Title: Insights into disease mechanisms and potential therapeutics for *C9orf72*-related amyotrophic lateral sclerosis/frontotemporal dementia.

Delia Gagliardi*[1], Gianluca Costamagna*[1], Michela Taiana[1], Luca Andreoli[1], Fabio Biella[1], Margherita Bersani[1], Nereo Bresolin[1,2], Giacomo Pietro Comi[1,2], Stefania Corti[1,2]

[1]Dino Ferrari Centre, Neuroscience Section, Department of Pathophysiology and Transplantation (DEPT), University of Milan, Via Francesco Sforza 35, 20122 Milan, Italy.
[2]Neurology Unit, IRCCS Foundation Ca' Granda Ospedale Maggiore Policlinico, Via Francesco Sforza 35, 20122, Milan, Italy.

Corresponding author: Prof. Stefania Corti, MD, PhD.
Dino Ferrari Centre, Neuroscience Section, Department of Pathophysiology and Transplantation (DEPT), University of Milan, Neurology Unit, IRCCS Foundation Ca' Granda Ospedale Maggiore Policlinico, Via Francesco Sforza 35, 20122 Milan, Italy.
Phone: +39 0255033817; Fax: +39 025503800; Email: stefania.corti@unimi.it.

*These authors contributed equally to this work.

Highlights

- Loss- and gain-of-function mechanisms are involved in C9-FTD/ALS.
- Different cellular downstream pathways are impaired in C9-FTD/ALS.
- Several disease models have provided new insights into the pathogenic mechanisms.
- Biochemical and neuroimaging biomarkers may represent useful tools for C9-ALS/FTD patients.
- Therapeutic strategies targeting *C9orf72* repeats are promising for clinical use.

Abstract

In 2011, a hexanucleotide repeat expansion (HRE) in the noncoding region of *C9orf72* was associated with the most frequent genetic cause of frontotemporal dementia (FTD) and amyotrophic lateral

sclerosis (ALS). The main pathogenic mechanisms in C9-ALS/FTD are haploinsufficiency of the C9orf72 protein and gain of function toxicity from bidirectionally-transcribed repeat-containing RNAs and dipeptide repeat proteins (DPRs) resulting from non-canonical RNA translation. Additionally, abnormalities in different downstream cellular mechanisms, such as nucleocytoplasmic transport and autophagy, play a role in pathogenesis. Substantial research efforts using *in vitro* and *in vivo* models have provided valuable insights into the contribution of each mechanism in disease pathogenesis. However, conflicting evidence exists, and a unifying theory still lacks.

Here, we provide an overview of the recently published literature on clinical, neuropathological and molecular features of C9-ALS/FTD. We highlight the supposed neuronal role of C9orf72 and the HRE pathogenic cascade, mainly focusing on the contribution of RNA foci and DPRs to neurodegeneration and discussing the several downstream mechanisms. We summarize the emerging biochemical and neuroimaging biomarkers, as well as the potential therapeutic approaches. Despite promising results, a specific disease-modifying treatment is still not available to date and greater insights into disease mechanisms may help in this direction.

Abbreviations:

AAV: adeno-associated virus; AD: Alzheimer's disease; ADAR2: adenosine deaminase acting on RNA 2; ALS: amyotrophic lateral sclerosis; ASOs: antisense oligonucleotides; BAC: Bacterial artificial chromosome; C9-ALS: *C9orf72* related ALS; C9-FTD: *C9orf72* related FTD; CARM1: coactivator-associated arginine methyltransferase 1; Cas: CRISPR associated protein; CBD: corticobasal degeneration; CHIT-1: chitotriosidase-1; CNS: central nervous system; CRISPR: Clustered Regularly Interspaced Short Palindromic Repeats; CSF: cerebrospinal fluid; DSBs: double-strand breaks; DENN: Differentially Expressed in Normal and Neoplasia; DPRs: dipeptide repeat proteins; DSIF: DRB sensitivity-inducing factor; DTI: Diffusion Tensor Imaging; ER: endoplasmic reticulum; fALS: familial ALS; FTD: frontotemporal dementia; G_4C_2: GGGGCC; GA: Gly-Ala; GP: Gly-Pro; GR: Gly-Arg; HD: Huntington's Disease; HRE: hexanucleotide repeat expansion; HSPs: heat shock proteins; iPSCs: induced Pluripotent Stem Cells; iPSNs: induced Pluripotent Stem cell derived Neurons; KO: knock-out; LCDs: low complexity sequence domains; LDH: lactate dehydrogenase; LLPS: liquid-liquid phase separation; miRNA: micro RNA; MND: Motor Neuron Disease; MNs: Motor Neurons; MQC: mitochondrial quality control; MRI: magnetic resonance imaging; mRNA: messenger RNA; MSD: mesoscale discovery; mTOR: mammalian target of rapamycin; N/C: nucleo/cytoplasmic; NCT: nucleo-cytoplasmic transport; NEFM: neurofilament medium polypeptide; NES: nuclear export signal; NfL: light chain neurofilament; Nfs: neurofilaments; NLS: nuclear localization sequence; NMD: nonsense-mediated mRNA decay; NPCs: nuclear pore complexes; NPTXR: neuronal pentraxin receptor; Nups:

Nucleoporins; P-bodies: processing bodies; p-NfH: phosphorylated neurofilament heavy chain; PA: Pro-Ala; PAF1C: polymerase II-associated factor 1 complex;
PD: Parkinson's disease; PFN1: profilin-1; PMA: Progressive Muscular Atrophy; PLS: Primary Lateral Sclerosis; PR: Pro-Arg; PreSxC9: Presymptomatic C9 expansion carrier; PSP: progressive supranuclear palsy; Rab-GEF: Rab-Guanosine Exchange Factor; RAN: repeat-associated non-ATG translation; RanGAP1: Ran GTPase-activating protein 1; RBP: RNA binding protein; RNAi: RNA interference; RNP: ribonucleoprotein; ROS: reactive species of oxygen; sALS: sporadic ALS; SGs: stre ss granules; shRNAs: short hairpin RNAs; SINEs: selective inhibitors of nuclear export; siRNA: short interfering RNA; SMA: Spinal Muscular Atrophy; SOD1: superoxide dismutase; SRSF1: serine/arginine-rich splicing factor 1; TDP-43: TAR-DNA binding protein 43; TMS: transcranial magnetic stimulation; TMX2: thioredoxin-related transmembrane protein 2; UCHL1: ubiquitin carboxyl-terminal hydrolase isozyme L1; UPS: ubiquitin-proteasome system.

Keywords: *C9orf72*; amyotrophic lateral sclerosis; RNA foci; dipeptide repeat proteins; downstream mechanisms; therapeutic approaches

1. Introduction

Amyotrophic lateral sclerosis (ALS) is a fatal neurodegenerative disorder affecting upper and lower motor neurons (MNs) that causes progressive and relentless muscular weakness and atrophy leading to death for respiratory insufficiency. Frontotemporal dementia (FTD) is the most common form of dementia before 65 years of age and is characterized by relevant changes in social behavior and personality or language disturbance, due to degeneration of the frontal and/or temporal lobes. The term frontotemporal lobar degeneration (FTLD) denotes a neuropathological spectrum encompassing different neurodegenerative disorders, such as ALS, FTD and various combinations of them. Although the majority of these clinical syndromes are sporadic, a conspicuous part of them (ranging from 10 to 25% in FTD and 5-10% in ALS) is familial.
In 2011 De Jesus-Hernandez et al. and Renton et al. independently discovered that the hexanucleotide GGGGCC (G_4C_2) expansion in a non-coding region of *Chromosome 9 Open reading frame 72* (*C9orf72*) gene on chromosome 9 is the most frequent genetic cause of both sporadic and familial forms of ALS and FTD in Western countries (DeJesus-Hernandez et al., 2011; Renton et al., 2011). The clinical phenotypes associated with *C9orf72* hexanucleotide repeat expansion (HRE) are markedly heterogeneous (Woollacott

and Mead, 2014), the number of repeats and the clinical presentation are not correlated and HRE length is highly variable in different tissues of the same patient and in parental-offspring transmission.

Although the function of *C9orf72* protein is not completely understood, both primary neurons from patients with *C9orf72*-related ALS/FTD (C9-ALS/FTD) and studies in *in vitro* and *in vivo* C9-models have improved our understanding of the etiology and pathogenesis of C9-ALS/FTD. Three possible, but not mutually exclusive pathomechanisms have emerged and are still debated in the field: loss-of-function due to *C9orf72* protein haploinsufficiency, gain of toxic function from repeat-containing RNAs, also known as RNA foci, generated by bidirectional transcription of HRE, and toxic gain-of-function of dipeptide repeat proteins (DPRs) translated from sense and antisense HRE transcripts by non-canonical translation of repetitive RNAs (Balendra and Isaacs, 2018). Further, alterations in various downstream cellular pathways, such as nucleocytoplasmic transport (NCT), RNA metabolism, ubiquitin-proteasome system (UPS) and autophagy, vesicle trafficking, DNA damage, and mitochondrial metabolism, might be involved in disease pathogenesis.

The precise contribution of each mechanism into C9-ALS/FTD pathogenesis is still controversial, but they all likely play a role to some extent. The discovery of *C9orf72* as the most common genetic cause of ALS/FTD has prompted the research towards an HRE-targeted therapy, opening the path for new promising pharmacologic approaches. However, though a phase I clinical trial on antisense oligonucleotides (ASOs) directed on HRE is now ongoing, an efficacious treatment for C9-ALS/FTD still lacks.

Here, we provide a literature overview on clinical, pathological and molecular aspects of C9-ALS/FTD. We report recent epidemiological data, clinical features and neuropathology underlying C9-ALS/FTD, highlighting the main similarities and differences between C9-ALS/FTD and sporadic form of ALS (sALS). We describe the gene structure and the supposed role of *C9orf72* protein in cellular machinery. Through the description of neuropathological findings and *in vitro* and *in vivo* studies, we focus on the main three mechanisms involved in disease pathogenesis, investigating proofs and controversies regarding the contribution of each of them in driving neurodegeneration, and widely exploring the downstream cellular pathways. Then, we highlight the most recently investigated biochemical, structural and functional biomarkers to assess disease progression and drug efficacy in clinical trials. Finally, we provide a synopsis of the possible therapeutic approaches in C9-ALS/FTD, including ASOs specifically targeting the *C9orf72*

HRE, RNA interference, small molecules, genome editing techniques and DPRs-directed strategies.

We are confident that a better comprehension of the molecular mechanisms underlying C9-ALS/FTD pathogenesis would provide major and promising advances in specific disease-modifying treatments.

2. Epidemiology and clinical features

HRE in *C9orf72* is the most common genetic cause of ALS and FTD all over the world and in particular in the Caucasian population, while it is very infrequent in the Asiatic population. Large population studies conducted by Majounie and colleagues in 2012 have shown that the prevalence of G_4C_2 repeat expansion in *C9orf72* accounts for 5–7% of sporadic ALS and FTD forms in white Europeans, Americans, and Australians (Majounie et al., 2012b). The pathogenic expansion is more frequent in white (7%) and Hispanic (8,3%) ALS patients than in the Afroamericans (4.1%). The rate of pathogenic expansion carriers among familial ALS (fALS) and FTD was higher, accounting for 37.6% and 25.1%, respectively (Majounie et al., 2012b). A recent meta-analysis based on 111 studies confirmed these findings: overall, the prevalence of G_4C_2 expansion in *C9orf72* in fALS (22,5%) was higher than sALS (3,1%) (Zou et al., 2017). Conversely, the prevalence of the HRE in healthy controls is estimated around 0.15-0.6% in different studies (Akimoto et al., 2013); (Beck et al., 2013); (Byrne et al., 2012); (Cooper-Knock et al., 2012); (M. Harms et al., 2013; M. B. Harms et al., 2013b); (Majounie et al., 2012a, 2012b); (Ratti et al., 2012); (Renton et al., 2011); (Simón-Sánchez et al., 2012); (Smith et al., 2013)).

Inheritance usually follows an autosomal dominant pattern, but some familiar forms may present as sporadic, probably due to variable penetrance of *C9orf72* expansion and small family sizes (Woollacott and Mead, 2014). The penetrance of the mutation in carriers increases with age: it is absent under 35 years of age, it is 50% by 58 years of age and complete over 80 years (Majounie et al., 2012b). Though controversial, there is little evidence in support of disease anticipation, such as a significant decrease in age at onset (Mossevelde et al., 2017), longer repeat size and increased CpG methylation in the offspring (Gijselinck et al., 2016). Another work reports a contraction in repeat length within successive generations, arguing against anticipation (Jackson et al., 2020).

The major risk factor for developing C9-ALS/FTD is a positive family history for ALS and FTD, with a higher risk for individuals with at least three affected family members

(Mahoney et al., 2012). Although not reproducible in all the cohorts, patients with ALS and *C9orf72* pathogenic expansion present an earlier age at onset (Byrne et al., 2012); (García-Redondo et al., 2013) and shorter survival in respect to non-C9-ALS (Sabatelli et al., 2012); (Stewart et al., 2012). Disease duration is shorter in patients with G_4C_2 expansion (Millecamps et al., 2012) and age-matched survival is reduced in C9-ALS compared with non-expansion carriers (20 versus 26 months) (Byrne et al., 2012).

Patients carrying *C9orf72* expansion have a marked phenotypic heterogeneity, which is due to the length and the variability of HRE and its interaction with genetic and environmental modifiers (Woollacott and Mead, 2014). Most patients present ALS, FTD or both. Bulbar presentation is more frequent in C9-ALS than in sporadic and other familial forms (Chiò et al., 2012); (Ratti et al., 2012); (Snowden et al., 2013). Further, among patients with C9-related motor neuron disease (MND), presentations with primary lateral sclerosis (PLS) and progressive muscular atrophy (PMA) are also observed. Cognitive impairment, until overt dementia, and behavioral dysfunction are more frequent in C9-ALS patients in comparison with other forms of MND (Patel and Sampson, 2015). Parkinsonism may present as well – either in isolation or in association with typical clinical ALS/FTD manifestations – as the akinetic-rigid variant poorly responsive to levodopa (Boeve et al., 2012); (Mahoney et al., 2012); (O'Dowd et al., 2012). Also, several studies have found *C9orf72* HRE in patients with a clinical diagnosis of other neurodegenerative disorders, such as Parkinson's disease (PD), parkinsonism, FTLD-associated parkinsonism, progressive supranuclear palsy (PSP), corticobasal degeneration (CBD), ataxic syndromes, and Alzheimer's disease (AD) (Beck et al., 2013; Lesage et al., 2013; Majounie et al., 2012a; Xi et al., 2012).

3. *C9orf72* gene and the G_4C_2 repeat expansion

The *C9orf72* gene is placed on the short arm of chromosome 9 (9p21), contains twelve exons and generates three transcription variants by using alternative transcription start and termination sites (Figure 1). The HRE is located in a non-coding portion of the gene, in the first intron of variants 1 and 3, and within the promoter region of the variant 2. Despite the intronic location of the G_4C_2 repeats in variants 1 and 3, they are likely retained in the mature messenger RNA (mRNA) and successfully translated. Central nervous system (CNS) tissue shows a higher expression of variant 2 compared with variants 1 and 3.

The minimum length that increases the risk of disease, and its relationship with the clinical phenotype are not clearly understood. The normal repeat size is variable, with more than

95% of healthy individuals who have fewer than 11 hexanucleotide repeats (Woollacott and Mead, 2014); 30 units are usually considered a threshold for normal phenotypes. C9-ALS/FTD patients typically harbor a number of repeats between several hundreds and thousands (from 400 to more than 4400 repeats), but some ALS cases present repeat lengths between 24 and 28 (Byrne et al., 2012; Millecamps et al., 2012; Ratti et al., 2012) and a part of FTD patients harbor a range of repeats between 20 and 22 (García- Redondo et al., 2013). On the other hand, healthy carriers may have expansions larger than 400 units, reduced disease penetrance and higher age at symptom onset (Beck et al., 2013). An intermediate-range of G_4C_2 repeats, varying between 7 and 30 repetitions, is over the typical healthy controls' values and may not be clearly pathological (van der Zee et al., 2013), though a recent meta-analysis shows an association between intermediate repeat expansion of 24 to 30 repeats and ALS (Iacoangeli et al., 2019).

The relationship between repeat size and age of onset is unclear. Paradoxically, larger repeat sizes correlate with later age at onset in C9-ALS and C9-FTD (Beck et al., 2013); so far, only one study suggests that longer pathogenic repeats are associated with earlier age of onset (Gijselinck et al., 2016).

C9orf72-related disorders present the phenomenon of somatic instability, which refers to the high variability of repeat expansion size within different tissues in the same patients (Nordin et al., 2015; van Blitterswijk et al., 2013). Somatic instability can explain why the detection of intermediate expansion in blood samples may correspond to massive repeats in the brain (Rohrer et al., 2015). Besides, fluctuations in repeat length have been reported in parental-offspring transmission as well, with a tendency towards a contraction in repeat length over successive generations (Jackson et al., 2020).

4. Protein function

C9orf72-related transcript variants encode for two different protein isoforms. Transcript variants 2 and 3 are translated into the full-length *C9orf72* protein (481 amino acids), and variant 1 yields a predicted short protein (222 amino acids) (Figure 1) (Woollacott and Mead, 2014). It is not clear whether the predicted short isoform *C9orf72* is present in nature. Xiao et al. have shown a decreased short isoform expression in nuclear membranes of C9-ALS/FTD cases compared with controls, by using an antibody selectively targeting the predicted short isoform of *C9orf72* protein (Xiao et al., 2015). *C9orf72* may be exclusively expressed as the full-length isoform in mouse and human tissues, as displayed

using antibodies against the N-terminal region of *C9orf72* able to recognize both the full-length and the predicted short isoform (Frick et al., 2018; Saberi et al., 2018).
Recently, the physiological function of the full-length *C9orf72* protein is emerging. Bioinformatic studies show that *C9orf72* has structural sequence homology with Differentially Expressed in Normal and Neoplasia (DENN) proteins, which work as Rab-Guanosine Exchange Factor (Rab-GEF) regulators of endosomal trafficking by activating Rab-GTPases (Levine et al., 2013; Zhang et al., 2016). Indeed, Rab proteins interact with vesicular components, regulate the intracellular transport of different molecules and alternate between two conformational states (the active GTP-bound form and the inactive GDP-bound form). Specifically, Rab-GEF regulators promote the release of GDP from Rab and exchange it for GTP. Consistent with these data, a study performed on neuronal cells and human spinal cord samples displayed that *C9orf72* colocalizes and coprecipitates with different Rab proteins involved in endosomal trafficking (Farg et al., 2014), supporting the role of *C9orf72* as Rab-GEF in regulating endosomal transport. Besides, the endosomal system is implicated in protein regulation, sorting and degradation through the UPS and the autophagy pathway. Farg and colleagues have demonstrated that *C9orf72* colocalizes with ubiquilin-2 and Rab proteins, co-travels with lysosome-stained vesicles in neuronal cell lines and regulates the autophagy process in neuronal cells, primary cortical neurons and human spinal cord cells (Farg et al., 2014). In support of this hypothesis, short interfering RNA (siRNA)-mediated silencing of *C9orf72* leads to endocytosis inhibition as well as autophagy dysregulation (Farg et al., 2014).
C9orf72 is important also in other cellular processes: it may regulate synaptic vesicles (Frick et al., 2018) and lysosomal function (Amick and Ferguson, 2017), drive the formation of SGs (Maharjan et al., 2017), as well as play a role in NCT (Xiao et al., 2015) and inflammatory response (O'Rourke et al., 2016) (see 7. Downstream mechanisms).

4.1. Possible neurodevelopmental role of *C9orf72*

Neurodevelopmental defects might contribute to neurodegeneration in repeat expansion disorders, such as in spinocerebellar ataxia (Edamakanti et al., 2018; Kovacs et al., 2014) and Huntington disease (Siebzehnrübl et al., 2018; van der Plas et al., 2019). As shown in rodent and fish models, *C9orf72* is expressed during nervous system development (Atkinson et al., 2015; Ciura et al., 2013). So far, no study has demonstrated a robust physiopathological link between *C9orf72* activation during early CNS development and late-onset neurodegeneration. However, a C9-ALS/FTD zebrafish model supports a

possible neurodevelopmental role of *C9orf72* in the pathogenesis of C9-ALS/FTD. Consistently with this hypothesis, morpholino-induced *C9orf72* knockdown decreases motility and axonogenesis and increases neuronal apoptosis in fish embryos (Yeh et al., 2018). Particularly, lack of *C9orf72* is associated with reduced GTPase activity and increased expression of TP53 and CCNG1 (encoding cyclin G1, a protein involved in the regulation of the cell division cycle). Accordingly, morpholino-induced downregulation of cyclin G1 rescues the axonal and motility defects (Yeh et al., 2018).
Although it is unclear how *C9orf72* regulates the transcription of cyclin G1, this study shows that reduced *C9orf72* expression might affect neural apoptosis. Programmed cell death is a crucial process for proper neurodevelopment and cyclin G1 could play a role in this process. Thus, at least in zebrafish embryos, the *C9orf72*-cyclin G1 cascade may be involved in C9-ALS/FTD at a neurodevelopmental level.

5. Neuropathology

Most ALS and approximately half of FTD cases show TAR-DNA binding protein 43 (TDP-43) inclusions in the nucleus and cytoplasm of neurons and oligodendroglia (Neumann et al., 2006), and are usually acknowledged as FTLD-TDP. These aggregates display abnormal TDP-43 phosphorylation and ubiquitination and truncated C-terminal fragments of TDP-43 (Arai et al., 2006; Neumann et al., 2006). Neuropathology and genetic alterations are correlated and C9-ALS/FTD cases are associated with type A and type B FTLD-TDP, according to morphology and distribution of dystrophic neurites and inclusions (Mackenzie et al., 2011). TDP-43 pathology is observed in the frontal and temporal cortices, including the hippocampus, and the primary motor cortex (Mackenzie et al., 2014).
Besides, brains of C9-ALS/FTD patients display cytoplasmic, and to a lesser extent nuclear, TDP-43-negative, p-62 and/or ubiquilin-2-positive inclusions (Al-Sarraj et al., 2011; Troakes et al., 2012). TDP-43-negative inclusions are composed of DPRs (Mori et al., 2013a; Zu et al., 2013). They are mainly found in the cerebellum, hippocampus, and neocortex (Al-Sarraj et al., 2011; Troakes et al., 2012), where TDP-43 pathology is absent or less common. DPRs derive from repetitive RNAs translated via an unconventional mechanism called repeat-associated non-ATG (RAN) translation to form five different DPRs (Ash et al., 2013; Gendron et al., 2013; Mori et al., 2013a, 2013b; Zu et al., 2013). Sense transcripts generate poly-Gly-Ala (GA) and poly-Gly-Arg (GR), while antisense

transcripts yield poly-Pro-Arg (PR) and poly-Pro-Ala (PA); poly-Gly-Pro (GP) are predominantly translated from the sense transcripts (Gendron et al., 2013), but can also be produced from the antisense strand with a different C terminus (Figure 2) (Zu et al., 2013). The most frequent subtype of DPR in post-mortem brain tissues is poly-GA, followed by poly-GP and poly-GR (Davidson et al., 2016; Gomez-Deza et al., 2015; Mackenzie et al., 2015). DPRs are mainly distributed in the cerebellum, hippocampus and neocortex, while they are rare in subcortical regions, brainstem and spinal cord. Further, poly-GP and poly-GA are present both in CNS tissue (Gendron et al., 2015) and in cerebrospinal fluid (CSF) (Su et al., 2014) of C9-patients, being potentially useful biomarkers for diagnosis and evaluation of treatment efficacy.

The role of DPRs as drivers of toxicity and neurodegeneration in C9-ALS/FTD is still debated. The neuropathological load of DPRs and TDP-43 inclusions do not correspond and DPRs and TDP-43 rarely coexist in the same cell (Davidson et al., 2014; Mackenzie et al., 2013). Moreover, DPR concentrations are lower in tissues most affected by ALS, such as spinal MNs, reach maximum levels in less affected tissues, i.e. the cerebellum (Gomez-Deza et al., 2015; Mackenzie et al., 2015), and, unlike TDP-43 pathology, DPR inclusions do not strongly correlate with neurodegeneration (Mackenzie et al., 2013). One possible interpretation is that DPRs-mediated toxicity may occur in an early stage of the disease, and might be less evident in post-mortem samples since most affected neurons are dead in advanced stages. Soluble proteins may drive toxicity and lead to neuronal loss, whereas aggregates may represent protective species; thus, the absence of correlation with DPR inclusions may be misleading. A recent neuropathological study supports the pathogenic role of DPRs in C9-ALS/FTD; poly-GR, but not other DPR subtypes, are significantly higher in affected regions compared with unaffected ones, and form aggregates co-localizing with phosphorylated TDP-43 in the motor cortex (Saberi et al., 2018).

The relationship between TDP-43 pathology and DPRs is still unclear. DPR-mediated toxicity might predate TDP-43 pathology in *C9orf72* carriers, as shown in some studies reporting DPR aggregates but mild or absent TDP-43 pathology in post-mortem diseased brains (Baborie et al., 2015; Gami et al., 2015). Accordingly, TDP-43 pathology correlates closer with neurodegeneration, since it is likely to occur downstream of DPR pathology.

Other than TDP-43 and DPRs, RNA foci pathology is also a typical finding in C9-ALS/FTD and it is mostly represented in the frontal cortex, hippocampus, cerebellum and spinal cord (Zu et al., 2013). RNA foci are mainly found in neuronal nuclei, while they are sporadically reported in the cytoplasm (Lagier-Tourenne et al., 2013, p.; Mizielinska et al.,

2013). Sense RNA foci are predominant compared with antisense ones (Mizielinska et al., 2013). The highest portion of RNA foci is located in cerebellar Purkinje cells (DeJesus-Hernandez et al., 2017). Sense transcripts and inclusions containing sense-derived DPRs are most abundant in cerebellar granule neurons, while antisense foci and their derived DPR inclusions are found mainly in MNs (Cooper-Knock et al., 2015). As shown in a large-scale study, RNA foci neuropathological burden is not correlated with clinical variables and neurodegeneration (DeJesus-Hernandez et al., 2017).

C9orf72 transcripts are reduced in several brain regions and *C9orf72* protein levels are decreased within the frontal cortex (Waite et al., 2014) and cerebellum of C9-carriers compared with non-carriers (van Blitterswijk et al., 2015). Consistently, the long isoform of C9orf72 protein is reduced to 80% in the cerebellum of C9-carriers compared with controls (Frick et al., 2018).

Taken together, these findings provide evidence of TDP-43, DPR and RNA foci pathology and post-mortem brain tissues in C9-ALS/FTD patients. *C9orf72* protein expression is reduced both in affected and unaffected brain areas, and its role in pathogenesis is still debated.

Recently, many *in vitro* and *in vivo* models have elucidated the potential mechanisms involved in C9-ALS/FTD pathogenesis.

6. Mechanisms of neurodegeneration

Three different but not mutually exclusive mechanisms contribute to *C9orf72*-mediated neurodegeneration: loss of function of *C9orf72* protein due to haploinsufficiency, toxic gain of function from sense and antisense RNA foci, which are bidirectionally transcribed from HRE into repetitive RNAs, and toxic gain of function from DPRs, derived from repetitive RNA non-canonical translation. All these processes play a role in C9-ALS/FTD pathogenesis to some extent, and they are likely responsible for the clinical, pathological and prognostic heterogeneity observed in C9-ALS/FTD cases. However, their relative contribution in different patients and at different disease stages is still unclear.

6.1. Loss of function

Consistent with the loss-of-function hypothesis, C9-ALS patients present decreased *C9orf72* transcripts in blood lymphocytes, frontal cortex, spinal cord and cerebellum compared with controls (Ciura et al., 2013; Gijselinck et al., 2012; van Blitterswijk et al.,

2015; Xi et al., 2013). Accordingly, *C9orf72* HRE carriers have reduced concentrations of *C9orf72* protein, probably as a consequence of loss of transcription from the mutant allele (Waite et al., 2014).

Epigenetic modifications may partially explain the reduced expression of *C9orf72*. DNA analysis from C9-ALS patients has revealed hypermethylated HRE in almost the totality of cases (Xi et al., 2015). HRE binds to trimethylated histone H3 at lysine 9 (H3K9) and 27 (H3K27) inducing epigenetic repression and reducing *C9orf72* mRNAs (Belzil et al., 2013); the non-pathogenic repeats show no histone binding. Moreover, approximately one-third of cases display hypermethylation in the CpG island in the *C9orf72* promoter region upstream of the HRE (Xi et al., 2014, 2013). Particularly, hypermethylation is associated with increased repeat length and reduced transcription of *C9orf72*, explaining how HRE could lead to loss of function, without excluding a role for length-dependent toxic gain of function mechanisms (Gijselinck et al., 2016). Moreover, the degree of hypermethylation is associated with a later age of onset in C9-ALS/FTD patients (Russ et al., 2015) and with reduced disease duration in C9-ALS patients (Xi et al., 2013).

Researchers have used different *in vivo* and *in vitro* models to investigate the haploinsufficiency hypothesis, yielding conflicting evidence. *C9orf72* loss-of-function causes MN degeneration and motor deficits in *C. elegans* and zebrafish models (Ciura et al., 2013; Therrien et al., 2013). Similarly, haploinsufficient human-induced MNs exhibit impaired vesicle trafficking and lysosomal biogenesis, two cellular downstream mechanisms disrupted in C9-ALS/FTD (Shi et al., 2018). Conversely, divergent evidence exists for rodent models. *C9orf72* haploinsufficient mice lack ALS- or FTD-like phenotypes (Koppers et al., 2015), show a dysregulated immune system (Sudria-Lopez et al., 2016), possibly through *C9orf72* effect on autophagosome, and exhibit neurodegeneration, when depleted of genes involved in autophagy (e.g. ATG5 and ATG7) (Hara et al., 2006; Komatsu et al., 2006), suggesting a non-relevant role for *C9orf72* protein in regulating autophagy and neuronal survival. On the contrary, in another study, haploinsufficient rodents display alleviated neurodegeneration by restoring *C9orf72* levels or increasing its function with modulators of Rab5 protein (Shi et al., 2018).

Clinical evidence still argues against haploinsufficiency as the main mechanism of the disease. Indeed, C9-ALS patients lack loss-of-function mutations in *C9orf72* (M. B. Harms et al., 2013a) and patients homozygous for *C9orf72* repeat expansion do not present with a more severe phenotype (Fratta et al., 2013).

6.2. Gain of function

6.2.1. Toxic gain-of-function from repeat-containing RNAs

Repeat-containing RNAs transcribed from HRE aggregate into sense and antisense RNA foci, mainly located in the nucleus and occasionally in the cytoplasm (Donnelly et al., 2013; Lagier-Tourenne et al., 2013; Mizielinska et al., 2013; Zu et al., 2013). Sense and antisense RNA foci accumulate in neurons, fibroblasts, glial cells, astrocytes, microglia, and oligodendrocytes (Gendron et al., 2013; Lagier-Tourenne et al., 2013; Mizielinska et al., 2014; Zhang et al., 2014) and their RNAs can form high-order secondary structures, such as G-quadruplexes, hairpins, and i-motifs (Fratta et al., 2013; Haeusler et al., 2014; Kovanda et al., 2015; Reddy et al., 2013; Šket et al., 2015). Further, repeat-containing RNAs hybridize with HRE containing DNA, creating aberrant R-loops (Haeusler et al., 2014; Reddy et al., 2014), three-stranded nucleic acid structures which affect transcription of surrounding regions (O'Rourke et al., 2015) and lead to genome instability (O'Rourke et al., 2015). Different iPSN lines have displayed the high variability of RNA foci levels without showing a direct correlation with the number of repeats, thus suggesting that RNA foci formation can be influenced by other genetic or epigenetic factors (Almeida et al., 2013).

The main hypothesis supporting the RNA foci–mediated toxicity in MNs involves RNA metabolism impairment. RNA foci may sequester RNA binding proteins (RBPs), inducing their depletion, and subsequently resulting in neurodegeneration (Fratta et al., 2012; Haeusler et al., 2014). A large number of RBPs potentially interact with expanded repeat RNAs *in vitro*, including ADARB2, RanGAP, hnRNPA1, hnRNPA3, Pur-α, FUS, TDP-43, nucleolin, SRSF1, SRSF2, ALYREF (Donnelly et al., 2013; Lee et al., 2013; Sareen et al., 2013). However, only ADARB2 and hnRNP-H seem to colocalize with intranuclear RNA foci with high frequency (Donnelly et al., 2013). Two landmark publications have shown that brain tissues from C9-ALS patients present intranuclear G_4C_2 RNA foci, gene expression dysregulation and RBPs sequestration; these phenotypes can be mitigated by ASOs targeting the *C9orf72* transcript or the HRE (Donnelly et al., 2013; Sareen et al., 2013). Altogether, these findings support the contribution of RNA toxicity in causing neurodegeneration through a gain-of-function mechanism.

Although the pathogenic role of RNA foci is still debated, both *in vitro* and *in vivo* studies have provided evidence of RNA foci pathology in C9-ALS (Table 1). Primary cortical MNs expressing increased G_4C_2 repeats exhibit RNA foci accumulation and decreased survival (Wen et al., 2014). A Drosophila model harboring 30 G_4C_2 repeats shows RNA-mediated

eye and motor neuron degeneration (Xu et al., 2013). C9-ALS/FTD zebrafish embryos exhibit sense and antisense RNA repeats toxicity as well as motor axonopathy (Swinnen et al., 2018). Conversely, adult models of Drosophila do not confirm these findings, lacking either nuclear or cytoplasmic sense and antisense RNA mediated toxicity, when DPR expression is prevented (Moens et al., 2018). These results may likely reflect higher susceptibility of developing neurons to RNA toxicity compared with adult neurons or the expression of different types of RBPs in human tissues, thus limiting the use of flying models to observe RNA toxicity.

Despite the unclear pathogenic role of RNA-mediated toxicity, a therapy targeting the repeat-containing transcripts could potentially reduce RNA foci (Hu et al., 2015; Jiang et al., 2016; Martier et al., 2019a), alleviating disease progression.

6.2.2. Toxic gain-of-function from DPRs

Repeat RNAs from *C9orf72* HRE are localized into the nucleus, but they can escape and associate with ribosomal complexes in the cytoplasm. As a consequence, RAN translation of transcribed HRE leads to the accumulation of cytoplasmic DPR proteins (Ash et al., 2013; Mori et al., 2013a; Zu et al., 2013). Arginine-rich DPRs, most of all poly-PR, are considered the main effectors of DPR toxicity (Moens et al., 2017; O'Rourke et al., 2015; Wen et al., 2014); poly-GA, and to a lesser extent poly-PA, are also responsible for neurotoxicity (Jovičić et al., 2015). Poly-GP is unlikely to be a toxic species. However, the exact length and composition of different DPR proteins in C9-patients is not completely known and other unconventional translational mechanisms beside RAN might be involved in DPR generation (Gao et al., 2017), suggesting that DPR diversity could be much larger than theoretically expected.

Since G_4C_2 repeats can generate both RNA foci and DPR proteins, the toxicity of DPRs and their relative contribution to C9-ALS pathogenesis are still debated. In support of a DPRs-driven toxicity, *in vitro* (Mizielinska et al., 2014; Wen et al., 2014; Zhang et al., 2014; Zu et al., 2013) and *in vivo* studies in Drosophila (Boeynaems et al., 2016; Freibaum et al., 2015; Lee et al., 2016, p. 201; Mizielinska et al., 2014), Zebrafish (Ohki et al., 2017; Swaminathan et al., 2018; Swinnen et al., 2018) and mouse models (Hao et al., 2019; O'Rourke et al., 2015; Schludi et al., 2017) have shown neurotoxicity related to DPR protein accumulation. In particular, pure repeats expression, but not RNA-only repeats (i.e. stop codon enriched repeats, unable to be translated into DPRs) leads to neurodegeneration in Drosophila models (Mizielinska et al., 2014). For instance, a fly model expressing 160

G_4C_2 repeats lacks neurodegeneration and presents normal lifespan, when only RNA foci — and not DPRs — are produced (Tran et al., 2015). Conversely, when increasing transgene transcription and RNA translocation in the cytoplasm (Drosophila model with 36 G_4C_2 repeats transcribed as a poly(A) mRNA), Tran and colleagues have observed DPR toxicity and reduced lifespan without an increase in RNA foci generation, supporting the hypothesis of a DPRs rather than RNA foci mediated-neurodegeneration (Tran et al., 2015). In parallel, accumulated DPRs may disrupt downstream cellular processes, such as NCT (Jovičić et al., 2015) and RNA processing (Jovičić et al., 2015; O'Rourke et al., 2015). As a consequence, protein translation (Hartmann et al., 2018; O'Rourke et al., 2015), nucleolar stress (Tao et al., 2015), alteration in UPS (O'Rourke et al., 2015) and SGs formation (Boeynaems et al., 2017) are dysregulated. Further, DPRs, and not RNA foci accumulation, can prompt cytoplasmic mislocalization and accumulation of TDP-43, which in turn can increase DPR levels in a feedback cycle, contributing to G_4C_2-related toxicity (Solomon et al., 2018).

Despite numerous *in vitro* and *in vivo* evidence, analysis of post-mortem tissues from C9-ALS/FTD patients still challenges a gain-of-toxic function mechanism by DPR species. Indeed, DPRs are less accumulated in the brain regions most affected in ALS/FTD and their levels do not correlate with clinical phenotype, disease severity and neuronal loss (Davidson et al., 2014; Gomez-Deza et al., 2015, p.; Mackenzie et al., 2013, 2015). This may depend on a precocious involvement of DPR proteins in disease pathogenesis, suggesting that neurons carrying toxic DPRs are almost entirely dead in the later stages of the disease.

Although DPR overexpression might not necessarily reflect patients' endogenous pathogenesis, taken together, these findings support gain-of-function toxicity in C9-ALS.

6.3. Loss- and gain-of-function mechanisms in mouse models

So far, scientists have failed to develop a reliable murine model that entirely encompasses *C9orf72*-related pathophysiology. The greatest challenge is how to recapitulate the three features of C9-ALS/FTD pathogenesis (i.e. haploinsufficiency of *C9orf72* protein, RNA toxicity and DPR toxicity) in one model, and to observe a clinical phenotype as similar as possible to disease in humans. On the one hand, loss-of-function models have failed to demonstrate neurodegeneration and ALS or FTD phenotype, suggesting that haploinsufficiency is insufficient to trigger and precipitate the disease; on the other hand,

gain-of-function models are highly heterogeneous and do not reflect the exact genetic background observed in patients.

Murine *C9orf72* shares 98% homology with human ortholog and is expressed in embryonic and early postnatal neurons, glial cells and various non-neuronal tissues (Atanasio et al., 2016; Ferguson et al., 2016; O'Rourke et al., 2015; Suzuki et al., 2013). Ubiquitous knockout of *C9orf72* causes lymphadenopathy and splenomegaly, due to immune system dysregulation (Atanasio et al., 2016; O'Rourke et al., 2015; Sudria-Lopez et al., 2016) Even if some models have shown reduced lifespan (Atanasio et al., 2016; O'Rourke et al., 2015; Sudria-Lopez et al., 2016) or mild motor and cognitive impairment (Atanasio et al., 2016; O'Rourke et al., 2015), an overt neurodegenerative phenotype in knock-out (KO) mice still lacks (Burberry et al., 2016; Jiang et al., 2016; Koppers et al., 2015; O'Rourke et al., 2015). Otherwise, conditional neuronal and glial *C9orf72* knockout (O'Rourke et al., 2015) and transient reduction of *C9orf72* expression in CNS by ASOs (Lagier-Tourenne et al., 2013) do not lead to cognitive or motor phenotype.

Researchers have generated several transgenic mouse models, evaluating RNA foci, DPR and TDP-43 pathology as well as behavioral and motor phenotypes (Table 1). Adeno-associated virus (AAV)-mediated CNS delivery of 66 G_4C_2 repeats (Chew et al., 2015) and poly-GA (O'Rourke et al., 2015; Schludi et al., 2017) leads to motor and behavioral phenotype, RNA foci and DPR pathology. In the model proposed by Chew et al, mice exhibit TDP-43 pathology and cortical and cerebellar neuronal loss (Chew et al., 2015), while others fail to show TDP-43 inclusions (O'Rourke et al., 2015) and neurodegeneration (Schludi et al., 2017), suggesting that poly-GA might not be the main drivers of TDP-43 accumulation in C9-ALS. A transgenic poly-PR mouse model displays motor phenotype, decreased survival time and neuropathological abnormalities in the cerebellum and spinal cord, supporting a role for poly-PR in C9-related neurodegeneration (Hao et al., 2019). Bacterial artificial chromosome (BAC) transgenic mice enable the expression of the human *C9orf72* sequence, surrounded by regulatory regions and flanking sequences. Different BAC transgenic mouse models show RNA foci and DPR pathology, but neither TDP-43 aggregates nor reduced survival and neurodegeneration (O'Rourke et al., 2015; Peters et al., 2015). HRE-directed ASO administration reduces RNA foci and DPR accumulation and ameliorates behavioral deficits (O'Rourke et al., 2015). Another transgenic model displays frank neurodegeneration, encompassing both C9-related pathology (e.g. RNA foci, DPR aggregate, and TDP-43 inclusions) and motor paralysis with reduced survival (Y. Liu et al., 2016).

So far, a unified and reproducible rodent model that conveys each aspect of *C9orf72*-related disease does not exist. This is likely due to the large heterogeneity of mice in terms of genetic background, flanking *C9orf72* sequences and other possible unknown contributors.

6.4. Interaction between loss- and gain-of-function disease mechanisms

C9-ALS is largely considered a gain of function disease. Supporting this hypothesis, ASOs binding HRE rescue toxicity and ameliorate pathologic phenotype in iPSNs (Donnelly et al., 2013)), while ASO-mediated knock-down does not affect C9-ALS/FTD-related neuropathology *in vitro* (Sareen et al., 2013). Moreover, knockout mouse models lack neurodegeneration.

However, a growing body of evidence suggests that loss- and gain-of-function disease mechanisms might cooperate in addressing C9-mediated neurodegeneration (Abo-Rady et al., 2020; Shi et al., 2018). Reduced *C9orf72* activity can trigger MNs' death by impairing DPR clearance and modulating vulnerability of human MNs to degenerative stimuli (Shi et al., 2018). Consistently, restored *C9orf72* levels rescue MN survival and ameliorate neurodegeneration in both gain- and loss-of-function C9 mouse models (Shi et al., 2018). Interestingly, a double-hit mechanism involving haploinsufficiency and DPRs-mediated toxicity may play a role in neuronal death in C9-ALS (Boivin et al., 2020).

Given that autophagy prevents DPR accumulation (Cristofani et al., 2018), reduced expression of *C9orf72* protein, which regulates autophagy, leads to DPR accumulation, resulting in cell death. Thus, Boivin and colleagues suggest that *C9orf72* protein deficiency, by impairing autophagy, could increase DPR toxicity (Boivin et al., 2020). Accordingly, pharmacological activators of autophagy, e.g. mammalian target of rapamycin (mTOR) inhibitors and phenothiazine derivatives, reduce neuronal cell death caused by DPR accumulation (Boivin et al., 2020).

Additional proof supporting a gain- and loss-of-function interaction model has emerged from a recent work conducted *in vitro* using isogenic human iPSCs-derived MNs (Abo-Rady et al., 2020). Compared to MNs harboring HRE alone, MNs having both HRE and *C9orf72* KO show disrupted axonal trafficking, increased apoptosis, altered transcriptome expression and decreased levels of HSP70 and HSP40 (Abo-Rady et al., 2020). Conversely, *C9orf72* KO lines do not display any significant changes. Other than suggesting a possible role for heat shock proteins (HSPs) in driving C9 pathogenesis, these results point at a model where loss-of-function mechanisms exacerbate the effects of gain of function toxicity (Abo-Rady et al., 2020).

Thus, these data provide new insights into C9-ALS/FTD pathogenesis, suggesting that either DPR expression or *C9orf72* loss-of-function is not sufficient to induce the pathology alone. A loss-of-function mechanism might contribute to disease pathogenesis and enhance gain-of-function toxicity. As if one model could not explain neurodegeneration alone, the two pathogenic mechanisms may coexist and interact synergistically.

7. Downstream mechanisms

In addition to *C9orf72* loss- and gain-of-function mechanism, various studies have focused on downstream cellular alterations resulting from the HRE pathogenic cascade, including altered nucleocytoplasmic transport and RNA metabolism, disrupted proteostasis as well as other cellular functions. Researchers have used multiple human and non-human model systems for this purpose (Figure 3).

7.1. Nucleocytoplasmic transport

NCT refers to the active transport of proteins and RNAs through NPCs, an essential process for cellular functions (Knockenhauer and Schwartz, 2016). Different evidence shows that impairment of NCT can play a key role in neurodegeneration, likely representing the most relevant common pathway downstream to a variety of ALS-causing mutations (Kim and Taylor, 2017).

The first evidence of NCT involvement in MNDs derives from the discovery of a mutation in *GLE1*, an NPC component that causes lethal arthrogryposis and anterior horn motor neuron loss (Nousiainen et al., 2008) and that may play a role in adult-onset MND (Kaneb et al., 2015). Other evidence includes ALS-causing mutations in the nuclear localization sequence (NLS) of *FUS* and *hnRNPA1* and the cytoplasmic mislocalization of TDP-43, FUS and hnRNPA1 proteins (Dormann et al., 2010; Q. Liu et al., 2016) and decreased importin β1 and importin β2 mislocalization, two regulators of NCT, in post-mortem brain and spinal cord tissues of C9-ALS/FTD patients (Nishimura et al., 2010). Finally, 18 genes encoding for the NPC complex and proteins involved in NCT act as phenotypic modifiers in Drosophila models, as shown in a large-scale, unbiased genetic screening. Two genes, *Nup50* and *Ref1*, can differentially act on neurodegeneration when knocked-down; the first by increasing and second by lowering neuronal loss (Freibaum et al., 2015).

The definitive association between defects in NPC and C9-ALS derives from the demonstration of an mRNA export defect with RNA nuclear retention in C9-ALS *in vitro* models and Drosophila (Freibaum et al., 2015; Zhang et al., 2015). In a fly model overexpressing 30 G_4C_2 repeats, Ran GTPase-activating protein 1 (RanGAP1), an effector of protein transport from cytoplasm to nucleus, binds to the HRE and is consequently sequestered into sense RNA foci. As a result, RanGAP1 nucleo/cytoplasmic (N/C) distribution is reduced, impairing nuclear RNA export and nuclear protein import (Zhang et al., 2015). Increasing RanGAP1 activity rescues toxicity from HRE and ASOs targeting *C9orf72* HRE recovers altered N/C Ran gradient, suggesting that RNA foci-mediated NCT deficit is a relevant mechanism underlying C9-ALS pathogenesis (Zhang et al., 2015).
In addition to RNA foci, also DPR-mediated toxicity may impair NCT (Jovičić et al., 2015). Jovicic et al. have engineered yeast cells to express five DPR species, showing that arginine-containing DPRs, i.e. poly-GR and poly-PR, are highly neurotoxic (Jovičić et al., 2015). Mechanistically, poly-PR and poly-GR combine with nucleopore proteins (Nups), that compose the central channel of the nuclear pore, and decrease the transport of macromolecules (Shi et al., 2017). Besides, poly-GA aggregates can sequester NCT proteins, such as Pom121, and lead to mislocalization of RanGAP1 in rodents (Zhang et al., 2016).
Although the main cause for the NCT impairment in C9-ALS/FTD is unclear, NCT defects seem to play a role in other neurodegenerative disorders, such as AD (Eftekharzadeh et al., 2018) and Huntington's Disease (HD) (Gasset-Rosa et al., 2017; Grima et al., 2017), possibly acting as a more global factor involved in neurodegeneration.

7.1.1. Nucleocytoplasmic transport and mislocalization of TDP-43

The cytoplasmic accumulation of hyperphosphorylated and ubiquitinated TDP-43 is the pathological signature of TDP-43 proteinopathies, including C9-ALS/FTD (Neumann et al., 2006). TDP-43 is a mainly nuclear RBP, formed by two RNA-recognition motifs, an NLS, and a nuclear export signal (NES), that drive the transfer between the nucleus and cytosol. Its C-terminal fragment contains a prion-like domain enabling protein-protein interactions. As a consequence, mislocalized TDP-43 can form pathologic aggregates (Nonaka et al., 2009). Also, RBPs aberrantly sequestered in the cytosol can impair RNA metabolism and proteostasis, finally resulting in neurodegeneration (Ling et al., 2013).
NPC regulates NCT and is composed of highly conserved nucleoporins that are rich in low complexity sequence domains (LCDs). LCDs are responsible for selective nucleic acid and

protein transport between the nucleus and the cytosol. In the work of Woerner et al., cytoplasmic, and not nuclear TDP-43 aggregation *in vitro* induces sequestration and mislocalization of LCD-containing proteins, including factors of nuclear import and export machinery (Woerner et al., 2016). In addition, NPC components are the main constituents of pathological TDP-43 accumulations, which are responsible for triggering Nup cytoplasmic sequestration in both wild type and TDP-43 mutant cell models (Chou et al., 2018). TDP-43 clusters induce morphological defects in NPCs, nuclear protein import and mRNA export dysfunction, that are potentially rescued by pharmacological inhibition of TDP-43 toxicity (Chou et al., 2018). Similarly, spinal MNs from a C9-ALS zebrafish model display perturbed NCT with TDP-43 accumulation and subsequent neurodegeneration (Svahn et al., 2018), supporting the potential pathogenic role of NCT in C9-ALS *in vivo*.

Recently, NCT dysfunction and TDP-43 mislocalization have gained attention also from a therapeutic point of view. Indeed, Williamson et al. have successfully rescued mislocalized TDP-43 in the cytoplasm by overexpressing the oxidation resistance 1 (Oxr1) protein in primary MNs derived from TDP-43-mutated mice (Williamson et al., 2019)

Taken together, these studies provide *in vitro* and *in vivo* evidence that NCT impairment represents an important mechanism underlying *C9orf72*-related neurodegeneration. Moreover, TDP-43, an important pathological hallmark of ALS-related disorders, might contribute to NCT dysfunction and represent a therapeutic target. However, whether a primary defect in NCT is responsible for TDP-43 mislocalization or TDP-43 aggregation drives secondary NCT impairment is still debated.

7.2. RNA metabolism

Impairment of RNA metabolism in *C9orf72* models involves nucleolar dysfunction, alterations in SGs and processing bodies (P-bodies).

7.2.1. Nucleolar dysfunction

Nucleolus plays an important role in RNA biogenesis and works as a sensor for various types of stress. In response to stress, the nucleolus can change morphology and composition (Boulon et al., 2010). Human brain samples, human iPSCs, Drosophila, mammalian neuronal and nonneuronal cells have served as models to study *C9orf72*-related nucleolus dysfunction. For example, nucleolin, a nucleolar phosphoprotein involved in ribosome

biogenesis that binds G-quadruplexes (Haeusler et al., 2014), is aberrantly localized in iPSC-derived MNs and patients' motor cortex (Conlon et al., 2016), as well as in *C9orf72* BAC transgenic mice (O'Rourke et al., 2015).

DPR toxicity seems to play a role in nucleolar stress. Overexpressed poly-GR and poly-PR induce nucleolar enlargement in Drosophila and mammalian neurons and nonneuronal cells (Kwon et al., 2014; Mizielinska et al., 2017a; Wen et al., 2014; Yang et al., 2015). Moreover, these arginine-rich DPRs impair RNA splicing and ribosomal maturation and accumulate in the nucleolus of cultured astrocytes (Kwon et al., 2014). Overexpressed poly-GR and poly-PR colocalize with nucleoli and induce abnormal nucleolar morphology, as shown in primary neurons, iPSNs and Drosophila (Wen et al., 2014). Similarly, *C9orf72* patient brains exhibit bidirectional changes of nucleolus volumes, with larger nucleolar volumes in neurons containing poly-GR inclusions in the context of generally smaller nucleoli (Mizielinska et al., 2017a). However, suggesting an indirect role in nucleolus dysfunction, DPR proteins do not localize within the nucleolus in *C9orf72* patient brains (Schludi et al., 2015). Thus, the impact of nucleolar dysfunction in *C9orf72* diseased brains still needs to be elucidated (Mizielinska et al., 2017b; Schludi et al., 2015).

7.2.2. Alterations in stress granules

Under stressful conditions, cytoplasmic ribonucleoprotein (RNP) granules, e.g. P-bodies and SGs, assemble to segregate mRNAs, translation initiation factors and other RBPs. When cellular stress releases, RNP granules are degraded by the autophagy pathway or disassemble (Guo et al., 2014). RBPs associated with SGs, such as FUS (Murakami et al., 2015), hnRNP1/2 (Molliex et al., 2015) and TDP-43 (Conicella et al., 2016) tend to fibrilize forming irreversible hydrogels and constitute droplets in vitro, undergoing liquid-liquid phase separation (LLPS) (Molliex et al., 2015; Murakami et al., 2015; Patel and Sampson, 2015). In addition, RBPs incorporate prion-like LCDs that are particularly prone to aggregate in the context of ALS-related mutations (Elbaum-Garfinkle, 2019; Patel and Sampson, 2015).

A relevant pathogenic mechanism in C9-ALS/FTD involves altered assembly or clearance of SGs (Li et al., 2013). Arginine-rich DPRs, such as poly-PR and poly-GR, affect the function of SGs by interacting with LCDs and altering LLPS (Boeynaems et al., 2017; Lee et al., 2016) as confirmed *in vitro* and animal studies. Cortical neurons overexpressing poly-PR exhibit increased SG generation (Wen et al., 2014), while knock-down of different

LCDs results in important toxicity in murine neurons and severe eye degeneration in Drosophila expressing poly-GR (Lee et al., 2016).
Besides, altered SG dynamics impair NCT by sequestering nuclear pore protein in human cultured cells expressing poly-GR and poly-PR (Zhang et al., 2018). Consistently, inhibition of SG assembly reverts NCT alteration and neurodegeneration in patient-derived neurons and *in vivo* (Zhang et al., 2018).

7.2.3. P-bodies and nonsense-mediated mRNA decay

P-bodies are membraneless RNP granules involved in RNA degradation (Balendra and Isaacs, 2018). Particularly, P-bodies have been considered reservoirs for untranslated RNA as well as proteins regulating RNA decay and potentially the site for nonsense-mediated mRNA decay (NMD) (Hubstenberger et al., 2017; Protter and Parker, 2016). NMD eliminates defective mRNA with premature codon stop (He and Jacobson, 2015), regulates expression of genes with long 3′ untranslated regions (Lykke-Andersen and Jensen, 2015) and requires regulatory up-frameshift proteins (UPF1, UPF2, and UPF3) to work properly (He and Jacobson, 2015). Although P-body formation is not entirely necessary for NMD (Eulalio et al., 2007), these key up-frameshift proteins localize in P-bodies (Shukla and Parker, 2016).
Dysregulation of P-bodies through impairment of NMD may play a role in C9-ALS/FTD. Arginine-rich DPRs inhibits NMD in cultured cells (Sun et al., 2019; Xu et al., 2019) and this may be caused by reduced formation of P-bodies (Xu et al., 2019). *C9orf72* cellular and Drosophila models exhibit decreased arginine-rich DPR-related neurotoxicity when genetic (UPF 1 and UPF2 overexpression) and pharmacological approaches (the NMD-activating drug tranilast) reactivate the NMD pathway (Xu et al., 2019).
NMD exerts a protective role in HRE-mediated toxicity through the activation of eukaryotic translation termination factor 1 (eRF1). When HRE is present, RNA binding proteins are sequestered by RNA foci and DPR aggregates (Boeynaems et al., 2017; Hautbergue et al., 2017), impairing mRNA splicing; this cascade activates mRNA surveillance pathways, including NMD (Zhang et al., 2009). In C9-HRE MNs, the overexpression of eRF1 inhibits protein translation and triggers NMD-mediated mRNA degradation, reducing toxic RNA transcripts and thus the formation of RNA foci and DPR aggregates. The overexpression of NMD master regulator UPF-1 – which is recruited by eRF1 when mRNA splicing is altered – yields similar results (“Nucleocytoplasmic Proteomic Analysis Uncovers eRF1 and Nonsense-Mediated Decay as Modifiers of

ALS/FTD C9orf72 Toxicity," 2020). Thus, these findings suggest that NMD-mediated mRNA decay may play a protective role in C9-ALS/FTD and pinpoint NMD as a possible therapeutic target.
Altogether, these studies show that dysregulation of P-bodies and SG dynamics and function may be an important pathogenic mechanism in C9-ALS/FTD.

7.3. Proteostasis

The protein homeostasis or proteostasis refers to a complex cellular regulatory network involving protein biosynthesis, folding, translocation, assembly/disassembly, and clearance. Cell survival is inseparably tied to protein quality control, and dysregulation of protein metabolism can play a pathogenic role in C9-ALS/FTD affecting different mechanisms (Medinas et al., 2017). Here, we focus on the role of autophagy and lysosomes as well as the UPS.

7.3.1. Translation inhibition and Ubiquitin proteasome system

Both arginine-rich DPRs and G_4C_2 repeat-containing RNAs can impair protein translation. Overexpressed poly-GR and poly-PR halt translation in an *in vitro* translation assay and in cell lines by directly binding to mRNA, thus disrupting the translational apparatus (Kanekura et al., 2016). Poly-PR and poly-GR block elongation factors and ribosome subunits as well as translation initiation (Kanekura et al., 2016). G_4C_2 repeat-containing RNAs sequester ribosomal subunits and trigger SGs, inhibiting in turn global translation (Fay et al., 2017; Green et al., 2017).
UPS plays a fundamental role in degrading unfolded, misfolded, or aggregated proteins and is impaired in C9-ALS/FTD (Guo et al., 2018). Poly-GA-induced toxicity is associated with UPS impairment (Yamakawa et al., 2015; Zhang et al., 2016) and leads to reduced proteasome activity (Zhang et al., 2014). Consistently, poly-GA aggregates sequester multiple UPS components and may functionally impair poly-GA-associated proteasomes (Guo et al., 2018; Zhang et al., 2016). Indeed, 3D imaging of the cell interior using cryo-electron tomography reveals that poly-GA accumulation sequesters subunits of UPS intracellularly, i.e. 26S proteasome complexes (Guo et al., 2018). This segregation depletes proteasome from other cellular functions critical for proteostasis, e.g. endoplasmic reticulum (ER)-associated protein degradation (Zhang et al., 2014), damaging neurons. In

addition, when proteasomes interact with poly-GA adopt a highly transient intermediate state of activation that reduces their functionality (Guo et al., 2018).
These results suggest that G_4C_2 repeat-containing RNAs and DPRs impair protein translation and UPS function affecting cellular proteostasis and contributing to C9-ALS/FTD disease pathomechanisms.

7.3.2. Autophagy

Autophagy refers to a cytoplasmic clearance pathway in which lysosomes degrade dysfunctional proteins and damaged organelles, a process altered in several neurodegenerative diseases (Menzies et al., 2017). A partial *C9orf72* loss-of-function reduces *C9orf72* protein binding to autophagy regulating proteins, such as ULK1 and SMCR8, impairing autophagy (Amick et al., 2016; Jung et al., 2017; Sellier et al., 2016). Consistent with this evidence, lack of *C9orf72* and SMCR8 alters the expression of the autophagy markers LC3 and p62 (Sellier et al., 2016; Ugolino et al., 2016; Yang et al., 2015).
A recent study has elucidated the role of *C9orf72* protein and p62 in regulating autophagy-mediated SG clearance (Chitiprolu et al., 2018). Physiologically, *C9orf72* protein interacts with the autophagy receptor p62 and drives SG clearance by autophagy (Chitiprolu et al., 2018); this process depends upon the binding between p62 and proteins that are symmetrically methylated on arginines, such as SMN and FUS. Rodents lacking p62 exhibit altered FUS-dependent splicing and accumulate arginine-methylated proteins (Chitiprolu et al., 2018). Patients' neurons accumulate symmetric arginine dimethylated proteins that recruit p62 and are unable to eliminate them in SGs by autophagy.
Thus, these results suggest that *C9orf72* regulates C9-ALS-related proteins (p62, SMN, FUS) and contributes to SG degradation by autophagy.

7.3.3. Lysosomes and cell starvation

Lysosomes are fundamental components of the autophagy degradative pathway. Recently, different researches have linked C9-ALS/FTD with altered immune response in macrophages as well as in microglia (O'Rourke et al., 2016; Sullivan et al., 2016) and lysosomal dysfunction (Corrionero and Horvitz, 2018; Zhang et al., 2018). *C9orf72*-knockout mice display defective microglia – which may contribute to disease progression and lead to age-related inflammation in the spleen and CNS – and exhibit altered lysosomal

accumulation (O'Rourke et al., 2016). Similarly, brain and spinal cord patients' samples display up-regulated pathways linked to neuroinflammation and lysosome accumulation in microglia (O'Rourke et al., 2016). Consistent with these results, microglia cells in *C9orf72*-knockout mice exhibit enlarged lysosomes (Sullivan et al., 2016). In parallel, *C9orf72*-deficient macrophages present increased lysosomal exocytosis (Zhang et al., 2018). Altogether, these results show that *C9orf72* may impact on the survival of MNs by regulating inflammation through the autophagy-lysosomal pathway in microglia and macrophages.

Recent studies have suggested a role for *C9orf72* in regulating the autophagy-lysosome pathway during nutrient stress conditions (Amick et al., 2016; Liu et al., 2018; Liu and Wang, 2019). *C9orf72* promotes the lysosomal degradation of an important epigenetic regulator of lipid metabolism and autophagy–lysosomal functions, the coactivator-associated arginine methyltransferase 1 (CARM1) (Liu and Wang, 2019). As a consequence, C9-ALS/FTD patient-derived neurons with reduced *C9orf72* expression show altered levels of NADPH oxidase NOX2, fatty acids and CARM1 (Liu et al., 2018). In particular, upon glucose starvation, *C9orf72* localizes to the surface of lysosomes (Amick et al., 2016) and drives the delivery of CARM1 to the lysosome for degradation. In cells lacking *C9orf72* the recruitment of CARM1 to the lysosome is dysfunctional under stress conditions and CARM1 accumulates in the lysosome, leading to impaired lipid metabolism and autophagy (Liu and Wang, 2019).

Taken together, these studies suggest a role of *C9orf72* during starvation conditions as a key regulator in the negative feedback control of the autophagy-lysosome pathway.

7.4. Other cellular processes:

7.4.1. Vesicle trafficking

Recently, studies using different models have proposed vesicle trafficking as a potential disease mechanism in C9-ALS/FTD (Aoki et al., 2017; Coyne et al., 2017; Dickson et al., 2019). C9 ALS patient-derived fibroblasts and MNs display dysfunctional intracellular and extracellular vesicle trafficking, as well as impaired trans-Golgi network phenotype, similar to SH-SY5Y neuroblastoma cells lacking *C9orf72* (Aoki et al., 2017). Both *C9orf72* overexpression and ASOs targeting HRE to upregulate variant 1 transcript levels rescue the abnormal vesicle phenotype (Aoki et al., 2017). In another study, inducing early endosomal protein Rab5A increases *C9orf72* expression, rescues neurodegeneration and ameliorates vesicle trafficking in haploinsufficient mice (Shi et al., 2018).

C9orf72 HRE carriers exhibit impaired synaptic vesicular trafficking in fly models (Coyne et al., 2017). Heat shock protein Hsc70-4 regulates synaptic exo- and endocytosis in neurons (Bronk et al., 2001) and its post-transcriptional reduction plays a major role in vesicular trafficking. Indeed, *C9orf72* HRE flies show reduced colorant uptake in dye uptake experiments at the fly neuromuscular junction, whereas a co-overexpression of Hsc70-4 restores its levels (Coyne et al., 2017).

Studies using extensive RNA sequencing on human frontal cortex samples support the role of impaired vesicle trafficking in C9-ALS/FTD pathogenesis (Dickson et al., 2019). First, differentially expressed genes in expansion carriers are enriched for endocytosis compared with controls. Secondly, co-expression analysis reveals a module enriched for vacuolar transport, lysosome function, as well as Golgi vesicle transport (Dickson et al., 2019) Thirdly, low expression of SGSM3 (that interacts with Rab-8A (Yang et al., 2007), a Rab-GTPase regulated by the *C9orf72*-SCMR8 complex (Sellier et al., 2016)) affects vesicular trafficking (Zhang et al., 2018) and is associated with reduced survival in *C9orf72* expansion carriers (Dickson et al., 2019). These results suggest that SGSM3 may counteract the detrimental role of *C9orf72* in expansion carriers and maintain a functional vesicular network.

Altogether, these findings provide evidence that disruption of vesicle trafficking and its functional network may be linked to *C9orf72* repeat expansion and contribute to C9-ALS/FTD pathomechanisms.

7.4.2. DNA Damage

Since neurons have high metabolic activity, they are particularly vulnerable to DNA alterations and oxidative stress, a major factor inducing DNA damage (Mata-Garrido et al., 2018). Many neurodegenerative diseases, including AD, PD and ALS, are characterized by increased DNA damage and impaired DNA damage response (Madabhushi et al., 2014). Both *in vitro* and *in vivo* models have tried to recapitulate DNA alterations in C9-ALS/FTD.

Spinal MNs from C9-ALS patients and *C9orf72* iPSC-derived MNs exhibit increased DNA double-strand breaks (DSBs) (Lopez-Gonzalez et al., 2016; Madabhushi et al., 2014) and iPSNs and mice models expressing poly-GR display poly-GR-induced DNA damage (Choi et al., 2019). Particularly, poly-GR binds to Atp5a1, a mitochondrial complex V component, impairs mitochondrial function, increases oxidative stress and induces the Ku80-dependent DNA repair pathway. By reducing poly-GR levels in mice or inducing

ectopic Atp5a1 expression in vitro it is possible to rescue poly-GR-induced DNA damage. In parallel, poly-GR-induced DNA breakage increases apoptosis that can be prevented by a knockdown of Ku80 (Choi et al., 2019). This study shows that poly-GR-induced-DNA damage plays a role in C9-ALS/FTD pathogenesis, and partial inhibition of overactivated Ku80-dependent DNA repair pathway represents a potential therapeutic target.
Other studies have conveyed similar results. Poly-GR overexpressed in human MNs and neuroblastoma cells induce DNA damage (Farg et al., 2017; Lopez-Gonzalez et al., 2016) by increasing oxidative stress. The inhibition of reactive species of oxygen (ROS) partially reduces poly-GR-related DNA damage in human MNs and flies (Lopez-Gonzalez et al., 2016). Poly-GR activates the p53 pathway and increases the level of γH2AX, a marker of early response to DSBs (Mah et al., 2010), resulting in increased DNA strand breaks, while partial inhibition of the p53 pathway reduces poly-GR-induced DNA alterations (Lopez-Gonzalez et al., 2016).
Although the exact role of DNA damage in C9-ALS/FTD is still elusive, these studies show that poly-GR-induced nucleic acid toxicity can contribute to disease mechanisms in C9-ALS/FTD models.

7.4.3. G-quadruplexes and R-loops

Double-stranded, right-handed helix is the typical conformation of DNA and RNA molecules. However, non-canonical alternative configurations, such as hairpins and quadruplexes exist (Madabhushi et al., 2015). G-quadruplexes and R-loops are examples of alternative nucleic acids organizations: G-quadruplexes consist of four guanine bases organized in stable tetrads stacked on top of one another; R-loops are physiological hybrids between DNA and RNA, generated during the transcription of repetitive sequences by RNA displacement of the complementary DNA strand, producing a three-stranded nucleic acid structure (Groh and Gromak, 2014). The presence of the G-rich G_4C_2 sequence in the *C9orf72* repeat expansion determines the stability of R-loops and G-quadruplexes (Chan et al., 2014; Walker et al., 2017), reflecting the tendency of G-rich structures to organize into steady secondary conformations (Konopka and Atkin, 2018).
The pathogenic role of G-quadruplexes in C9-ALS/FTD is not clear, but various researches have identified small molecules binding to both DNA and RNA G-quadruplexes (Asamitsu et al., 2019) and their potential therapeutic role *in vitro* and *in vivo* (Biffi et al., 2014; Di Antonio et al., 2012; Jiang et al., 2016; Simone et al., 2018). Non-conjugated aromatic diamidines specifically binding to *C9orf72* repeat RNA G-quadruplexes reduce RNA foci

and DPRs levels in C9-patients derived neurons and increase survival as well as suppress toxic poly-GR in *C9orf72* flies (Simone et al., 2018). In another study, a porphyrin compound (TMPyP4) stabilizes G-quadruplexes, reduces the affinity of RanGAP1 to G_4C_2 repeats and decreases nuclear import deficits in Drosophila (Zhang et al., 2015) Although the pathogenic role of G-quadruplexes is unknown, these studies indicate that small molecules selectively binding RNA G-quadruplexes are potential therapeutics for C9-FTD/ALS.

R-loops tend to be thermodynamically stable structures, but their persistent accumulation causes genome instability by inducing DNA DSBs (Bhatia et al., 2014). R-loop-mediated genome instability may play a role in neurodegeneration associated with *C9orf72* repeats (Walker et al., 2017). C9-ALS patient brain tissues display higher levels of R-loops than control and human fibroblasts as well as rat cortical neurons expressing G_4C_2-repeat expansions and poly-GA exhibit a length-dependent increase in R-loops (Walker et al., 2017). Accordingly, the overexpression of the R-loop helicase senataxin reduces the expression of γH2AX+ suggesting that R-loops induce DSBs in cells with *C9orf72* repeat expansions. This study shows that increased R-loops expression contributes to genome instability in *C9orf72* models and that targeted modulation of R-loop homeostasis by specific helicases may provide new therapeutic opportunities.

7.4.4. Mitochondrial damage

Mitochondria are mainly responsible for cellular energetic metabolism as well as calcium homeostasis, signaling transduction and cell death. Mitochondrial quality control (MQC) system maintains mitochondrial homeostasis both by repairing damaged organelles and proteins and by eliminating mitochondria through mitophagy (Khalil and Liévens, 2017), coordinates mitochondria biogenesis, fission, and fusion, and regulates mitochondria size and morphology to preserve their normal function. Defects in mitochondria lead to excessive ROS production, impairment of cellular processes and neuronal death (Gao and Zhang, 2018). Mitochondrial dysfunction has been investigated in superoxide dismutase 1 (*SOD1)*-related ALS (Fukada et al., 2004), but other ALS-related genes may interfere with the MQC system (Khalil and Liévens, 2017)

C9-ALS models exhibit altered mitochondrial fission, mitophagy and reduced activity of complex I (Khalil and Liévens, 2017). TDP-43 accumulates within mitochondria in neurons from patients with sporadic and *TDP-43*-mutated ALS/FTD (Wang et al., 2016). Both wild-type and mutated *TDP-43* bind to mRNA of ND3 and ND6, encoding respiratory

complex I subunits, impairing their expression and disassembling complex (Wang et al., 2016). Suppression of TDP-43 mitochondrial localization rescues mitochondrial dysfunction and neuronal death and ameliorates the phenotype of transgenic mutant *TDP-43* mice (Wang et al., 2016).

While the study by Wang and colleagues indicates mitochondria-directed TDP-43 toxicity, another work has failed to demonstrate a link between mitochondrial dysfunction and TDP-43 protein localization in *TDP-43* and *C9orf72*-mutated fibroblasts (Onesto et al., 2016). In oxidative conditions, *C9orf72*-mutated fibroblasts display increased oxygen consumption as well as ROS and ATP production, mitochondrial hyperpolarization, mitophagy impairment, perturbed mitochondrial morphology and dynamics and increase in mitochondrial DNA and mitochondriogenesis (Onesto et al., 2016). Since colocalization between TDP-43 and mitochondrial markers lacks, Onesto et al. argue against a direct association between TDP-43 and mitochondrial impairment and speculate that compensatory proliferation mechanisms of aberrant mitochondria could be responsible for such molecular findings (Onesto et al., 2016). However, TDP-43 can interact with mitochondrial proteins encoded by both nuclear (e.g. Mfns) (Khalil et al., 2017; Sephton et al., 2011) and mitochondrial DNA (e.g. Tom family proteins and Tim77, mitochondrial membrane translocases) (Wang et al., 2016). Thus, whether TDP-43 toxicity may drive mitochondrial dysfunction needs to be elucidated.

Morphological changes in mitochondria (e.g. alterations in crystal structures, swelling and abnormalities of the outer mitochondrial membrane) and release of apoptotic factors have been reported in *C9orf72* iPSC-derived MNs in association with calcium overload in ER (Dafinca et al., 2016). Bcl-2 family proteins regulate calcium homeostasis and exchange between ER and mitochondria. Since calcium is a key regulator of ER-mitochondria interplay, increased calcium in ER induces ER stress, Bcl-2 family dysregulation (i.e. low levels of antiapoptotic Bcl-2 and high levels of pro-apoptotic BAK), cytochrome c release from the mitochondria and caspases activation, which affect mitochondria morphology and trigger neuronal death (Dafinca et al., 2016).

Taken together, these findings suggest a role of mitochondrial dysfunction in ALS/FTD pathogenesis.

8. Biomarkers

8.1. Biochemical biomarkers

Biomarkers in ALS may help to make an earlier diagnosis, to enroll patients in clinical trials and to monitor the efficacy of therapeutic interventions. Biochemical biomarkers include DPRs, neurofilaments and other proteins that are related to the pathogenesis of C9-ALS/FTD and can be measured in biofluids or biological tissues.

CSF poly-GP levels discriminate symptomatic and pre-symptomatic carriers from non-carriers, thus they may be useful as pharmacodynamic biomarkers (Floeter and Gendron, 2018) and as including criteria for patients in clinical trials. CSF poly-GP levels are elevated both in symptomatic subjects and asymptomatic carriers (though at lower concentrations), consistent with the brain C9-RAN protein pathology arising before symptom onset (Gendron et al., 2017a). Lehmer et al. have compared poly-GP levels in CSF of C9-asymptomatic and symptomatic subjects, revealing no association with age at onset, disease duration, disability, and neuronal damage markers, i.e. neurofilaments (Nfs) and neuroimaging alterations (Lehmer et al., 2017; Meeter et al., 2018). Finally, longitudinal studies have shown that CSF poly-GP levels are stable over 6 to 18 months (Gendron et al., 2017a), though a modest increase in CSF poly-GA is present in pre-symptomatic carriers (Meeter et al., 2018). In vitro and in vivo models have yielded similar results. Extracellular poly-GP proteins reflect the intracellular G4C2 RNA accumulation in lymphoblastoid and iPSN lines. (Gendron et al., 2017a). C9-ASO intrathecal administration leads to a decrease of CSF poly-GP levels that correlate with DPR-protein pathology and RNA foci in C9orf72 mice models' brains (Gendron et al., 2017a).CSF phosphorylated neurofilament heavy chain (p-NfH) provides a useful prognostic indicator in C9-ALS patients. P-NfH concentrations are associated with shorter survival in C9-ALS patients and are higher than in non-C9 ALS patients, thus reflecting a higher burden of neurodegeneration in C9-related diseases. Moreover, they are stable in longitudinal samples, potentially playing a role in monitoring the efficacy of pharmacological therapies (Gendron et al., 2017b). A cut-off value of 176 pg/ml in CSF discriminates C9-ALS/FTD patients from asymptomatic carriers with high sensitivity and specificity (98.8% and 96.4%) (Gendron et al., 2017b). Serum and CSF light chain neurofilament (NfL) are elevated in symptomatic compared with asymptomatic C9-carriers and their levels may predict the phenoconversion in pre-symptomatic individuals (Benatar et al., 2018). Complementarily, serum and CSF Nfs might assume a prognostic role, providing information about disease activity and progression.Biochemical biomarkers may also help to differentiate between C9-ALS and C9-FTD patients and suggest new pathogenic mechanisms, as recently shown by a deep proteomic

approach on CSF (Barschke et al., 2020). C9-FTD patients dispplay decreased neuronal pentraxin receptor (NPTXR) levels, whereas C9-ALS patients express higher neurofilament medium polypeptide (NEFM), chitotriosidase-1 (CHIT1), profilin-1 (PFN1) and ubiquitin carboxyl-terminal hydrolase isozyme L1 (UCHL1). Increased Nfs and CHIT1 CSF levels have been previously described in ALS (Steinacker et al., 2018, 2016), and seem to reflect neurodegeneration and neuroinflammation, respectively. PFN1 is an actin-binding protein whose mutations are known to be causative of ALS (Yang et al., 2016). Since *C9orf72* interacts with cofilin and other actin-binding proteins to regulate axon dynamics, increased CSF PFN1 levels could shed new light on profilin1 and microtubules dynamics in C9-ALS (Barschke et al., 2020).
UCHL1 is involved in hydrolase and ubiquitin ligase activity (Liu et al., 2002); by playing a role in ubiquitination, which is a key process in autophagy, UCHL1 may explain diverging mechanisms underlying C9-FTD and C9-ALS (Barschke et al., 2020). On the contrary, NPTXR is a protein organizing excitatory and inhibitory synapses and its decrease might indicate different synaptic patterning between the two groups.

8.2. Neuroimaging biomarkers

Structural neuroimaging represents another biomarker since it traces the macroscopic consequences in the patients' brains — mainly atrophy distribution — of the C9-related neuropathological changes, particularly neuronal loss and TDP-43 pathology.
Gray matter atrophy on magnetic resonance imaging (MRI) is more prominent in extra-motor cortical and subcortical regions (including cerebellum and thalamus) and less evident in the precentral motor cortex in C9-ALS patients (Floeter et al., 2016; Omer et al., 2017). Whether a specific C9-related MRI-signature exists remains a challenge. Indeed, a large study on C9- and non-C9 ALS patients has failed to identify neuroimaging specific markers for C9-carriers, since approximately 20% of C9-negative patients share the same widespread structural brain involvement found in C9-carriers (Westeneng et al., 2016).
MRI scans coupled with neuropsychological investigations have shown that presymptomatic *C9orf72* expansion carriers (PreSxC9) display early cognitive and structural abnormalities. PreSxC9 present abnormally low gyrification, i.e. cortical folding, and microstructural anomalies on MRI imaging as early as their 30s, decades before expected symptom onset (Bertrand et al., 2018; Caverzasi et al., 2019). Besides, PreSxC9 exhibit declined gyrification in regions atrophied during the symptomatic phase (Caverzasi

et al., 2019) as well as decreased grey matter volume, impaired white matter integrity and early disruption of connectivity network (Bertrand et al., 2018; Lee et al., 2017; Rohrer et al., 2015). Whether these alterations represent early neurodegeneration or neurodevelopmental defects is unclear. However, these results suggest that *C9orf72* expansion may increase the susceptibility of specific brain areas to future neurodegeneration and be associated with reduced gyrification and grey matter volume, possibly useful early structural imaging biomarkers.

Multimodal approaches combining two CSF measures (i.e. poly-GP and NfL) with quantitative neuroimaging are also possible. Meeter and colleagues have explored the relationship between poly-GP and axonal damage biomarkers in a cohort of *C9orf72* symptomatic and asymptomatic carriers, suggesting their potential clinical use as complementary biomarkers (Meeter et al., 2018).

Functional connectivity MRI studies (Agosta et al., 2017) and Positron Emission Tomography (PET) studies (Cistaro et al., 2014) have investigated dynamic differences between C9-carriers and non-carriers. Moreover, the use of transcranial magnetic stimulation (TMS) has revealed cortical hyperexcitability in C9-population (Geevasinga et al., 2015), though reporting no difference between asymptomatic and symptomatic carriers. Taken together, these findings suggest that the combination of biochemical and neuroimaging biomarkers could improve early diagnosis and patients' enrollment in clinical trials, as well as prognosis estimation and evaluation of therapy efficacy.

9. Therapeutics

To date, no cure exists for C9-ALS/FTD. Different studies using ASO technology, small molecules, CRISPR-Cas9 as well as RNA interference (RNAi) strategies have targeted various pathogenic pathways, including *C9orf72* RNA and DNA, DPRs, TDP-43 and downstream mechanisms (Table 2).

9.1. Targeting *C9orf72* DNA and RNA

9.1.1. Antisense oligonucleotide therapies

Recently, ASO-based therapies for neurological disorders have gained interest after FDA approval of Nusinersen, an ASO selectively modulating the splicing of *SMN2* pre-mRNA in spinal muscular atrophy (SMA). Also, phase I-II trials using ASOs against *huntingtin* in HD (Tabrizi et al., 2019) and *SOD1* in familial ALS (Miller et al., 2013) have provided

encouraging preliminary results. Upcoming phase III trials are planned to evaluate the potential clinical benefits of ASOs in these pathologies.

In C9-ALS, ASOs targeting *C9orf72* RNA reduce sense RNA foci deposition and glutamate excitotoxicity, rescue downstream aberrant gene expression, NCT defects and TDP-43 mislocalization in *C9orf72* fibroblasts and iPSNs (Donnelly et al., 2013; Gendron et al., 2015; Lagier-Tourenne et al., 2013; Sareen et al., 2013)(Donnelly et al., 2013; Gendron et al., 2015; Lagier-Tourenne et al., 2013; Sareen et al., 2013). *In vivo* models have yielded similar results. Particularly, ASOs mitigate NCT defects and neurodegeneration in flies (Moens et al., 2018), alleviate behavioral and cognitive impairments as well as reduce RNA foci and DPR deposition in mice (Gendron et al., 2015; Jiang et al., 2016; Lehmer et al., 2017). Following these promising results, a phase I clinical trial with ASO against sense *C9orf72*-derived RNA has started in 2018 (NCT03626012).

9.1.2. Small molecules

An alternative approach for targeting *C9orf72* repeat DNA/RNA includes small molecules. These compounds can bind the secondary structure of *C9orf72* repeat RNA (Alniss et al., 2018; Simone et al., 2018; Su et al., 2014; Zamiri et al., 2014; Zhang et al., 2018). TMPyP4, a G quadruplex binder, interacts with G_4C_2 RNA, prevents sequestration of RBPs (Zamiri et al., 2014) and rescues transport defects, RAN translation and neurodegeneration in Drosophila (Zhang et al., 2015). Non- conjugated aromatic diamidines bind the hairpin configuration of G_4C_2 repeats, reducing RNA foci and poly-GR in iPSNs (Simone et al., 2018; Su et al., 2014) and Drosophila (Simone et al., 2018). In another study, a small molecule selectively binding the G_4C_2 hairpin inhibits both RAN translation — DPR poly-GP production — and prevents the sequestration of RBPs (Wang et al., 2019). However, it is not clear whether this molecule is beneficial in reducing other toxic DPRs, i.e. poly-GR and poly-PR.

Following these promising results, high-throughput screening methods for drug development have yielded other RAN translation inhibitors potentially valuable as therapeutics to test *in vitro* (Green et al., 2019).

9.1.3. Targeting the RNA transcription machinery

The RNA transcription machinery is involved in the production of toxic RNA transcripts in C9-ALS/FTD and its components may be therapeutic targets. DRB sensitivity-inducing factor (DSIF) complex and the polymerase II-associated factor 1 complex (PAF1C) interact

with RNA polymerase II during transcript elongation (Van Oss et al., 2017). Particularly, subunits of DSIF complex, SUPT4H and SUPT5H, and PAF1C critically contribute to the production of toxic transcripts (Goodman et al., 2019; Kramer et al., 2016). The inhibition of SUPT4H reduces both sense and antisense RNA foci and their DPR products as well as mitigates neurodegeneration in Drosophila and iPSNs (Kramer et al., 2016). Similarly, C9-ALS/FTD patients present upregulated expressions of PAF1 and LEO1, components of PAF1C, that correlate with an increase of G_4C_2-rich transcripts. Downregulation of PAF1C reduces RNA and poly-GR dipeptide production in a Drosophila model overexpressing G_4C_2 (Goodman et al., 2019). Although potentially valuable, this therapeutic strategy requires further investigations since the reduced expression of SUPT4H induces a global reduction in RNA (Naguib et al., 2019).

Another potential therapeutic approach targeting the transcription machinery of *C9orf72* involves RNA helicases. RNA helicases are a family of highly conserved enzymes implicated in almost all aspects of RNA metabolism. DDX3X, a member of the DEAD-box family of RNA helicases, regulates RAN translation by binding the G_4C_2 repeat RNA (Cheng et al., 2019). Reduced expression of DDX3X by short hairpin RNAs (shRNAs) leads to a dramatic increase of poly-GP accumulation in yeast and in human iPSNs. Conversely, overexpression of DDX3X induces a decrease of DPR levels, rescues NCT abnormalities, glutamate-mediated excitotoxicity and improves survival in patient-derived iPSNs (Cheng et al., 2019). Thus, these results suggest that specific RNA helicases, by regulating DPR production from RAN translation, contribute to C9-related pathogenesis and might be future therapeutic targets.

9.1.4. CRISPR

CRISPR/Cas refers to a genome-editing system naturally present in bacteria that can be designed to cut target genes following gene repair through nonhomologous end-joining (Heidenreich and Zhang, 2016). CRISPR-induced knock-down of AFF/FMR2, an elongation factor involved in *C9orf72* transcription, reduces the levels of C9-RNA, RNA foci, and DPR proteins in iPSNs from C9-ALS/FTD patients (Yuva-Aydemir et al., 2019). These CRISPR-edited neurons show decreased axonal degeneration and TDP-43 pathology (Yuva-Aydemir et al., 2019). In another study, the use of CRISPR/Cas9 system to target G_4C_2 repeat DNA (Pinto et al., 2017), by reducing repeat translation, can decrease the level of RNA foci and DPR levels in cell lines. An approach involving RNA-targeting Cas9 with RNA endonuclease lowers RNA foci and DPR protein levels (Batra et al., 2017). Although

the clinical use of CRISPR-Cas9 is still at its infancy, these findings suggest the potential efficacy of this technology in targeting some key *C9orf72*-related pathomechanisms.

9.1.5. RNA interference strategies

RNAi represents an alternative system targeting RNA/protein-mediated gain-of-function toxicity in C9-ALS. The three most common RNAi mechanisms include short interfering RNAs (siRNAs), shRNAs and artificial microRNAs (miRNAs). One of the major challenges in C9-ALS/ FTD drug development is to target the *C9orf72* transcripts in the nucleus. SiRNAs can decrease *C9orf72* mRNA in patient fibroblasts, though not reducing nuclear RNA foci (Lagier-Tourenne et al., 2013). Other studies indicate that both single-stranded and double-stranded silencing RNAs reduce sense and antisense RNA foci formation (Hu et al., 2017, 2015).
AAV5-delivered miRNAs engineered to target *C9orf72* decrease the abundance of RNA foci in the cytoplasm and the nucleus of iPSC-derived MNs and in mice (Martier et al., 2019a, 2019b). Altogether, these findings highlight the feasibility of RNAi-based approaches as a therapeutic strategy for C9-ALS.

9.2. Targeting DPRs

DPRs may spread from cell to cell according to the principle of the prion-like propagation of misfolded/aggregated proteins (Chang et al., 2016; Khosravi et al., 2020; Ravits, 2014). Coculture cell assays indicate that poly-GP, poly-PA and poly-GA are susceptible to cell-to-cell transmission (Chang et al., 2016; Zhou et al., 2017). Active and passive immunization target toxic proteins that spread from cells to cells, such as α-synuclein in PD (Masliah et al., 2011) and p-Tau (Boutajangout et al., 2011) in AD, ameliorating pathology and disease phenotype in mouse models.
Consistent with this rationale, treatment with anti-GA antibodies significantly reduces intracellular poly-GA levels in DPR-expressing cells (Zou et al., 2017) and prevents poly-GA seeding and spreading in *C9orf72* brain extracts (Zhou et al., 2017). Active immunization with ovalbumin-$(GA)_{10}$ elicit a strong antibody response in GA-fluorescent protein (CFP) transgenic mouse models and reduce poly-GA inclusions in the spinal cord (Zhou et al., 2020). Moreover, poly-GA vaccination lowers TDP-43 mislocalization, neuronal damage and inflammatory response and ameliorates motor deficits in GA-CFP mice (Zhou et al., 2020).

Though exciting, these DPR-oriented therapeutics present some potential limitations to consider. First, current approaches in other neurodegenerative diseases target the extracellular pool of toxic proteins, which is presumably involved in prion-like propagation. Thus, the success of this therapeutic rationale depends on whether there is a pathological relevant extracellular pool of DPRs to target. Otherwise, techniques to deliver antibodies intracellularly would be necessary. Secondly, since poly-GR and poly-PR are more toxic than poly-GA, poly-GP and poly-PA in model systems, it is still unclear which DPR proteins and conformations to target. Other important factors to consider involve the ideal timing of these treatments, the specificity, and the safety of the immunization, as well as the risk of therapy-related autoimmune responses.
Another way to directly alleviate DPR protein-mediated toxicity is to increase the DPR protein turnover rate. The overexpression of the small heat shock protein HSPB8 induces the autophagy-mediated reduction of different DPR aggregates in MNs (Cristofani et al., 2018).

Taken together, these findings suggest that active and passive immunization, as well as the modulation of physiological DPR turnover represent valuable therapeutic strategies in rescuing *C9orf72*-related pathology and in mitigating disease severity in animal models.

9.3. Targeting downstream mechanisms

9.3.1. Nucleocytoplasmic Transport and stress granules

Therapeutic approaches that correct downstream cellular pathways might represent useful strategies for C9-ALS/FTD. So far, different studies have focused on drugs modulating NCT. *C9orf72* repeat transcripts sequester serine/arginine-rich splicing factor 1 (SRSF1) or exportin 1, triggering transcript nuclear export and subsequent RAN translation, thus leading to toxic accumulation of DPRs (Hautbergue et al., 2017). Accordingly, SRSF1 knockdown inhibits the nuclear transport of *C9orf72* repeat transcripts, reduces DPRs and RNA foci and mitigates neurodegeneration in Drosophila and patient-derived neurons (Hautbergue et al., 2017). The underlying therapeutic mechanism seems to reduce cytoplasmic repeat RNA (hence, DPRs) (Hautbergue et al., 2017) or to rescue NCT impairment and consequently a mislocalization of RBPs, such as TDP-43 (Zhang et al., 2015).

Small molecules known as selective inhibitors of nuclear export (SINEs) that inhibit exportin 1 can alleviate NCT defects and neurodegeneration in a Drosophila model (Zhang et al., 2015). Though not able to revert mislocalized TDP-43 distribution, these SINE compounds increase primary neuron survival and partially rescue motor function in rodents overexpressing mutant TDP-43 (Archbold et al., 2018). Notably, one SINE compound, KPT-350, may soon be tested by Karyopharm Therapeutics and its partner Biogen Inc in human clinical trials (Jiang and Ravits, 2019). Since NCT is a fundamental cellular process, it is still unclear whether the therapeutic window for SINE compounds is adequately large when addressing nuclear export.

Other studies indicate that the inhibition of SG formation might rescue NCT defects. Knockdown of ataxin 2, an essential component of SGs, leads to decreased SG assembly, suppressing NCT deficits in iPSNs from C9-ALS patients and alleviating neurodegeneration in a C9-ALS Drosophila model (Zhang et al., 2018). Similarly, ASOs targeting ataxin-2 in TDP-43 transgenic mice reduce accumulation of TDP-43, improve motor function and expand rodents' lifespan (Becker et al., 2017). Altogether, these studies highlight the potential therapeutic role of molecules targeting NCT in C9-ALS/FTD.

9.3.2. Autophagy

In addition to NCT, other cellular downstream mechanisms explored in drug development for C9-ALS are the modulation of autophagy and ER stress. Bosutinib, an Src/c-Abl pathway inhibitor that increases autophagy, improves the survival of iPSNs from C9-ALS/FTD patients (Imamura et al., 2017). mTOR inhibitors and various phenothiazine derivatives, known to activate autophagy, decrease accumulation and toxicity of DPR proteins *in vitro* and reduce neuronal cell death caused by synergistic DPR accumulation and C9orf72 protein depletion (Boivin et al., 2020).

9.3.3. Endoplasmic reticulum stress

Knockdown of the ER protein thioredoxin-related transmembrane protein 2 (TMX2) has beneficial effects *in vitro* (Kramer et al., 2018); suppression of TMX2 modulates ER stress signatures and improves the survival of iPSNs from C9-ALS/FTD patients (Kramer et al., 2018). Similarly, tauroursodeoxycholic acid (TUDCA) and salubrinal, two ER stress inhibitors, reduce lactate dehydrogenase (LDH) activity in culture media and caspase-3 activation in neurons expressing GFP-$(GA)_{50}$ by downregulating phospho-PERK/CHOP

signaling and by increasing eIF2-alpha phosphorylation, respectively (Zhang et al., 2014). Given that an increase in phospho-PERK activity and consequent eIF2-alpha dephosphorylation are mediators of ER stress-induced cell death (Han et al., 2013), these two compounds protect against poly-GA-induced toxicity *in vitro* (Zhang et al., 2014).

Collectively, these findings suggest that therapeutic approaches targeting cellular downstream mechanisms, though at their infancy, may be beneficial in C9-ALS/FTD.

10. Closing remarks

Since the identification of *C9orf72* repeat expansion, many studies have focused on unraveling the underlying pathogenic mechanisms in C9-ALS/FTD. At the same time, several disease models *in vitro* and *in vivo* have provided insights into the loss/gain of function-related neurotoxicity, as well as the downstream cellular function abnormalities. Despite thorough investigations of both biomarkers and new therapies have proceeded enthusiastically, disease-modifying therapies for C9-ALS/FTD still lack. However, the recent use of ASO-based therapeutics selectively targeting repeat-containing RNA in phase I clinical trial for C9-ALS/FTD patients might be promising. Thus, constant research efforts towards the study of disease mechanisms and therapy development using different approaches could lead to new interventions for C9-ALS/FTD and, hopefully, other MNDs as well.

Author statement

Gagliardi Delia and Costamagna Gianluca: Conceptualization, Investigation, Writing - Original Draft. Taiana Michela: Investigation, Writing - Reviewing and Editing. Andreoli Luca: Visualization. Biella Fabio: Investigation, Visualization. Bersani Margherita: Visualization. Bresolin Nereo: Supervision. Comi Giacomo Pietro: Supervision. Corti Stefania: Conceptualization, Writing - Reviewing and Editing, Supervision. All authors have approved the final draft of the article.

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Figure 1: *C9orf72* gene structure, transcript variants, and protein isoforms. The *C9orf72* gene (A) is composed of eleven exons, it is spliced in three different transcript variants (B) and it is translated into two protein isoforms (C). In this figure, green boxes indicate coding exons and blue boxes represent non-coding exons. The GGGGCC (G_4C_2) hexanucleotide repeat expansion mutation localizes within the promoter sequence of transcript variant 2 and in the first intron of transcript variants 1 and 3. Transcript variants 2 and 3 encode the full-length *C9orf72* isoform, (481-amino acid protein) and variant 1 encodes a short isoform (222 amino acids). The existence of the repeat expansion facilitates transcription from exon 1a, expanding the fraction of transcripts including the repeat expansion.

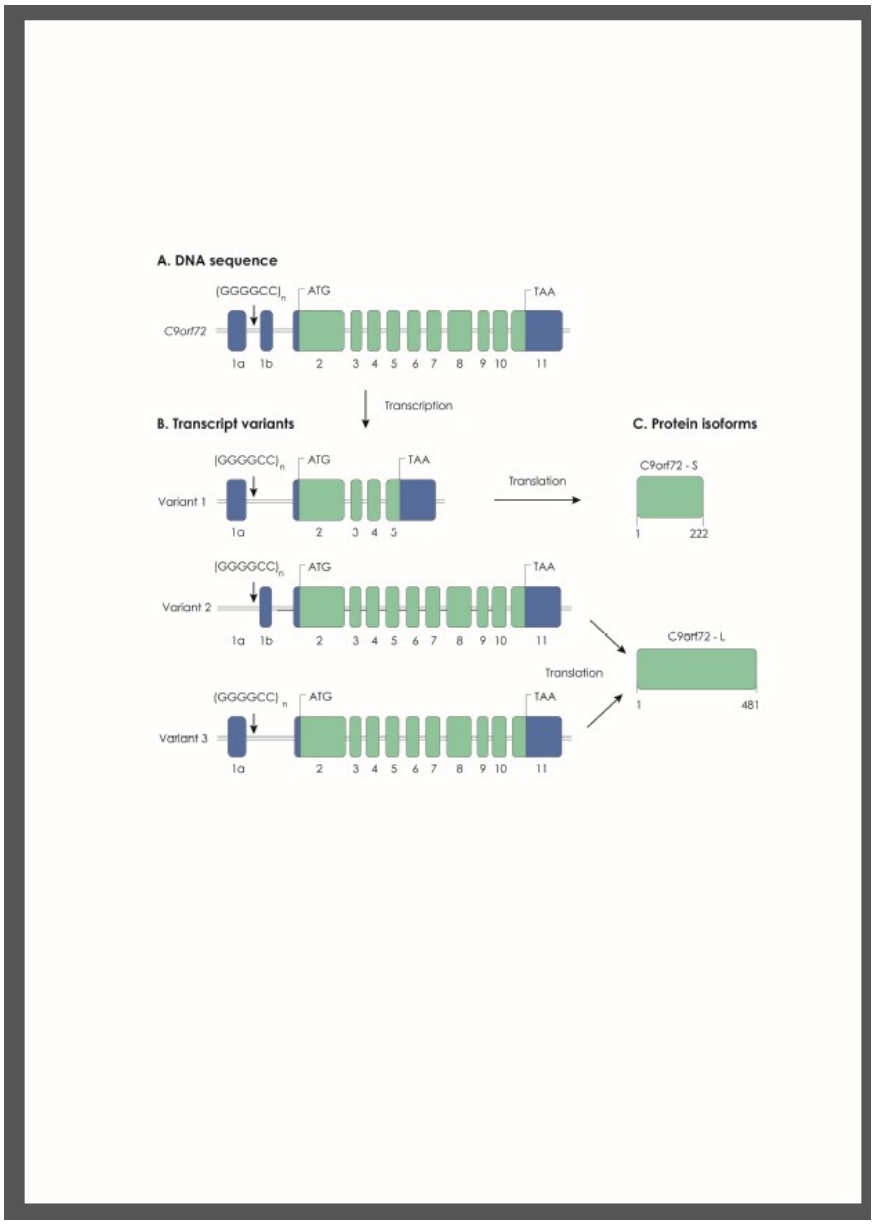


Figure 2: *C9orf72* repeat-associated non-ATG translation and dipeptide repeat proteins. The *C9orf72* gene can be translated into sense (GGGGCC) and antisense (CCCCGG) RNA strands. Dipeptide repeat proteins (DPRs) are formed following G_4C_2 repeat-associated non-ATG (RAN) translation of sense and antisense RNAs. The sense RNA strand encodes poly-GA, poly-GP and poly-GR, while the antisense RNA strand generates poly-GP, poly-PA and poly-PR.

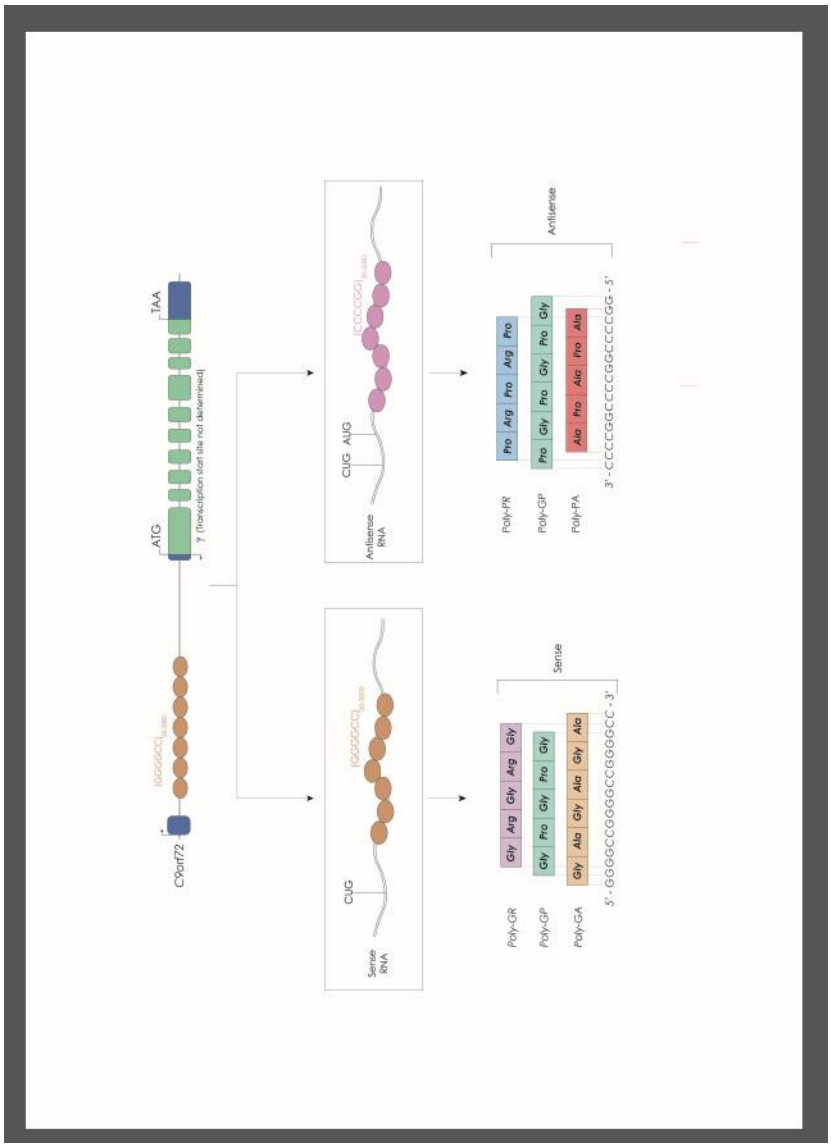
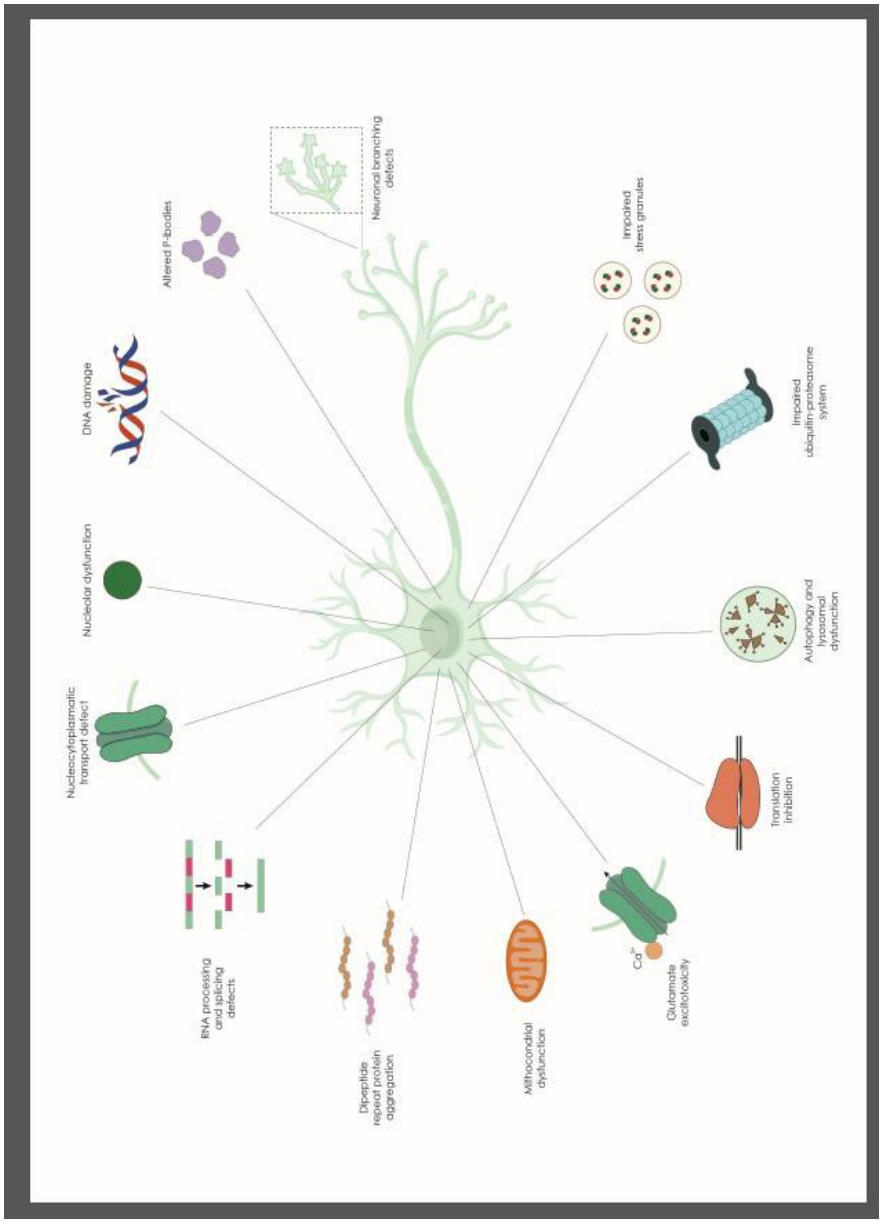


Figure 3: Cellular processes involved in C9-ALS/FTD. Different cellular downstream disease mechanisms are investigated in *C9orf72*-related amyotrophic lateral sclerosis/frontotemporal dementia (C9-ALS/FTD). Both *C9orf72* toxic gain- and loss-of-function impair RNA metabolism, leading to alterations of stress granules (SGs), processing-bodies (P-bodies) and the nucleolus, disrupt RNA splicing and translation and

induce DNA damage. Proteostasis-related pathways seem to be involved in C9-ALS/FTD pathogenesis, such as autophagy, lysosomal function and the ubiquitin-proteasome system (UPS). Other pathomechanisms implicated in C9-ALS/FTD include altered nucleocytoplasmic transport (NCT) and dysfunctional mitochondria. C9-ALS/FTD may be also associated with altered neuron-specific processes, such as glutamate excitotoxicity and



neuronal branching defects.

Table 1. In vivo models of C9orf72 gain- and loss-of-function from selected studies

Model	Reference	Methodolody/ strain	RNA Foci	DPRs	TDP-43 pathology	Other phenotypes
Zebrafish (gain-of-function)	(Ohki et al., 2017)	Transgenic fish carrying 80 G_4C_2 repeats in the GA reading frame alone or with ATG codon	Sense foci in spinal cord neurons	GFP- $(GA)_{30}$ inclusions in muscles	No TDP-43 pathology	Pericardial edema Reduced circulation of blood cells
	(Swaminathan et al., 2018)	Embryos injected with DNA constructs overexpressing 100 GR repeats using Tol2 transposon-based system	Not reported	GFP- $(GR)_{100}$ inclusions in MNs	Not reported	Mild cardiac edema Reduced swimming behavior Retarded MN growth Slightly increased MN death
	(Swinnen et al., 2018)	Embryos injected with sense G_4C_2 and antisense C_24_4repeats RNA of variable length	Sense and antisense foci in the cytoplasm of neural and non-neural tissue	Poly-GA both in neural and non-neural tissue	Not reported	Axonopathy in MNs
	(Shaw et al., 2018)	Embryos injected with a DNA containing 89 G_4C_2 repeats	Antisense foci in nuclei of muscle cells	poly-GP, -GA, -PA and -PR in the nuclei of muscle cells	Not reported	Early mortality Altered swimming behavior Reduced weight gain Muscle atrophy and MN loss
Zebrafish (loss-of-function)	(Ciura et al., 2013)	ASO knock-down of C9orf72 orthologue	Not applicable	Not applicable	Not reported	Axonopathy Reduced touch-evoked escape response
	(Sellier et al., 2016)	ASO knock-down of C9orf72 orthologue and expression of Ataxin-2 with intermediate size polyQ	Not applicable	Not applicable	Not reported	Axonopathy Reduced touch-evoked escape response
	(Yeh et al., 2018)	ASO knock-down of C9orf72 orthologue	Not applicable	Not applicable	Not reported	Decreased motility Reduced axonogenesis Increased neuronal apoptosis
Mouse (gain-of-function)	(Chew et al., 2015)	C57BL/mice, AVV-mediated expression of G_4C_2 repeats	Sense foci in CNS, antisense foci less represented	Poly-GP expression in $(G_4C_2)_{66}$ repeats	pTDP-43 inclusions in nuclei and occasionally in cytoplasm	Rotarod poor performance from day 2, anxiety-like behavior and hyperactivity
	(O'Rourke et al., 2015)	C57BL/6J mice BAC transgenic, full gene and 100-1000 G_4C_2 repeats	Sense and antisense RNA foci in CNS	Poly-GP inclusions increasing with age	No TDP-43 pathology	Normal motor phenotype and behavior
	(Jiang et al., 2016)	C57BL/6 mice BAC transgenic, exons 1-5 and 110 or 450 G_4C_2 repeats	Sense and antisense foci	Poly-GA, -GP and -GR inclusions	Increased levels of pTDP43; no aggregation or mislocalization	Normal motor phenotype; working memory impairment and anxiety disorders
	(Schludi et al., 2017)	C57BL/6 mice, neural expression of $(GA)_{149}$-CFP and 31 carboxyterminal amino acids from endogenous human locus	Not applicable	$(GA)_{149}$- CFP inclusions throughout CNS increasing with age	Higher pTDP-43 Levels; no TDP43 inclusions or mislocalization	Impaired gait; normal muscle strength and spatial memory
	(Hao et al., 2019)	C57BL/6 mice: neuron-specific GFP-$(PR)_{28}$ overexpression	Not applicable	GFP-$(PR)_{28}$ inclusions throughout CNS	No TDP-43 pathology	Impaired motor phenotype; hyperactivity and anxiety-like behavior; neuronal loss; increased gliosis
	(Khosravi et al., 2020)	C57BL/6N mice expressing $(GA)_{149}$-GFP (protocol by Schludi et al, 2017)	Not applicable	Not reported	Higher p-TDP-43 levels Increased cytoplasmic TDP- 43 in MNs	Not reported

Mouse (loss-of-function)	(Lagier-Tourenne et al., 2013)	Somatic brain transgenesis with antisense oligonucleotide against C9orf72	Not applicable	Not applicable	Not reported	Normal motor phenotype. No cognitive or behavioral impairment
	(Jiang et al., 2016)	Non-conditional knockout of exons 2–6	Not applicable	Not applicable	Not reported	Mild motor deficits and behavioral abnormalities Splenomegaly and lymphadenopathy
	(Ugolino et al., 2016)	Non-conditional knockout of exons 2–6	Not applicable	Not applicable	Not reported	Splenomegaly and lethargy
	(Shao et al., 2019)	C9orf72 loss-of-function heterozygous mice crossed with C9-BAC mice	Not applicable	Not applicable	Not reported	Early motor deficits Impaired balance Normal body weight Normal behavior
***Caenorhabditis elegans* (loss-of-function)**	(Therrien et al., 2013)	*C9orf72* orthologue (alfa-1) mutant worms with a deletion in exons 3 and 4	Not applicable	Not applicable	Not reported	Progressive paralysis Neurodegeneration of GABAergic motor neurons Increased sensitivity to osmotic stress
***Caenorhabditis elegans* (gain-of-function)**	(Kramer et al., 2016)	Synaptobrevin promoter driving neuronal expression of $(G_4C_2)_{66}$	Sense and antisense foci	Poly-GP	Not reported	Reduced life span
***Drosophila* (gain-of-function)**	(Mizielinska et al., 2014)	Transgenic flies expressing $(G_4C_2)_{36}$ and $(G_4C_2)_{104}$ repeats	Sense and antisense RNA foci in the nuclei of salivary glands cells	Poly-GR, -GP accumulation in neurons and eye cells	Not reported	Eye degeneration, increased lethality Neurodegeneration
	(Freibaum et al., 2015)	Transgenic flies expressing $(G_4C_2)_8$ $(G_4C_2)_{28}$, $(G_4C_2)_{58}$ and GFP coding sequence	Not reported	Poly GR and poly GP	Not reported	Rough eye phenotypes; decreased larval growth, reduced locomotion
	(Zhang et al., 2016)	Transgenic flies that expresse a $(G_4C_2)_{49}$-GFP repeats; overexpression of TDP-43 with or without added ATXN2 with an intermediate poly-Q	Not reported	Poly GR-GFP accumulation	TDP-43 accumulation	Eye pigment loss and ommatidial disorganization; neurodegeneration
	(Hautbergue et al., 2017)	fly expressing $(G_4C_2)_{36}$ repeats (Mizielinska et al, 2014) crossed with two transgenic RNAi lines targeting SRSF1 or ALYREF	Not reported	Poly-GR, -GP and GA	Not reported	Compound eye disruption, climbing deficits, increased neuronal cell death
	(Simone et al., 2018)	fly expressing $(G_4C_2)_{36}$ repeats (Mizielinska et al, 2014)	Not reported	Increased poly-GR levels	Not reported	Reduced poly-GR and increased survival by small molecule administration

AV: adeno-associated virus; ASOs: antisense oligonucleotides; ATXN2: Ataxin-2; BAC: Bacterial artificial chromosome; CNS: central nervous system; DPRs: dipeptide repeat proteins; G_4C_2: GGGGCC; GA: Gly-Ala; GABA: Gamma-aminobutyric acid; GFP: Green fluorescent protein; GP: Gly-Pro; GR: Gly-Arg; MNs: Motor Neurons; PA: Pro-Ala; PolyQ: polyglutamine; pTDP-43: phosphorylated TAR-DNA binding protein 43; RNAi: RNA interference; SRSF1: serine/arginine-rich splicing factor 1; TDP-43: TAR-DNA binding protein 43;

Table 2. Possible therapeutic strategies in C9-ALS/FTD

Therapeutic strategies	Read out	Model	Reference
Targeting C9Orf72 DNA and RNA			
ASOs targeting C9Orf72 RNA	Reduction of sense RNA foci deposition and glutamate excitotoxicity, rescue of downstream aberrant gene expression, NCT defects and TDP-43 mislocalization	C9Orf72 fibroblasts and iPSNs	(Donnelly et al., 2013; Lagier-Tourenne et al., 2013; Moens et al., 2018; Sareen et al., 2013)
	Mitigation of NCT defects and neurodegeneration	Drosophila	(Moens et al., 2018)
	Reduction of RNA foci and DPR deposition, amelioration of behavioural and cognitive impairment	BAC C9Orf72 transgenic mouse	(Jiang et al., 2016; Lehmer et al., 2017)
Small molecules			
TMPyP4 (G-quadruplex binder)	Rescue of transport defects, RAN translation and neurodegeneration	Drosophila	(Zhang et al., 2015)
	Prevention of RBP sequestration	*in vitro*	(Zamiri et al., 2014)
Several compounds binding the hairpin configuration of G_4C_2 repeats	Reduction of DPR production, particularly poly-GP	Patient- derived fibroblast and neurons Drosophila	(Simone et al., 2018; Su et al., 2014; Wang et al., 2019) (Simone et al., 2018)
Targeting the RNA transcription machinery			
Spt4 inhibitor (transcription elongation factor of DSIF complex)	Reduction of both sense and antisense repeat transcripts and DPRs; mitigation of neurodegeneration	Drosophila, *C. elegans*, yeast and *C9Orf72* fibroblasts	(Kramer et al., 2016)
PAF1C downregulation	Reduction of RNA and poly-GR dipeptide production	Drosophila	(Goodman et al., 2019)
DDX3X (RNA helicase) overexpression	Decrease of DPR levels, rescue of NCT abnormalities and glutamate-mediated excitotoxicity, survival improvement	iPSNs	(Cheng et al., 2019)
CRISPR-mediated editing			
AFF/FMR2 (elongation factor involved in *C9Orf72* transcription) knock down	Decrease in levels of C9-RNA, RNA foci and DPRs; decreased axonal degeneration and TDP-43 pathology	iPSNs	(Yuva-Aydemir et al., 2019)
G_4C_2 repeat DNA	Decrease in repeat translation, levels of RNA foci and DPRs	HeLa cells	(Pinto et al., 2017)

RNA-targeting Cas9	Decrease in RNA foci and DPRs levels	Human cells	(Batra et al., 2017)
RNA interference			
siRNA against C9Orf72 RNA	Decrease in C9Orf2 mRNA but not RNA foci	Patient-derived fibroblasts	(Lagier-Tourenne et al., 2013)
ss-siRNA against HRE	Reduction of sense and antisense RNA foci	Patient-derived fibroblasts	(Hu et al., 2017)
AAV5-delivered miRNA targeting *C9Orf72* RNA	Decrease in both nuclear and cytoplasmic RNA foci	iPSC-derived MNs and transgenic C9Orf72 mouse	(Martier et al., 2019a, 2019b)
Targeting DPRs			
Anti-GA antibodies	Decrease in intracellular poly-GA levels	DPRs-expressing cells	(Zhou et al., 2017)
Active immunization with ovalbumin-$(GA)_{10}$	Poly-GA inclusions reduction in the spinal cord	GA-CFP mouse	(Zhou et al., 2020)
Targeting downstream mechanisms			
SRSF1 knockdown	Nuclear transport of repeat transcripts inhibition, DPR and RNA foci decrease and mitigation of neurodegeneration	Patient-derived neurons and Drosophila	(Hautbergue et al., 2017)
SINEs inhibiting XPO1	Amelioration of NCT defects and neurodegeneration Increase in survival Partial rescue of motor function	Drosophila Rodent primary cortical neurons Rat TDP-43 models	(Zhang et al., 2015) (Archbold et al., 2018) (Archbold et al., 2018)
Knockdown of ataxin 2 (component of SGs)	Decrease in SG assembly and NCT deficits suppression Mitigation of neurodegeneration	iPSNs Drosophila	(Zhang et al., 2018) (Zhang et al., 2018)
Bosutinib (autophagy activator)	Survival improvement	iPSNs	(Imamura et al., 2017)
mTOR inhibitors and phenothiazine derivatives	Decrease accumulation and toxicity of DPRs and reduction of cell death caused by synergistic DPR accumulation and C9Orf72 protein depletion	DPRs-transfected HEK 293 cells	(Boivin et al., 2020)
TMX2 (ER protein) knockdown	ER stress signature modulation and survival improvement	iPSNs	(Kramer et al., 2018)
TUDCA and salubrinal (ER stress inhibitors)	Decrease in LDH activity in media and caspase-3 activation and protection against poly-GA-induced toxicity	Neurons expressing GFP-GA	(Zhang et al., 2014)

AAV-5: Adeno-Associated Virus 5; ASOs: Antisense-Oligonucleotides; BAC: bacterial artificial chromosome; C9-ALS: C9Orf72 related-ALS; C9Orf72: Chromosome 9 Open reading frame 72; Cas9: CRISPR associated protein 9; CFP: cyan fluorescent protein; CRISPR: Clustered Regularly Interspaced Short Palindromic Repeats; DPRs: dipeptide repeat proteins; DSIF: Double-Strand Break sensitivity-inducing factor; ER: endoplasmic reticulum; G_4C_2: GGGGCC; GA: glycine-alanine; GP: glycine-proline; GR: glycine-arginine; HEK 293: Human embryonic kidney 293; HRE: hexanucleotide repeat expansion; iPSNs: induced-Pluripotent Stem Cells-derived Neurons; LDH: lactate dehydrogenase; mRNA: messenger RNA; mTOR: mammalian target of rapamycin; NCT: nucleocytoplasmic transport; PAF1C: polymerase II-associated factor 1 complex; RAN: Repeat Associated Non-AUG translation; RBPs: RNA-binding proteins; SFSF1: serine/arginine-rich splicing factor 1; SGs: stress granules; SINEs: selective inhibitors of nuclear export; siRNA: short interfering RNA; ss-siRNA: single-stranded silencing RNA; TDP-43: TAR DNA-binding protein 43; TMX2: thioredoxin-related transmembrane protein 2; TUDCA: Tauroursodeoxycholic acid; UPS: ubiquitine-proteasome system; XPO1: exportin 1.