated CCL2 serum levels, showing a link to an accelerated disease progression and a more severe disease stage. The results of this work further support that elevated CCL2 levels indicate an unfavorable prognosis.

5.3 Changes in TNF-α Serum Levels in ALS Patients Receiving G-CSF Treatment, the Possible Impact of TNF-α on Disease Progression, and its Potential as a Biomarker in ALS

5.3.1 Evaluation of the Results Regarding Baseline TNF-α Levels in Short- and Long-Term Survivors

The fact that the medians of baseline serum TNF-α levels in the short-term and long-term survivor groups do not differ significantly indicates that the TNF-α baseline serum level has no effect on the course of the disease in patients under G-CSF therapy and is also not suitable as a prognostic parameter for the course of the disease and survival (Figure 1).

5.3.2 Evaluation of the Results Regarding Absolute TNF-α Serum Levels During Treatment

5.3.2.1 Comparison of Median Absolute TNF-α Serum Levels of Short- and Long-Term Survivors

It is found that the central tendencies of absolute TNF-α serum levels of short-term survivors and long-term survivors do not differ significantly (Figure 2, Figure 3). This indicates that the absolute TNF-α serum level is not suitable as a biomarker of predictive value for disease progression and survival in ALS patients under G-CSF therapy.

5.3.2.2 Analysis of the Slope of the Simple Linear Regression of Absolute TNF-α Serum Levels in Short- and Long-Term Survivors and Comparison of the Medians of Absolute TNF-α Follow-Up Serum Levels and Absolute TNF-α Baseline Levels in Short- and Long-Term Survivors

In the short-term survivor group, the slope of the simple linear regression of absolute TNF-α serum levels over time under G-CSF therapy does not deviate significantly from zero (Figure 2), indicating that there is no continuous change in the cytokine serum levels of short-term survivors. Accordingly, there is no evidence of a continuous, increasing treatment effect upon repeated G-CSF administrations regarding their absolute TNF-α serum levels.

In contrast, in the long-term survivor group, the slope of the simple linear regression of both absolute TNF-α serum levels immediately after G-CSF treatment (Figure 2) and absolute TNF-α serum levels without considering the G-CSF treatment status (Figure 3) decreases significantly. This might suggest that there is a continuously increasing treatment effect in long-term survivors, with absolute TNF-α serum levels measured directly after G-CSF injections decreasing steadily over time with treatment duration.

Thus, if one attributes this change in TNF-α serum levels to G-CSF administration, the finding might indicate that in terms of a continuous decrease in absolute serum TNF-α levels, long-term survivors respond better to G-CSF administration than short-term survivors, albeit only in the short-term, directly after G-CSF application. It can be assumed that G-CSF administration has an overall anti-inflammatory effect and that TNF-α, as a pro-inflammatory cytokine, is neurotoxic in the context of the inflammatory response in ALS and promotes aggressive disease progression. Therefore, the fact that long-

term survivors respond better to G-CSF administration than short-term survivors in terms of TNF-α serum levels suggests that the anti-inflammatory effect of G-CSF is more effective in the long-term survivors, at least in terms of decreasing TNF-α serum concentrations. However, it is questionable whether such a short-term effect of G-CSF on TNF-α serum levels can represent an actual relevant treatment effect and maybe it is just reflecting the positive treatment effect, coincident not causal. A long-term effect would certainly have greater relevance.

The fact that the response of long-term survivors in terms of TNF-α serum levels is evident both when considering absolute serum TNF-α levels immediately after G-CSF administration (Figure 2) and when considering absolute serum TNF-α levels in general without considering the G-CSF treatment status (Figure 3), but no significant change in the slope of the simple linear regression of absolute TNF-α serum levels before the G-CSF administrations can be found (Figure 2), may indicate that the anti-inflammatory effect of G-CSF on absolute TNF-α serum levels in long term-survivors is only short- rather than long-term.

Moreover, it is found that the central tendencies of absolute TNF-α serum levels are significantly higher during the disease course under G-CSF treatment than the central tendencies of absolute TNF-α serum baseline levels in both short- and long-term survivors. This finding supports the hypothesis that the absolute TNF-α serum level therapy is a suitable biomarker for the severity of neuroinflammation in ALS under G-CSF since the TNF-α serum levels appear to increase during the disease progression and accordingly rise with increasing neurodegeneration and -inflammation.

5.3.3 Evaluation of the Results Regarding Relative TNF-α Serum Levels During Treatment

5.3.3.1 Comparison of Median Relative TNF-α Serum Levels of Short- and Long-term Survivors

As with absolute TNF-α serum levels, also in terms of relative TNF-α serum levels, there are no significant differences in the central tendencies of short- and long-term survivors. In general, it is more useful to consider the absolute rather than the relative TNF-α levels to compare cytokine serum levels of short- and long-term survivors and to draw conclusions on the prognostic value of serum cytokine levels. However, the

difference in baseline values between groups is not as large for TNF-α as for CCL2, and therefore the reference to baseline does not bias the significance of relative TNF-α values as much as for CCL2.

5.3.3.2 Analysis of the Slope of the Simple Linear Regression of Relative TNF-α Serum Levels in Short- and Long-term Survivors and Comparison of the Medians of Relative TNF-α Serum Levels and the Relative TNF-α Baseline in Short- and Long-Term Survivors

Looking at the relative TNF-α serum levels of the short-term survivors, no significant change in the slopes of the simple linear regression (Figure 4, Figure 5) and, accordingly, no significant continuous change in the relative TNF-α serum levels over time after the start of G-CSF treatment is observed.

If one assumes that G-CSF treatment has a continuous anti-inflammatory effect on patients, one might expect that relative serum TNF-α levels would continuously decrease during disease progression. In contrast, if one assumes that TNF-α, as a pro-inflammatory cytokine, is an indicator of the extent of neuroinflammation, one might expect that relative serum TNF-α levels would increase continuously during disease progression, especially in the short-term survivor group. The finding that the slope of the simple linear regression of relative serum TNF-α levels in short-term survivors does not deviate from zero may be because these two trends offset each other so that overall, there is no significant continuous trend in relative serum TNF-α levels in short-term survivors.

Another explanation for the lack of a significant change in the slope of the simple linear regression of relative serum TNF-α levels could be that G-CSF therapy does not have a significant effect sustained over time on relative serum TNF-α levels in short-term survivors because these patients do not respond sufficiently well to the administration of the anti-inflammatory G-CSF in terms of TNF-α serum levels. However, in this case, one might expect a continuous increase in relative serum TNF-α levels due to the progressive neuroinflammation if G-CSF treatment has no decreasing anti-inflammatory effect on relative serum TNF-α levels in short-term survivors. In this case, the small size of the patient cohort could be a possible reason for the lack of a significant increase in the slope. Another reason for the absence of a slope in the linear regression of relative serum TNF-α levels in short-time survivors could be that a potential change

in TNF-α serum levels may not be gradual and continuous, and therefore not detectable through linear regression analysis.

Looking at the relative TNF-α serum levels of long-term survivors, the simple linear regression of relative TNF-α serum levels measured before the individual G-CSF administrations has a significantly positive slope (Figure 4). In contrast to this, the simple linear regression of relative TNF-α serum levels measured after G-CSF administration has a significantly negative slope (Figure 4). The slope of the simple linear regression of relative TNF-α serum levels of long-term survivors without considering the G-CSF treatment status did not significantly deviate from zero (Figure 5).

If, as hypothesized, serum TNF-α levels are a suitable biomarker of progressive neuroinflammation, the positive slope of the simple linear regression of relative serum TNF-α levels of long-term survivors before G-CSF administration could be indicative of the increasing inflammatory response. In contrast, the negative slope of the simple linear regression of relative serum TNF-α levels of long-term survivors after G-CSF administration could be due to a response to the anti-inflammatory G-SF treatment effect. This could indicate that long-term survivors respond better to the G-CSF administration in terms of TNF-α serum levels than short-term survivors, since a negative slope of relative TNF-α serum levels after G-CSF treatment can only be observed in the group of long-term survivors.

The fact that a negative slope of relative TNF-α serum levels is only detected in the long-term survivors when measured after the G-CSF applications but not when measured before the G-CSF applications, probably indicates that the anti-inflammatory effect of G-CSF is exhausted by the time of the next G-CSF administration and only has a short-term effect on TNF-α serum levels in the long-term survivors. Thus, it can be hypothesized that - at least as for a continuous effect on relative TNF-α serum levels - repeated G-CSF applications might be essential for the anti-inflammatory effect of G-CSF.

The fact that the slope of the simple linear regression does not significantly deviate from zero when relative TNF-α serum levels of long-term survivors are considered without taking into account the G-CSF treatment status (Figure 5) could be because the observed positive slope of the simple linear regression of relative TNF-α serum levels before G-CSF administration and the negative slope of the simple linear

regression of TNF-α serum levels after G-CSF administration cancel each other when the G-CSF treatment status is not considered and all relative TNF-α serum levels of long-term survivors are taken together for analysis.

Where the analysis of the slope of the simple linear regression differs between absolute and relative TNF-α values, the consideration of relative TNF-α values seems to be more valid, because the reliance on absolute values may expose the analysis to the influence from outliers or extreme values, which can distort the interpretation of overall trends. By attenuating the impact of interindividual variability, relative TNF-α values provide a better way to compare proportional changes between different individual patients. The results of the analysis of the slope of the simple linear regression differ between absolute and relative values when examining long-term survivors before G-CSF administration. Here, the linear regression of absolute TNF-α serum values has no significant positive or negative slope, whereas the linear regression of the relative TNF-α values has a positive slope. Moreover, the results differ regarding the slope of the simple linear regression of long-term survivors without considering the G-CSF treatment status. The analysis of the simple linear regression of absolute TNF-α serum values detects a negative slope whereas the analysis of the simple linear regression of relative TNF-α values finds no significant slope.

As for the comparison of the median of relative TNF-α serum values and baseline, the relative TNF-α serum levels are significantly higher during the disease course under G-CSF treatment than the relative TNF-α serum baseline levels in both short- and long-term survivors. (Figure 4, Figure 5).

As already mentioned previously regarding absolute TNF-α levels, the finding that relative TNF-α serum levels are significantly higher in the course of the disease after the start of G-CSF therapy compared to the baseline serum level measurement is most likely indicative of the increasing neuroinflammation in the disease course of ALS. However, theoretically, this result could also indicate that G-CSF treatment increases serum TNF-α levels in the long term. In this case, the anti-inflammatory effect of G-CSF in ALS patients does not result from a decrease in the levels of the pro-inflammatory cytokine TNF-α but rather occurs despite a G-CSF-induced increase in TNF-α levels.

However, the relative TNF-α serum levels of the long-term survivors are on average somewhat less elevated relative to the TNF-α baseline serum value in comparison to the baseline than the average TNF-α serum levels of the short-term survivors (Figure 4, Figure 5). This finding may indicate that the extent of neuroinflammation is greater in short-term survivors and despite the progressive neuroinflammation is also present in long-term survivors, the inflammatory response is less pronounced in the long-term survivors.

5.3.4 Summary Evaluation of the TNF-α Serum Level as a Biomarker in ALS With Special Reference to Literature Data Regarding Other Neurodegenerative Diseases

In general, numerous studies support the notion that the TNF-α serum level plays an important role as a biomarker in neuroinflammatory processes, and the results of this study are consistent with previous findings about neurodegenerative diseases.

To be able to discuss the results of the present work in light of the findings from the literature data, the most relevant results of the literature research (see also section 2.3.3.3.) are briefly summarized again.

In vitro studies using animal models of ALS found increased baseline levels of TNF-α mRNA in primary cultured astrocytes derived from presymptomatic transgenic animals, which showed also significantly higher TNF-α mRNA levels than nontransgenic cells when stimulated with pro-inflammatory cytokines (292). Similarly, another study found that TNF-α induced significant NO_2^- production and that the TNF-α-mediated effect was further enhanced in the presence of other pro-inflammatory cytokines (291). Both findings suggest that even comparatively small increases in concentrations of synergistically acting cytokines trigger a disproportionate inflammatory response in ALS, and TNF-α is a major contributor to this. In vivo studies using animal models of ALS found an upregulation of TNF-α mRNA and protein expression already prior to signs of disease onset (293–295) and in end-stage disease (293, 295), and the levels were observed to increase from asymptomatic to end-stage (293). Also, the upregulation of proteins associated with the TNF-α system was found in the transgenic animals (297) and inhibition of TNF-α slowed down motor dysfunction and prolonged life expectancy (298).

In human ALS, elevated TNF-α levels were found in the CNS (62) and also repeatedly reported in the CSF (62, 201, 211). However, of course, literature data on TNF-α levels in serum or plasma in ALS are of particular interest in the present work, and several studies have found increased serum TNF-α concentrations in ALS patients (300–305). Moreover, a recent meta-analysis also reported elevated TNF-α levels in the blood of ALS patients (217). The elevated TNF-α blood levels were reported to be accompanied by higher blood values of its soluble receptors sTNFR1 (217, 300) and sTNFR2 (300). Here too, however, there are also study results that show no differences in the circulating TNF-α concentrations in ALS patients compared to controls, although it must be pointed out that the controls in this study were not healthy subjects, but patients with other neurological diseases including various neurodegenerative disorders (348).

Against the background of the investigations and results of the present work, it is particularly interesting whether other researchers found a correlation between peripheral TNF-α values and survival or disease course and disease severity. One study found no correlation between serum TNF-α levels and disease duration or severity and no differences in clinical disease parameters between subjects with high and low TNF-α levels (300), and another report also found no association between TNF-α levels in plasma samples and ALSFRS-R scores or disease duration (303). Contrary, other authors reported a positive correlation between serum TNF-α levels and disease duration, i.e. increasing TNF-α serum levels were associated with disease progression and therefore with more severe disease stages (301). Another study reported that in one subgroup of the investigated cohort of ALS patients, which didn´t show specific known genetic mutations, TNF-α plasma levels correlated negatively with survival (306). The results of these latter two reports seem plausible since high levels of TNF-α are known to be cytotoxic. Nevertheless, they are in contradiction to the results of the present work, which did not observe a correlation between TNF-α serum levels and disease progression. This could be because the study conditions were different and the patients in this study received treatment with G-CSF, which may likely have influenced the TNF-α serum levels and, accordingly, their prognostic value.

However, although no correlation between serum TNF-α levels and survival duration was found in the present work, both long-term and short-term survivors were found to have significantly augmented serum TNF-α levels during disease progression compared to baseline levels. Moreover, as compared to baseline, TNF-α serum levels were

even more elevated in short-term survivors than in long-term survivors. The increase in TNF-α serum levels as compared to baseline measurements observed in the present work fits well with the above-mentioned observation from an animal model of ALS that the TNF-α serum levels rose from presymptomatic to the terminal stage (293), even though a gradual and continuous increase was only found in the slope of the simple linear regression of relative TNF-α serum levels of long-term survivors before G-CSF administrations in the present work. In summary, despite some partly contradictory literature on TNF-α in ALS, the present study's results and conclusions are largely consistent with the majority of existing data and discrepancies could potentially be attributed to variations in study design.

Against this background, the question arises as to what extent literature data on other neurodegenerative diseases are useful for interpreting the results of the present work. Therefore, the most relevant findings from literature (see also section 2.3.3.) are briefly recapitulated to assess whether the patterns reported in TNF-α levels as a biomarker in diseases like PD and AD offer insights into the expected behavior of TNF-α levels in ALS and either reinforce or challenge the conclusions drawn in the present work.

In animal models of PD, elevated TNF-α levels were observed in the nigrostriatal dopaminergic brain regions (241). Nigral degeneration was found to be attenuated by neutralization (242) or by simultaneous (243) or delayed (244) inhibition of sTNF-α and behavioral deficits were reported to be reduced by sTNF-α inhibition (243). Interestingly, early TNF-α inhibition was observed to be neurotoxic whereas late inhibition had neuroprotective effects (245). Studies with other animal models of PD similarly reported an upregulation of TNF-α mRNA in the substantia nigra (246–248) and elevated TNF-α plasma levels even one year after injection (249) and found a positive correlation between PD symptom severitiy and TNF-α plasma levels (249). In human PD elevated concentrations of TNF-α were found in the striatum (253) and in the CSF (253). Additionally, augmented numbers of TNF-α expressing glial cells were observed in the substantia nigra (252), and the numbers of TNF-α-positive activated microglia in the substantia nigra were reported to increase with disease progression (349). As for TNF-α serum concentrations, most of the current studies reported markedly increased TNF-α levels in patients with PD (254–257) and a positive correlation between TNF-α blood levels and cognitive impairment/disease severity (255–260).

In AD, increased microglial TNF-α expression was reported in the brain (274) and in the CSF (275). While some reports show no disparity in TNF-α serum levels between AD patients and controls (275, 277–280), multiple studies either reported increased TNF-α serum concentrations in AD (281–285). Moreover, some studies link higher TNF-α levels to a more advanced disease stage (279, 286), with one study suggesting a prognostic significance of elevated TNF-α baseline levels, which correlated with a more rapid disease progression (287).

The existing literature on TNF-α PD and AD along with the observations of increasing TNF-α serum levels during the progression of PD and the positive association between TNF-α serum levels and the severity of PD and AD align closely with the outcomes of the current study. Similarly, this study also identified elevated TNF-α serum levels over the disease course of ALS compared to baseline measurements. However, no direct correlation between TNF-α levels and disease severity was found in this work. Overall, according to the current literature, the TNF-α values seem to behave quite similarly in ALS, PD, and AD. In summary, the study results reported from the literature on TNF-α levels in PD and AD corroborate the assumption of elevated TNF-α levels with increasing neurodegeneration. They support the relevance of TNF-α as a potential biomarker of neuroinflammation and the hypothesis of this work that serum TNF-α levels tend to increase with increasing neurodegeneration associated with disease progression in ALS.

5.4 Evaluation of CCL2 and TNF-α Baseline Serum Levels as Potential Tools for Methodological Validation of the Chosen Cut-Off Time for Classification as Short- and Long-term Survival

It was found that when regarding the medians of the CCL2 baseline levels, the P value is minimal at a threshold between 29 and 31 months survival duration after treatment start (Figure 6). This means that the patients can best be classified as short- or long-term survivors if the cut-off value is between 29 and 31 months after the start of therapy. Thus, the CCL2 baseline level appears to be a suitable biomarker for methodological validation of the clinically chosen cut-off in this study.

As for TNF-α, no significant difference was found between short- and long-term survivors (Figure 6). Thus, the baseline serum levels don´t prove to be a useful biomarker for validating the chosen cut-off at 30 months survival duration after the start of G-CSF

treatment as a threshold between long-term and short-term survivors under G-CSF therapy in the present work. Still, it can be speculated that TNF-α, as an important neuroinflammatory biomarker, might theoretically be useful as a validation for the cut-off if the entire neuroinflammatory process could be treated in the context of ALS disease, which is not the case with G-CSF therapy.

In summary, the chosen cut-off of 30 months survival duration after treatment start seems reasonable overall, even though the medians of TNF-α baseline serum levels show no significant difference between the two groups at this threshold. Contrary to the chosen cut-off in this work, a cut-off of 30 months after diagnosis was chosen in a previous publication (338), as already mentioned in the Material and Methods section. Nevertheless, the chosen cut-off of 30 months after treatment initiation seems more appropriate for the investigations of the present work. In this work the change of the cytokine levels during disease progression under repeated G-CSF applications is examined, so treatment is a relevant factor. Moreover, it has to be taken into account that the response to G-CSF treatment in some patients of this cohort is of great importance for their survival duration under G-CSF treatment (339). Because of this, the threshold for classifying patients into short- and long-term survivors should be set sufficiently long enough after the initiation of G-CSF treatment, to consider potential treatment effects. Therefore, a later cut-off for group differentiation was chosen here than in an earlier publication with the same patient cohort (339). Still, valid arguments for the cut-off chosen in the earlier publication can also be found. In principle, the cut-off can be clinically justified in different ways and chosen accordingly.

5.5 Limitations

It must be pointed out that the present study has some limitations, which may impact the extent to which the data can be generalized and replicated, as well as the overall impact of the findings.

One of the most central limitations of the current work is, of course, the small size of the patient cohort and the lack of a control group. This is because ALS is a relatively rare disease and therefore only a limited number of patients could be recruited until the database was closed, and also because this was not a prospective clinical trial. The small size of the patient cohort on the one hand makes it difficult to obtain valid significant results, on the other hand, it leads to the fact that no control group could be

formed. The lack of one or even several control groups means that the present work only investigates the development of the CCL2 and TNF-α serum levels in G-CSF-treated ALS patients who also receive riluzole and a direct comparison to untreated patients or patients treated only with riluzole is not possible. Also, no direct comparison to patients treated with edaravone is possible because this drug was not administered in the study. As a result, only limited conclusions can be drawn from the results of the present study to the disease course in the ALS patient population in general. One way to counteract this in future research would be to conduct large multicenter studies, since this way sufficiently large patient cohorts can be recruited, which allows a control group to be formed, even in the case of a relatively rare disease like ALS.

An additional constraint is that there is no clinical definition of short- and long-term survival in ALS. The cut-off time point as a threshold between short- and long-term survivor groups was determined based on clinical considerations and can be justified accordingly. In retrospect, even a methodological validation of the chosen cut-off can be found due to the significant differences in the CCL2 serum baseline values of the groups. Nevertheless, comprehensible justifications for a differently chosen cut-off could also have been found, as in an earlier publication investigating the same patient cohort (339). Another cut-off would have led to a different group composition and accordingly, of course, to different results. Other factors, such as patient age, gender, or life circumstances, could also be investigated to analyze whether these factors serve to methodologically validate the chosen cut-off between the short- and long-term survivor groups and whether these factors support or weaken the chosen cut-off for differentiation.

Another limitation of this study pertains to the constrained inter-individual comparability of cytokine serum levels. This limitation arises from two main factors. Firstly, the data acquisition process lacks a high degree of standardization, leading - at least in part - to cytokine serum level measurements occurring at different treatment time points for each patient. Secondly, the varying individualized therapeutic regimens and diverse dosages of G-CSF administered contribute to this lack of comparability. The timing of measurements after G-CSF application often differed by several days among different patients. This may affect the levels of the measured values since it can be assumed that the serum levels can change significantly just a few days after G-CSF application. In addition, in some patients, especially at later stages of the disease, only

measurements either before or after G-CSF administration were performed. This means that measurements before and after G-CSF administration at fixed intervals are not available for every patient, so serum levels in some patients may contribute primarily to serum levels measured either before or after G-CSF injection, which could bias some of the results. Moreover, some patients had more serum level measurements than other patients over a comparable period of time, which could also distort the results. Due to all these factors, even relative serum cytokine levels can only be compared to each other to a limited extent. Accordingly, in certain investigations, the cytokine levels of the patients cannot undergo sample pairing for statistical analysis. Consequently, for the assessment of a potential treatment effect, in addition to calculating linear regression, a comparison was made between follow-up cytokine levels and baseline values. However, it is important to note that this comparison comes with certain limitations. Derivation of mean disparities between cytokine levels post-treatment and pre-treatment offers merely an initial insight into the general alteration direction. A constraint of this methodology lies in its inability to accommodate individual variability among patients or the irregular temporal spacing between measurements. It disregards the temporal context and could overlook subtle fluctuations in cytokine levels that manifest discretely over time. Moreover, it might amalgamate sporadic, isolated modifications with continuous, progressive patterns, potentially resulting in premature deductions.

An additional relevant limitation with regard to the analysis of the P value of the difference in CCL2 and TNF-α baseline serum levels in relation to the respective cut-off time point for group classification in short- and long-term survivors ist the difference in the numbers of patients belonging to the respective groups, which reduces the informative value and validity of the analysis.

So overall, due to the small patient cohort, the lack of a control group, and limitations in standardization of the data acquisition process, general conclusions from the results of this work on the entire patient population of ALS disease are only possible to a limited extent.

6 Conclusion and Outlook

Overall, the results of this work are consistent with theories stating that ALS pathogenesis involves inflammatory activation. The cytokine results are also consistent with the

overall concept that an immune-mediated inflammatory response with microglial activation leads to neuronal inflammation and is one of the key components of ALS pathogenesis.

As for CCL2, the results of the present work suggest that CCL2 serum level, in general, might be a suitable biomarker for ALS disease under G-CSF treatment. The CCL2 baseline serum level appears to be a good biomarker for predicting short- and long-term survival under G-CSF treatment and is suitable as a methodological validation of the chosen cut-off in the present work. The observations indicate that higher baseline CCL2 serum levels before the start of G-CSF therapy correlate with a more severe disease course in ALS patients receiving G-CSF therapy. The findings also indicate that under G-CSF therapy absolute CCL2 serum levels are higher in patients with a shorter survival. Therefore, CCL2 serum levels in general might have a predictive value for survival under G-CSF therapy. Moreover, the results suggest that in terms of a continuous decrease in CCL2 serum levels, patients with short-term survival might respond even more strongly to G-CSF administration than patients with long-term survival. Comparison of relative follow-up CCL2 values with baseline values moreover indicates that CCL2 serum levels are overall lower in the long term under G-CSF therapy than before the start of treatment. However, one of the limitations of the present work is that it remains unclear what the predictive value of CCL2 would be without G-CSF administration.

As for TNF-α, known for its pro-inflammatory signaling mechanisms, it can be concluded that in this work, in contrast to the CCL2 serum level, the TNF-α baseline serum level and the TNF-α follow-up serum levels during treatment have no association with the severity of the disease course and the survival in patients under therapy with G-CSF. Therefore the TNF-α serum level is not suitable as a prognostic parameter for the course of the disease and survival. Moreover, the TNF-α baseline serum level is not suitable as a methodological validation of the chosen cut-off. However, the results indicate that the TNF-α serum level is a suitable biomarker for the severity of neuroinflammation in ALS patients under G-CSF therapy. The fact that relative TNF-α serum levels are significantly higher during the disease course under G-CSF treatment than the relative TNF-α serum baseline levels is most likely indicative of the increasing neuroinflammation in the course of the progression of the disease. Theoretically, however, this result could also indicate that G-CSF treatment increases TNF-α

serum levels in the long term. It could be speculated that theoretically, the TNFα serum level might also serve as a predictive biomarker for the severity of disease progression and survival under G-CSF therapy in ALS patients if the entire neuroinflammatory process could be addressed by the G-CSF therapy, which, however, is not the case.

The small size of the patient cohort and the lack of a control group limit the validity of the results of the present work so that conclusions from the results of the present work on CCL2 and TNF-α levels in the disease progression of ALS, in general, can only be drawn to a limited extent. Nevertheless, considering the results of the present work and also taking into account the findings from the extensive literature review, it seems obvious that CCL2 and TNF-α signaling pathways play a fundamental role in the development and progression of ALS, and the chemokine CCL2, as well as the cytokine TNF-α, seem to be relevant mediators of the injury response in the disease process of ALS disease. Taken together, the results strengthen the clinical evidence that ALS is accompanied by an increased inflammatory response. They also support the suitability of the CCL2 serum level, and especially of the CCL2 baseline serum level, as a biomarker with predictive value regarding survival in ALS patients receiving G-CSF therapy. They also indicate the suitability of the TNF-α serum level as a biomarker with predictive value regarding survival in ALS patients receiving G-CSF therapy and as a biomarker of progressive neuroinflammation in ALS patients receiving G-CSF therapy. However, further research will be necessary to confirm or question the results found in this work by analyzing larger patient cohorts and control groups and to further elucidate the suitability of the CCL2 serum level and the TNF-α serum level as biomarkers in ALS disease in patients without G-CSF treatment.

As outlined above, various studies investigating different cytokine levels in CSF have identified several potential biomarkers for ALS, including TNF-α and CCL2, and in many cases, comparable results have been obtained for the same cytokines in serum. It could be that the same biomarkers and similar changes in their levels are present in both fluids during ALS. However, the finding that the levels of potential biomarkers detected in blood only sometimes correlated with the levels of the same potential biomarker in CSF in prior studies, suggests that these two fluids are partially regulated independently. This assumption is reasonable considering the existence of the blood-brain barrier, although it has to be said that in the course of neurodegenerative

diseases, the blood-brain barrier is often increasingly compromised. Nevertheless, it is not conclusively clear to what extent study results on the levels of certain cytokines in CSF regarding their suitability as biomarkers can be transferred to the suitability of serum levels of the same cytokines as biomarkers.

Plasma or serum, as used in this work, are generally more suitable biofluids for biomarker discovery and validation than CSF, as their easy availability due to the ease of blood collection makes blood levels a more efficient, practical, and suitable option for detection and establishment of biomarkers. Single serum cytokine levels in small studies of ALS may not be sufficiently robust biomarkers, but larger studies and multiplex analyses of inflammatory, growth factors, and pro-angiogenic factors in serum could likely better identify a peripheral signature of ALS pathogenesis and progression, so future research is needed to further explore the complex interactions between CCL2 and TNF-α and the various other immunological and non-immunological factors in ALS.

This work investigates the development and changes in the central tendencies of serum values of CCL2 and TNF-α in ALS patients treated with G-CSF. The neuroprotective properties and the neuro-regenerative potential of the hematopoietic growth factor G-CSF are described, focusing on its effect on neurodegenerative diseases, especially in ALS. Many arguments speak for G-CSF as a new type of neurotrophic drug. The main advantage of G-CSF is the well-established pharmacological profile of the protein and its high treatment safety. The clinical trials and studies conducted so far show promising hints but there is a lack of adequate randomized controlled and double-blinded studies to draw sound conclusions about the true effect of G-CSF, so the efficacy of G-CSF in altering disease progression is not clear at the current state. Moreover, there are still many unanswered questions regarding the optimal use of G-CSF treatment. For example, the optimal timing of G-CSF treatment (acute or delayed), the G-CSF dosage (low-dose, high-dose, or ascending-dose), and the regimen of treatment (intermittent for a few times, cyclically repeated at fixed intervals, or chronic continuous) as well as localization of application (subcutaneous, intravenous, or intrathecal/local) are still unclear and may differ from one disease to another. Also, the safety of prolonged treatment over several years should be further investigated in future studies.

7 Supplements

7.1 Demographics

Pat. No.	Age at Dx [years]	Sex	Cause of death	Survival after Dx [months]	Survival after start of Tx [months]	Time Dx to Tx [days]	Mean of G-CSF Dose (Range) [Mio IE/ month]
1	50	F	natural	36,2	19,6	498	150 (150-150)
2	42	M	natural	52,2	31,5	619	280 (150-300)
3	77	M	natural	33,4	8,1	759	173 (150-240)
4	68	F	natural	3,9	2,9	29	150 (150-150)
5	67	M	natural	56,6	42,0	439	260 (150-300)
6 ■ #	26	M	not recorded	not recorded	not recorded ■	486	485 (150-1170)
7	50	F	natural	25,4	7,6	536	240 (240-240)
8	73	M	natural	21,4	12,4	270	166 (150-240)
9	50	M	natural	21,4	8,3	393	133 (90-150)
10	56	M	natural	40,0	14,4	770	242 (150-300)
11	41	M	natural	36,3	35,5	24	287 (150-300)
12 + □	35	F	not recorded	not recorded	not recorded □	115	296 (240-300)
13	48	F	natural	29,7	28,5	38	216 (150-300)
14	43	M	natural	47,3	45,3	61	561 (192-768)
15	65	F	natural	18,3	15,6	81	192 (192-192)
16	51	F	natural	6,4	3,0	101	225 (150-300)
17	60	F	natural	11,5	10,8	21	192 (192-192)
18	58	M	natural	25,4	23,9	45	311 (240-480)
19	46	M	natural	34,7	26,4	249	150 (150-150)
20	50	M	natural	8,0	8,0	1	198 (192-240)
21 ■ #	27	M	not recorded	not recorded	not recorded ■	53	301 (150-600)
22 ■ #	45	M	not recorded	not recorded	not recorded ■	26	666 (240-816)
23 □ +	55	M	not recorded	not recorded	not recorded □	26	263 (150-300)
24 □	61	M	suicide	9,2	7,0	66	375 (150-510)
25	60	M	natural	23,0	19,0	135	602 (240-816)
26	65	F	natural	11,3	7,3	122	563 (240-900)
27	43	F	not recorded	not recorded	not recorded	338	628 (240-720)

□ +					□		
28	60	M	not recorded	12,1	11,4	23	589 (480-720)
29	45	F	natural	29,6	13,2	493	535 (150-720)
30	47	M	natural	19,3	6,9	396	585 (450-720)
31	50	M	not recorded	13,7	12,9	23	667 (240-720)
32 □ #	39	M	not recorded	not recorded	not recorded □	343	1015 (450-1170)
33 □	56	M	suicide	35,6	18,1	525	744 (450-1056)
34 □ #	59	M	not recorded	not recorded	not recorded □	52	1044 (450-1440)
35 □ #	69	M	not recorded	not recorded	not recorded □	62	1344 (450-2160)
36 □ #	35	M	not recorded	not recorded	not recorded □	288	1141 (300-1440)
Mean (SD)	52 (12,2)	11 F 25 M		Numbers cannot be given as not recorded in every patient	Numbers cannot be given as not recorded in every patient	236,3 (231,4)	

Legend:
#: the patient was still alive upon closure of data admission
+: the patient's date of death and survival duration were not recorded
□: the patient had been observed for less than 30 months from treatment start upon closure of data admission or suicide, therefore not long enough for group assignment
■: the patient had been observed for more than 30 months from treatment start, therefore long enough for assignment to the long-term survivor group

7.2 Comparison Between Medians of Cytokine Serum Levels of Short- and Long-Term Survivors (cf. Chapters 4.2 - 4.5)

absolute CCL2 serum levels	relative CCL2 serum levels	Absolute TNF-α serum levels	relative TNF-α serum levels
median of short-term survivors before G-CSF: **780.69 pg/ml serum**	median of short-term survivors before G-CSF: **0.98**	median of short-term survivors before G-CSF: **3.00 pg/ml serum**	median of short-term survivors before G-CSF: **1.00**
median of short-term survivors after G-CSF: **327.34 pg/ml serum**	median of short-term survivors after G-CSF: **0.66**	median of short-term survivors after G-CSF: **3.91 pg/ml serum**	median of short-term survivors after G-CSF: **1.57**
median of short-term survivors without considering the G-CSF treatment status: **522.74 pg/ml serum**	median of short-term survivors without considering the G-CSF treatment status: **0.74**	median of short-term survivors without considering the G-CSF treatment status: **3.25 pg/ml serum**	median of short-term survivors without considering the G-CSF treatment status: **1.29**
median of long-term survivors before G-CSF: **249.23 pg/ml serum**	median of long-term survivors before G-CSF: **0.99**	median of long-term survivors before G-CSF: **2.81 pg/ml serum**	median of long-term survivors before G-CSF: **1.06**
median of long-term survivors after G-CSF: **218.39 pg/ml serum**	median of long-term survivors after G-CSF: **0.82**	median of long-term survivors after G-CSF: **3.37 pg/ml serum**	median of long-term survivors after G-CSF: **1.43**
median of long-term survivors without considering the G-CSF treatment status: **227.23 pg/ml serum**	median of long-term survivors without considering the G-CSF treatment status: **0.89**	median of long-term survivors without considering the G-CSF treatment status: **3.14 pg/ml serum**	median of long-term survivors without considering the G-CSF treatment status: **1.24**
median of short-term survivors before G-CSF **>** **median of long-term survivors before G-CSF** ****** The median of short-term survivors before G-CSF is significantly higher than the median of long-term survivors before G-CSF ($p \leq 0.01$).	**median of short-term survivors before G-CSF does not differ significantly from median of long-term survivors before G-CSF** **ns** The median of short-term survivors before G-CSF is not significantly different from the median of long-term survivors before G-CSF ($p > 0.05$).	**median of short-term survivors before G-CSF does not differ significantly from median of long-term survivors before G-CSF** **ns** The median of short-term survivors before G-CSF is not significantly different from the median of long-term survivors before G-CSF ($p > 0.05$).	**median of short-term survivors before G-CSF does not differ significantly from median of long-term survivors before G-CSF** **ns** The median of short-term survivors before G-CSF is not significantly different from the median of long-term survivors before G-CSF ($p > 0.05$).
median of short-term survivors after G-CSF **>** **median of long-term survivors after G-CSF** ***** The median of short-term survivors after G-CSF is significantly higher than the median of long-term survivors after G-CSF ($p \leq 0.05$).	**median of short-term survivors after G-CSF** **<** **median of long-term survivors after G-CSF** ***** The median of short-term survivors after G-CSF is significantly lower than the median of long-term survivors after G-CSF ($p \leq 0.05$).	**median of short-term survivors after G-CSF does not differ significantly from median of long-term survivors after G-CSF** **ns** The median of short-term survivors after G-CSF is not significantly different from the median of long-term survivors after G-CSF ($p > 0.05$)	**median of short-term survivors after G-CSF does not differ significantly from median of long-term survivors after G-CSF** **ns** The median of short-term survivors after G-CSF is not significantly different from the median of long-term survivors after G-CSF ($p > 0.05$).
median of short-term survivors **>** **median of long-term survivors** ****** The median of short-term survivors without considering the G-CSF treatment status is significantly higher than the median of long-term survivors without considering the G-CSF treatment status ($p \leq 0.01$).	**median of short-term survivors** **<** **median of long-term survivors** ***** The median of short-term survivors without considering the G-CSF treatment status is significantly lower than the median of long-term survivors without considering the G-CSF treatment status ($p \leq 0.05$).	**median of short-term survivors does not differ significantly from median of long-term survivors** **ns** The median of short-term survivors without considering the G-CSF treatment status is not significantly different from the median of long-term survivors without considering the G-CSF treatment status ($p > 0.05$).	**median of short-term survivors does not differ significantly from median of long-term survivors** **ns** The median of short-term survivors without considering the G-CSF treatment status is not significantly different from the median of long-term survivors without considering the G-CSF treatment status ($p > 0.05$).

Legend:

ns: $p > 0.05$

*****: $p \leq 0.05$

******: $p \leq 0.01$

7.3 Comparison Between Medians of Cytokine Serum Levels of Follow-up Cytokine Serum Levels and Medians of Baseline Cytokine Serum Levels (cf. Chapters 4.2 - 4.5)

absolute CCL2 serum levels of short-term survivors	**absolute CCL2 serum levels of long-term survivors**	**absolute TNF-α serum levels of short-term survivors**	**absolute TNF-α serum levels of long-term survivors**
median of baseline cytokine levels: 956.79 pg/ml serum median of follow-up cytokine levels without considering the G-CSF treatment status, excluding baseline: **349.69 pg/ml serum**	median of baseline cytokine levels: **225.31 pg/ml serum** median of follow-up cytokine levels without considering the G-CSF treatment status, excluding baseline: **267.49 pg/ml serum**	median of baseline cytokine levels: **2.82 pg/ml serum** median of follow-up cytokine levels without considering the G-CSF treatment status, excluding baseline: **3.46 pg/ml serum**	median of baseline cytokine levels: **2.68 pg/ml serum** median of follow-up cytokine levels without considering the G-CSF treatment status, excluding baseline: **3.18 pg/ml serum**
median without considering the G-CSF treatment status excluding baseline **<** **median of baseline** **** The median of the absolute follow-up CCL2 serum values of short-term survivors under G-CSF therapy is significantly lower than the median of the absolute baseline levels ($p \leq 0.0001$).	**median without considering the G-CSF treatment status excluding baseline** **<** **median of baseline** * The median of the absolute follow-up CCL2 serum values of long-term survivors under G-CSF therapy is significantly lower than the median of the absolute baseline levels ($p \leq 0.05$).	**median without considering the G-CSF treatment status excluding baseline** **>** **median of baseline** *** The median of the absolute follow-up TNF-α serum values of short-term survivors under G-CSF therapy is significantly higher than the median of the absolute baseline levels ($p \leq 0.001$).	**median without considering the G-CSF treatment status excluding baseline** **>** **median of baseline** * The median of the absolute follow-up TNF-α serum values of long-term survivors under G-CSF therapy is significantly higher than the median of the absolute baseline levels ($p \leq 0.05$).
relative CCL2 serum levels of short-term survivors	**relative** CCL2 serum levels of long-term survivors	**relative** TNF-α serum levels of short-term survivors	**relative** TNF-α serum levels of long-term survivors
baseline: **1.00** median of follow-up cytokine levels without considering the G-CSF treatment status, excluding baseline: **0.66**	baseline: **1.00** median of follow-up cytokine levels without considering the G-CSF treatment status, excluding baseline: **0.89**	baseline: **1.00** median of follow-up cytokine levels without considering the G-CSF treatment status, excluding baseline: **1.33**	baseline: **1.00** median of follow-up cytokine levels without considering the G-CSF treatment status, excluding baseline: **1.25**
median without considering the G-CSF treatment status excluding baseline **<** **median of baseline** *** The median of the relative follow-up CCL2 serum values of short-term survivors under G-CSF therapy is significantly lower than the baseline ($p \leq 0.001$).	**median without considering the G-CSF treatment status excluding baseline** **<** **median of baseline** * The median of the relative follow-up CCL2 serum values of long-term survivors under G-CSF therapy is significantly lower than the baseline ($p \leq 0.05$).	**median without considering the G-CSF treatment status excluding baseline** **>** **median of baseline** *** The median of the relative follow-up TNF-α serum values of short-term survivors under G-CSF therapy is significantly higher than the baseline ($p \leq 0.001$).	**median without considering the G-CSF treatment status excluding baseline** **>** **median of baseline** * The median of the relative follow-up TNF-α serum values of long-term survivors under G-CSF therapy is significantly higher than the baseline ($p \leq 0.05$).

Legend:
ns: $p > 0.05$
*: $p \leq 0.05$
**: $p \leq 0.01$
***: $p \leq 0.001$
****: $p \leq 0.0001$

7.4 Overview of the Slopes of the Simple Linear Regressions of Cytokine Serum Levels (cf. Chapters 4.2 - 4.5)

CCL2	**simple linear regression of absolute CCL2 serum levels of short-term survivors**	**simple linear regression of relative CCL2 serum levels of short-term survivors**	**simple linear regression of absolute CCL2 serum levels of long-term survivors**	**simple linear regression of relative CCL2 serum levels of long-term survivors**
before G-CSF	**slope < 0** * (slope = -0.747927, p **= 0.0294**)	slope = 0 **ns** (slope = 0.000139, p **= 0.5480**)	slope = 0 **ns** (slope = -0.040825, p **= 0.5958**)	slope = 0 **ns** (slope = 0.000074, p **= 0.3896**)
after G-CSF	slope = 0 **ns** (slope = 0.024259, p **= 0.9425**)	slope = 0 **ns** (slope = 0.000045, p **= 0.8615**)	slope = 0 **ns** (slope = -0.064545, p **= 0.2617**)	slope = 0 **ns** (slope = 0.000052, p **= 0.4984**)
without considering the G-CSF treatment status	slope = 0 **ns** (slope = -0.370768, p **= 0.1196**)	slope = 0 **ns** (slope = -0.000011, p **= 0.9538**)	slope = 0 **ns** (slope = -0.057368, p **= 0.2258**).	slope = 0 **ns** (slope = 0.000048, p **= 0.4167**)
TNF-α	simple linear regression of **absolute** TNF-α serum levels of short-term survivors	simple linear regression of **relative** TNF-α serum levels of short-term survivors	simple linear regression of **absolute** TNF-α serum levels of long-term survivors	simple linear regression of **relative** TNF-α serum levels of long-term survivors
before G-CSF	slope = 0 **ns** (slope = 0.000003, p **= 0.9975**).	slope = 0 **ns** (slope = 0.000334, p **= 0.4505**)	slope = 0 **ns** (slope = -0.000285, p **= 0.1979**).	**slope > 0** * (slope = 0.000357, p **= 0.0193**)
after G-CSF	slope = 0 **ns** (slope = -0.000335, p **= 0.7835**)	slope = 0 **ns** (slope = -0.000509, p **= 0.4524**)	**slope < 0** *** (slope = -0.001155, p **= 0.0007**)	**slope < 0** ** (slope = -0.000487, p **= 0.0014**)
without considering the G-CSF treatment status	slope = 0 **ns** (slope = 0.000278, p **= 0.7288**).	slope = 0 **ns** (slope = 0.000108, p **= 0.8033**)	**slope < 0** ** (slope: -0.000672, p **= 0.0018**)	slope = 0 **ns** (slope = -0.000065, p **= 0.5554**)

Legend:

ns: $p > 0.05$

*: $p \leq 0.05$

**: $p \leq 0.01$

***: $p \leq 0.001$

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9 List of Figures

Figure 1 Boxplots of absolute baseline CCL2 and TNF-α levels of short-term and long-term survivor group, measured before the start of G-CSF injections at a threshold for grouping into short-term and long-term survivors 30 months after initiation of G-CSF treatment.

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10 Conflicts of Interest

The author has no conflicts of interest to declare.

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