

Review

# Pathogenesis of Multiple Sclerosis with Special Consideration of Iron Metabolism, Ferroptosis and Other Forms of Programmed Cell Death

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## Abstract

The pathogenesis of multiple sclerosis (MS) is a complex topic that involves many individual aspects, several MS risk factors and various alterations in the immune system. In addition to the aspects still being researched in MS today, such as the activation of pro-inflammatory lymphocytes and macrophages, a particular scientific interest refers to the dysregulation of iron and to ferroptosis as an important form of programmed cell death (PCD), which is closely associated with this dysregulation. These two aspects are particularly significant because the new McDonald criteria include the diagnostic determination of so-called “paramagnetic rim lesions” (PRLs) in MS, which are areas of abnormal iron accumulation visible on magnetic resonance imaging (MRI) scans. Along with other diagnostic criteria, these PRLs are an important criterion for the diagnosis of MS. This review begins with an overall description of pathogenic risk factors and the dysregulation of the immune system. However, the priority of the review is the dysregulation of the cerebral iron balance and ferroptosis, as well as other forms of programmed cell death, which have also been demonstrated in MS: apoptosis, pyroptosis, necroptosis and NETosis. One of the main current findings on the forms of PCD in MS is their non-physiological occurrence in the brain’s own cells such as oligodendrocytes and neurons, and the extensive protection of pro-inflammatory cells from the occurrence of PCD. Emerging evidence indicates that ferroptosis occurs at an early stage in MS and appears to play a role in its pathogenesis. This review will present the distinct characteristics of the various PCDs in MS, alongside other pathological mechanisms, genetic factors, and current experimental approaches of therapeutic concepts that affect especially ferroptosis and pyroptosis. The aim is to provide an overview on iron dysregulation and forms of PCD as key aspects for understanding the pathogenesis of MS.

**Keywords:** multiple sclerosis; immunology; iron metabolism; ferroptosis; pyroptosis; apoptosis; necroptosis; NETosis

## 1. Introduction

The etiopathogenesis of multiple sclerosis (MS) has been the subject of uninterrupted and intensive research since the 19th century. After MS was first described independently by Robert Carswell and Jean Cruveilhier in the years 1838 to 1841 [1], it was subsequently regarded as a multifactorial autoimmune disease. Despite recent advances, the pathogenesis of MS remains incompletely understood. The currently increasing prevalence of MS worldwide is an additional factor that emphasizes the need for continued research into all known pathophysiological factors. Based on a comparison of epidemiological data from 81 countries from the years 2013 and 2020, a marked increase in the global prevalence of MS from 29.26 to 43.95 cases per 100,000 people was observed between 2013 and 2020. This increase in prevalence affects all regions of the world [2,3].

In addition to the long-known risk factors such as vitamin D deficiency or genetic profiles, as well as pathophysiological alterations in the immune system, the dysregulation of the iron balance has currently become a major focus of MS research. Ferroptosis, a type of cell death associated with iron imbalance and first identified by Dixon *et al.* in 2012 [4], is also relevant here. Therefore, a re-

view of ferroptosis in MS is needed, but should be preceded by an overview of MS risk factors and pathophysiology to provide context. This review also describes other forms of cell death, namely apoptosis and autophagic cell death. Although known for a long time, the role of apoptosis, autophagy and autophagic cell death in MS is still not fully understood. Pyroptosis, first described in 2001 by Cookson and co-workers [5], is another form of cell death that is also attracting increasing scientific interest in MS, which also applies to other forms of programmed cell death such as necroptosis and NETosis. This review will present and discuss the current knowledge on all these aspects, including genetic factors and current strategies to new therapeutic options that could influence various forms of cell death in MS.

## 2. Important Risk Factors and Pathophysiological Aspects of MS

### 2.1 Risk Factors

Approximately 2.9 million people worldwide suffer from MS, the prevalence is particularly high in the countries of North America and Europe, but lower in Latin America, Africa, East Asia and generally in countries near the equator. Statistically, the prevalence in the various coun-



tries correlates with the distance from the equator to the north and south, whereby the reduced endogenous vitamin D production as a result of less sunlight exposure is considered to be an important factor [2,6]. Vitamin D is involved in neuroprotection through various signaling pathways, such as the stimulation of anti-inflammatory T regulatory cells (Tregs), the suppression of pro-inflammatory Th17 cells and M1 microglia, the promotion of myelin and neuronal acid synthesis in oligodendrocytes, and the down-regulation of the human leukocyte antigen (HLA) risk allele *HLA-DRB1\*15:01*. The totality of these effects of vitamin D also explains the increased risk of developing MS as a result of vitamin D deficiency [7,8]. In addition, over 200 genetic risk factors for MS, mostly specific alleles or single-nucleotide polymorphisms, have been identified and mainly influence immune cell pathways. Despite the sex-specific ratio of 3:1 between women and men, the role of sex hormones and the importance of the X chromosome in the development of MS is controversially discussed [9–11]. An important indication of the significance of genetic factors is an increased risk of disease of 20–30% in identical twins of MS patients and 2–5% in fraternal twins [12]. The risk alleles of the *HLA-II* genes, such as *HLA-DRB1\*15:01*, which have been known for decades, are important examples of MS risk factors. There is now a consensus that not the *HLA* genes and their alleles alone, but only a polygenic risk score, taking into account numerous other genes, shows a significant correlation with the risk of MS [13–15]. Important examples are risk alleles of the gene for the vitamin D receptor, as well as risk alleles of genes that are directly associated with the regulation of immune cells, such as the *forkhead box P3 (FOXP3)* gene, which is an important marker of T-regulatory cells [16,17]. Epigenetic changes such as aberrant methylation status of *HLA* genes and aberrant expression of several micro RNAs (miRNAs) are also discussed as risk factors and biomarkers of MS [18,19]. Current evidence suggests that considering both genetic and environmental or lifestyle factors provides a better correlation with MS risk than genetics alone. One example is the increased risk of MS in smokers in combination with the presence of *HLA* risk genes [20]. However, even without considering genetic findings, nicotine consumption, lack of physical activity and obesity are considered risk factors for MS [21,22]. Dysbiosis of the bacterial intestinal flora is also a significant etiological factor due to the detection of a reduction in anti-inflammatory species such as bacteroides or parabacteroides, as well as an increased presence of pro-inflammatory species such as *Akkermansia* in MS patients. The detection of reduced immune globulin A (IgA) coating of individual bacterial species is also considered pathogenetically significant. The authors emphasize the need for further research to better understand the range of species with pro- and anti-inflammatory effects, as well as the underlying pathomechanisms [23–25]. Infection with the Epstein-Barr virus (EBV), which is seroposi-

tive in the cerebrospinal fluid (CSF) of almost 100% of all MS patients, is considered one of the most important environmental factors. A variety of signaling pathways have been described for the pathological influence of EBV on the immune system in MS. A major influence of the EBV is the immortalization of infected B-cells by means of the so-called latent member protein 1 (LMP1), as well as the induction of continued antigen presentation by the B-cells with production of cytotoxic autoantibodies and cytokines [9,26–28]. A causative association with MS is also discussed for infections with other virus species such as human herpesvirus-6 (HHV-6) and human endogenous retroviruses (HERVs). In contrast to EBV, not all MS patients are seropositive for these viruses [29–31].

## 2.2 Pathophysiological Aspects of MS

The cumulative influence of all the risk factors mentioned above promotes the peripheral development of CD4+ Th1 and Th17 T cells, which increasingly express pro-inflammatory cytokines such as interferon- $\gamma$  (IFN- $\gamma$ ), interleukin-17 (IL-17) and tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ). The underlying mechanism for this is still the subject of ongoing research, the reduced activity of the T-regulatory cells with reduced inhibition of Th1 and Th17 cells and reduced expression of the anti-inflammatory cytokines interleukin-10 (IL-10) and transforming growth factor  $\beta$  (TGF- $\beta$ ) are considered major causes [9]. Another aspect is the overexpression of the transcription factor zinc finger E-box-binding homeobox (ZEB1) in T-cells, which is an activator of the Janus kinase/signal transducers and activators of transcription (JAK/STAT) pathway with forced differentiation of Th1 and Th17 cells to the detriment of anti-inflammatory Th2 cells [32]. The nerve injury-induced protein 1 (NINJ1) is also an early mediator in the development of MS, which is involved in the transmigration of inflammatory cells through the blood-brain barrier (BBB), in the promotion of the expression of inflammatory cytokines and in the rupture of cell membranes with the subsequent formation of danger associated molecular patterns (DAMPs) [33]. Th1 and Th17 cells are essential for the differentiation of encephalogenic and myelin-reactive CD8+ T cells, which are often more numerous in MS lesions than CD4+ T cells. A special feature in this context are the clonally developing CD8+/CD20+ T cells, which have been shown in experimental models to develop through interaction with CD20+ B-cells in the form of trogocytosis, i.e. an exchange of the surface molecule CD20+. These cells also show a high pathogenic potential in MS through the expression of proinflammatory cytokines and adhesion molecules [9,34]. The already mentioned reduced activity of T-regulatory cells is also a cardinal cause for the expansion of myelin-reactive B-cells, which are often located perivascularly in lesions with ongoing myelin degradation. In addition to the involvement of B-cells in myelin damage by auto-reactive antibodies, increased pathogenic cy-

tokine expression can also be observed in B-cells, which leads to further differentiation of pathogenic T cells [35]. The pathogenic role of B-cells in MS has been clearly confirmed by the promising therapeutic application of selective B cell depletion in MS patients [36].

Macrophages are generally present in MS lesions and are recruited from peripheral monocyte-derived macrophages, which infiltrate the central nervous system (CNS) at an early stage of MS, as well as from resident mononuclear phagocytes of the CNS, which include microglia and CNS border-associated macrophages (BAMs). These BAMs are prominently localized perivascularly, in the meninges, and in the area of the choroid plexus. Before infiltrating the CNS, the peripheral macrophages together with dendritic T cells are involved in the presentation of myelin-specific antigens to the immunoreactive Th1 and Th17 cells that subsequently infiltrate the CNS. These T cells are also stimulated by the expression of IL-1 $\beta$ , IL-6, and IL-23 by macrophages [33,37]. Additionally, the above-mentioned expression of inflammatory cytokines such as IFN- $\gamma$  and IL-17 by Th1 and Th17 cells is considered an essential factor for the activation of macrophages in the CNS [9,33]. In the resident macrophages of the central nervous system, autopsy using transcriptome analysis showed that even in the non-pathologically altered white matter, there was increased expression of genes associated with macrophage-related inflammation and stress responses. This was further confirmed by single-cell RNA analysis [38]. In MS, most macrophages have a pro-inflammatory phenotype with expression of CD40, but many macrophages are also involved in neuroprotective tasks such as phagocytosis of myelin debris and recruitment of oligodendrocyte progenitor cells. In contrast to pro-inflammatory CD40<sup>+</sup> macrophages, these macrophages express homeostatic markers such as CD163. This divergent differentiation of macrophages into pro-inflammatory and anti-inflammatory phenotypes has coined the term “intermediate activation status” of macrophages in MS [37,39]. Microglia play an additional role in the dysregulation of iron balance in MS, which is discussed in the following chapter.

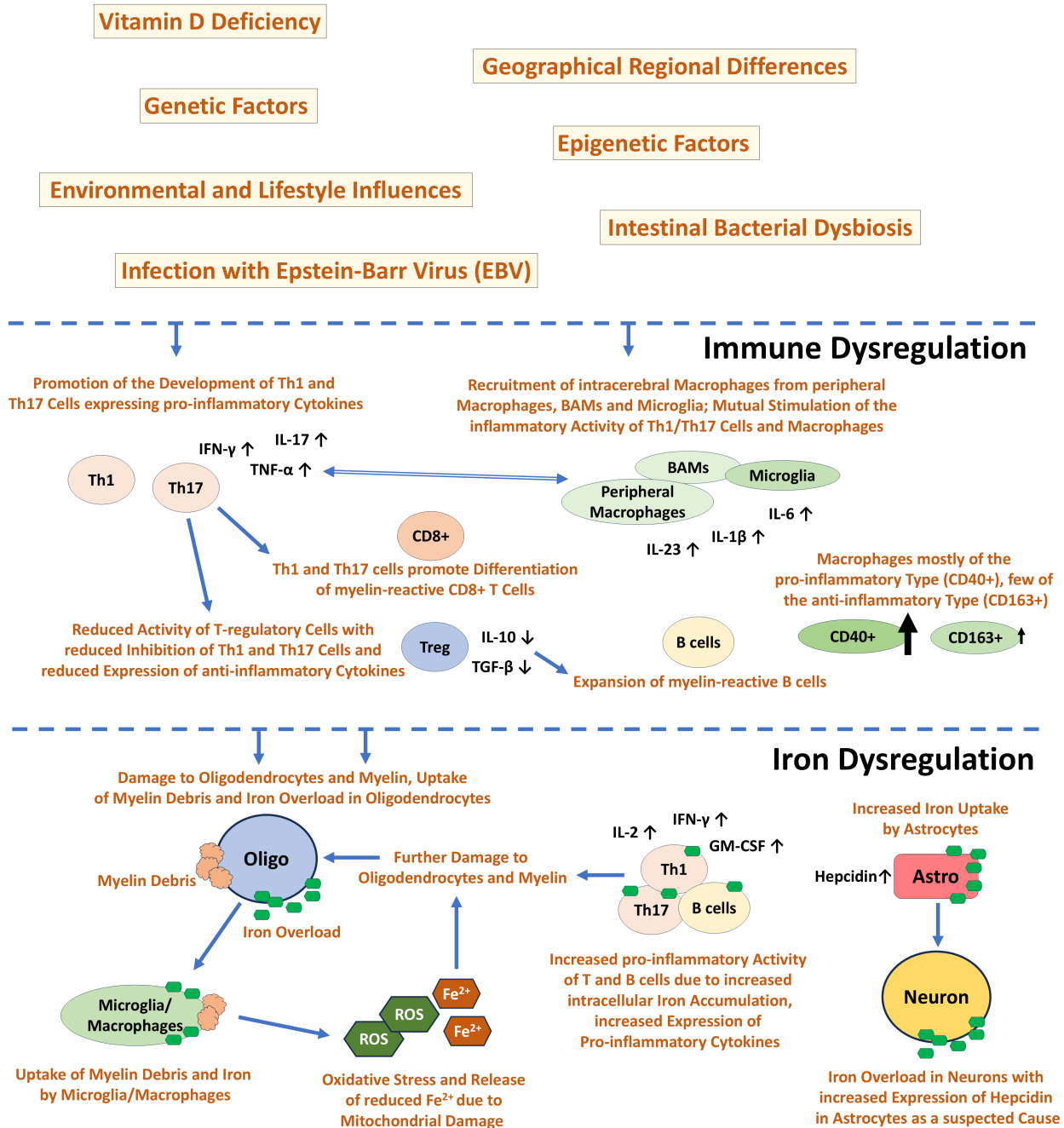
### 3. Dysregulation of the Iron Balance and Ferroptosis

#### 3.1 Iron Dysregulation

Evidence of iron dysregulation and regional redistribution in MS has primarily been obtained through 3T or 7T brain magnetic resonance imaging (MRI) scans, including echo-planar diffusion-weighted (DWI) and multi-planar susceptibility-weighted (SWI) MR sequences. A key finding is an increased iron concentration in the deep grey matter in early stages of MS compared to control collectives, as well as in patients with clinically isolated syndrome (CIS) [40–42]. In later stages, an increased iron concentration was found at the edges of the MS foci with a re-

duced concentration in the core area of the lesions [37]. The early increase of iron concentration in the deep grey matter has been reported to correlate with the severity of neurological deterioration, but no differences were found between healthy individuals and MS patients with regard to the iron content in the entire cerebrum [40]. Furthermore, a reduced iron content in the unaffected white matter of MS patients has been reported in comparison with healthy persons [43]. Oligodendrocytes have the highest iron content of all CNS cells, as it is an important cofactor in the synthesis and homeostasis of myelin [44]. Current evidence suggests that inflammation-related damage to myelin and oligodendrocytes is a main cause of increased local iron levels in MS [45]. Microglia are already found in the early stages of MS lesions and, together with the peripherally infiltrated macrophages, are significantly involved in the pro-inflammatory activity and the subsequent uptake of myelin detritus and iron (Fig. 1, Ref. [7,9,14,19,21,23,26,30,33,34,37,39,45–49]) [46]. The increased iron accumulation radiologically detectable in the form of the so-called “paramagnetic rim lesions” at the edge of MS lesions corresponds to a neuropathologically confirmed accumulation in activated microglia [50,51]. This intracellular excess of iron damages in particular the mitochondrial membranes and the iron-sulphur clusters with increased release of reduced Fe<sup>2+</sup> and the generation of free radicals and oxidative stress, which further increases the damage to myelin and oligodendrocytes (Fig. 1) [47]. Furthermore, astrocytes also play a role in the increased uptake of iron under pathological conditions by increasingly expressing the receptor for the iron transport protein transferrin in demyelinating diseases. It is discussed that an increased intracellular iron concentration in astrocytes could have a pro-inflammatory effect [45,48,52]. The significance of increased iron accumulation in neurons and lymphocytes is also the subject of current scientific interest. Increased iron in neurons impairs their function, a central cause appears to be the increased expression of hepcidin by astrocytes (Fig. 1). Hepcidin is a negative iron regulator that leads to the degradation of the iron exporter ferroportin1 and thus to increased intracellular iron accumulation. A role of polymorphisms of the *hepcidin* gene and other *hepcidin-regulating* genes is also being discussed as a possible cause of this dysregulation [45,49,53]. In T and B lymphocytes, higher intracellular iron levels are thought to promote proliferation and increased production of pro-inflammatory cytokines such as IL-2, IFN $\gamma$ , and granulocyte-macrophage colony-stimulating factor (GM-CSF) (Fig. 1) [44,48]. Overall, recent literature also repeatedly emphasizes that the role of dysregulation of the iron balance in MS is only incompletely understood, and additionally mentions the significance of ferroptosis and other forms of programmed cell death [37,45,54].

## Multiple Sclerosis - Key Risk Factors

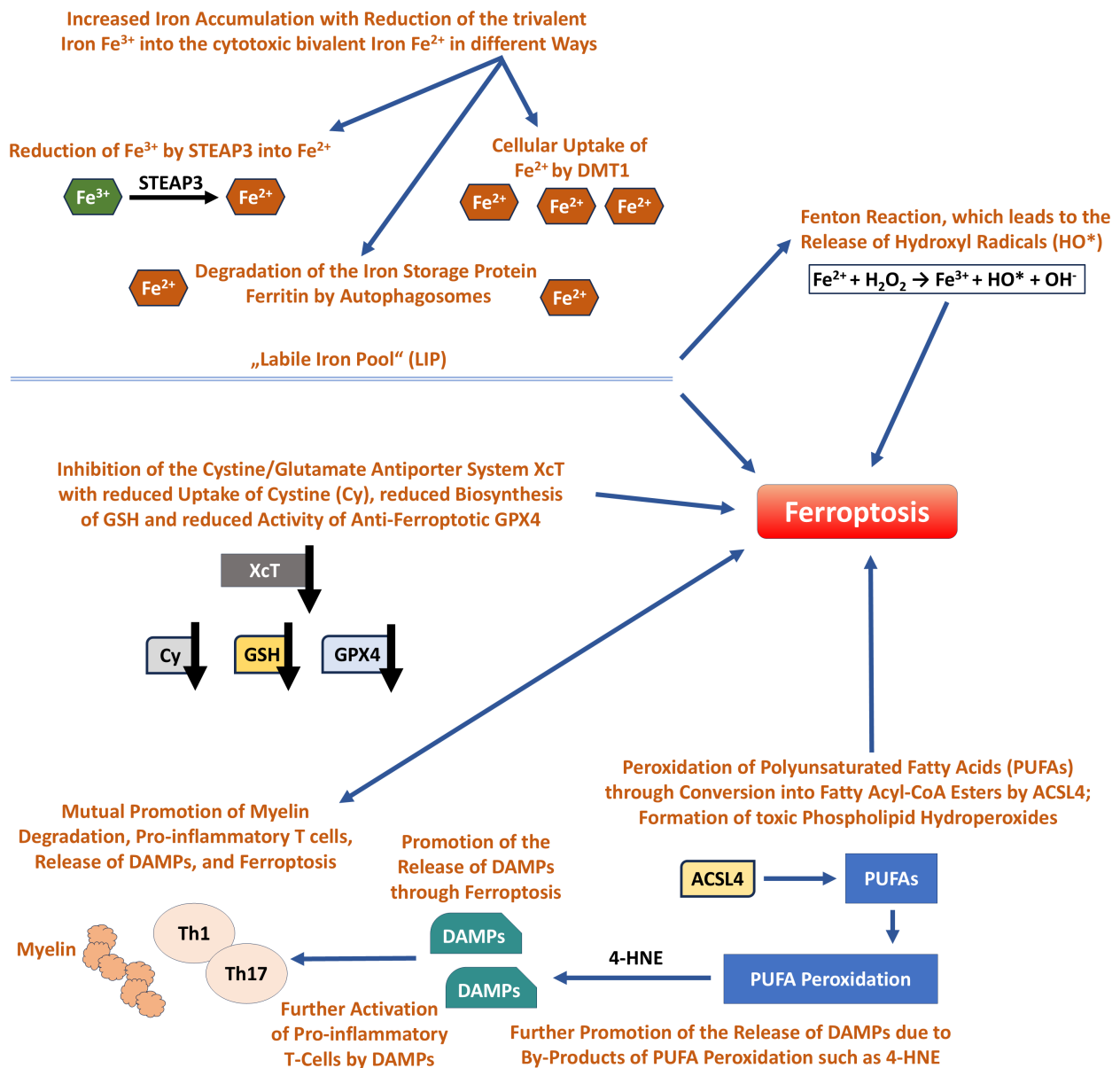


**Fig. 1. Multiple Sclerosis - key factors of disease risk [7,9,14,19,21,23,26,30], immune dysregulation [9,33,34,37,39], and iron dysregulation [45–49] as described in the text.** IFN- $\gamma$ , interferon- $\gamma$ ; IL-17, interleukin-17; TNF- $\alpha$ , tumor necrosis factor  $\alpha$ ; Tregs, T regulatory cells; BAMs, border-associated macrophages; IL-10, interleukin-10; TGF- $\beta$ , transforming growth factor  $\beta$ ; GM-CSF, granulocyte-macrophage colony-stimulating factor; ROS, reactive oxygen species.

### 3.2 Ferroptosis

Ferroptosis is a form of programmed cell death first described by Dixon and colleagues in 2012 [4], which differs completely from apoptosis in terms of morphology and

pathobiochemistry. In contrast to apoptosis, there is no disintegration of the chromatin and the cell nucleus, but there is damage to the cell membranes and the mitochondria with rupture of their membrane and degeneration of the mitochondrial cristae [55]. It is now known that ferroptosis



**Fig. 2. Ferroptosis in Multiple Sclerosis - the main changes and pathways and their cumulative effect on initiation of ferroptosis [55–61] as described in the text.** STEAP3, six-transmembrane epithelial antigen of prostate 3; DMT1, divalent metal transporter 1; GSH, glutathione; GPX4, glutathione peroxidase 4; DAMPs, danger-associated molecular patterns; 4-HNE, 4-hydroxynonenal; PUFAs, polyunsaturated fatty acids; ACSL4, acyl-CoA synthetase long-chain family member 4.

frequently occurs in MS and plays a role in the development of the disease [56]. A key promoter of ferroptotic cell death is increased iron accumulation in the cytosol with additional reduction of the trivalent iron Fe<sup>3+</sup> by the ferric reductase six-transmembrane epithelial antigen of prostate 3 (STEAP3) into the reactive and cytotoxic bivalent iron Fe<sup>2+</sup>. This reduction can occur intracellularly, but Fe<sup>2+</sup> can also be taken up across the cell membrane by the divalent metal transporter 1 (DMT1), and it can occur after degradation of the redox-inactive iron storage protein ferritin by autophagosomes (ferritinophagy) [57]. This ex-

cess of divalent iron, also known as the “labile iron pool” (LIP), also triggers the Fenton reaction, in which Fe<sup>2+</sup> is oxidized again by hydrogen peroxide, releasing hydroxyl radicals and hydroxide ions (Fig. 2, Ref. [55–61]) [57,58]. An additional effect of LIP is the catalysis of the peroxidation of lipids, in particular polyunsaturated fatty acids (PUFAs). This peroxidation occurs mainly through the conversion of free long-chain fatty acids into fatty acyl-CoA esters by acyl-CoA synthetase long-chain family member 4 (ACSL4). The subsequent oxidation by iron leads to the formation of toxic phospholipid hydro-peroxides, which

destroy the cell membranes and lead to ferroptosis [56,59]. The expression of the pro-ferroptotic protein ACSL4 is significantly increased in MS [60]. The triggering of ferroptosis is further promoted by a reduced expression of the cystine/glutamate antiporter system (XcT), which leads to a reduced cellular uptake of cystine, which is involved in the biosynthesis of the antioxidant glutathione (GSH). The reduced availability of cysteine and GSH results in lower levels and activity of the anti-ferroptotic enzyme glutathione peroxidase 4 (GPX4), which reduces the inhibition of lipid oxidation and ultimately leads to ferroptosis (Fig. 2) [61]. Furthermore, ferroptosis in MS is triggered by increased proinflammatory T-cell activity and associated myelin degradation. It also accelerates lipid peroxidation and inflammation by releasing danger-associated molecular patterns (DAMPs), which further activate inflammatory T cells (Fig. 2) [55,57]. The release of DAMPs is accompanied by an increase in by-products of lipid peroxidation such as 4-hydroxynonenal (4-HNE), which itself arises from the peroxidation of PUFAs and is often used as an immunohistological marker in the context of histological detection of ferroptosis (Fig. 2) [55,57]. Ongoing research continues to add new insights into the molecular cascade involved in ferroptosis. Based on experimental analyses and RNA expression analysis in MS patients, there is evidence that ferroptosis is a very early event in MS and experimental autoimmune encephalomyelitis (EAE), and occurs earlier than T-cell activation. It is hypothesized that ferroptosis may promote T-cell activation through T-cell receptor (TCR) signaling *in vitro* and *in vivo* [60]. Another key finding relates to the endoplasmic protein stimulator of interferon genes (STING), which contributes to the release of interferon 1 (IFN1) in microbial infections through canonical activation. In MS, non-canonical activation of STING has been described, which leads to degradation of the anti-ferroptotic GPX4 by autophagy. In EAE, increase in the phosphorylated activated form of STING has been described with increasing exacerbation of EAE [62]. The protein ninjurin-1 (NINJ1), which plays no role in the early phase of ferroptosis in the context of lipid peroxidation, has been shown to be involved in the late phase. It is involved in the rupture of the plasma membrane and the release of DAMPs, and it has also been shown that its reduced expression in macrophages protects these cells from ferroptosis [63]. Another newly described inducer of ferroptosis in MS and EAE is the histone methyltransferase G9a, which leads to a repression of anti-ferroptotic genes and contributes to the reduction of intracellular glutathione levels and thus to the initiation of ferroptosis [64].

Research on cell-type-specific ferroptosis in MS is still in its early stages, with genetic and expression studies providing new insights. Single cell analyses in defined regions of the central nervous system of MS patients and control cases showed a particularly pronounced change in the expression of ferroptosis-relevant genes (ferroptosis score)

at the edges of active MS lesions, mainly affecting the phagocytizing microglia and macrophages. Compared to these peripherally localized macrophages, the macrophages in the center of the lesions showed a lower ferroptosis score. In contrast, a significantly higher ferroptosis score was detected in the neurons of the grey matter [65]. Single cell analysis revealed that ferroptosis occurs in all primary cell types of the CNS, with evidence of reduced expression of several ferroptosis-inhibiting proteins such as glutathione peroxidase 4 (GPX4) or ferritin heavy chain 1 (FTH1). Anti-ferroptotic gene expression was reduced in neurons, oligodendrocytes, oligodendrocyte precursor cells and astrocytes of MS patients compared to controls [66]. Further studies have confirmed ferroptosis in all these cell types, and reduced expression of anti-ferroptotic proteins such as GSH and GPX4 has been identified as a significant contributing cause [48,61,67]. The situation is different for CD4+ T lymphocytes, which show a high expression of the anti-ferroptotic protein GPX4 in MS patients and are therefore largely protected from ferroptosis. This observation was verified by an experimental knockout of GPX4, which resulted in improved symptoms in mice with EAE [68]. Single cell analysis of human cells from MS patients also showed that the ferroptosis score is low in both T and B lymphocytes, confirming that the high ferroptosis score in MS lesions affects macrophages in contrast to lymphocytes [65]. Genetic analyses are usually based on a large data set of many genes and their changes in certain diseases. Methods such as “weighted gene co-expression network analysis” and “receiver operating characteristic curve analysis” can be used for that purpose. These are data analysis methods for detecting changes in specific genes in defined diseases, in this case: changes concerning ferroptosis-related genes in MS [69]. These ferroptosis-related genes are involved in numerous functions such as mitochondrial translation, microglia activation, inflammation and oxidation, but the need is emphasized that the exact involvement of individual genes in MS has yet to be investigated [69]. In addition to the already mentioned reduced expression of anti-ferroptotic genes such as GPX4, the downregulation of the gene of the *anti-inflammatory hemoxigenase1 (HMOX1)* should be emphasized [69]. Ferroptosis-associated genes that are overexpressed in MS include the genes of *arachidonate lipoxygenases 5 and 12 (ALOX5, ALOX12)*, which are involved in the lipid peroxidation of PUFAs [70], as well as the mitochondrial protein voltage-dependent anion-selective channel protein 3 (VDAC3). This protein is involved in the interaction with pro-apoptotic proteins, and has a number of other functions that are still poorly understood and which are evidently related to PUFAs [70].

#### 4. Apoptosis

Apoptosis can be induced via two main pathways: the extrinsic pathway, mediated by receptors of the tumor necrosis factor (TNF) family and by the effector cas-

pases 3, 6 and 7; and the intrinsic (mitochondrial) pathway, initiated by pro-apoptotic proteins like Bcl-2-associated X protein (BAX), Bcl-2 homologous antagonist/killer (BAK), caspase 3 and the release of cytochrome C. Other factors may be involved in the induction of apoptosis, in particular the increased expression of p53 and complement-mediated apoptosis [71,72]. The role of defective apoptosis in multiple sclerosis was already suspected at the turn of the millennium. Studies revealed that expression of anti-apoptotic proteins such as myeloid cell leukemia sequence 1 (MCL1) was increased in T-cells, while pro-apoptotic proteins such as BAK were reduced [73,74]. Inspired by these results, increased expression of histone deacetylase 3 (HDAC3) was discovered in MS patients. HDAC3 is involved in various processes of cell proliferation and inhibition of apoptosis, whereby the downregulation of p53 appears to play a role. *In vitro* inhibition of HDAC3 resulted in an increased expression of p53 in T-cells from healthy individuals. In contrast, T-cells from MS patients showed no increased expression of p53, but instead an increased resistance to apoptosis. Therefore, a role of HDAC3 in the prevention of apoptosis of autoreactive T-cells and thus an involvement in the promotion of autoimmunity is suspected [71]. The Fas ligand (CD95) is a member of the TNF family and is involved in the initiation of apoptosis. In MS, its expression on the surface of CD4+ T-cells is reduced. Moreover, in mice with *Fas* gene knockout, maintenance of EAE due to the lack of Fas expression was confirmed experimentally [75]. Expression of the Fas ligand (Fas-L) by astrocytes is necessary for the induction of apoptosis by Fas, which was demonstrated in knockout mice without expression of astrocytic Fas-L by the lack of expression of caspase 3 and the absence of apoptosis of autoreactive CD4+ T-cells [76]. The nuclear receptor 4A1 (NR4A1) is involved in cellular proliferation, DNA repair, the regulation of T-cell-mediated immunity and the initiation of apoptosis. When comparing MS patients with a mild clinical course and patients with active relapsing-remitting MS, a reduced expression of NR4A1 with suppression of apoptosis of activated T-cells was found in the latter group, as well as a suppression of regulatory T-cell activity. With regard to cell type specificity, it is noteworthy that when comparing the transcriptional profiles of both patient groups, 75% of the genes were linked to CD4+ T-cells, but not to CD8+ T-cells, CD14+ macrophages or CD20+ B-cells. The authors conclude that these mechanisms collectively contribute to an augmented autoimmune response and increased disease activity, and that targeting the NR4A1-pathway in reactive T-cells could be a viable therapeutic strategy to prevent MS progression [77].

Apoptosis in MS and in EAE has been demonstrated in oligodendrocytes (OL), although it has been pointed out that their cell death in MS can also be caused by other factors such as metabolic stress or ferroptosis. It must therefore still be emphasized today, that the exact mechanism responsible for OL cell death in human MS remains elusive [78].

Apoptosis has been detected in oligodendrocytes of MS brains within morphologically intact brain areas, in the grey matter near intact neurons, and at the edge of areas with active demyelination. It is therefore hypothesized that apoptosis of oligodendrocytes precedes or even induces myelin damage in MS [79]. Pathological changes in mouse models with induced apoptosis of oligodendrocytes revealed demyelination and migration of microglia, which were similar to the changes in human MS [80,81]. Apoptosis of cerebral neurons in a mouse model with defective autophagy induced by suppressed autophagy-related protein 5 (Atg5) also demonstrated the role of oligodendrocytes. The defective autophagy in oligodendroglia led to myelin defects in the animals with reduced expression of basic myelin protein (MBP) and cyclic nucleotide phosphodiesterase (CNP), cognitive impairment in the context of behavioral tasks, as well as degeneration of axons and apoptosis of neurons with increased expression of apoptosis markers such as caspase 3 [82]. To date, the contribution of neuronal apoptosis to the clinical course of MS is controversial. However, there is evidence, that microglia trigger neuronal apoptosis by increased expression of cytotoxic molecules such as TNF $\alpha$ , IL-1 $\beta$  and IL-6, and by increased oxidative stress [83,84]. Furthermore, it is assumed that neuronal apoptosis and the activation of microglia are functionally interlinked. It has been shown that neuronal apoptosis is a key factor for driving multiple states of microglia with distinct transcriptional profiles. In particular, these microglia showed resistance to the absence of the otherwise necessary cellular survival signal through the colony-stimulating factor 1 receptor (CSF1R) [85]. Complement-induced activation of neuronal apoptosis was also confirmed by experimental treatment of neurons with isolated IgG from MS patients, which led to activation of C1q, C3b, C5b9, as well as caspase 3 in the treated neurons with subsequent apoptosis [72].

## 5. Pyroptosis

This form of cell death was described by Cookson and co-workers in 2001 [5]; the name refers to the detection in macrophages in the context of a *Salmonella* infection. Pyroptosis represents an independent form of PCD, characterized by its own signaling pathways. It has been reported in various inflammatory reactions, but also in several neurodegenerative diseases and tumors. The typical activation occurs through the contact of danger-associated molecular patterns (DAMPs) and/or pattern-associated molecular patterns (PAMPs) with the inflammasome. The inflammasome is a protein complex of the innate immune system. This protein complex consists of sensor complexes such as the protein family “nucleotide binding oligomerization domain leucine rich repeat and pyrin domain containing” (NLRP). These NLRPs recognize DAMPs and PAMPs mediated by Toll-like receptors (TLR) and subsequently lead to activation of the “nuclear factor kappa-light-chain-enhancer of activated B-cell complex” (NF- $\kappa$ B), which leads to the ac-

tivation of caspase 1 [86,87]. Another component of the inflammasome is the adaptor protein “apoptosis-associated speck-like protein” (ASC), which acts as a link between pro-caspase 1 and the inflammasome. The subsequent activation of caspase 1 in turn leads to increased expression of the inflammatory cytokines IL-1 $\beta$  and IL-18, as well as to the activation of gasdermin D (GSDMD), which causes porification of the cell membrane and thus initiates pyroptosis (canonical pathway of pyroptosis) [86,87]. An alternative (non-canonical) pathway of pyroptosis consists of the activation of caspases 4, 5 and 11 by liposaccharide (LPS) with subsequent oligomerization of the caspases and activation of GSDMD equal to the canonical pathway [86]. In MS, evidence for the occurrence of pyroptosis is provided by increased concentrations of ASC and caspase-1 in the serum [88], and by the detection of increased concentrations of NLRP3 and caspase-1 in peripheral fibroblasts of MS patients [89]. In addition, genetic analyses using whole exome sequencing of peripheral blood DNA showed a significantly higher incidence of rare allele variants of the NLRP1, NLRP3 and caspase-1 genes in MS patients compared to a control group. There was also a higher incidence of allele variants in genes mediating inflammasome inactivation via auto- and mitophagy, especially the genes for *receptor interacting protein kinase 2 (RIPK2)*, and *MEFV innate immunity regulator (MEFV)*. Compared to a control group, allele variants of these genes are more common in MS patients [90]. Pathologically altered gut flora in MS patients has also been discussed as an additional factor contributing to inflammation and pyroptosis; the increased occurrence of short-chain fatty acids, lipopolysaccharides and neuroactive compounds in MS patients appears to be of causal importance [91].

Microglia and neurons were found to be the main cell types in which pyroptosis occurs in MS [92,93]. More and more activation pathways of pyroptosis affecting these cell types were discovered. The protein “absent in melanoma 2” (AIM2) is a sensor for double-stranded DNA and is involved in the activation of the inflammasome and caspase-1 after recognition of aberrant DNA. In cortical neurons, activated AIM2 not only triggers caspase 1 but also increases the expression of IL-1 $\beta$  and mediates pyroptosis [93]. Application of IFN- $\beta$  prevents pyroptosis through the downregulation of AIM-2, NLRP1 and NLRP3, so that IFN- $\beta$  is considered a potential therapeutic agent in MS regarding the prevention of this form of PCD [86,93]. GSDMD-initiated pyroptosis was demonstrated by *in vitro* analysis in microglia from MS patients and from an EAE mouse model; experimental inhibition of caspase-1 using the small-molecule inhibitor VX-765 demonstrated a suppression of pyroptosis in each case [94]. Overexpression of caspases 3 and 7 in GSDMD-immunopositive microglia was also described as an additional signaling pathway in microglia from MS patients and from EAE transgenic mice. Here, as well, a decline in pyroptosis was observed in the

form of a decrease in typical GSDMD-positive cytological changes with rounding and fragmentation of the cell membrane following experimental inhibition of caspases 3 and 7 [92]. Caspases 3 and 7 are also activated by caspase 1 and are then in turn involved in the activation of GSDMD, whereby in particular a co-localization of activated caspase 3 and activated GSDMD in the microglia from the MS lesions has been demonstrated [92]. Irrespective of pyroptosis and inflammatory diseases such as MS, extensive research into the inflammasome and its components, particularly NLRPs, should be noted. This research complex is all the more important for microglia in MS, as the mere activation of NLRPs does not necessarily result in pyroptosis [95]. Of the 14 NLRP proteins, NLRP1, NLRP3 and NLRP4 in particular have been described as being involved in the activation of an aberrant immune response in autoimmune diseases, as well as in the activation of pyroptosis [93,95]. The best-researched protein, NLRP3, has multiple roles. In addition to activating inflammatory reactions including pyroptosis, it is involved in the recruitment of activated T-cells and in the prevention of cell death of Th17 cells. In microglia, it is involved in promoting the expression of inflammatory cytokines and in the initiation of pyroptosis [65,86,88]. The expression of NLRP1 and AIM-2 was detected in neurons, but no neuronal expression of NLRP3 was observed [96]. NLRP1 also activates caspase-1, but it is pointed out that its exact cascade of action is still poorly understood [88,96]. NLRP4, like NLRP3, is present in microglia and is increasingly expressed in MS foci. Therefore, NLRP3 and NLRP4 are considered to be the actual regulators of inflammatory activity and also of pyroptosis in activated microglia in MS [88].

## 6. Other Forms of Programmed Cell Death

In addition to the three most commonly studied forms of cell death in MS (ferroptosis, apoptosis, pyroptosis), there are already findings on necroptosis and NETosis. The role of autophagy in MS is also still the subject of research. The special case of autophagic cell death is subject to a strict definition with the need to exclude all other forms of cell death. Proof of the occurrence of autophagic cell death in MS is still pending.

### 6.1 Necroptosis

Degterev and co-workers first described necroptosis in a mouse model with cerebral ischemia. It was given its name because of its cytological similarity to necrosis with rupture of the plasma membrane [97,98]. Central and diagnostically relevant molecules in the activation cascade are receptor-interacting protein kinase 1 and 3 (RIPK1/3) and the protein mixed lineage kinase domain-like (MLKL), which is activated by RIPK1 and RIPK3 and subsequently induces necroptosis as a result of oligomerization. The receptor-interacting protein kinases RIPK1 and RIPK3 are serine/threonine protein kinases, which in turn

are activated by osmotic stress, tumor necrosis factor  $\alpha$  (TNF $\alpha$ ), interferon- $\gamma$  (IFN- $\gamma$ ) or toll-like receptors 3 and 4 (TLR-3/4) [66,99]. RIPK1 and RIPK3 can be involved in the induction of various inflammatory reactions, apoptosis or necroptosis. Inactivation of caspase 8 by the anti-apoptotic protein “FLICE-like inhibitory protein” (c-FLIP) is a prerequisite for the absence of apoptosis and the initiation of necroptosis [99,100]. In cortical MS lesions, inactivated caspase 8 combined with overactivated RIPK1, RIPK3 and MLKL was detected in autoptic MS brains, but not in healthy controls. This finding led to the author’s conclusion, that the necroptotic machinery is activated in MS. The expression of c-FLIP was significantly elevated in the MS samples, which also suggests an inhibition of caspase 8 in favor of the induction of necroptosis in the MS lesions [99]. In the same study, necroptosis in oligodendrocytes was stimulated by TNF- $\alpha$  in an animal model, which could be inhibited by the necroptosis inhibitor 7N-1 [99]. A comparable result was found in an EAE model by inhibiting RIPK1 expressed in microglia by necrostatin-1, which decreased necroptosis of oligodendrocytes [101]. The authors conclude that increased microglial expression of RIPK1 promotes oligodendrocyte necroptosis and inflammation in MS [99,101]. Another study used an EAE model to demonstrate increased expression of RIPK1 in microglia and astrocytes, which correlates with the level of expression of inflammatory genes [102]. Experimental induction of demyelination together with immunocytochemical differentiation of several microglia populations showed that necroptosis of pro-inflammatory microglia resulted in lesser demyelination and gradual regeneration of myelin with predominance of pro-regenerative microglia. The subpopulation of pro-inflammatory microglia was discussed as a potential therapeutic target to induce necroptosis, especially since necroptosis of other cell populations and cell types is associated with demyelination [103]. In addition to the necroptosis of microglia, astrocytes and oligodendrocytes in MS, necroptosis has also been demonstrated in cortical neurons of MS patients. Neuronal necroptosis was mainly observed in cortical layers II and III and was particularly pronounced in cases with prominent meningeal inflammation [104].

## 6.2 Autophagy and Autophagic Cell Death

The term “autophagy” refers to the self-preservation of a cell through the degradation of its own cellular components under physiological conditions. Since the first description of autophagy by Clark in 1957 and by Ashford and Porter in 1962 [105,106], the molecular cascade has been investigated in detail. Of the three forms of autophagy, macroautophagy with the formation of an autophagosome surrounded by a double membrane is often used in the literature as a synonym for the term “autophagy”. Two further forms are microautophagy with typical changes in the lysosomal membrane such as protrusion or invagination, and

chaperone-mediated autophagy with direct lysosomal uptake of the substances to be degraded as a result of the activity of the chaperone “heat shock protein family A member 8” (HSPA8) and the lysosome-associated membrane protein 2 (LAMP2) [107]. Macroautophagy, referred to below as “autophagy” in accordance with the literature, is essentially initiated by the activation of autophagy-related genes (ATGs). A central cascade consists of the activation of ATG8 and the Unc-51-like kinase complex (ULK) by AMP-activated protein kinase (AMPK), which, together with the activation of additional ATGs, leads to the formation of an autophagosome and, as a result of its fusion with lysosomes, to an autolysosome [87,108]. In contrast to autophagy, much less is known about “autophagic cell death”. For its detection, genetic or experimental evidence is required that (1) it occurs as a result of activation of the autophagy cascade, and (2) that all other forms of programmed cell death can be excluded [107]. In MS, autophagy has a multifaceted significance regarding the promotion of other forms of cell death such as apoptosis or ferroptosis. This should be distinguished from autophagic cell death, whose role in MS has not yet been clearly established. Autophagy makes an important protective contribution in the CNS due to increased disposal of damaged mitochondria with consequently reduced production of ROS [108–110]. This applies in particular to astrocytes and oligodendrocytes with corresponding promotion of cellular survival of both cell types. It is still unclear whether autophagy is dysregulated in astrocytes or in neurons of MS patients [111]. ATG expression was reduced in oligodendrocytes of MS lesions, but increased in microglia. These findings indicate an inhibited protective effect of autophagy in oligodendrocytes, but an intensified cell-preserving function of autophagy in the case of pro-inflammatory microglia [108]. As with pro-inflammatory microglia, intensified autophagy plays an important role in the maintenance of pro-inflammatory T-cells, as well as B-lymphocytes, in which an activation of autophagy as a result of citrullination of peptides in the context of EBV infection is suspected [111]. Autophagy in microglia, in pro-inflammatory T-cells and in B-cells thus appears to be a central factor in the progression of MS [109,111,112]. Dysregulation of autophagy can also lead to the induction of various forms of cell death. Examples include the promotion of ferroptosis by degradation of the anti-ferroptotic protein GPX4 during autophagy [62], and indirectly by damage to myelin and neuronal axons due to reduced autophagic activity in oligodendrocytes with secondary induction of apoptosis of neurons [82]. As already mentioned, autophagic cell death must be distinguished from these examples of induction of other forms of cell death; a role for autophagic cell death in MS has not yet been conclusively confirmed.

NETosis is a form of cell death that affects neutrophil granulocytes (NGs) and, together with the general role of NGs, is attracting increasing scientific interest in the field of autoimmune diseases. With regard to NGs, it has been shown in MS that, in contrast to a control group of healthy volunteers, NGs exhibit a primed status, i.e., the status of primary activation as a result of the proinflammatory environment. The significant changes in the NGs were shown by higher expression of TLR-2, IL-8, CD43, and the formyl peptide receptor (fMLP-receptor), and also by enhanced degranulation and oxidative stress [113]. In addition, an increased proportion of so-called “low-density granulocytes” was detected in the peripheral blood of MS patients, which are characterized by a typical expression pattern with a lack of CD14-expression and increased CD15-expression. They occur more frequently in various pathologies such as inflammatory diseases or in patients with heart disease compared with healthy persons [114,115]. It remains unclear whether this cell fraction of NGs plays a causal pathophysiological role or is more of an epiphenomenon [114]. Following contact of NGs with pathogens or as a result of diverse stimulation of NGs by immune complexes, cytokines or chemokines, the formation of so-called “neutrophil extracellular traps” (NETs) can occur. These NETs consist of various components such as nuclear proteins like histones, granules, cytoplasmic components like neutrophil elastase (NE), high-mobility-group protein B1 (HMGB1), myeloperoxidase (MPO), and proteinase-3 (PR3), which are released by the NGs into the extracellular space [116]. The first of the two basic forms of this “NETosis” is the “suicidal NETosis” in which lysis and thus cell death of the NGs occurs because of chromatin decondensation, increased calcium release, increased expression of protein kinase C (PKC), induction of the nicotinamide adenine dinucleotide phosphate oxidase (NADPH-oxidase) complex, and an increased production of reactive oxygen species (ROS). In “non-lytic NETosis”, NETs are formed without subsequent lysis of the NGs [116]. In the peripheral blood of MS-patients, significantly higher levels of NETs and increased concentrations of neutrophil-activating chemokines, cytokines, and neutrophil-derived enzymes were detected [113,117]. Importantly, elevated plasma levels of several NET-associated proteins such as neutrophil elastase (NE) and myeloperoxidase (MPO) correlate with lesion burden and the extent of demyelination (Table 1, Ref. [55–104,113–120]) [117,118]. In addition, NETs promote the activation of pro-inflammatory Th17 lymphocytes in autoimmune diseases [117,119,120]. Overall, previous experience confirms a significant role of NGs and NETs in promoting an inflammatory environment in various diseases and a potential role in MS, but it is also emphasized in the latest literature that research on NETs and NETosis is still at a very early stage [116,117]. This also applies to the differences between the two forms of “vital non-lytic NE-

Tosis” and “suicidal NETosis” associated with cell death, which also still need to be investigated in MS and its experimental models.

## 7. Discussion

Most of the risk factors for MS such as vitamin D deficiency and genetic influences have been well known for a long time. Currently, there is increasing interest in epigenetic factors in MS, including the methylation status of genes regulating myelin homeostasis [18], and the altered expression behavior of non-coding RNA molecules such as micro-RNAs (miRNAs) or circular RNAs (cRNAs) and their influence on the inflammatory activity of T and B-cells [19,121]. Despite the known significance of EBV virus infection and pathological intestinal bacterial flora, the need for further research is also emphasized for these two aspects [23,24,26,29]. The cumulative influence of all these risk factors contributes to cerebral immunological dysregulation; the scientific focus is still on the increased activity of pro-inflammatory CD4+ T-lymphocytes, peripheral monocyte-derived macrophages and resident mononuclear phagocytes of the central nervous system such as microglia, but also on B-lymphocytes (Fig. 1) [9,33–35,37]. An additional aspect of MS pathology is the dysregulation of the cerebral iron balance, mainly caused by inflammatory damage to the oligodendroglia and the iron-containing myelin [44,45]. Increased uptake of iron by pro-inflammatory T-cells, B-cells and macrophages, and phagocytosis of myelin debris by macrophages seems to intensify pro-inflammatory activity, oxidative stress with damage to the mitochondria and increased production of cytotoxic  $\text{Fe}^{2+}$ , causing damage to oligodendrocytes and macrophages [44–48]. Neuronal damage because of increased cellular uptake of iron has also been proven (Fig. 1) [45,53]. The currently increased scientific interest in iron dysregulation in MS coincides with the forthcoming renewal of the McDonald criteria for the diagnosis of MS, especially the paramagnetic rim lesions (PRLs) in the brain. MRI scans will be a diagnostic criterion alongside other innovations such as the central vein sign or the detection of kappa free light chains as biomarkers. PRL is a paramagnetic effect detectable in T2\*- and susceptibility-weighted MR sequences with central extinction of the MR signal due to iron-loaded macrophages at the edge of MS lesions. The sensitivity and specificity of this sign in MS is considered very high at 90% and therefore diagnostically significant [50,51,122]. Although it is currently repeatedly emphasized that the dysregulation of iron balance in MS is only incompletely understood, the imminent introduction of PRL as a diagnostic criterion represents an important step and ultimately proves that imaging evidence of dysregulation of iron balance in MS is diagnostically and clinically significant [50,51,122].

In addition to the direct promotion of pro-inflammatory activity of lymphocytes and macrophages,

**Table 1. Detected forms of programmed cell death (PCD) in MS.**

Type of PCD	TT: Typical Triggers MS: Major Impacts on MS and its Experimental Models	Major Components of the Molecular Cascade
Ferroptosis [55–70]	TT: Increased iron accumulation, increase of ferrous iron (Fe <sup>2+</sup> ); inhibition of the cellular antioxidant system; peroxidation of poly-unsaturated fatty acids MS: Early event in MS, preceding the activation of pro-inflammatory CD4 <sup>+</sup> T-cells; further acceleration of inflammation and demyelination through a release of danger-associated molecular patterns (DAMPs)	STEAP3, DMT1, PUFAs, ACSL4, GSH, GPX4
Apoptosis [71–85]	TT: Different triggers such as increased expression of receptors of the tumor necrosis factor (TNF) family, pro-apoptotic proteins such as BAX and BAK, p53, or complement-mediated activation MS: Dysregulation of apoptosis in reactive T-cells; evidence for a functional link between neuronal apoptosis and altered activation status of microglia	TNF- $\alpha$ , Fas-L, TRAIL, MCL1, BAX, BCL2, BAK, Caspases
Pyroptosis [86–96]	TT: Contact between DAMPs and/or PAMPs with the inflammasome, detected by NLRPs; activation of GSDMD by ASC and Caspase-1 or by LPS and Caspases 4, 5 and 11 MS: Cortical neurons are affected by pyroptosis due to activation of AIM2; pyroptosis can also occur in microglia	DAMPs, PAMPs, NLRPs, ASC, GSDMD, LPS, AIM2, Caspases
Necroptosis [97–104]	TT: Inactivation of caspase 8 and overactivation of RIPK1/3 and MLKL in MS-lesions are an indication for the activation of the necroptotic machinery in MS MS: With additional consideration of experimental results, necroptosis was detected in all primary cell types of the CNS: neurons, oligodendrocytes, astrocytes, microglia; evidence for induction of necroptosis in oligodendrocytes and promotion of inflammation due to overexpression of RIPK1 in microglia	RIPK1/3, TNF- $\alpha$ , IFN- $\gamma$ , TLR3/4, MLKL, c-FLIP
NETosis [113–120]	TT: Contact between neutrophil granulocytes (NGs) and pathogens such as DAMPs and PAMPs leads to the expression of NETs consisting of numerous proinflammatory components such as NE or MPO MS: A higher concentration of NETs has been detected in the peripheral blood of MS patients; a significant pro-inflammatory role of NGs and NETs in the context of MS is suspected	NETs, NE, MPO, HMGB1, PR3, PKC, ROS

Abbreviations of molecules: - Ferroptosis: STEAP3, ferric reductase six-transmembrane epithelial antigen of prostate 3; DMT1, divalent metal transporter 1; PUFAs, polyunsaturated fatty acids; ACSL4, acyl-CoA synthetase long-chain family member 4; GSH, glutathione; GPX4, glutathione peroxidase 4. - Apoptosis: TNF- $\alpha$ , tumor necrosis factor  $\alpha$ ; Fas-L, Fas-ligand; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand; MCL1, myeloid cell leukemia 1; BAX, Bcl-2 associated X protein; BCL2, B-cell lymphoma 2; BAK, Bcl-2 homologous antagonist/killer, apoptosis regulator BAK. - Pyroptosis: DAMPs/PAMPs, danger-associated / pattern-associated molecular patterns; NLRP1–4, nucleotide binding oligomerization domain leucine rich repeat and pyrin domain containing 1–4; ASC, Apoptosis-associated speck-like protein; GSDMD, gasdermin D; LPS, liposaccharide; AIM2, Absent in melanoma 2. - Necroptosis: RIPK1/3, receptor-interacting protein kinase 1 and 3; TNF- $\alpha$ , tumor necrosis factor  $\alpha$ ; IFN- $\gamma$ , interferon- $\gamma$ ; TLR3/4, toll-like receptors 3 and 4; MLKL, mixed lineage kinase domain-like; c-FLIP, FLICE-like inhibitory protein. - NETosis: NETs, neutrophil extracellular traps; NE, neutrophil elastase; MPO, myeloperoxidase; HMGB1, high-mobility-group protein B1; PR3, proteinase-3; PKC, protein kinase C; ROS, reactive oxygen species.

the promotion of ferroptosis is one of the most important aspects of focal iron overload in MS. The main findings on ferroptosis in MS are currently that it is a common form of programmed cell death (PCD), and that it occurs very early during MS. Ferroptosis plays a direct role in the development of MS and in the activation of pro-inflammatory lymphocytes, with ferroptosis and the pro-inflammatory process apparently promoting each other (Fig. 2) [56,60]. Experimental and genetic analyses in MS brains revealed that ferroptosis occurs in all brain cell types: neurons, astrocytes, oligodendrocytes, and oligodendrocyte precursor cells [48,61,66,67]. In contrast, pro-inflammatory T and B-lymphocytes showed a reduced ferroptosis score, and CD4<sup>+</sup> T-cells additionally showed increased expression of the anti-ferroptotic protein GPX4, and thus extensive protection of these cells against ferroptosis [65,68]. Very similar, inhibited apoptosis in T-cells of MS brains was found due to decreased expression of several pro-apoptotic proteins such as Fas-ligand (CD95) and “nuclear receptor subfamily 4 group A member 1” (NR4A1), as well as increased expression of anti-apoptotic proteins such as MCL1 and HDAC3 [71,75–77]. In contrast to the reduced apoptosis of pro-inflammatory T-cells, a significant occurrence of apoptosis in oligodendrocytes (OLs) and in neurons has been demonstrated in MS, with apoptosis occurring very early and in morphologically intact brain areas, particularly in the case of OLs. Consequently, the hypothesis is being discussed that apoptosis of OLs contributes significantly to myelin damage at an early stage of the disease [78,79]. In neurons, there is evidence for the occurrence of apoptosis in MS because of microglial activation with expression of inflammatory cytokines, as well as evidence for complement-activated apoptosis [72,83,84]. In MS, autophagy primarily makes a protective contribution as a result of the degradation of damaged mitochondria with the avoidance of increased oxidative stress. However, reduced expression of autophagy-regulating genes with a correspondingly reduced protective effect on endogenous brain cells has been demonstrated in MS lesions, whereas an increased expression in pro-inflammatory microglia with corresponding protection of these cells by autophagy was observed [108,109,111,112]. The detection of autophagic cell death requires the reliable exclusion of other forms of programmed cell death, which has not yet been demonstrated in MS. In contrast, the occurrence of pyroptosis in MS has been described with certainty; it mainly affects microglia and neurons. However, the functions of both the gasdermins and the NLRPs as the main components of the inflammasome are still incompletely understood and the subject of research. According to current knowledge, NLRP1 is a component of the inflammasome in neurons, while NLRP3 and NLRP4 are involved in the regulation of the inflammatory activity of microglia [65,86,88,96]. Like ferroptosis, necroptosis was also detected in autaptic and experimental studies in all primary cell types of the CNS in MS or

EAE [99,101–104]. In addition, increased expression of the necroptosis-inducing protein RIPK1 in pro-inflammatory microglia was detected in MS, which led to necroptosis in oligodendrocytes [101]. Based on previous experience, the occurrence of necroptosis in MS can be considered certain, although experience in this regard is still relatively limited compared to the proven significance of ferroptosis (Table 1).

Concerning other forms of PCD, recent findings include the importance of neutrophil granulocytes and their formation of “neutrophil extracellular traps” (NETs) in MS, whose concentration in the serum of MS patients is significantly increased and correlates with the severity of the disease [113,117,118]. Further investigation is needed to determine whether this expression of NETs in MS occurs as a result of vital or suicidal NETosis, and whether the increase of low-density granulocytes is an epiphenomenon or a causal factor in the pathogenesis of MS [113,114,117,118]. Parthanatos, another type of PCD, has already been shown to play a role in other neurodegenerative diseases such as amyotrophic lateral sclerosis or Parkinson’s disease, but also in EAE and MS [123,124]. The central regulatory protein of parthanatos, poly-ADP-ribose polymerase-1 (PARP-1), leads to cellular protection when moderately activated, but to cell death as a result of nicotinamide adenine dinucleotide (NAD) depletion when more strongly activated. Therefore, it is currently unclear whether therapeutic manipulation of PARP-1 is beneficial or detrimental to disease progression [125,126]. Panoptosis is a form of cell death in which key enzymes of apoptosis, necroptosis and pyroptosis and thus all three forms of PCD are activated. The exact mechanism of panoptosis has not yet been clarified. A panoptosome is postulated, which leads to the initiation of cell death in a similar way to the inflammasome and apparently contains various components of the inflammasome such as NLRP2 [127]. With regard to the interaction of different forms of PCD, panoptosis is therefore an important example due to the involvement of key molecular components of apoptosis, pyroptosis, and necroptosis. It is believed that the panoptosome plays a central role in this interaction due to its simultaneous activation of the molecular components of these PCDs. While research into panoptosis in tumors is already at an advanced stage, there is also some experience with neurodegenerative diseases such as Alzheimer’s disease; research into panoptosis in the context of MS is still pending [87,127–130]. Another example for the interaction of different PCDs is the role of caspase 8, which is an obligate promoter of apoptosis but, in the event of its inactivation and the simultaneous activation of RIPK1/3, enables the initiation of necroptosis [99,100]. Research into the interaction of different PCDs is more advanced in the case of tumors such as gliomas, an important finding concerns cysteine as a cofactor of necroptosis and, at the same time, as an inhibitory factor of ferroptosis [87,131]. However, it is explicitly pointed out that findings

## Forms of Programmed Cell Death in Multiple Sclerosis

### - Promising Additional Aspects for Further Research -

#### Neutrophil Granulocytes and Neutrophil Extracellular Traps (NETs)

- What is the exact Role of „Low-Density Granulocytes“?
- Differences between Suicidal and Non-Lytic NETosis in MS
- Diagnostic Significance of NETs in the peripheral Blood

#### Parthanatos

- Further Understanding of this Form of PCD in general
- Assessing the Split Role of PARP-1 between Cellular Protection and Cell Death
- Is there a Cell Type-specific Role of PARP-1 and Parthanatos?

#### Panoptosis

- Does Panoptosis occur in MS, and if so, in which Cell Types?
- Detailed Analysis of the “Panoptosome” and its possible Variants
- Concepts for therapeutic Targeting

#### Epigenetic Research

- Importance of Methylation Status and Histone Modifications in PCDs in MS
- Significance of individual ncRNAs (microRNAs, circRNAs, lncRNAs)
- Identification of ncRNAs with particular pathophysiological Significance

#### New therapeutic Concepts and their Experimental Testing

- Which PCD Forms should be therapeutically influenced in which Cell Type?
- Is a certain PCD Form predestined as a therapeutic Target in MS?
- Should Targeting of PCDs be combined with additional molecular Concepts?

**Fig. 3. Additional important aspects of research into various forms of PCD in multiple sclerosis, following on from research priorities already initiated: Neutrophil granulocytes and neutrophil extracellular traps (NETs), parthanatos, panoptosis, epigenetic research concepts, and special considerations for therapeutic concepts targeting PCDs.**

on the role of PCDs and their interactions are only specific to the respective disease or group of diseases and cannot necessarily be transferred to other diseases [87,131]. In MS, in-depth research into these interactions between different PCDs is still in its infancy.

The same holds for the question concerning the involvement of different PCDs in different cell types. As explained in the previous chapters, the current state of knowledge is that ferroptosis and necroptosis can occur in all primary cell types of the CNS in the context of MS and its models: neurons, oligodendrocytes, astrocytes, and mi-

**Table 2. Important recent studies (years 2021 to 2025) with experimental influencing on forms of PCD, ferroptosis and pyroptosis, in mouse models of EAE.**

Type of targeted PCD	First Author, Year	Name and Type of the Agent
Ferroptosis Treatment lowered the overall clinical disease score with amelioration of disease progression and significant reduction in demyelination; this effect was more pronounced when compared with the use of other ferroptosis inhibitors	Van San 2023 [56]	UAMC-3203 (Ferrostatin-analogue)
Ferroptosis miR-367-3p delivered by stem cell-derived exosomes led to alleviation of EAE by suppressing ferroptosis via activation of the antioxidant system cystine/glutamate antiporter system (XcT, SLC7A11) with activation of GPX4; the extent of inflammation and demyelination was significantly reduced	Fan 2023 [138]	miR-367-3p (micro-RNA)
Ferroptosis Treated mice showed clinical improvement with reduced paralysis; histology showed reduction in myelin loss and lesion size, as well as increased protein levels of anti-ferroptotic proteins from the antioxidant system (XcT, GSH, GPX4)	Jhelum 2023 [139]	UAMC-3203 (Ferrostatin-analogue)
Ferroptosis Reduction of demyelination, astrogliosis, and microgliosis in the optic nerve of EAE mice; reduction of axonal damage and ganglion cell loss in the retina; increased expression of the anti-ferroptotic protein GPX4; the iron deposition in the optic nerve was significantly diminished according to histological confirmation	Rayatpour 2022 [140]	Deferiprone (Iron chelator)
Pyroptosis Application of the vectors via lentivirus infection led to inhibition of the HMGB1/TLR4/NF- $\kappa$ B signaling pathway, as well as inhibition of pyroptosis with reduced expression of NLRP3, caspase-1, and GSDMD; in addition, reduced infiltration with inflammatory cells was confirmed	Feng 2025 [141]	Sh-HMGB1 and Sh-NLRP3 (short hairpin RNA vectors targeting HMGB1 and NLRP3)
Pyroptosis The glucagon-like peptide-1 receptor (GLP-1R) agonist is known to have neuroprotective capacity; after application, the disease score was ameliorated with reduction of inflammation and demyelination, the expression of the pyroptosis-associated proteins caspase-1, NLRP3 and GSDMD was also reduced	Song 2022 [142]	Liraglutide (GLP-1R agonist)
Pyroptosis The severity of the clinical course was attenuated; the degree of demyelination and inflammation was also reduced with lowered amount of inflammatory CD4 <sup>+</sup> cells and glial activity; the expression of NLRP3 and GSDMD was decreased	Wang 2025 [143]	Forsythoside B (natural phenylethanoid glycoside)
Pyroptosis Severity of EAE was significantly reduced after treatment, as well as the expression of pro-inflammatory cytokines (TNF- $\alpha$ , IL-6, IL-17); in addition, there was increased expression of anti-inflammatory cytokines (IL-10) and reduced tissue levels of NLRP3 and caspase 1	Kiasalari 2021 [144]	Ellagic acid (natural polyphenol)
Pyroptosis Remyelination was enhanced as a result of upregulation of microglial SLC2A4-regulated autophagy; likewise, downregulation of NLRP3 led to inhibition of microglial pyroptosis, which was confirmed <i>in vitro</i> in a microglial cell line	Zhao 2025 [145]	Evodiamine (natural tryptamine indole alkaloid)

croglia [48,61,66,67,99–104]. The occurrence of apoptosis has been described primarily in oligodendrocytes and neurons, whereas neurons and microglia were found to be the main cell types in which pyroptosis occurs in MS [78–84,92,93]. It should be noted that in the case of microglia, their significance in the induction of neuronal apoptosis in MS is suspected to be due to increased expression of inflammatory cytokines [83,84]. Another important aspect men-

tioned in this context is the extensive prevention of PCD of pro-inflammatory lymphocytes, particularly concerning ferroptosis and apoptosis [65,68,76,77]. When considering the significance of individual cell types in PCDs, it must be taken into account that the current literature often highlights the as yet incomplete but constantly growing knowledge for almost all cell types, for example, the important aspect of the occurrence of apoptosis in oligodendrocytes [78,79]. It

is important to note that the complex topics of iron dysregulation, PCDs and their molecular components, as well as the cell types involved in MS, is still dominated by an ever-growing number of individual observations. One example of new research findings in this area is the experimental blocking of MLKL, which leads to an improvement in clinical symptoms and, as expected, to the blocking of necroptosis. In addition, however, it has been shown that MLKL, apart from its importance as a necroptosis activator, has additional significance in the development of an inflammatory response as a result of the activation of Th1 and Th17 cells, as well as in the activation of several signaling pathways such as the nuclear factor kappa-light-chain-enhancer of activated B-cells (NF- $\kappa$ B). This is an important indication that the influence of the components of the individual PCDs in MS is complex and not limited to the PCDs themselves [132]. The situation is similar with regard to the significance of defective proteasomes such as proteasome 20S beta 8 (PSMB8), which leads to dysregulation of neuronal homeostasis, increased oxidative stress, and ferroptosis. It is important to note that PSMB8 directly leads to the downregulation of the anti-ferroptotic protein GSH [133]. In a review of this study, the question is explicitly raised as to whether these changes observed in neurons will also be detectable in oligodendrocytes [134]. Another important finding is the significance of the plasma  $\alpha$ -dimorphecolic acid ( $\alpha$ -DIPA), a linoleic acid derivative that is downregulated in MS and has a primarily anti-inflammatory effect. Experimental treatment of EAE with  $\alpha$ -DIPA reduced the activation of microglia and the transcriptional level of pro-inflammatory genes, such as the NF- $\kappa$ B pathway, as well as the occurrence of ferroptosis [135]. These highly topical studies also repeatedly point out that the interrelationships between different PCDs, individual molecular components, and individual cell types in MS are still elusive. For example, the aforementioned study emphasizes that it is unclear whether endogenous  $\alpha$ -DIPA is capable of regulating microglial inflammation in the CNS mainly through the ferroptosis pathway [135].

Another important aspect of research into PCDs in MS will be the role of non-coding RNA molecules (ncRNAs). In the case of micro-RNAs, there are approaches to understanding their role in the pathogenesis of MS and in influencing certain forms of PCDs such as ferroptosis [136,137], as well as in the suppression of ferroptosis in the EAE model using micro-RNA miR-367-3b delivered by stem-cell derived exosomes [138]. Investigation of the influence of ncRNAs is therefore an essential aspect of current experimental research into potential therapeutic approaches targeting forms of PCD (Fig. 3). Also, all other aspects mentioned with regard to the forms of PCD point to a variety of possibilities for experimental concepts for the development of new treatment modalities for patients with MS. Current studies mainly relate to the experimental targeting of ferroptosis or pyroptosis in the mouse model of

EAE and show remarkable results on clinical and morphological improvements due to the inhibition of these PCDs (Table 2, Ref. [56,138–145]). In the case of ferroptosis, in addition to the aforementioned application of microRNAs, current therapeutic approaches using ferrostatin analogues and iron chelators are also noteworthy [56,138–140]. It should be emphasized that differences in *in-vivo* success have been reported in the context of the use of various ferroptosis inhibitors [56]. All of these approaches resulted in the inhibition of ferroptosis, mainly due to the promotion of the expression of anti-ferroptotic proteins such as GSH and GPX4. Histologically, reduced gliosis and a significant attenuation of demyelination were demonstrated, as well as reduced loss of oligodendrocytes, together with reduced iron deposition (Table 2) [56,138–140]. Pyroptosis was targeted experimentally by influencing pro-inflammatory and pyroptosis-promoting signaling pathways such as the HMGB1/TLR4/NF- $\kappa$ B pathway by an agonist of the neuroprotective glucagon-like peptide-1 receptor (GLP-1R), and by naturally occurring compounds such as polyphenols [141–145]. These approaches have shown an improvement in disease scores as a result of reduced expression of pyroptosis-associated components such as NLRP3 and GSDMD. Therefore, experimental therapeutic approaches are currently focused primarily on reducing ferroptosis and pyroptosis (Table 2). The diversity of the forms of PCD and their molecular components involved in MS suggests that further therapeutic approaches will be explored in the future with the aim of blocking central pathophysiological processes in MS that are directly or indirectly related to the various forms of PCD.

## 8. Conclusions

Dysregulation of the iron balance and different types of programmed cell death (PCD), in particular ferroptosis, apoptosis and pyroptosis, play a major role in the pathophysiology of MS. An important aspect of the forms of cell death is their ambivalent role in this disease due to their non-physiological occurrence in the brain's own cells and their widespread absence in pro-inflammatory cells. Research of different forms of PCD in MS is at the beginning, and approaches to understand NETosis, parthanatos, and the role of individual micro-RNAs will bring further insights in the near future. Experimental results on ferroptosis and pyroptosis in animal models encourage a further intensive search for potential therapeutic approaches in MS. In addition, iron dysregulation has become increasingly important in neuroradiological imaging diagnostics, and the new diagnostic McDonald criteria will include the imaging correlate of iron dysregulation as a sign of MS disease. Based on current knowledge, it must be emphasized that both the dysregulation of iron metabolism and the individual forms of PCD in MS are not yet fully understood. This also applies to the diverse functions of the various molecular components of PCDs, as well as to the

interactions between the individual forms of PCD and their occurrence and significance in the different cell types of the brain and in immune cells. Current studies show that more and more details are being discovered about all these aspects and that it is still not possible to comprehensively or even definitively explain the precise role and mechanism of these individual aspects in MS, such as the dysregulation of iron metabolism. In addition to the aspects mentioned above, the central tasks for future research include investigating additional forms of PCDs, of which NETosis and parthanatos have already been described in MS, while research into other forms such as panoptosis is still pending. The increasing importance of epigenetics in medical research is also becoming apparent in the context of MS and its models in relation to the forms of PCDs. In particular, it seems important here to investigate the significance of individual non-coding RNA molecules in the different forms of PCD and thus their role as potential therapeutic targets or even as therapeutic agents. Finally, it is likely that future research on forms of PCD will also focus on cell type specificity, for example, with regard to therapeutic targeting (Fig. 3).

### Author Contributions

RN and EH designed the review. RN performed the literature research. RN and EH wrote the initial draft of the article. Both authors contributed to editorial changes in the manuscript. Both authors read and approved the final manuscript. Both authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

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Not applicable.

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### Conflict of Interest

The authors declare there are no conflicts of interest.

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