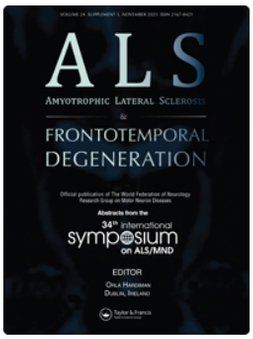

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THEME 04 *IN VIVO* EXPERIMENTAL MODELS

IVV-01 AIT-101 improves functional deficits in a human TDP-43 animal model of ALS

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Background: PIKfyve is a lipid kinase that catalyzes the phosphorylation of phosphatidylinositol-3-phosphate at the 5-position to produce phosphatidyl-inositol-(3,5)-bisphosphate. Inhibition activates the transcription factor TFEB, which, in turn, upregulates the autophagosomal/lysosomal clearance of toxic protein aggregates (1–4). AIT-101 (INN: Apilimod, aka LAM-002A) is a first-in-class, potent and highly selective inhibitor of PIKfyve that has been safely administered to over 750 human subjects. TDP-43 is an RNA binding protein that functions in the nucleus to regulate pre-mRNA splicing but is mis-localized to the cytoplasm where it forms aggregates in >90% of ALS patients. Experiments in patient-derived, induced motor neurons from both sporadic and familial ALS subjects have shown an improvement in survival in response to stress in the presence of AIT-101 and a concomitant correction in the nuclear:cytoplasmic ratio of TDP-43 (5,6).
Objective: To test AIT-101 for its ability to slow or halt functional deficits similar to ALS in a transgenic mouse model inducibly expressing human TDP-43 lacking a nuclear localization signal.
Methods: 24 rNLS8 (7) mice, in which human TDP43ΔNLS is induced in neurons by removal of doxycycline, were maintained on a doxycycline diet, and at ~10 weeks-of-age, 12 mice per group were treated with 60mg/kg AIT-101 or vehicle b.i.d. by oral gavage. After 3 days, doxycycline was removed and after 1 week reintroduced at an intermittent low dose to extend the timing of clinical deterioration. The effects of AIT-101 on body weight were documented daily, various functional endpoints were assessed 3 times per week, and, after 24 days, animals were sacrificed, and tissues and fluids assessed for biomarkers.
Results: AIT-101 treatment of rNLS8 mice led to significant improvements in body weight ($p < 0.0001$ two-way ANOVA interaction term), clasping ($p = 0.001$), hindlimb paralysis ($p = 0.015$) and grill agility ($p = 0.001$) at Week 3, and a composite score (clasping, hindlimb paralysis, grill agility, tremor and overall wellbeing) after Day 6 ($p \leq 0.048$) and after Weeks 2 and 3 ($p \leq 0.01$). There were also significant decreases in CSF ($p < 0.00001$) and plasma ($p = 0.03$) levels of neurofilament light (NfL). Quantitative IHC staining for hTDP-43 and GFAP, a marker of neuroinflammation, showed significant decreases with AIT-101 in several areas of the cortex and corticospinal tract.
Discussion: These data show that AIT-101 reduces functional deficits in a TDP-43 mouse model with accompanying decreases in plasma and CSF NfL, TDP43 aggregates and neuroinflammation.

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IVV-02 Cytoplasmic interactions of TDP with core paraspeckle proteins are enriched in ALS vulnerable brain regions in the mouse

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Background: Paraspeckles are membraneless organelles found within the cell nucleus, and often increase in response to cellular stress. They have been identified in the spinal motor neurons of sporadic ALS patients (1), and a number of paraspeckle proteins have been linked to ALS and FTD (frontotemporal dementia). TDP-43, a core pathogenic protein in around 97% of ALS cases, is known to associate with key paraspeckle proteins, and a loss of TDP-43 is sufficient to stimulate paraspeckle formation (2). It has hence been hypothesized that aberrant paraspeckle protein function may play a role in ALS.
Objectives: To investigate potential differences in the interaction of TDP-43 with selected paraspeckle proteins in disease vulnerable (cortex) versus disease resistant (cerebellum) brain regions in healthy control mice.
Methods: Cortical and cerebellar brain sections (30μm) from 6-month-old C57Bl6/J mice ($n = 3$) were assessed for TDP-43 and paraspeckle protein (PSPC1, SFPQ, NONO, FUS) interactions using proximity ligation assays. Relative expression levels and localisation of each protein in the cortex and

cerebellum were assessed using western blot analysis and immunohistochemistry.
Results: A marked interaction of TDP-43 with all four paraspeckle proteins was identified in the nucleus and cytoplasm of large neurons in layer V of the motor cortex, and the Purkinje and granular layer neurons of the cerebellum. PSPC1, SFPQ and NONO displayed significantly increased cytoplasmic interactions with TDP-43 in the motor cortex compared to the cerebellum ($p < 0.05$), coupled with a significantly larger 'spot' size ($p < 0.05$). Similar findings were obtained with FUS and TDP-43 interactions, although differences were only observed between cerebellar Purkinje cells and motor cortical neurons, and increased spot size did not reach significance. In general, SFPQ and NONO expression were increased in the cerebellum relative to the cortex, driven at least in part by relative increases in cytoplasmic protein levels ($p < 0.05$). A similar increase in TDP-43 was also observed in the cerebellum, again driven primarily by increased cytoplasmic expression ($p < 0.05$). No significant change in PSPC1 or FUS levels was identified in either region, although there was a relative shift towards nuclear localisation of PSPC1 in the cortex.
Discussion: Despite lower cytoplasmic levels of TDP-43, SFPQ and NONO in the motor cortex compared to the cerebellum, there was a marked increase in TDP-43:paraspeckle protein interactions in the cytoplasm of neurons within the motor cortex compared to the cerebellum, and the size of these puncta were larger, suggesting that there may be cell/region specific differences in the cytoplasmic behaviour of these proteins. Exploring these functional differences may provide new insight into ALS disease mechanisms and yield new avenues for therapeutic development.

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IVV-03 Cell stress and apoptotic activation even prior to disease onset in a TDP-43 mouse model of ALS/FTD

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Background: As a critical pathological marker, TAR DNA binding protein 43 (TDP-43) accumulation occurs in >95% of amyotrophic lateral sclerosis (ALS) cases and ~50% of frontotemporal dementia (FTD).
Objective and methods: In this study, we sought to define the molecular pathways crucial for disease pathogenesis in ALS and FTD using the rNLS8 cytoplasmic TDP-43 mouse model, and to modulate identified gene or pathway to prevent disease progression.
Results: Using RNA expression screening, we identified dramatic dysregulation in several cell stress pathways, such as integrated stress response (ISR) effectors, neuroinflammatory, DNA damage, cellular metabolism, and apoptosis. Notably, even prior to disease onset, ISR effectors, including activating transcription factor 4 (Atf4) and CCAAT/enhancer-binding homologous protein (Chop/Ddit3), were upregulated along with anti-apoptotic gene Bcl2 and diverse pro-apoptotic genes including BH3-interacting domain death agonist (Bid), Bcl2 homology 3 (BH3)-only pro-apoptotic initiator (Bim) and phorbol-12-myristate-13-acetate-induced protein 1 (Pmaip1/Noxa), suggesting that ISR activation-induced apoptosis drives the neurodegenerative process. We further confirmed the cleavage of caspase-3, the executor of the apoptosis cascade, in the rNLS8 mouse cortex and spinal cord. Interestingly, the predominant pro-apoptotic and impaired anti-apoptotic signalling at later stages of disease suggests suppression of anti-apoptotic responses as the disease progresses. To modulate ISR signalling to prevent neurodegeneration, we employed antisense oligonucleotide-mediated silencing that successfully suppress Chop expression in the brain and spinal cord, however, this down-regulation of Chop did not affect disease progression in rNLS8 mice.
Discussion: These findings suggest that precise temporal modulation of cell stress and death pathways at early disease phases may be beneficial to protect against neurodegeneration in ALS and FTD. More targeted therapeutic strategies that fine-tune the ISR and other stress pathways may benefit therapy development for ALS and FTD caused by TDP-43 dysfunction.

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IVV-04 Imaging the spinal cord neurodegeneration of the TDP-43-A315T ALS mouse model: relationship between MRI and TDP-43 aggregates

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Background: ALS diagnosis delays around 12 months after symptoms' onset due mostly to the absence of biomarkers for diagnosis. Few studies showed Magnetic Resonance Imaging (MRI) alterations in the spinal cord of ALS patients

(1), and suggest their relation with disease duration and progression (2,3). TDP-43 aggregates, the hallmark of ALS, were also shown to be related with disease progression (4). So far, no study reported the relation between MRI alterations and the occurrence of TDP-43 aggregates.
Objectives: To perform MRI analysis in the TDP-43-A315T mice model of ALS and to correlate such analysis with the presence of TDP-43 aggregates, before and after symptoms' onset.
Methods: Female transgenic TDP-43-A315T (TDP; $n = 10$) and wild-type C57BL/6J (WT; $n = 10$) mice were used. Weight and motor function were assessed weekly, and MRI was performed at 3 or 6 months ($n = 5$ for each group). Mice were euthanized immediately after MRI for samples collection. Data is presented as mean ± standard error of the mean (SEM) and statistical analysis was realized with Student's *t*-test.
Results: At 3 months-old, there was no difference regarding body weight (TDP: 22.0g ± 1.1, WT: 23.2g ± 0.8; $p = 0.4$) or grip strength (TDP: 1.11N ± 0.07, WT: 1.29N ± 0.05; $p = 0.06$) between groups. TDP-43-A315T mice start to show a decline in body weight and motor function at 4 months-old. At 6 months, TDP-43-A315T mice presented significant decrease in hindlimb grip strength (TDP: 0.43 ± 0.04, WT: 0.70 ± 0.02; $p = 0.004$) and body weight ($p < 0.001$). MRI analysis of spinal cord showed no differences between TDP-43-A315T and wild-type mice at 3 or 6 months. Western blot confirmed the expression of human TDP-43 only in the TDP-43-A315T mice. Western blot analysis also revealed an increase in soluble and insoluble TDP-43 and in insoluble phosphorylated TDP-43 in TDP-43-A315T mice compared to wild-type.
Discussion: As described in the literature and confirmed in this study, symptoms' onset for the TDP-43-A315T mice start around 4 months of age. The absence of MRI alterations at 3 months correlates with a normal motor function in analyzed mice. Despite the presence of motor impairment and TDP-43 pathology, mice at 6 months of age did not show differences in MRI analysis. Increase in insoluble phosphorylated TDP-43 could be a marker for motor impairment in the TDP-43-A315T mice.

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IVV-05 TAR DNA binding protein-43 KDa (TDP-43) pathology causes differential expression of retrotransposons in a TDP-43-Q331K mouse model

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Background: Pathological aggregates of TDP43 are seen in patients with ALS, FTD and AD. TDP43 is a nucleic acid binding protein with important functions such as translational regulation, stress granule formation and retrotransposon repression. Retrotransposable elements (RTE) are mobile elements capable of inserting copies into different genomic locations. Studies in flies have established the causal role of RTEs in mediating both the intracellular toxic effects of TDP43, and the intercellular spread of that toxicity from glia to neurons (1). The role of TDP43 in RTE regulation has also been replicated in postmortem human tissue.
Objectives: Here, we establish the first rodent model to examine the effects of TDP43 pathology on RTEs.
Methods: We look at TDP43 proteinopathy in a mouse model where the human TDP43 transgene, with or without the Q331K familial ALS mutation, is overexpressed 1.5 times. We used hindlimb clasping and rotarod to quantify motor deficits in 1.5, 3, 6, 10 and 15 mo mice. To investigate the RTE transcript levels, we sequenced total RNA from the motor cortex (MC) of TDP43-Q331K Tg, TDP43-WT Tg and nTg littermates. To explore the number, location and cell types in which retrotransposition events occur, we used the L1-EGFP reporter mouse that expresses EGFP after a retrotransposition event occurs. We crossed the two transgenic lines with L1- EGFP animals and imaged GFP positive cells in multiple brain regions at different ages.
Results: The TDP43-Q331K Tg animal shows hindlimb clasping and lower latency to fall on the rotarod starting at 3 months, while the TDP43- WT Tg animal shows a delayed onset of motor deficits starting at 15 months. We see a significant upregulation of RTE at 3 months in the TDP43-Q331K Tg animals and at 15 months in the TDP43-WT Tg animals. This upregulation of RTEs coincides with the onset of motor defects in each of these transgenic lines. The TDP43-Q331K Tg animals show significantly higher GFP positive glia and neurons, which occur in large clusters in the striatum(Str) and nucleus accumbens(NA) at 3 months and in the MC at 6 months. The TDP43-WT Tg animals show similar clusters in the NA and Str at 10 months. Thus, both the transgenes drive retrotransposition of L1, that first appear in NA and Str, and later appear in MC.
Discussion: Interestingly, both the RTE transcript levels in the MC and the clusters of GFP positive cells within each brain region appear transiently, becoming undetectable at

later time points, consistent with the hypothesis that the cells that contain these de novo transposition events are not viable. In conclusion, we show evidence of RTE expression and retrotransposition events in a cell type, brain region specific, and age- dependent manner in TDP43 pathology mice.

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IVV-06 Modulation of mutant TDP-43 within astrocytes ameliorates disease progression in a mouse model of amyotrophic lateral sclerosis

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Background: ALS shares with Frontotemporal dementia (FTD), some causative genes, and pathologic hallmarks, like the accumulation of a protein called TDP-43 in intracellular inclusions (1). ALS is a non-cell-autonomous disease, with glial cells playing a key role in pathogenesis and contributing to the progression of the disease (2,3). In this context, we studied the role of astrocytes in the propagation of neuronal damage, but further research is needed to fully understand this complex interplay.
Objectives: In recent years, our lab has been investigating the role of astrocyte-to-neuron miscommunication in a mouse model of ALS. In this work, we wanted to unravel the contribution of these cells to disease progression and discover possible genetic markers of the pathology process.
Methods: Cohorts were tested for Three-Chambers Social Task, accelerated Rotarod, and Grip Strength at different age stages to evaluate possible ameliorations after TDP-43 selective removal through an inducible Cre-loxP system, under the control of the astrocyte-specific GLAST promoter. Selective removal was verified through an immunofluorescence assay. Submandibular blood collection was performed to collect plasma samples used for neurofilament light chain (NfL) quantification by SiMoA technology. Finally, we performed RNA-sequencing at three age stages on astrocyte and oligodendrocyte cell populations, purified with Miltenyi Isolation Kits.
Results: To understand the contribution of astrocytes, mutated TDP-43 Q331K was removed selectively from astrocytes in our mouse model. This was sufficient to improve cognitive function and motor function compared to TDP-43 Q331K mice at different age stages. To validate the effective removal of the TDP-43 Q331K protein only from astrocyte populations, not neuronal ones, we performed an immunofluorecence assay. Moreover, decreased axonal damage can be noticed also by NfL quantification from plasma samples at early symptomatic stages (6 months; p-value =0.0128).
Discussion: In this preliminary work, we reported a delayed progression of typical symptoms in our Q331K TDP43 mouse model, by simply removing TDP-43 from astrocytes. Once again, this result confirms the hypothesis that glial cells are key drivers for the maintenance of a physiological environment. For this reason, to further investigate their contribution, we performed RNA-sequencing at different time points on astrocytes and oligodendrocytes derived from the brain and spinal cord of our mouse model. This sequencing will allow us to identify new markers for disease progression and possibly find potential therapeutic targets that could halt or slow down the progression of the disease.

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IVV-07 A new ALS/FTD mouse model with widespread expression of C9orf72 nucleotide repeats throughout the CNS

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Amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD) exist on a continuum, with overlapping clinical presentation, pathology, and genetic causes. One such genetic cause is a hexanucleotide (G4C2)n repeat expansion in the C9orf72 gene, which has three proposed mechanisms for the deleterious effects of the C9orf72 mutation (C9-NRE): loss of function of the endogenous C9orf72 protein and gain of toxicity through RNA-mediated nuclear protein sequestration and/or dipeptide repeat proteins (DPRs). Current C9-NRE mouse models have not recapitulated complete ALS/FTD disease phenotypes, suggesting a combination of these mechanisms may underlie the diseases, and no existing model has shown an ALS motor phenotype. Here we present a novel C9-NRE mouse model created by intrathecally injecting repeat-containing AAV9 into C9orf72 knockout mice. Our results show this model achieves robust, widespread expression throughout the CNS, particularly throughout the pathophysiologically relevant spinal cord ventral horns and cortex. We are performing a rigorous, longitudinal characterization of this C9-NRE model, assessing pathology markers, ALS-relevant phenotypes (including muscle strength and function),

and FTD-relevant phenotypes (including behavioral assays of executive function and memory). Furthermore, because neuroinflammation has been implicated as a potential disease modifier, we are evaluating the neuroinflammatory signature of this C9-NRE model, as well as inducing chronic inflammation to determine if this will further exacerbate C9-NRE related pathology and disease. Through this work, we will provide a new model that recapitulates a more complete ALS/FTD phenotype, as well as further determine the contributions of all three C9-NRE pathogenic mechanisms and neuroinflammation to disease.

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IVV-08 Investigating the molecular basis for selective vulnerability in FET-linked amyotrophic lateral sclerosis (ALS) and frontotemporal dementia (FTD)

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Background: Fused in Sarcoma (FUS), Ewing Sarcoma (EWSR1) and TATA-Box Binding Protein Associated Factor 15 (TAF15) are conserved RNA binding proteins that make up the FET family. Genetic and molecular dysfunction of these proteins have been linked to pathological phenotypes in ALS and Frontotemporal Dementia (FTD). In ALS, mutations in FUS lead to its cytoplasmic mislocalisation and the formation of cytoplasmic inclusion in neurons (1,2). However, in FTD, inclusions are made up of all three FET proteins along with their nuclear import receptor TNPO1 (1) in the absence of any mutation.
Objectives: This project aims to characterise the spatio-temporal expression of the FET proteins in the central nervous system (CNS) of non-transgenic mice through aging, ultimately forming the basis for comparative analysis of FET expression between disease vulnerable and resistant FUS-ALS and FET-FTD patient tissues and healthy controls.
Methods: A combination of high-throughput microscopy and quantitative reverse transcription polymerase chain reaction (RT-qPCR) were used to map the spatio-temporal expression of FET proteins in 3, 12 and 24 month non-transgenic C57BL6 mice CNS as well as mouse embryonic stem cells. This was accompanied by comparisons of FUS-ALS, FET-FTD vulnerable and resistant patient tissues and healthy controls.
Results: We have seen clear differences in FET protein levels and sub-cellular localisation across the whole brain and spinal cord of non-transgenic mice throughout aging, along with differential FET gene expression across different brain regions. Endogenous EWSR1 and TAF15 show differential localisation when FUS inclusions are seen in ALS patient tissue. While we observe mislocalisation of FET proteins in FTD, we also notice differential inclusions signatures being displayed.
Discussion: There is a clear difference in endogenous expression and physiological functions between FET proteins. This project provides insights into why protein inclusions differ between the diseases and helps us to understand the specific cell vulnerability in FUS-ALS and FET-FTD.

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IVV-09 Early bioenergetics hypothalamic dysfunction in SOD1G93A mice

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Background: A growing body of evidence highlighted as metabolic dysfunctions underlies the neurodegenerative process in ALS patients. In this regard, metabolic alterations are associated with a poor prognosis and a faster decline of locomotor performances. Indeed, hypermetabolism was observed in about one third of patients, who show a decrease in body mass index, often during a premorbid state (1). The increased energy expenditure generally came with hyperlipidemia and glucose handling reduction. A large set of data in patients and in animal models, described a drop in energy production possibly ascribable to mitochondrial failures (2). Central nervous system (CNS), controls metabolism and energy balance of the whole body, interacting with hormones and nutrients. Within the CNS, the hypothalamus plays a pivotal role in regulating metabolism through the functionality of different neuronal populations, especially the anorexigenic POMC and orexigenic AgRP neurons in the Arcuate Nucleus (ARC). Particularly, energy metabolism dysfunctions in these two neuron populations and the neuroinflammatory status of ARC seem to affect the whole body energy expenditure (3,4). Currently, an increasing amount of evidence indicates that the metabolic derangements observed in ALS patients may be linked to changes in hypothalamic function. In line with this, both ALS patients and individuals at ALS genetic risk have been reported to exhibit hypothalamic atrophy (5).

Methods: Bioenergetic analysis was performed with Seahorse xF96 Technology on N-43/5 cells (POMC cell model) expressing some ALS related protein and on hypothalamus punches of SOD1G93A mice. The morphological analysis was carried out with immunofluorescence on the ARC area of SOD1G93A mice staining POMC and AgRP neurons as well as astrocytes and microglia.
Results: Our study describes the morphological and metabolic alterations in the hypothalamus of SOD1G93A mice at presymptomatic stage. Firstly, we observed a decrease of oxidative metabolism and ATP production in a cell model of POMC neurons expressing different ALS related proteins. Consistently, the analysis of energy metabolism performed by Seahorse xF96 Technology on punches of hypothalamus of SOD1G93A mice revealed a significant impairment of mitochondrial functionality specifically in this area, since Hippocampus punches of the same mice did not show metabolic alterations. Furthermore, the immunofluorescence analysis highlighted changes in morphology of the ARC area. In detail we observed a decrease in the POMC neurons and a concomitant increase in the AgRP neurons that came with a sustained neuroinflammation with microglia and astrocytes activation.
Conclusions/Discussion: On the whole our results suggest that the bioenergetics and inflammatory alterations in hypothalamus of SOD1G93A mice are detectable in a presymptomatic stage and thus could affect the disease course.

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IVV-10 Antibiotic mediated modulation of gut microbiome affects survival in SOD1 mice

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Background: While there has been significant progress in understanding the genetic underpinnings of ALS, environmental factors that modulate genetic disease risk are unknown. Dysbiosis has already been implicated in the pathogenesis of several neurological disorders including Parkinson's disease, multiple sclerosis, and autism spectrum. Recently, the gut microbiome has been shown to protect against disease progression in ALS, as a recent study that ablated the gut of SOD1+/− mice with oral antibiotics cocktail significantly reduced survival and neurological function.
Objectives: To modulate the gut microbiome by administering single antibiotic treatment and determine the relative contribution of specific bacterial populations to disease progression in SOD1 mice.
Methods: We modulated the gut microbiome by administering low dose oral vancomycin and metronidazole to SOD1+/− mice over lifespan and measured survival and overall motor function with neurological score and the body weights over time.
Results: While we found that metronidazole did not impact disease progression, vancomycin improved mortality ($p - 0.02$) and neurological function significantly, implying that the bacterial populations affected by the drug have a deleterious effect on disease progression.
Ongoing work in progress: We now will be sequencing the microbiota to study the altered bacterial populations in these SOD mice and add males to the study to further evaluate sex-specific effects of the single antibiotic treatment. Repeating the experiments with different antibiotics such as neomycin will help broadening the scope of evaluation of single antibiotic therapy for ALS. We will also be incorporating TDP-43 models in the experiments to compare the effects across different mutations associated with the pathogenesis of the disease.
Discussion: Through this investigation, we strive to deepen our understanding of the complex interactions between the gut microbiome and ALS, with the prime goal of identifying these specific disease ameliorating microbes and microbial products as well as developing innovative strategies to mitigate the devastating effects of the progressive neurodegenerative diseases.

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IVV-11 Pan-neuronal expression of human SOD1 mutations in Drosophila induces early neuroinflammation

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Background: Superoxide Dismutase 1 (SOD1), coding for the antioxidant enzyme SOD1, is the second most common ALS-associated gene, with more than 200 identified point mutations. Since most of the mutant proteins retain their enzymatic function, SOD1-associated pathogenicity is thought to be due to a toxic gain-of-function leading to motor neuron death. Several causes sustain ALS neurodegeneration when a certain, still undefined, threshold of damage is exceeded and generates a pathogenic cascade culminating in motor neurons depletion. Despite the generation of many in vitro and in vivo models and the huge effort of research (1), our knowledge about the events that precede this neurodegenerative cascade remains elusive.
Objectives: Based on these premises, our main objective was to better characterize the very early cellular mechanisms that

are deregulated in SOD1-ALS and induce motor neuron degeneration with clinically evident symptomatology.
Methods: Drosophila is a well-established model for studying ALS, not only because transgenic flies recapitulate several symptoms, but also because they can be easily adopted for screening disease progression and novel therapeutic compounds (2). Here we took advantage of two Drosophila models expressing different mutant human SOD1 transgenes (hSOD1-A4V or hSOD1-G85R) (3).
We expressed the transgenes pan-neuronally and performed: qRT-PCR, immunofluorescence and western blot analysis to assess the expression of several inflammatory and antioxidant markers; cytological preparations to score chromosomal aberrations; flow cytometry to quantify global ROS measurement; finally climbing and survival assays.
Results: Our results show that pan-neuronal expression of mutant SOD1 induces a strong neuroinflammatory phenotype in Drosophila, resulting in glial activation, innate immunity stimulation, antimicrobial peptides up-regulation and oxidative stress, in turn sustaining genomic instability and impaired motor performance. Interestingly, the observed phenotypes manifest in the very early stages of adult life when neuronal depletion is not still detected. However, at later time points, ALS flies show reduced survival respect to healthy controls.
Discussion: Taken together, our results reinforce the concept that SOD1-ALS pathogenesis is characterized by a very precocious neuroinflammatory component that precedes, and maybe sustains, the progressive cascade of motor neuron damage, overall leading to the typical ALS symptoms.

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IVV-12 Drosha-dependent microRNAs modulate FUS-mediated amyotrophic lateral sclerosis *in vivo*

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Mutations in Fused in Sarcoma (FUS) gene cause the familial and progressive form of amyotrophic lateral sclerosis (ALS). FUS is a nuclear RNA-binding protein involved in RNA processing and the biogenesis of a specific set of microRNAs. Here we report that Drosha and two previously uncharacterized Drosha-dependent miRNAs are strong modulators of FUS expression and prevent the cytoplasmic segregation of insoluble mutant FUS in vivo. We demonstrate that depletion of Drosha mitigates FUS-mediated degeneration, survival, and motor defects in Drosophila. Mutant FUS strongly interacts with Drosha and causes its cytoplasmic mis-localization into the insoluble FUS inclusions. Reduction in Drosha levels increases the solubility of mutant FUS. Interestingly, we found two Drosha dependent microRNAs, miR-378i and miR-6832-5p, which differentially regulate the expression, solubility, and cytoplasmic aggregation of mutant FUS in iPSC neurons and mammalian cells. More importantly, we report different modes of action of these miRNAs against mutant FUS. Whereas miR-378i may regulate mutant FUS inclusions by preventing G3BP-mediated stress granule formation, miR-6832-5p may affect FUS expression via other proteins or pathways. Overall, our research reveals a possible association between ALS-linked FUS mutations and the Drosha-dependent miRNA regulatory circuit, as well as a useful perspective on potential ALS treatment via microRNAs.

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IVV-13 Characterising novel, humanised and physiological mouse models of FUS-ALS

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Background: Despite extensive research into ALS, there is still no cure, and little in the way of efficacious treatments. This is in part due to a lack of physiologically relevant models of human disease. Mouse models of ALS, and other neurodegenerative diseases, have historically been transgenic and as such, the phenotypes they display may represent artefacts of overexpression, rather than disease relevant mechanisms.
Objectives: To characterise novel, humanised knock-in mouse models of FUS-ALS. Humanised wildtype mice were generated whereby the mouse Fus gene from the ATG start codon through to the 3'UTR, including all introns and exons, was replaced with the human FUS sequence at the endogenous Fus locus. An ALS patient mutation, P525L, was then introduced into the human FUS gene sequence using CRISPR/Cas9 techniques.
Methods: Humanised FUSP525L mice were studied through longitudinal phenotyping pipelines including several motor and cognitive tests. Alongside overt phenotyping, relevant histopathological changes were assessed in ALS-relevant

tissues such as the spinal cord and the predominantly fast twitch tibialis anterior muscle.
Results: This humanised FUS-ALS model expresses the mutant FUS protein at physiologically relevant levels, and in a normal pattern of expression. Heterozygous humanised FUSP525L male mice show disease relevant phenotypes, including progressive late-onset reduction in muscle strength from 1 year, hyperactivity and metabolic impairment. This is alongside cellular and molecular changes from 4 months, as shown by RNA sequencing data from the spinal cord and tibialis anterior muscle. There is also evidence of histopathological changes, such as changes in fibre type composition of the tibialis anterior muscle of 15-month-old male mice.
Discussion: The phenotypes displayed by this model highlight the potential of fully humanised knock-in mice to aid in unravelling early disease mechanisms, and ultimately produce therapies targeted towards the human gene and protein, in the context of, but not limited to, ALS.

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IVV-14 A novel modulator of modified proteins reduced pathogenetic stress in *in vivo* ALS models

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Background: Amyotrophic lateral sclerosis (ALS) is a complicated neurodegeneration with a modest cure of riluzole (1,2) linked to the most pathogenic superoxide dismutase 1 SOD1 and repeat expansion of Chromosome 9 open reading frame 72 C9orf72 genes (3,4).
Objectives: To identify a potential novel neuromodulator of ALS.
Methods: The sod1G93Ros10 mutant zebrafish was utilized. It shows earlier neural stress in an interneuron at 72 hpf, neuromuscular junctions (NMJ) weakness, mobile performance defects, muscle atrophy, and an essential hsp70-DsRed stress fluorescent readout (5,6). The LP0927 compound was investigated. It is believed to act on modified protein toxicity. The (G4C2)45-C9-ALS zebrafish evaluated the work. It shows hexanucleotide repeat expansions of dipeptide protein repeats (DPRs) that are cellular toxicities related to ALS.
Results: Dose-response screen of LP0927 of 1 μM, 3 μM, and 10 μM showed a significant reduction effect on hsp70-DsRed in treated sod1G93Ros10 zebrafish. It was 13.61, 25.43, & 51%, respectively. The evaluation of 3 μM and 10 μM of LP0927 showed inhibition effects of DPR expressions in treated (G4C2)45-C9 zebrafish of 24.93 and 41.76% compared to DMSO.
Discussion: LP0927 compound showed an efficacy reduction of neuronal stress agreement with previous work (5,7). The evaluation stage delivers a potent neuroprotective treatment. It identifies a potential protein modulator of ALS-developed drugs.

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Acknowledgements

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IVV-15 Do glial cells contribute to the differential vulnerability of neuromuscular junctions in amyotrophic lateral sclerosis?

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Background: Neuromuscular denervation (1,2) precedes the apparition of symptoms and motor neuron degeneration. Perisynaptic Schwann cells (PSCs), glial cells at the neuromuscular junction (NMJ), regulate the maintenance and repair of the synapse. This is in part under the control of muscarinic receptors (mAChRs) whereby a reduced muscarinic activation prevails during denervation and repair. However, PSCs show a hypermuscarinic activation at a presymptomatic stage of

the disease which persists at disease onset at innervated and denervated NMJs of SOD1G37R mice (3–5). Importantly, NMJ innervation of the extraocular muscle (EOM) is preserved in patients (6,7) and in mouse models of ALS (8). Yet, despite the importance of NMJ denervation, mechanisms of NMJ vulnerability and resistance remain unclear.
Objectives: We posit that the hyperactive muscarinic excitation of PSCs contributes to NMJ instability at vulnerable NMJs, while PSCs at EOM would regulate NMJ maintenance and repair owing to a normal muscarinic activation.
Methods: Experiments were performed using SOD1G37R mice. Properties of PSCs at NMJs of vulnerable and resistant muscles were determined using calcium imaging and morpho-functional analyses of NMJ innervation. Muscarinic activity of PSCs of vulnerable muscles were dampened by the overexpression of the third intracellular loop of mAChRs while PSCs hyperactivity of EOM will be induced using daily activation of a DREADD receptor. PSCs were selectively targeted using an AAV 5/8 under a human GFAP promotor injected IP or in the periocular cavity.
Results: PSCs at NMJs of EOM had a normal muscarinic activation compared to their WT littermate's ($p = 0.44$, unpaired *t*-test), while PSCs at vulnerable NMJs of the extensor digitorum longus (EDL), exhibited an increased muscarinic activation. Overexpressing the M3iLoop receptor reduced the muscarinic activation of PSCs of EDL ($p = 0.0006^{***}$, unpaired *t*-test) and preserved NMJ innervation, muscle strength and motor functions. The overexpression of a scrambled peptide as control had no effect. We posit that PSCs hyperactivation of EOM will cause NMJ instability, denervation and altered motor functions.
Discussion: Determining the contribution of PSCs in the resistance of NMJs would unravel an important factor underlying ALS pathophysiology. Understanding NMJ resistance and vulnerability could pave the way towards novel therapeutic strategies targeting glial cells in ALS, to preserve neuromuscular innervation and motor functions.

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IVV-16 Divergent pathologies in novel knockin mutant matrin 3 mouse models

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Background: Matrin 3 (MATR3) mutations were first linked to familial forms of ALS in 2014 with the discovery of 4 gene mutations segregating in families (1). Additional MATR3 mutations have since been found in Canadian, Italian and Taiwanese ALS population, for a total of 15 MATR3 mutations linked to ALS. Matrin 3 is a highly conserved nuclear matrix protein (98.5% between human and mouse) that functions in binding and stabilizing mRNAs, DNA damage response, and anchoring A-to-I edited RNA within the nucleus. However, it remains unknown how MATR3 mutations cause human disease or alter protein function.
Objectives: In order determine the effects of matrin 3 mutations *in vivo* without the possibility of overexpression artifact, we generated two novel matrin 3 knockin mouse models that expressed the P154S and the S85C MATR3 mutations.
Methods: Both these models used CRISPR-Cas technology to generate and were controlled under the endogenous promoter. To determine longitudinal phenotypic effects induced by MATR3 mutations, motor and cognitive assays were performed. CNS and muscle tissue were collected at early and late time points to determine the effects of MATR3 mutations on neuromuscular pathology and changes in gene expression.
Results: Behavioral analysis demonstrated that homozygous S85C mice caused significant motor impairment detectable as early as 3 months of age. These impairments remained steady until 12 months of age when mice began to decline in performance. This drop in motor performance in mice also corresponded with drops in matrin 3 levels, in the spinal cord, cortex, and muscle. Conversely, the P154S mutation did not produce significant motor impairment. Pathological analysis demonstrated neuronal loss in the S85C mouse model in distinct cellular populations. In addition, transcriptomic analysis demonstrated gene dysregulation and splicing alterations in spinal cord, muscle, cortex, and cerebellum in both mouse models. Further, we performed Gene Set Enrichment Analysis (GSEA) to get a broader sense of biological processes affected in our S85C mouse model. We found 288 significantly enriched biological processes gene ontology terms. Examples of gene terms that were most enriched included: vesicle mediated transport in synapse, synaptic vesicle cycle, cation transport, and transynaptic signaling.
Discussion: This study presents two separate models that maintain physiologically relevant levels of matrin 3 while producing a strong motor phenotype in the S85C mutant mice. We propose that identifying the differing molecular changes in both models could lead to a deeper understanding of the role of matrin 3, and its mutations, in pathogenesis. These

new models represent potentially valuable tools to neuromuscular disease field.

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IVV-17 Investigating the regeneration competence of peripheral motor neurons in ALS

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Background: Amyotrophic lateral sclerosis (ALS) is a spectrum of diseases with adult onset, multiple aetiologies, wide phenotypical heterogeneity, and inadequate understanding of pathological mechanisms, which hampers the development of efficacious therapeutical interventions. The neuromuscular junction (NMJ) is the first target of the pathology in both patients and several animal models (1,2). Distal denervation begins very early, prior to the onset of symptoms, and precedes motor neuron death in the spinal cord. Motor unit loss is preceded by a plastic remodelling of the NMJs, which undergo cycles of denervation/re-innervation (3), until regenerative processes are progressively overwhelmed by degenerative events.
Working hypothesis: While motor axon terminal degeneration in ALS has been widely studied, very little is known about the regenerative capability of the system that we addressed in the present study. The ability of ALS NMJs to remodel for a time suggests that supporting/prolonging synaptic plasticity and regeneration can be therapeutically exploitable to delay denervation.
Methods: We assessed the regeneration competence of ALS MNs by exposing SOD1G93A mice at different disease stages to the acute and reversible degeneration of their motor axon terminals by the presynaptic neurotoxin α-Latrotoxin (α-LTx) (4), injected in the hind limbs. We then followed over time the morphological and functional recovery of their NMJs by electrophysiology and imaging (5,6).
Results and discussion: We found that the regenerative capability of SOD1G93A NMJs is maintained for a certain period and therefore we identified a convenient time window to stimulate the regeneration competence of ALS motor axon terminal (see abstract by Dr Giorgia D'Este), in order to stabilize the NMJs, to delay denervation and to counteract disease progression.

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IVV-18 Nuclear import defects in a NEMF mutant mouse model of neurodegeneration

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Background: Nucleocytoplasmic transport defects are increasingly being identified as a factor in multiple forms of neurodegenerative diseases, including amyotrophic lateral sclerosis (ALS). A pathological hallmark of motor neuron disease is the accumulation of cytoplasmic amyloid-like aggregated proteins. These cytoplasmic inclusions have been characterized in forms of neurodegeneration such as TAR DNA-binding protein 43 (TDP-43) and C9ORF72 sporadic and familial ALS, as well as other neurodegenerative diseases such as Huntington's and Parkinson's.
Objectives: To determine how nuclear import defects in a mutant Nuclear Export Mediator Factor (NEMF) mouse model contribute to disease progression in a motor neuron disease.
Methods: *In vitro* investigations into NEMF R86Snucleocytoplasmic defects were performed in mouse embryonic fibroblasts by exogenously expressing GFP in frame with different nuclear import reporters, such as the classical SV-40 Import-β nuclear localization signal (cNLS), Proline-Tyrosine Transportin-1 nuclear localization signal (PY-NLS), and the PY-NLS in frame with a pKINES exportin-1 nuclear export signal. Further investigations into nuclear import defects involved immunostaining for nuclear transport factors and observing the colocalization of these factors with NEMF. *In vivo* investigations followed immunofluorescence staining

of nuclear transport factors in lumbar spinal cord of 21-day old Wild type (WT) NEMF and early onset NEMF R86SC57/Bl6J mice. To determine disease onset, WT NEMF and late onset NEMF R487Gmice were aged to 21 days, 6 months, and 12 months and then immunostained for nuclear transport factors. Brain, lumbar spinal cord, and quadricep tissue was isolated and RNA was extracted, and reverse transcribed for qPCR of downstream TDP-43 transcriptional targets.
Results: NEMF R86S MEFs display defective Importin-β nuclear import, but not transportin-1 nuclear import or exportin-1 nuclear export. This loss of nuclear import is consistent with the cytoplasmic accumulation of nuclear transport factors such as NEMF, Importin-β, Ran, and TDP-43. *In vivo* investigations in NEMF R86S mice revealed that these factors are also lost from the nucleus in lumbar spinal motor neurons and accumulate in cytoplasmic aggregates in the soma. In the late onset NEMF R487G mice, NEMF is temporally retained in the nucleus but is eventually lost with age, preceding the nuclear loss of TDP-43. This nuclear loss and cytoplasmic gain are consistent with disease onset in both the early onset NEMF R86S mice and the late onset R487G mice.
Conclusions: Our study highlights that nucleocytoplasmic transport defects are a prevalent pathological hallmark in neurodegeneration and may serve as a potential therapeutic target for motor neuron disease.

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IVV-19 DNAJC7 interaction prevents tau fibril formation in vitro and loss of interaction with tau occurs after phosphorylation of threonine 175 and PAD exposure of tau in a rat model of traumatic brain injury

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Background: DNAJC7 is a highly conserved heat shock protein involved in protein folding surveillance. DNAJC7 has been described to interact with tau proteins, maintaining tau in a natively folded conformation by interacting with the amyloid motif (275VQIINK280) of tau and preventing tau fibril formation (1). Recently, mutations in DNAJC7 have been detected in ALS (2) strongly suggesting that this protein may have a role in disease pathogenesis. We have employed a rat model of traumatic brain injury (TBI) to examine the sequence of events leading to tau fibril formation (3). Using this model we have determined that phosphorylation of threonine 175 (pThr175) of tau occurs rapidly after injury, then PAD exposure occurs, Thr231 phosphorylation increases and fibril formation becomes evident.
Objective: To examine the expression of DNAJC7 after traumatic brain injury in rats and determine when DNAJC7 dissociation from tau occurs, and if this is driven by the phosphorylation of Thr175 of tau.
Methods: HEK293T and Neuro2A cells were used to examine coimmunoprecipitation of exogenously or endogenously expressed tau and DNAJC7. Rats were subjected to a single open-skull controlled impact (injured) or to sham surgery (control) and brains examined by western blot and confocal microscopy for DNAJC7 expression, cellular localization (neurons (NeuN), microglia (Iba1) and astrocytes (GFAP)) and colocalization with tau, pThr175 tau, and TNT1 (PAD exposure).
Results: DNAJC7 is expressed in neurons, microglia and astrocytes in both control and injured brain, with increased expression after injury. Colocalization with pThr175 tau was evident, but once PAD exposure was detected colocalization with DNAJC7 dropped significantly from colocalized to showing a separation of signals when PAD exposure was detected. Complimentary *in vitro* experiments showed that overexpression of DNAJC7 could prevent tau fibril formation. DNAJC7 immunoprecipitates with wildtype tau, and pThr175 null tau (Thr175Ala) but not with Thr175Asp tau. However, pThr175 tau elicited with sodium arsenite treatment of cells did co-immunoprecipitate, showing that DNAJC7 dissociation occurs after phosphorylation of Thr175.
Discussion: After a single TBI in rats, DNAJC7 rapidly dissociates from tau. This dissociation temporally follows pThr175 tau immunoreactivity and coincides with PAD exposure. However, DNAJC7 can be immunoprecipitated from cells expressing pThr175 tau, suggesting that either a downstream phosphorylation event or structural change of tau is likely responsible for DNAJC7 dissociation.

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IVV-20 Phenotyping of the rNLS8 mouse model of amyotrophic lateral sclerosis

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Background: The SOD1-G93A model has been used as a gold standard in preclinical ALS research for over a decade. However, the model lacks clear TDP-43 pathology, including phosphorylated and ubiquitinylated TDP-43 protein with cytoplasmic inclusions, which is evident in most sporadic and familial ALS patients. Therefore, development of TDP-43 targeting therapies and increased translatability to humans call for alternative murine models of ALS.
Objective: In this work we describe the phenotyping of the rNLS8 mouse model, which is a double transgenic model characterized by doxycycline (Dox)-suppressible expression of human TDP-43 with a defective nuclear localization signal (1). The aim of the work was to reproduce published data to verify the behavioral and biomarker phenotype of the model to support future preclinical efficacy studies.
Methods: The animal work was conducted in accordance with European Union directive 2010/63 and approved by the national Project Authorization Board. Male ($n = 6$) and female ($n = 5$) rNLS8 (B6;C3-Tg(NEFH-tTA)8Vle Tg(tetO-TARDBP*)4Vle/J, The Jackson Laboratory, ID: 028412) double transgenic mice were used. In addition, 3 male and 4 female tTA mice lacking the tetO-hTDP-43-ΔNLS transgene were used as controls. Clinical scores and body weights were recorded three times per week. Limb strength and coordination were tested with the wire hang and rotarod tests at the baseline and 2 and 4 weeks after Dox discontinuation. At 4 weeks after Dox withdrawal, the animals were euthanized. Anterior horn motor neuron counts and TDP-43 expression in the lumbar spinal cord were determined by cresyl fast violet histological staining or immunohistochemistry, respectively. Neuromuscular junction (NMJ) integrity was analyzed by pre- and postsynaptic staining of the gastrocnemius muscle. Cerebrospinal fluid (CSF) levels of neurofilament light chain (NfL) were determined using the Simoa® (Quanterix) assay.
Results: Rotarod and wire hang performance of rNLS8 mice were significantly compromised at 2 weeks after Dox discontinuation, and further deteriorated by 4 weeks. At 2 weeks, body weights of the rNLS8 mice started to decline and limb weakness was observable by eye. Ex vivo analyses revealed markedly increased anterior horn total and phosphorylated TDP-43 expression in rNLS8 mice as compared to the tTA control mice. In addition, the proportion of nuclear versus cytoplasmic total TDP-43 was significantly reduced in rNLS8 mice. The pathology was also evident at the motor endplate, with an increase in denervated NMJs. Markedly elevated NfL levels suggested extensive axonal damage. However, no significant loss of motor neurons was observed at this disease stage.
Discussion: In conclusion, our results are in line with published literature and show that the rNLS8 mouse model manifests an ALS-like *in vivo* phenotype accompanied by motor neuron TDP-43-pathology and NMJ disintegration.

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