**MIF modulation in different subtypes of ALS: from basic mechanisms to therapeutic potential**

**Detailed Description of the Research Program**

1. **Scientific Background**

Amyotrophic lateral sclerosis (ALS), the most prevalent motor neuron disease affecting adults, is a devastating neurodegenerative disorder characterized by the progressive degeneration of both upper and lower motor neurons (MNs) [[1](#_ENREF_1), [2](#_ENREF_2)]. Approximately 90% of ALS cases manifest sporadically, while the remaining cases are familial and typically inherited in an autosomal dominant manner [[3](#_ENREF_3)]. Notably, nearly 20% of familial ALS cases are linked to missense mutations in the gene encoding cytoplasmic Cu/Zn superoxide dismutase (SOD1) [[3](#_ENREF_3)]. Although the precise mechanisms driving MN degeneration in ALS remain elusive, mounting evidence suggests a tight correlation between the aberrant accumulation of misfolded SOD1 and the severity of the disease [[4-6](#_ENREF_4)]. Transgenic animal models, including mice [[7-10](#_ENREF_7)] and rats [[11](#_ENREF_11), [12](#_ENREF_12)], engineered to express SOD1 harboring ALS-associated mutations, exhibit late-onset MN degeneration and muscle atrophy, mirroring ALS symptoms. Intriguingly, this degeneration occurs in a non-cell autonomous manner [[13-22](#_ENREF_13)] and is accompanied by the selective accumulation of misfolded SOD1 within the spinal cord, specifically at the cytoplasmic surface of intracellular organelles including mitochondria [[23](#_ENREF_23), [24](#_ENREF_24)] and the endoplasmic reticulum (ER) [[25-27](#_ENREF_25)]. Our research has revealed that misfolded SOD1 interacts with the mitochondrial protein VDAC1 [[23](#_ENREF_23), [28](#_ENREF_28), [29](#_ENREF_29)], hampering its conductance across the outer mitochondrial membrane and potentially compromising energy supply [[23](#_ENREF_23)]. Furthermore, mutant SOD1 alters the composition of mitochondrial proteins and disrupts the protein import mechanism [[30](#_ENREF_30)], although these effects are confined to spinal cord mitochondria and are not observed in unaffected tissues.

To date, the most effective strategies for mitigating the toxicity of mutant SOD1 in animal models or ALS patients involve the suppression of SOD1 expression using microRNAs, shRNAs, or antisense oligonucleotides (ASOs) [[31-33](#_ENREF_31)]. While the US Food and Drug Administration (FDA) has recently approved ASO-based therapy for ALS patients with SOD1 mutations, this approach is limited by its inability to distinguish between misfolded (toxic) and intact SOD1. Given the critical role of SOD1 as a vital antioxidant enzyme, its long-term depletion in the central nervous system (CNS) has the potential to impact neuron physiology, particularly under various disease-related stresses. Indeed, recent reports have linked a loss-of-function SOD1 variant associated with undetectable SOD1 activity to a debilitating neurological phenotype characterized by hyperekplexia, ataxia, muscular hypotonia, and severe psychomotor retardation [[34-36](#_ENREF_34)]. Therefore, there is an urgent need for alternative therapeutic approaches that selectively target misfolded SOD1 while preserving intact SOD1 proteins.

In light of the ubiquitous expression of SOD1, a major unanswered question in the field revolves around the structural determinants that underlie the selective misfolding of mutant SOD1. Our laboratory aims to identify the factors responsible for mutant SOD1 misfolding and its exclusive association with mitochondria in specific cell types, particularly those affected in ALS. In pursuit of this goal, we previously investigated whether this association is governed by mitochondria or the cytosol and found that the cytosol plays a pivotal role in the mitochondrial association of mutant SOD1 in affected tissues in the SOD1 ALS model [[25](#_ENREF_25)]. Moreover, through the inhibition of the mitochondrial deposition of misfolded SOD1, we identified and purified a 12 kDa multifunctional protein with chaperone activity known as macrophage migration inhibitory factor (MIF), which is abundant in non-neuronal tissues. MIF exhibits ATP-independent protein folding chaperone activity [[25](#_ENREF_25)], despite its primary role as one of the earliest-described cytokines [[37](#_ENREF_37)] that plays a crucial role in the immune response [[38-40](#_ENREF_38)]. Although a portion of MIF can be sequestered into vesicles and released extracellularly in response to various signals, MIF is primarily synthesized as a soluble cytoplasmic protein. Notably, MIF has been previously been implicated in intracellular protein chaperone activities, transitioning from a multimeric to a monomeric form and exposing a hydrophobic surface that facilitates ATP-independent chaperone activity [[41](#_ENREF_41)]. Our research has demonstrated that recombinant MIF directly binds to mutant SOD1, inhibiting its misfolding in NSC-34 cells and its association with mitochondria and the ER [[25](#_ENREF_25)]. Elevating MIF levels in neuronal cells reduced the accumulation of misfolded SOD1 and its association with intracellular membranes, while also extending the survival of mutant SOD1-expressing MNs [[25](#_ENREF_25)]. Additionally, our follow-up study revealed that the absence of endogenous MIF accelerates disease onset and late disease progression, shortening the lifespan of mutant SOD1G85R mice [[42](#_ENREF_42)]. These results thus emphasize the critical role of MIF in mitigating mutant SOD1 toxicity.

It is noteworthy that MIF protein levels are extremely low in the spinal MNs of rats and mice [[25](#_ENREF_25), [42](#_ENREF_42)], implying that low levels of chaperone activity may contribute to the selective vulnerability of MNs to mutant SOD1 misfolding and toxicity. Consequently, therapies aimed at enhancing intracellular MIF chaperone activity hold promise. In fact, our studies have demonstrated that overexpressing MIF in the spinal cord of newborn transgenic mutant SOD1G93A and SOD1G37R mice delayed disease onset and significantly extended their lifespan, accompanied by reduced misfolded SOD1 accumulation in the spinal cord [[43](#_ENREF_43)].

Apart from its role as a protein-folding chaperone for misfolded SOD1, MIF plays a crucial role in the immune response [[38-40](#_ENREF_38)]. Hence, to mitigate potential side effects when elevating MIF levels, it is imperative to decouple MIF's chaperone activity from its extracellular role as a cytokine. Additionally, MIF possesses tautomerase and thiol-oxidoreductase activities [[44-47](#_ENREF_44)]. Importantly, by abolishing these activities using site-directed mutagenesis, we demonstrated that they are not essential for MIF's chaperone function, [[48](#_ENREF_48)]. Lastly, MIF has been proposed to act as an endonuclease under specific circumstances [[49](#_ENREF_49), [50](#_ENREF_50)].

The MIF superfamily encompasses MIF and its recently identified homologue, D-dopachrome tautomerase (D-DT or MIF-2), which are closely located on chromosome 22q11.23 [[51](#_ENREF_51)]. MIF-2 shares tautomerase activity with MIF [[52](#_ENREF_52)] and exhibits biological similarities [[53-56](#_ENREF_53)]. Moreover, MIF-2 has shown receptor binding to CD74 [[55](#_ENREF_55)] and a potential ability to activate an inflammatory cascade downstream of CD74 [[57-60](#_ENREF_57)]. However, further investigations are needed to elucidate and compare the roles of MIF and MIF-2. Our recent studies revealed that although MIF-2 was able to inhibit the formation of SOD1 amyloid aggregates *in vitro*, it had no impact on misfolded SOD1 accumulation or toxicity in cellular models [[61](#_ENREF_61)]. Furthermore, MIF and MIF-2 expression patterns vary among tissues, suggesting distinct roles for these MIF homologues and underscoring the importance of focusing on MIF but not MIF-2 upregulation in the context of ALS in future research endeavours. Top of Form

1. **Research Objectives & Expected Significance**

**Objectives:** The primary objective of this proposal is to comprehensively investigate various facets of MIF, including its functions and properties, with the aim of understanding the mechanisms by which MIF exerts its protective capacities in the context of ALS. To address MIF's pivotal role as a modifying factor in ALS, we will focus on the following specific research objectives: 1) Augment MIF expression in various subtypes of ALS, other than SOD1, with the intent of elucidating its mechanism of action and to determine whether its function may be variable within the context of different ALS-based mutations; 2) Further expand our knowledge of MIF’s function in a human-relevant model of ALS by overexpressing MIF in MNs derived from induced pluripotent stem cells (iPSCs) sourced from both sporadic and familial ALS patients carrying distinct mutations; 3) Reveal and characterize novel MIF variants with enhanced stability and decreased immune reactivity, and to determine their effects on ALS pathology; 4) Unveil whether MIF exhibits endonuclease activity in the context of ALS pathogenesis and its potential involvement in a cellular process of cell death known as parthanatos, shown to be involved in dopaminergic neurodegeneration. These research pursuits will employ a multifaceted approach, encompassing molecular genetics, gene targeting, biochemical methodologies, and advanced imaging techniques. This comprehensive investigation will be conducted using a combination of mouse models and iPSC-derived cells, facilitating a comprehensive exploration of these four primary research aims, as elaborated below.

**Specific aims**

**Aim 1. Evaluate the impact of MIF overexpression on different subtypes of ALS.** Our prior research has demonstrated that MIF serves not only as a chaperone influencing SOD1 misfolding and aggregation, but also exerts its influence on diverse cellular pathways, including those related to inflammation, neurogenesis, and metabolism (manuscript under review). Furthermore, treatment with MIF has facilitated significant improvement in motor and neurological functions, leading to extended survival in mutant SOD1 mice. Given that diminished MIF protein levels have been observed not only in iPSC-derived MNs from SOD1-related cases but also in cases associated with C9orf72 and TDP43, as well as in the spinal cords of sporadic ALS patients, our aim here is to assess whether MIF overexpression may confer benefits in other ALS subtypes and whether MIF may demonstrate mechanistic variability across the various ALS subtypes. We aim to replicate the successful gene-targeting approach previously employed by us in SOD1 models, utilizing adeno-associated virus (AAV)-mediated delivery of MIF into the central nervous system (CNS) of various ALS mouse models, including those linked to TDP43 and Profilin1. This investigation holds the potential to determine which common pathways play a crucial role in the pathogenesis of various forms of ALS, whether MIF has a protective role not only for familial SOD1 cases but potentially for ALS cases across the spectrum, and whether its mechanism may vary depending on the ALS model.

**Aim 2.** **Determine the effect of MIF overexpression in MNs derived from iPSCs of sporadic and familial ALS patients carrying various mutations.** The recognition of MIF's significant role in slowing disease progression in mutant SOD1 mouse models of ALS has underscored the necessity to elucidate MIF's role in the context of human disease. Given the limitations and disparities between animal models and the manifestation of ALS in humans, we aim to address this gap by utilizing MNs derived from induced pluripotent stem cells (iPSCs) obtained from ALS patients carrying different mutations (in collaboration with Prof. Gad Vatine). Our objective is to investigate whether the observed low levels of MIF protein in both familial and sporadic ALS patients may signify a common underlying mechanism rendering MNs vulnerable. To this end, we seek to determine whether the overexpression of MIF can ameliorate their diseased phenotype and whether this effect involves a different mechanism of action between human and mouse models as well as different ALS subtypes. This endeavour holds the potential to further expand our comprehension of MIF's likely protective role, not only in mouse models of ALS but also in patient-derived cells, and will provide useful mechanistic insight in a more relevant human setting.

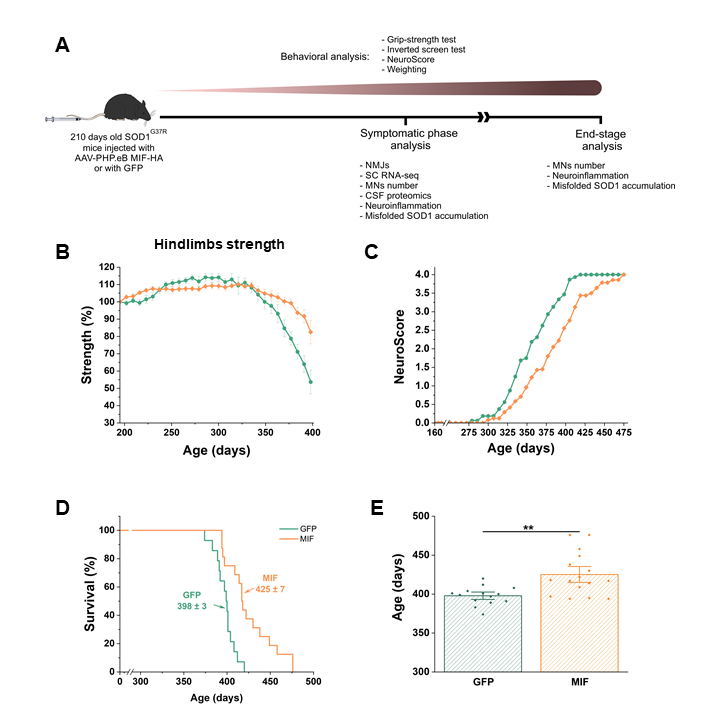
**Aim 3.** **Discover and characterize MIF variants with enhanced stability and decreased immune reactivity.** Our previous studies revealed that MIF protein level is extremely low in spinal MNs [[25](#_ENREF_25), [62](#_ENREF_62)] implicating low chaperone activity as a component of selective vulnerability of MNs to mutant SOD1 misfolding and toxicity. A clearer understanding of the cause for these low MIF levels, may be beneficial in optimizing future therapeutic approaches. MIF instability and subsequent degradation may be a contributing factor, thus utilizing directed evolution techniques such as yeast surface display (YSD) screening (in collaboration with Prof. Amir Aharoni), we aim to identify specific amino acids which contribute to the stability of the protein. In addition, apart from MIF's protective function as a chaperone for misfolded SOD1, we must take into consideration the inflammatory effects of MIF when overexpressed in a tissue for prolonged periods [[63](#_ENREF_63)]. Therefore, we will use directed evolution techniques to screen for MIF variants with reduced affinity to CD74 receptor, thus dissociating between these functions of MIF with the further aim of determining whether these functions are mutually exclusive. Ultimately, our plan involves employing a gene targeting strategy utilizing CNS-targeted AAV delivery to facilitate the expression of these novel MIF variants in mouse models of ALS. This critical step will pave the way for a better understanding of MIF’s protective mechanism of action in the context of ALS.

**Aim 4.** **Investigate the impact of MIF endonuclease activity on ALS pathogenesis.** Recently, Dawson and his research team reported that MIF plays a role in triggering cell death through an endonuclease activity that hinges on Poly (ADP-ribose) polymerase (PARP) activation. This process involves the release of Apoptosis-Inducing Factor (AIF) from mitochondria to the cytosol, the formation of an AIF-MIF complex, and its subsequent translocation to the nucleus, resulting in a distinctive form of cell death termed "parthanatos" [[50](#_ENREF_50)], shown to be a fundamental mechanism in the neurodegenerative process in mouse models of Parkinson’s disease [[49](#_ENREF_49)]. In this aim, we will use primary neurons and employ advanced biochemical and imaging techniques to ascertain whether the neurodegeneration observed in different forms of ALS, including those associated with SOD1, TDP-43, and C9orf72, involves the activation of this specific cell death pathway. This will provide crucial mechanistic insights into whether MIF acts as a protective or detrimental factor in various forms of neurodegeneration. Additionally, it will shed light on the commonalities or distinctions between the pathways implicated in different subtypes of ALS, and potentially, in other neurodegenerative diseases as well.

**Expected Significance**: The fundamental mechanisms underlying neurodegeneration in ALS remain incompletely understood, highlighting the need to uncover these mechanisms as the initial step toward developing therapeutic interventions. In light of accumulating evidence, which includes the harmful effect of misfolded SOD1, the potential risks associated with prolonged reduction of total SOD1 levels, and the multifaceted protective role of MIF—acting not only as a chaperone for misfolded SOD1 but also as an agent with broader effects impacting numerous pathways linked to neurodegeneration—we believe that modulating MIF levels holds promise for making a constructive impact. This impact may extend beyond SOD1-associated ALS cases to encompass other ALS forms and potentially even offer broader applications within the realm of neurodegenerative diseases.

**Detailed Description of the Proposed Research**

**C.1. Working hypothesis**



**Fig. 1. Peripheral delivery of AAV-PHP.eB-MIF to mutant SOD1G37R mice after disease onset improves motor function, neurological symptoms, and survival.** (**A**) Schematic representation of the experiment. AAV PHP.eB-MIF-HA or AAV PHP.eB–eGFP were delivered via tail vein injection after disease onset.  (**B**) Hindlimb strength (%) measured with a grip-strength meter. (**C**) Neuroscore measurement. (**D-E**) Survival (%) (**D**) and mean survival + SE (**E**) of SOD1G37R mice injected with AAV-PHP.eB-eGFP: green, n=16 or SOD1G37R mice injected with AAV PHP.eB-MIF: orange, n=15. Statistics were performed using multiple t-tests. \*\*p < 0.01.

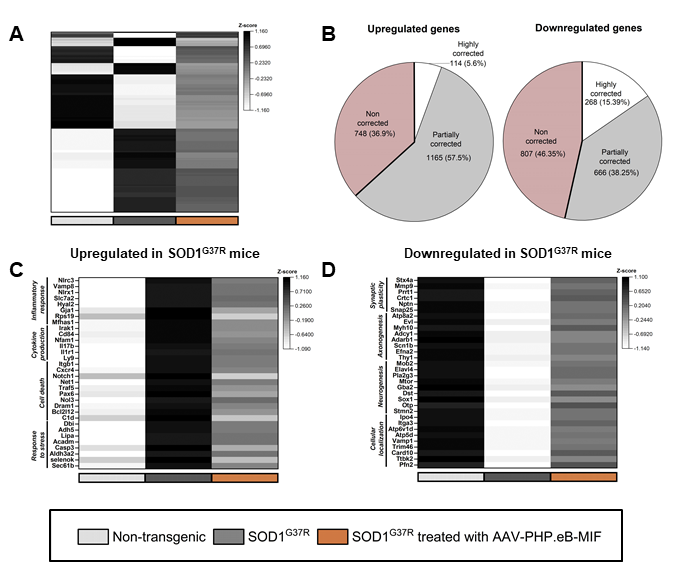
Our underlying hypothesis posits that the neurodegeneration observed in ALS may stem from a diminishment or impairment of specific chaperones, notably MIF, as supported by our previous findings. This insufficiency results in the accumulation and subsequent toxicity of misfolded proteins, initially within MNs, commencing even before the onset of the disease. As the disease advances, the buildup of misfolded proteins escalates, culminating in their association with intracellular organelles, causing organelle dysfunction. Ultimately, this cascade leads to MN demise, triggering an overactivation of glial cells, the release of toxic factors, and further MN loss, thereby hastening the progression of the disease. We believe that by suppressing the buildup of misfolded proteins and influencing various biological pathways within MNs through the elevation of MIF levels, we can potentially retard the progression of the disease. Consequently, we postulate that modulating MIF levels may provide substantial benefit not only to SOD1-related ALS cases but also to other ALS forms and neurodegeneration in general.

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**C.2. Experimental Design & Methods, Preliminary Results and Expected Outcomes**

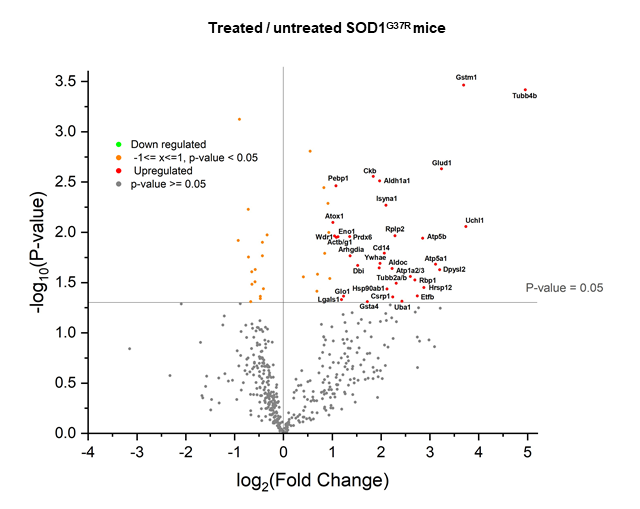
**Aim 1. A gene targeting approach to evaluate the impact of MIF overexpression across different subtypes of ALS.** We have previously demonstrated that MIF levels are extremely low in spinal MNs and that overexpressing MIF in mutant SOD1 neuronal cultures or primary MNs extends cell survival [[25](#_ENREF_25), [62](#_ENREF_62)]. Moreover, we have shown that the injection of AAV9 carrying MIF into the spinal cord of newborn mutant SOD1G93A and SOD1G37R mice reduced the degree of misfolded SOD1 accumulation, delayed disease onset, and extended survival [[43](#_ENREF_43)]. Importantly, the Gradinaru group has recently taken a step forward and elegantly developed novel engineered AAV capsids (AAV-PHP.eB) for efficient noninvasive gene delivery to the central and peripheral nervous systems [[64](#_ENREF_64)]. AAV-PHP.eB vectors were shown not only to easily cross the BBB following intravenous injection, but to express their targets at levels ~40-fold higher than those for normal AAV9 [[64](#_ENREF_64)]. We thus exploited this novel capsid to determine the effect of inhibiting misfolded SOD1 accumulation starting at a symptomatic point. Specifically, we aimed to determine whether reducing misfolded SOD1 within the CNS can slow disease progression following peripheral administration of AAV-PHP.eB to drive MIF expression in a mouse model of ALS. Cohorts of mutant SOD1G37R mice were injected via the tail vein with the AAV-PHP.eB vector carrying MIF at 210 days of age (disease onset). Control cohorts were injected with an AAV-PHP.eB-GFP virus, and both experimental groups were followed and analyzed (**Fig. 1A**). Mutant SOD1G37R mice injected with MIF maintained their hindlimb (**Fig. 1B**) and total limb strength longer than the GFP-injected group, who started to lose their hindlimb strength around 330 days of age. Moreover, the inverted screen test revealed that the MIF-treated mice were able to suspend longer than the GFP-injected mice from 350 days of age. NeuroScore of the mice shows a slower disease progression in the MIF treated mice (**Fig. 1C**). Finally, mutant SOD1G37R mice treated with MIF showed an extended survival of about four weeks compared to the GFP-injected group (**Fig. 1D*,* E**). For the first time, we performed RNA sequencing of lumbar spinal cord samples from these MIF-treated SOD1G37R mice and compared them to their untreated littermates. A total of 3,856 genes were significantly differentially expressed between the non-transgenic (NT) and SOD1G37R mice, of which 2,115 were upregulated and 1,741 were downregulated (**Fig. 2A**). MIF treatment was able to at least partially correct 63.1% of the upregulated and 53.6% of the downregulated genes in SOD1G37R mice, as indicated by gene hierarchical clustering (**Fig. 2B**). Gene ontology (GO) enrichment analyses performed using ShinyGO [[65](#_ENREF_65)] indicated that MIF overexpression reverted the upregulation of genes related to the inflammatory response, cytokine production, cell death, phagocytosis, gliogenesis, response to oxidative stress, and lipid oxidation (**Fig. 2C**), while MIF treatment reverted the downregualtion of genes including genes related to nervous system development, axonogenesis, neurogenesis, synaptic signaling, neurotransmitter transport, learning and memory (**Fig. 2D**). Notably, although MIF was mainly overexpressed in MNs, this treatment affected the expression of genes that were previously reported to be related to other cell types in single-cell RNA sequencing [[66](#_ENREF_66)], including astrocytes and microglia, which play essential roles in driving disease progression in the late phase [[67](#_ENREF_67), [68](#_ENREF_68)].

Proteomic analysis of the CSF profile of SOD1G37R mice further revealed the upregulation of inflammatory proteins and neurofilaments, which is a known hallmark of neurodegeneration. These mice also exhibited the downregulation of metabolic and CNS development-related proteins. Supporting these findings, are several studies suggesting defects in metabolic pathways as a mechanism for neurodegeneration in general, and ALS in particular, due to the high energy demands of the nervous system [[69](#_ENREF_69), [70](#_ENREF_70)]. MIF treatment was able to upregulate a portion of these neurogenesis and metabolic pathway-related proteins (**Fig. 3**). Notably, many metabolic-related upregulated proteins in the mouse CSF were highly correlated with MIF protein expression in the Answer-ALS iPSC-derived MN database, both in the whole dataset and specifically in the SOD1 cases (manuscript under review), suggesting MIF involvement in these metabolic pathways in cells derived from ALS patients as well, and implying its beneficial potential.



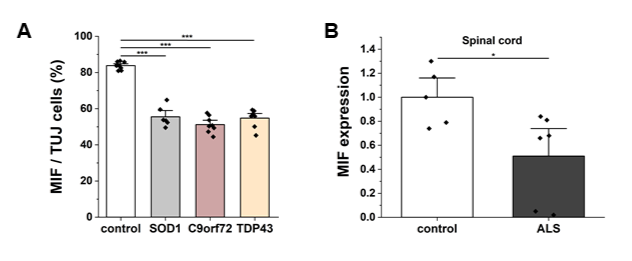
**Fig. 2. Overexpression of MIF in the nervous system partially corrects the expression of several genes that were altered in symptomatic mutant SOD1G37R mice. (A)** Heatmap of all the genes that were significantly altered in SOD1G37R (grey) compared to non-transgenic (NT) mice (white) and were at least partially corrected in the MIF-treated mice (orange). (**B**) Pie charts of all the upregulated or downregulated genes in the spinal cord of the mutant SOD1G37R compared to NT mice, representing the portion of highly corrected, partially corrected, and non-corrected genes in AAV-PHP.eB-MIF treated compared to untreated SOD1G37R mice. (**C-D**) Heatmaps of the expression of representative genes that were significantly upregulated (**C**) or downregulated (**D**) in SOD1G37R mice compared to NT, ordered according to their gene ontology (GO) pathways. Heatmaps were generated using gene expression Z-scores.

In summary, our recent findings shed light on the diverse roles of MIF in the CNS in general and its importance in MNs specifically, when administered in a therapeutically relevant manner. MIF not only functions as a SOD1 chaperone that modulates SOD1 misfolding and aggregation, but also affects diverse pathways, including the inflammatory response, neurogenesis, and metabolism, thus resulting in better motor and neurological functions leading to extended survival. These findings, together with the fact that low MIF protein levels were found in MNs derived from iPSCs of SOD1, C9orf72, and TDP43 cases (**Fig.** 4**A**) and in the spinal cord of the sporadic patients (**Fig.** 4**B**), suggest a therapeutic potential for MIF not only for SOD1 ALS but for ALS cases in general. Thus, in this Aim we will determine whether the overexpression of MIF in the CNS affects disease course in different subtypes of ALS using mouse models and a gene targeting approach as used above. Specifically, after disease onset, we will inject AAV-PHP.eB-MIF-HA via the tail vein into two different mouse models: hPFN1G118V [[71](#_ENREF_71)] and TDP-43Q331K [[72](#_ENREF_72)],. Following AAV-PHP.eB-MIF-HA or AAV-PHP.eB-GFP injection, we will monitor these mice through weight, NeuroScore, and grip strength measurements until they reach end-stage disease. After sacrificing these animals, we will examine MN numbers, neuroinflammation, neuromuscular junctions, and TDP-43 localization. In addition, we will isolate RNA from the lumbar spinal cord and CSF of symptomatic mice to perform RNA-seq and CSF proteomic analyses, respectively, comparing MIF-treated and untreated mice as we have done before with mutant SOD1 mice (**Figs. 2**and **3**). These experiments will provide us with important information regarding the pathways corrected or unaffected by MIF treatment in these different models.



**Fig. 3. Upregulation of MIF in the nervous system partially corrects the expression of altered proteins in the CSF of symptomatic mutant SOD1G37R mice.** Volcano plot of proteomics data. The –log (P-value) plotted against the log2 of the fold change of proteins in the CSF of AAV-PHP.eB-MIF treated compared to untreated SOD1G37R mice.

The AAV approach is a promising gene delivery modality. The small DNA genome of AAV can be replaced with a gene of interest that is packaged into replication-defective viral particles. In essence, the AAV-coated proteins provide an efficient mechanism for intracellular delivery of a gene of interest. Importantly, AAV vectors do not require integration for gene expression. This feature has made recombinant AAVs attractive as a therapeutic delivery tool in the nervous system, especially since wild-type AAV is known to be non-pathogenic and viral genes can be successfully removed and replaced [[73](#_ENREF_73)]. In addition to being efficient, AAV-mediated expression is also stable. For example, recombinant AAV expression has been shown to be sustained for over 3.5 years in the primate brain and up to 19 months in the rat brain with no obvious side effects [[74](#_ENREF_74)]. Encouragingly, the FDA has already approved AAV gene therapytreatment for inherited retinal disease (IRD), spinal muscular atrophy (SMA), hemophilia B, and Duchenne muscular dystrophy; and many other AAV clinical trials are currently underway, including trials for neurodegenerative diseases.

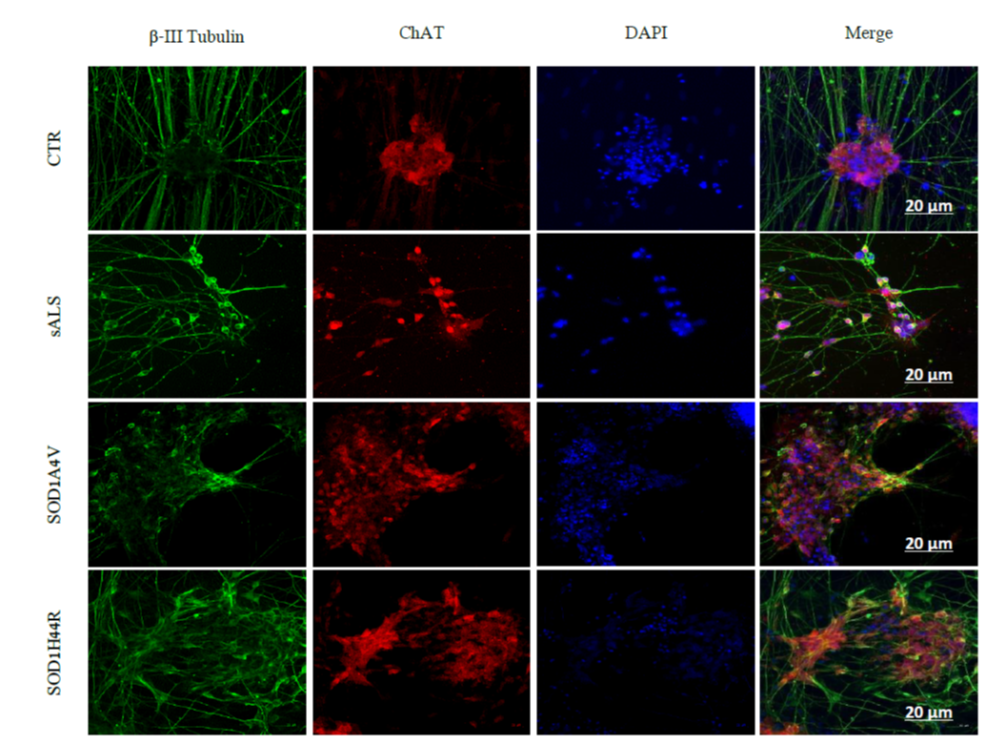


**Fig. 4. Reduced MIF protein expression in ALS patient-derived motor neurons and human ALS post mortem tissue. (A)** MIF expression in human iPSC-derived MNs from control, SOD1, C9orf71, and TDP-43 patients (**B**) MIF expression normalized to α-Tubulin in human post-mortem tissue samples from spinal cord (n=4-7) of sporadic ALS patients and healthy controls determined by immunoblot (unpaired two-tailed t-Test, \*p < 0,05).

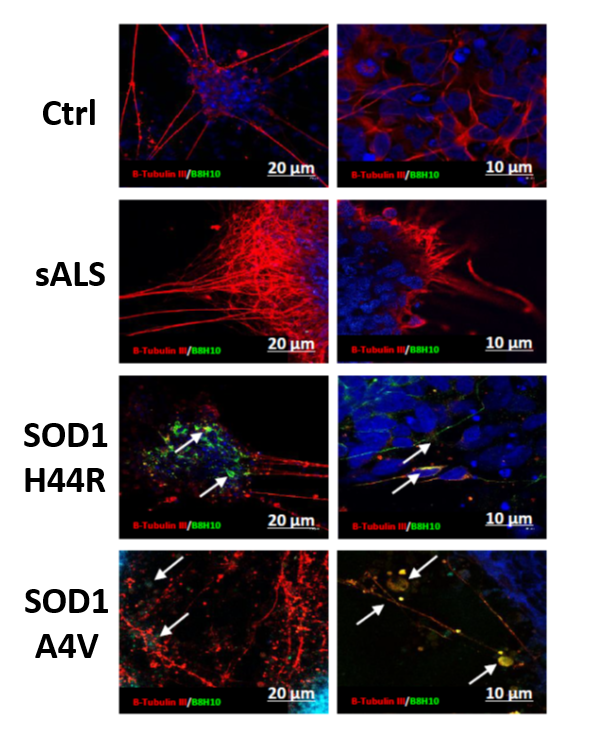
***Expected outcome and pitfalls:***

Even if MIF is not able to rescue the toxicity of any other form of ALS except mutant SOD1 disease, the experiments proposed herein will provide us with important mechanistic insight and useful information regarding the mechanisms through which the chaperone-like activity and general functions of MIF impact ALS pathogenesis. In addition, they will provide us with critical information about the common or differing pathways involved in different subtypes of ALS that will likely be used for the future development of therapeutics for this devastating disease.

**Aim 2. Determine the effect of MIF overexpression in MNs derived from iPSCs of sporadic and familial ALS patients carrying various mutations.** Revealing the important role of MIF in slowing ALS disease progression in SOD1 mouse models of ALS has resulted in a need to understand the role of MIF in human disease, given the limitations of animal models and their differences from human disease manifestation. Importantly, we also observed low MIF protein levels in iPSC MNs derived from SOD1, TDP43, and C9orf72 ALS cases (**Fig. 5A**). Additionally, we found that sporadic ALS patients, who have been reported to accumulate misfolded SOD1 according to several groups [[75-80](#_ENREF_75)], have lower MIF protein levels in the spinal cord (**Fig. 5B**). These low MIF protein levels found in familial and sporadic ALS patients may thus represent a common mechanism of MN vulnerability. We therefore aim to determine whether the upregulation of MIF is sufficient to revert disease phenotypes in a human-related model. To this end, we have begun modeling and studying ALS through the innovative utilization of patient-derived iPSCs in close collaboration with Prof. Gad Vatine (BGU). These iPSCs provide a unique platform that allows us to recapitulate the complex disease processes in a controlled laboratory setting [[81](#_ENREF_81)]. In iPSC-derived MNs from SOD1 ALS patients, studies have shown higher levels of misfolded or aggregated SOD1 in the cytosol displaying phenotypic toxicity and insolubility [[82-84](#_ENREF_82)], cellular degeneration, and impaired neural viability of cultured MNs besides morphological alterations of affected MNs[[82](#_ENREF_82), [85-87](#_ENREF_85)], decreased neurite length, and an overall decrease in the number of neurites per neuron consistent with degeneration and loss of connectivity [[82](#_ENREF_82), [85](#_ENREF_85), [86](#_ENREF_86)]. In addition, electrophysiological activity is the main functional output for neural cells. Thus, investigating the electrophysiological dysfunction in ALS cases is crucial and can provide data on the functionality of the neural systems (mainly MNs) in fALS and sALS. Clinically, one of the features of ALS is MN hyperexcitability, specifically at early stages of neurodegeneration in both fALS and sALS cases[[88-91](#_ENREF_88)]. While many studies conducted using transgenic mice with different ALS-related mutations show similar early-stage hyperexcitability [[92-97](#_ENREF_92)], other studies using different unrelated mouse models of ALS have reported neuronal hypo-excitability [[98](#_ENREF_98)]. Recordings from iPSC-derived MNs from ALS patients have similarly displayed neural hyperexcitability in some cases [[99](#_ENREF_99), [100](#_ENREF_100)], but hypoexcitable neurons in others [[87](#_ENREF_87)]. More recent studies recording iPSC-derived MNs at different stages of neural maturation *in vitro* have reported hyperexcitability to be an early-stage phenotype that later transitions into hypoexcitability, suggesting that these neural function phenotypes could be indicative of the disease progression and MN degeneration from a very early stage of neural development [[81](#_ENREF_81), [87](#_ENREF_87), [98-100](#_ENREF_98)]. This suggests that modeling ALS by differentiating patient iPSCs could be a very effective means of studying various aspects of this disease including molecular features, cell functionality and morphology, cell viability, and even neural connectivity. In this Aim, we plan to focus on studying distinct patient lines of iPSCs, with each line contributing to the understanding of a wide range of ALS cases. Working with a diverse set of patients will help us shed light on the mechanisms that may drive MN degeneration. We have so far successfully managed to differentiate cells from four lines (healthy control, sporadic ALS, SOD1A4V, and SOD1H44R) and we have characterized their neural and motor profiles. All lines showed positive staining for beta-III tubulin and ChAT (**Fig. 5**). In addition, all lines have been recorded for functional characterization and showed spontaneous activity as expected after the determination of their neural fate. Further experiments have shown that SOD1 mutant lines, but not the sporadic or healthy controls, show positive staining for misfolded SOD1 using the B8H10 antibody (**Fig. 6**). As for the functional phenotype, so far, we have managed to show a significant pattern of hyperexcitability at early neural development in SOD1A4V MNs. All experiments were performed following sequencing for both SOD1 mutations mentioned above.



**Fig. 5.** Immunocytochemistry staining representative images of differentiated MNs of all cell lines stained with b-Tubulin (green), ChAT (red) and DAPI (blue). CTR, sALS, familial ALS patient with A4V SOD1 mutation (SOD1A4V), and familial ALS patient with H44R SOD1 mutation (SOD1H44R), Scale bar: 20μm.

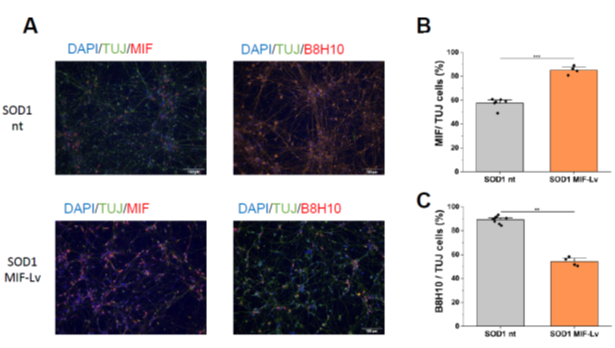


**Fig. 6.** Immunocytochemistry staining representative images of differentiated MNs for all cell lines stained with anti-β-Tubulin (red) and B8H10 (green). Scale bars: 10, 20μm.

In our proposed study we plan to increase MIF levels in MNs derived from different subtypes of ALS: SOD1, TDP43, C9orf72, and sporadic (we have kindly received the new lines from Dr. Michael Ward (iNDI initiative) with the aim of examining its effect on the ALS iPSCs MNs. In our preliminary results, we have shown that MIF overexpression using a lentiviral vector resulted in the reduction of misfolded SOD1 levels in iPSC-derived MNs from SOD1 patients (**Fig. 7**). Furthermore, we will determine whether MIF overexpression affects MN viability and MN morphology, including neurite length and number of neurites per neuron. Finally, we will use a microelectrode array (MEA) to measure the hyperexcitability pattern in untreated ALS MNs compared to MIF-overexpressing MNs. Ultimately, we aim to advance the understanding of ALS pathogenesis at the cellular, molecular, and functional levels, contributing valuable knowledge to the field of ALS research in general and more specifically the role of MIF in disease pathogenesis.

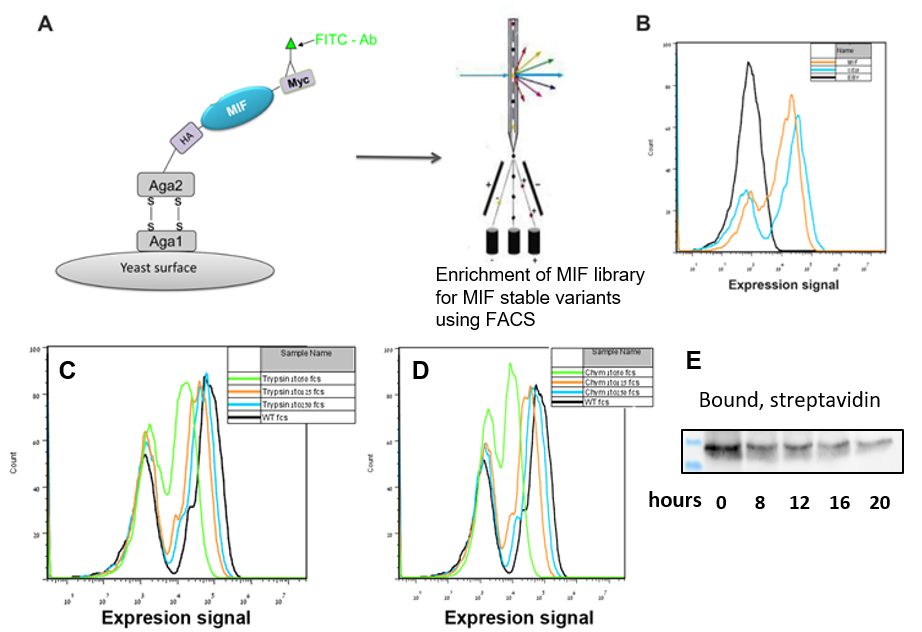
***Expected outcome and pitfalls:*** Although our model offers a promising avenue for research, it also comes with several limitations that are important to address. Firstly, since this is an isolated *in vitro* system, we cannot rule out the involvement of other cell types in disease pathogenesis. Secondly, disease heterogeneity poses a major challenge that iPSC models may not be able to fully capture. Finally, one of the greatest challenges in the field of disease modeling using iPSCs-derived cells, specifically with respect to late-onset diseases, is that iPSCs exhibit an embryonic rather than a senescent state, which makes it harder to replicate the clinical phenotypes of these diseases *in vitro*. To at least partially overcome these challenges, we will repeat our experiments using different lines and using isogenic controls for the familial forms of ALS. One of the major advantages of using the INDI cell lines (provided by Michael Ward) is that they have common isogenic controls, reducing dramatically the variability.

**Aim 3. Discover and characterize MIF variants with enhanced stability and decreased immune reactivity.** We have shown that MIF protein levels are extremely low in spinal MNs, [[25](#_ENREF_25), [62](#_ENREF_62)] implicating low chaperone activity as a component of the selective vulnerability of MNs in ALS. Deciphering the cause of these selectively low MIF levels in ALS-associated tissues may prove fundamental to our understanding of the role of MIF in ALS pathogenesis. One possible explanation for low MIF levels may be its short lifespan as a result of degradation. Thus, our first aim will be to identify variants with higher stability. The primary protein engineering approach that we will utilize for the generation of improved MIF variants is directed evolution. This methodology is based on the generation of large mutant libraries followed by powerful screening/selection assays to isolate proteins with desired properties [[101-103](#_ENREF_101)]. In this Aim, we will use a screening approach based on yeast surface display (YSD) of a large MIF library (in collaboration with Prof. Amir Aharoni), followed by the fluorescence-activated cell sorting (FACS) of mutants with enhanced stability (**Fig. 8A**). YSD is a powerful method for engineering proteins with increased affinity, specificity, and stability [[104](#_ENREF_104)] and it has been previously utilized by the Aharoni group for the display and engineering of different proteins, including PCNA, glucose oxidase, and IL-17 receptor [[105-107](#_ENREF_105)]. Our preliminary results using this system have achieved the optimization of YSD conditions to enable the display of MIF on the yeast cell surface at high levels (**Fig. 8B**). Following MIF library YSD, we will perform 3-5 rounds of library cell sorting by FACS to enrich MIF mutants with enhanced stability. These engineered MIF variants will then be characterized using biochemical and cell-based assays to assess MIF’s stability, inhibition of SOD1 misfolding, and rescue of MN cell death.



**Fig. 7. Lentiviral transduction of mutant SOD1 iPSC-derived MNs with MIF reduces SOD1 misfolding. (A)** ICC-staining for MIF and misfolded SOD1 before and after transduction of iPSC-derived mutant SOD1 MNs. **(B,C)** Quantitative analysis of MIF and misfolded SOD1 protein expression in IPSC-derived MNs. MIF expression **(B)** and misfolded SOD1 accumulation (**C**) after MIF transduction in relation to TUJ positive cells. Scale bars: 100 µm

**Generation of MIF mutant libraries:** DNA libraries based on the existing human MIF will be generated. We have already begun generating these MIF libraries using site saturation mutagenesis (SSM). Taking advantage of the small size of the MIF protein (115 amino acids) and the structure of its C-terminus, which seems to contribute less to overall MIF stability, we will mutate residues 1 – 99, while leaving the functional cysteine residues at positions 57, 60 and 81 intact. This library will allow us to systematically explore the effect of each mutation in residues 1-99 of MIF on the stability of the protein. Following library screening and the isolation of mutants with enhanced stability (see below), we will be able to combine beneficial mutations and generate a second comprehensive library with a high potential to dramatically improve MIF stability relative to the wild-type (WT) protein.



**Fig. 8. A.** Scheme describing the YSD approach. MIF is displayed on the yeast surface by fusion to Aga2 through its N-terminus and to a Myc tag on the C-terminus. MIF display levels are monitored using fluorescently labeled anti-Myc antibodies. The level of Myc display and the screening for MIF mutants with enhanced stability is performed by FACS. **B**. Histogram showing that MIF is displayed at a high level using the YSD approach. The display level of MIF (orange) was compared to the display levels of CD20 (blue) that is a known ScFv previously shown to display using the YSD approach. EBY100 (black) are yeast cells not displaying any tagged protein of interest. **C, D.** WT MIF was incubated with different concentrations of trypsin (**C**) or chymotrypsin (**D**). A stock solution of 100 µM trypsin or 40 µM chymotrypsin was diluted 1:250 (blue), 1:125 (orange), 1:50 (green), or no protease (black) for 5 min prior to labeling with α-myc-FITC antibody to assess the level of MIF that was un-cleaved on the yeast surface. **E**. Immunoblot showing MIF degradation as a function of time measured by the SPAAC pulse-chase assay.

**Screening of MIF mutant libraries:** To enable the screening of large MIF libraries for the isolation of variants with improved stability, we first established a protease stability assay for MIF. This assay enables the isolation of MIF variants with improved resistance to protease digestion. The assay is based on the incubation of yeast displaying MIF variants with different trypsin (**Fig. 8C**) and chymotrypsin (**Fig. 8D**) concentrations for a short duration. Following incubation, digested MIF will lose its C-terminal Myc tag and yeast cells will exhibit reduced a-Myc fluorescence intensity. Thus, increased protease concentrations lead to a decrease in the fluorescence signal obtained following labeling with the a-Myc antibody. We will utilize this assay to screen large MIF libraries for variants with increased stability and resistance to protease digestion.

**Characterization of improved MIF mutants:** To characterize the improved MIF mutants, we will estimate the half-life of these MIF variants compared to the WT form of MIF using the novel SPAAC pulse-chase method [[108](#_ENREF_108)]. Compared to other commonly used methods, this new technique overcomes several limitations. First, the SPAAC pulse-chase protocol is non-radioactive such that it minimizes DNA and cellular damage, and does not require the use of biohazardous radioisotopes or the need for specific permits and equipment. In addition, this non-toxic method does not require treatment with cycloheximide, a highly cytotoxic reagent that inhibits protein synthesis and may negatively influence cellular proteostasis. The SPAAC protocol allows for the live-labeling of newly synthesized proteins, with a non-toxic component conjugated to a reactive azide moiety. The labeled proteins are then reacted with dibenzocyclooctynol (DIBO) conjugated to biotin, and detected by using a streptavidin-HRP probe. We have already established the protocol in our group using SH-SY5Y cells transiently transfected to express MIFWT where MIF degradation can be clearly observed as a function of time (**Fig. 8E**). We propose to then use this assay to compare the half-life of MIFWT with the novel stable variants. In order to examine a possible superior functionality of these variants, we will determine their ability to inhibit SOD1 misfolding and the formation of amyloid aggregates. To this end, we will sequence, express, and purify the novel mutants using fast protein liquid chromatography (FPLC). Subsequently, we will use the ThT assay, as we have described previously, to examine amyloid aggregation [[48](#_ENREF_48), [61](#_ENREF_61), [109](#_ENREF_109)]. Furthermore, we will overexpress different MIF variants in SH-SY5Y or NSC-34 cells together with plasmids encoding SOD1 mutants and determine the levels of misfolded SOD1 accumulation by immunoprecipitation with the B8H10 antibody as well as cell viability using the XTT assay. These assays are routinely performed in our laboratory as described previously [[4](#_ENREF_4), [25](#_ENREF_25), [28](#_ENREF_28), [48](#_ENREF_48), [61](#_ENREF_61), [62](#_ENREF_62), [109](#_ENREF_109)]. The identification of those residues affecting MIF stability will provide mechanistic insight into the causes of low MIF levels in MNs while also providing us with target sites for improving MIF stability that may aid the future development of a MIF-based therapeutic approach.

In addition to its protective function as a chaperone for misfolded SOD1, we cannot ignore the inflammatory effects of MIF when overexpressed in a tissue for extended periods [[63](#_ENREF_63)]. Therefore, we will use directed evolution techniques to screen for MIF variants with reduced affinity for the CD74 receptor, with the goal of dissociating these two MIF functions. To this end, DNA libraries based on the existing human MIF will be generated. An effective approach for generating gene libraries highly enriched in mutants with reduced CD74 affinity entails the targeted mutagenesis of residues that deviate from the consensus sequence of the gene family [[107](#_ENREF_107), [110-112](#_ENREF_110)]. We will identify these residues based on phylogeny and MIF structure and will generate a MIF library via the partial mutagenesis of the targeted positions according to our previously reported protocols [[107](#_ENREF_107), [110-112](#_ENREF_110)]. Then, the screening will be performed in a similar manner as described above but we will screen for variants with lower CD74 affinity. After performing 3-4 rounds of FACS for the enrichment of these variants, we will combine the single mutants to identify the variant with the lowest affinity for CD74. Finally, these mutants will be characterized for MIF cytokine function. MIF binds to the extracellular domain of CD74, activating a signal transduction pathway that is followed by CD44 activation, which induces MIF-mediated phosphorylation of the ERK1/ERK2 signaling pathway [[113](#_ENREF_113)]. Accordingly, we aim to compare the CD74 binding affinity of the novel variants to that of MIFWT by testing MIF-mediated ERK activation. To do so, we will treat cells with MIF, collect cell lysates, and check for ERK1/2 phosphorylation by immunoblotting using phospho-specific anti-ERK antibodies as described previously [[114-116](#_ENREF_114)]. Lower ERK phosphorylation levels will indicate less activation of the pathway and therefore reduced CD74 binding affinity. Next, we will determine the chaperone function of these novel variants as described above.Once we have characterized both the stable variants and the mutants with reduced affinity for CD74, we will test our novel MIF variants both in cellular models of ALS and subsequently in a SOD1 mouse model of ALS using AAV-PHP.eB as we have recently done (**Fig. 1**). Cohorts of animals carrying a mutant SOD1G37R transgene (already in our colony) will receive tail vein injections of AAV-PHP.eB virions carrying the MIF variants at seven months of age. Control cohorts will be injected with AAV-PHP.eB-GFP virus, and the experimental groups will be followed and analyzed for behavior, disease onset and progression, survival, pathology, and viral distribution. The treated mice will be monitored for disease progression through weekly body weight and hind limb grip strength measurements. Survival curves for these groups will be compared to establish any effect on disease progression and survival (end-stage is defined by the inability of mice to right themselves 20 seconds after being placed on their sides). We will also test whether the administration of AAV-PHP.eB together with novel MIF variants inhibits the accumulation of misfolded SOD1 and neuroinflammation as we have previously shown. Additionally, MN loss will be quantified.

***Expected outcome and pitfalls:*** These experiments will provide us with important insights into how MIF’s stability affects MN death in the context of ALS. Even if the MIF variants with enhanced stability or lower affinity for CD74 are not able to delay disease progression and extend survival or are unable to further improve its protective effect as compared to MIFWT, we will still have a clearer understanding of MIF’s mechanism of action as a protective factor in ALS. For instance, if the MIF variant lacking CD74 binding is not as protective as MIFWT in the mouse model of ALS, it will confirm that the binding to CD74 plays a crucial role in MIF’s protective mode of function. In contrast, if the new MIF variants exert a protective effect similar to that of MIFWT, this will suggest that CD74 binding is not crucial for this protective activity.

**Aim 4. Investigate the impact of MIF endonuclease activity on ALS pathogenesis.** Recently, Dawson and colleagues reported that the neurodegeneration observed in mouse models of Parkinson’s disease occurs via a PARP-dependent cell death pathway (parthanatos), which involves AIF release from the mitochondria to the cytosol, AIF interaction with MIF, and translocation of the AIF-MIF complex to the nucleus followed by MIF nuclease activity [[49](#_ENREF_49)]. Moreover, parthanatos was suggested to be involved in the neurodegeneration observed in traumatic brain injury [[117](#_ENREF_117)], spinal cord injury [[118](#_ENREF_118)], or ceramide-induced reactive oxygen species levels [[119](#_ENREF_119)]. Parthanatos is a unique, caspase-independent cell death pathway distinct from apoptosis, necrosis, or other identified forms of cell death. Toxic stimuli that induce parthanatos activate nuclear PARP-1 excessively, causing it to synthesize a vast amount of PAR polymer. PAR polymer reaches a toxic level and translocates into the cytosol, where it constitutes a death signal through which mitochondrially localized AIF is forced out of its normal location into the cytosol and finally into the nucleus [[49](#_ENREF_49)]. In this Aim, we will determine whether the neurodegeneration observed in different forms of ALS also involves this cell death pathway. To this end, we will use SH-SY5Y neuronal cells and primary neurons. First, the endogenous expression and localization of AIF and MIF will be examined in control SH-SY5Y cells and SH-SY5Y MIF KO cells (kindly provided by the Dawson lab) by immunocytochemistry or by using a density gradient separation of intracellular organelles followed by immunoblotting. Then, the effect of MNNG, a carcinogenic substance that causes cellular stress (used as a positive control), will be tested. MNNG has been shown to drive the translocation of AIF and MIF in the context of cell death [[49](#_ENREF_49)]. Once the system is well established, we will transfect cells with plasmids encoding different ALS mutants (SOD1, TDP43, FUS, C9orf72). The transfected cells will then be tested for AIF and MIF localization and interaction using immunoblotting, immunocytochemistry, and immunoprecipitation approaches. As a negative control, the SH-SY5Y MIF KO cells will be used and co-transfection of MIF in the MIF KO cells will be performed to ultimately determine the role of MIF in this pathway. Finally, we will utilize mouse cortical neurons and MNs to determine the role of different ALS mutants in this cell death pathway. This will be achieved by infecting primary neurons with different mutants cloned into AAV expression vectors as we have previously reported [[43](#_ENREF_43)]. Then, the localization of AIF and MIF will be determined by immunocytochemistry. If we observe a MIF-dependent cell death pathway in one or more forms of ALS, we will then determine if this pathway is also relevant in a human disease context using iPSC lines from patients carrying different ALS mutations as described above in Aim 2.

***Expected outcome and pitfalls:*** These results will provide us with crucial insight into MIF’s role as a protective or detrimental factor within different contexts of neurodegeneration. Moreover, it will extend our knowledge regarding the common or distinct pathways involved in different subtypes of ALS in particular and other neurodegenerative diseases in general. Separating intracellular fractions is a delicate process that is not always successful even if the protocol is carried out with great precision, and this has the potential to impact the accuracy of the results. Thus, a combination of different experimental methods will be employed in order to ensure reliability. In addition, neuronal cell lines are not always the most precise model to investigate neurodegenerative phenotypes. Therefore, we propose to extend our study to primary cortical neurons and MNs in order to refine the quality of these results. Finally, the use of MNs derived from the iPSCs of ALS patients will give us a more reliable answer regarding the relevance of our results in a human context.

**C.4. Conditions available for the research.** The personnel involved in this project will include the principal investigator, four Ph.D. students and a technician. My laboratory is fully equipped, and has access to all equipment required for the proposed study of ALS pathogenesis including the described biochemical, molecular biology, imaging, and animal experiments. The laboratory is equipped with an FPLC system, real-time PCR machine, cryostat, low-speed and ultra-centrifuges, imaging systems including fluorescence and confocal microscopes, an ECL imaging apparatus, tissue culture room, incubators, and equipment for animal behavioral tests (Rotarod, hind limb grip strength). Institutional services at BGU include a new state-of-the-art animal facility equipped with animal surgical equipment, DNA sequencing unit, mass spectroscopy facility, and flow cytometers.

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