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Review

Omics-based exploration and functional validation of neurotrophic factors and histamine as therapeutic targets in ALS

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ABSTRACT

A plethora of genetic and molecular mechanisms have been implicated in the pathophysiology of the heterogeneous and multifactorial amyotrophic lateral sclerosis (ALS) disease, and hence the conventional "one target-one drug" paradigm has failed so far to provide effective therapeutic solutions, precisely because of the complex nature of ALS. This review intends to highlight how the integration of emerging "omics" approaches may provide a rational foundation for the comprehensive exploration of molecular pathways and dynamic interactions involved in ALS, for the identification of candidate targets and biomarkers that will assist in the rapid diagnosis and prognosis, lastly for the stratification of patients into different subgroups with the aim of personalized therapeutic strategies. To this purpose, particular emphasis will be placed on some potential therapeutic targets, including neurotrophic factors and histamine signaling that both have emerged as dysregulated at different omics levels in specific subgroups of ALS patients, and have already shown promising results in *in vitro* and *in vivo* models of ALS. To conclude, we will discuss about the utility of using integrated omics coupled with network-based approaches to provide additional guidance for personalization of medicine applications in ALS.

1. Introduction

Amyotrophic lateral sclerosis (ALS) is a rare progressive and chronically debilitating motor neuron disease with a new case diagnosed as fast as every 90 min. It is estimated that around 450,000 people worldwide are living with ALS and the number of cases is projected to increase as the population ages (Arthur et al., 2016). The etiology is not defined yet for most ALS patients. In particular, approximately 10 %–15 % of individuals with ALS have an inherited form of the disease thus classified as familial ALS (fALS) (Chiò et al., 2020), while the cause of the remaining percent of cases, known as sporadic ALS (sALS), is still unknown. Currently, no cure exists and only a small number of treatments (e.g., riluzole, edaravone, non-invasive ventilation) delay death (Mehta et al., 2018).

One of the reasons for this failure is the use of inappropriate recruitment of patients in clinical studies, without taking into account the medical and molecular heterogeneity of ALS. At present, the diagnosis is mainly based on clinical examination, but it is known that the disease starts with hidden symptoms initiating long before the appearance of any pathological sign, i.e. when an initial and peripheral disconnection of motor axons from the muscles gradually evolves into a loss of approximately one-third of the total motor neurons. This occurs long before any muscular weakness and/or atrophy is perceived. Because of this lag between disease onset and symptoms, ALS patients have to face

Abbreviations: ALS, amyotrophic lateral sclerosis; BDNF, brain-derived neurotrophic factor; CNS, central nervous system; CNTF, ciliary neurotrophic factor; CNV, copy number variants; CSF, cerebrospinal fluid; DAO, diamine oxidase; EGF, epidermal growth factor; ET-1, endothelin-1; G-CSF, granulocyte-colony stimulating factor; GDNF, glial-derived neurotrophic factor; HA, histamine; HDC, histidine decarboxylase; FGFs, fibroblast growth factors; HNMT, histamine *N*-methyltransferase; IGF-1, insulin-like growth factor 1; NTFs, neurotrophic factors; PACAP, pituitary adenylate cyclase-activating polypeptide; sALS, sporadic ALS; SNV, single nucleotide variations; SOD1, superoxide dismutase 1; TNF, tumor necrosis factor-α; TrkB.T1, tyrosine receptor kinase B.T1; VEGF, vascular endothelial growth factor

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with a first lost opportunity for therapeutic intervention. Due to a further underestimation of early symptoms by both patients and clinicians, and to a diagnosis often based on the exclusion of other possible diseases, another temporal delay of more than one year usually elapses before a definite ALS diagnosis can be formulated. Regrettably, this represents a second lost opportunity for initiating therapy.

Despite tremendous progress has been made during the past decade, there is yet lack of knowledge about ALS pathogenesis, but many hypotheses. It is now recognized that ALS is heterogeneous and multifactorial, characterized by the complex interplay between multiple genetic and environmental factors. Most consolidated pathogenic mechanisms include growth factor deprivation and glutamate toxicity, accompanied by oxidative stress, aberrant free radical handling, mitochondrial failing, metabolic alteration, proteasome dysfunction, protein aggregation, cytoskeletal impairment, and deranged axonal transport (Cook and Petrucelli, 2019; Chia et al., 2018). This complicated etiopathogenesis is obviously the summation of many pathological events and the main reason why ALS is undefeated today. We all agree that further research is necessary to shed light on the mechanisms underlying ALS, and to find effective and personalized treatments for patients.

Recent advances in high-throughput functional omics technologies and analytical tools for data integration, offer exciting opportunities to decipher the molecular landscape of ALS and the dynamics of disease manifestation. This review intends to highlight the perspective of integrative multi-omics approaches, with a particular emphasis on some candidate genes and pathways that might become useful for the development of a personalized genome-guided medicine for ALS.

2. Integrative “omics” approaches in the search for therapeutic targets in ALS

The pressing need for treating ALS has not yet met due to the complexity of disease mechanisms and multiple unknown genes and pathways. In the past decade, numerous high-throughput “omics” studies have been conducted on ALS to better understand the biology behind the disease, which will undoubtedly open new doors to therapeutics. Genome-wide association, whole genome/exome sequencing combined with functional genomics, transcriptomics, proteomics, metabolomics, and other omics have already enabled researchers to obtain comprehensive snapshots of biological systems at real time, and with single molecule resolution, thus identifying multiple genomic alterations related to ALS phenotypes, and providing biological insights to decipher the molecular underpinnings of the disease (Mitropoulos et al., 2018; Caballero-Hernandez et al., 2016). For instance, a recent study by Maniatis and coworkers has reported the use of RNA sequencing to define transcriptomic changes of mouse spinal cords over the course of the disease, as well as of postmortem tissues from ALS patients, identifying disease-associated pathways and establishing the key steps of motor neuron degeneration observed in ALS (Maniatis et al., 2019). Moreover, the growing number of ALS-linked genes (Babić Leko et al., 2019; Nowicka et al., 2019) has allowed researchers to identify a number of shared intracellular processes contributing to motor neuron degeneration in ALS, including oxidative stress, mitochondrial dysfunction, apoptotic mechanisms, disordered axonal transport, neuroinflammation, and autophagy (Cook and Petrucelli, 2019; Chia et al., 2018). In addition, some mutated RNA-binding proteins, including *TDP43, FUS, SOD1, UBQLN1* and *OPTN*, have been found in aggregates in the cytoplasm of motor neurons in ALS patients, leading to a broad range of deficits in RNA metabolism as well as to misfolding and mislocalizations (Blokhuis et al., 2013). Other interesting studies have shown that phosphorylated neurofilament heavy subunit protein concentrations in the cerebrospinal fluid (CSF) and blood of ALS patients represent candidate diagnostic and prognostic biomarkers, allowing to: (i) discriminate between patients with ALS and healthy individuals, and among different subtypes of motor neuron patients at an early stage of the diagnostic assessment, when the clinical signs are localized and subtle; (ii) provide monitoring during clinical trials to ensure target engagement (Wilke et al., 2019; La Cognata et al., 2018; Oeckl et al., 2016).

Although each layer of the omics profile offers exciting opportunities to identify important players in ALS pathogenesis, it is not sufficient by itself to acquire the precise picture of the composite molecular machinery involved in motor neuron degeneration. In this direction, integration of multi-omics measures with existing biological knowledge is essential for understanding how the interaction of multiple pathways is driving disease progression, providing a rational foundation for new potential therapeutic targets and biomarkers that will assist in the rapid diagnosis and prognosis of the disease, and for the stratification of patients into different subgroups with the aim of personalized therapeutic strategies. A good example of this approach is seen in previous works published by our research groups, in which we have established the foundation for a functional molecular taxonomy of ALS. In particular, we have firstly analyzed transcriptional profiles of motor cortex samples from sALS patients and identified two specific subgroups of patients (sALS1 and sALS2), depending on the combinations of genes and pathways that were found deregulated (Morello et al., 2017a, b; Aronica et al., 2015). We have identified antigen presentation/processing and extracellular matrix organization as the most representative subgroup-specific pathways in sALS1, while deregulated genes in sALS2 were associated with axonal guidance, oxidative stress and inflammatory intracellular signaling cascades. On one hand, our pathway-based analysis has confirmed the importance of molecular mechanisms that are known to be implicated in ALS pathogenesis. On the other hand and most importantly, it has been suggested for the first time the differential involvement of these processes in specific subsets of ALS patients. It is interesting to note that a similar stratification of ALS patients into different molecular subtypes has been confirmed in other recent studies (Tam et al., 2019; Vijayakumar et al., 2019; Jones et al., 2015). In particular, Tam and co-authors were able to stratify the transcriptomes of two independent cohorts of ALS postmortem cortex samples into three distinct molecular subtypes, two of which overlapped with the molecular signatures observed in our ALS patient samples (Tam et al., 2019).

To further elucidate the genomic events characterizing sALS pathology, we have recently characterized copy number variants (CNV) occurring in the same patients (Morello et al., 2019). Our findings have revealed distinct genomic signatures associated with the two previously characterized transcriptome-based sALS subgroups, suggesting a strong interaction between genomic and transcriptomic events in ALS. Beyond refining ALS molecular architecture, our goal was to identify and validate potential candidates for genomic-based patient stratification and individualized treatment. In other words, our aim was to identify genes that functionally drive or protect from ALS, and interrogate about their respective signaling pathways and therapeutic targets (Morello et al., 2019). Therefore, genes and pathways identified in the characterization of ALS subtypes were explored by using different pharmacological databases (i.e., PharmGkb, DrugBank), in order to identify compounds that inhibit or induce specific genes or proteins expression, or that block or stimulate specific pathways (Thorn et al., 2013; Wishart et al., 2008). Our analysis has revealed a good number of potential biomarkers and therapeutic targets that are differentially deregulated in specific subsets of ALS patients, bringing us a step closer to the establishment of a more efficacious and personalized genome-guided medicine for ALS. Besides identifying new potential pharmacological targets, our analysis might also provide a rational approach for “drug repositioning” for ALS. Under this perspective, many known drugs that were abandoned at clinical stages because of their low efficacy and/or toxicity might be re-evaluated in light of the emerging molecular taxonomy of ALS patients. Of note, some of the selected target genes that have emerged from our analysis exhibit conserved expression patterns in mouse and human ALS, thus providing a rationale to ensure their

preclinical trial success (Morello et al., 2017c).

Overall, we trust that a more complete analysis and evaluation of the molecular characteristics of the disease in each single patient is essential not only to reveal etiopathogenic mechanisms that are not clear by considering sALS pathology as a single entity (thus providing a powerful means for defining molecular signatures of the disease), but mostly to design more efficacious and individualized therapeutic interventions. In the following sections, we will provide a comprehensive multi-omics characterization of some of the most promising molecular targets and signaling mechanisms that have emerged as dysregulated in our works, focusing on those that have already shown results both in *in vitro* and *in vivo* models of ALS (Apolloni et al., 2019a, b; Maugeri et al., 2019; Bonaventura et al., 2018).

3. Omics-based exploration and functional validation of neurotrophic factors and neuropeptides as therapeutic targets in ALS

As potential targets for therapy, neurotrophic factors (NTFs) have been exploited in ALS, because of their large spectrum of biological effects, and because they regulate several physiological processes in the CNS, for instance neuronal differentiation and survival, axonal outgrowth and synapses maintenance, proliferation and differentiation of neural stem cells. Interestingly, several studies have already demonstrated the crucial role of NTFs to promote survival, and to be in part protective in models both *in vitro* and *in vivo* of motor neuron degeneration, thus representing a promising therapeutic strategy to treat ALS (Gouel et al., 2019; Tovar-y-Romo et al., 2014). In agreement with these works, our integrative omics analysis has identified a good number of potent neurotrophic and neuroprotective molecules as statistically deregulated in the same direction in ALS mouse models and human patients (Table 1) (Morello et al., 2017c, 2015; Aronica et al., 2015; Morello and Cavallaro, 2015). These include, among others, Ciliary Neurotrophic Factor (CNTF), Brain-derived Neurotrophic Factor (BDNF), Pituitary Adenylate Cyclase-Activating Polypeptide (PACAP), Epidermal Growth Factor (EGF) and Endothelin-1 (ET-1), Fibroblast Growth Factors (FGFs), Insulin-like Growth Factor 1 (IGF-1), Vascular Endothelial Growth Factor (VEGF), and Granulocyte-Colony Stimulating Factor (G-CSF), together with their corresponding receptors.

3.1. Ciliary neurotrophic factor

CNTF, a polypeptide hormone promoting neurotransmitter synthesis and neurite outgrowth, is one of the first NTFs to be investigated as potential drug target in ALS models, showing to improve motor function and survival and decreasing neuronal degeneration and muscle atrophy, when injected intraperitoneally or subcutaneously in the pmn/pmn and wobbler mice models of motor neuron disease (Stahl et al., 1994; Sendtner et al., 1992). Altered levels of *CNTF* expression are found in the brain and spinal cord of both ALS patients and animal models, and *CNTF* knockout mice develop atrophy and loss of motor neurons with aging, suggesting *CNTF* as a possible modifier gene for ALS (Laaksovirta et al., 2008; Gros-Louis et al., 2006; Giess et al., 2002). In addition, genetic alterations in the *CNTF* gene are also reported in individuals affected by ALS, including for example null mutations affecting splice sites (Gros-Louis et al., 2006). Despite these premises, in the 90's, two clinical phase I/II and phase III results obtained by subcutaneous administration of recombinant human CNTF (rHCNTF) in ALS patients, have demonstrated no statistically difference between rHCNTF-treated patients and placebo-treated patients, and even adverse effects at high doses (Bongioanni et al., 2004). The subsequent, phase I clinical trial with intrathecal pump delivery has suggested intrathecal administration as the potential preferred route for administrating CNTF but, also in this case, no significant clinical benefits were obtained for ALS patients (Penn et al., 1997; Group, 1996). Despite clinical trials in ALS patients have failed to show significant

Table 1
Neurotrophins and histamine signaling pathways differentially deregulated in cluster sALS patients.

Signaling pathway	Gene target	Expression	Therapeutic proposal
sALS1			
EGF signaling	*ErbB2*	Up-regulated	EGFR inhibitors (i.e., PKI166, BMS690514, Canertinib, Masoprocol, Gefitinib, Suramin, PD153035, Genistein, Erlotinib, Everolimus)
FGF-family signaling	*FGF1*	Up-regulated	FGFR agonist (i.e., PF-05231023)
	FGFR2	Down-regulated	
Histamine signaling	*H1R*	Up-regulated	H1-H4R modulators (i.e., Clozapine, Orphenadrine, JNJ7777120)
	H4R	Down-regulated	
sALS2			
BDNF signaling	*TrkB*	Up-regulated	TrkB inhibitors (i.e., Cyclotraxin-B and Ana-12)
PACAP signaling	*ADCYAP1*	Down-regulated	PACAP receptor agonists (i.e., Maxadilan)
	ADCYAP1R1		
EGF signaling	*EGF*	Up-regulated	EGFR/ErbB inhibitors (i.e., PKI166, BMS690514, Canertinib, Masoprocol, Gefitinib, Suramin, PD153035, Genistein, Erlotinib, Everolimus)
	EGFR		
	ErbB2		
EDN1 signaling	*EDN1*	Up-regulated	EDNR-B antagonists (i.e., BQ-788, Bosentan, IRL-2500)
	EDNRB		
FGF-family signaling	*FGF1*	Up-regulated	FGFR inhibitors (Orantinib, Brivanib, Dovitinib, Suramin, Pentosan polysulfate)
	FGFR1		
	FGFR2		
	FGFR3		
IGF-1 receptor signaling	*IGF1R*	Up-regulated	IGF1R inhibitors (ie., Masoprocol, BMS-754807 and Linsitinib)
VEGF-family signaling	*VEGFA*	Down-regulated	VEGF agonists (i.e., SB-509, Celecoxib)
G-CSF signaling	*CSF1*	Down-regulated	G-CSF modulators (i.e., pegfilgrastim, JNJ-40346527)
	CSF2RA	Up-regulated	
Histamine signaling	*H1R*	Up-regulated	Histamine receptor modulators (i.e., Orphenadrine, Ranitidine, Thioperamide, JNJ7777120)
	H2R	Down-regulated	
	H3R	Down-regulated	
	H4R	Up-regulated	
	HDC	Up-regulated	
	HNMT	Up-regulated	
	DAO	Up-regulated	

effects of CNTF on disease progression, promising results *in vivo* were obtained when CNTF was co-administrated with other NTFs, such as BDNF, suggesting that a synergistic action of these molecules (or their derivatives) may overcome side effects and increase the chances of success in arresting or reducing disease progression (Gouel et al., 2019; Henriques et al., 2010).

3.2. Brain-derived neurotrophic factor

BDNF is a neurotrophic factor that by binding two different receptors, respectively the low affinity p75NTR and the high affinity Tyrosine Receptor Kinase B.T1 (TrkB.T1), modulates glutamate receptor activity, synapse stability, dopaminergic, cholinergic, serotonergic, and GABAergic signaling, synaptogenesis, and dendritogenesis (Bathina and Das, 2015). Increased expression of *BDNF* and its *TrkB* receptor was found in the motor cortex of sALS patients, in accordance with the observation that a prolonged TrkB activation may render the motor neurons more vulnerable to pathophysiological insults, perhaps contributing to ALS (Morello et al., 2017c, 2015; Morello and Cavallaro, 2015). In fact, early synaptic hyper-excitability of motor neurons in ALS apparently enhances BDNF-mediated signaling, thereby causing glutamate excitotoxicity, and motor neuron death. In addition, the decrease of p75NTR expression, as well as the deletion of *TrkB.t1,* correlated with the delay of impairment and mortality in a mouse model of ALS (Yanpallewar et al., 2012; Turner et al., 2006). Despite the search for pharmacological compounds interacting with TrkB has been difficult, selective TrkB inhibitors (i.e., Cyclotraxin-B and Ana-12) have now shown promising effects by protecting motor neurons and decreasing neurotoxicity. The manipulation of BDNF/TrkB may thus give rise to neuroprotective therapeutic strategies in the treatment of diseases such as ALS (Pradhan et al., 2019; Carriedo et al., 2000).

3.3. Pituitary adenylate cyclase-activating polypeptide

Another interesting neurotrophic factor is PACAP, belonging to the vasoactive intestinal polypeptide/secretin/glucagon superfamily and involved in a wide range of physiological processes, including cell survival, stress response and cell division (Sherwood et al., 2000; Arimura et al., 1994). PACAP can initiate multiple signaling pathways by binding to three seven-transmembrane G protein-coupled receptors, PAC1R, VPAC1 and VPAC2, characterized by different ligand-binding specificities. Several *in vitro* and *in vivo* studies have revealed numerous biological activities of PACAP in the peripheral and central nervous system and its protective effects in neurodegenerative disorders including ALS (Chen et al., 2019; Maasz et al., 2017; Maugeri et al., 2017; Yang et al., 2015; Lee and Seo, 2014; Reglodi et al., 2011). For instance, neuroprotective activities of PACAP are already demonstrated against glutamate-induced excitotoxicity *in vitro* and *in vivo* in the most exploited animal model of ALS that recapitulates many features of the disease, the SOD1-G93A mice (Weihe et al., 2014; Ringer et al., 2013; Waschek, 2013; Tomimatsu and Arakawa, 2008). In our studies, PACAP and its receptor PAC1R exhibit a completely different mRNA and protein expression profile in the two sALS subgroups, perhaps due to different turnover rates or translation efficacy. While in sALS2 both PACAP and PAC1R are downregulated, in sALS1 they show opposite expression levels (Morello et al., 2017c; Aronica et al., 2015; Morello et al., 2015; Morello and Cavallaro, 2015). To validate PACAP as therapeutic target in ALS, we have then investigated the potential contribution of PACAP/PAC1R axis in motor neuron survival by using two well-characterized *in vitro* models of ALS, the motor neuron-like hybrid cell line NSC-34 expressing human SOD1-G93A, and human induced Pluripotent Stem Cells -derived motor neurons (Maugeri et al., 2019; Bonaventura et al., 2018). Our data have demonstrated that PACAP is able to rescue neuronal cells from apoptosis following neurodegenerative stimuli induced by growth factors deprivation, suggesting the involvement of the PACAP-PAC1R pathway in ALS pathology, and its role as a potential drug target to enhance motor neuron viability.

3.4. Epidermal growth factor

The mechanism underlying neuroprotective properties of PACAP could involve EGF pathway by activating its receptor EGFR and stimulating survival, proliferation, maturation, and migration of different cell types (Maugeri et al., 2019; Ayuso-Sacido et al., 2010; Junier et al., 1993). Despite its neuroprotective role, several lines of evidence suggest that the activation of EGFR signaling pathway may trigger quiescent astrocytes to become reactive astrocytes and, consequently, to play a role in the pathophysiology of many neurodegenerative disease including ALS (Liu and Neufeld, 2007; Liu et al., 2006). Consistent with the view that EGFR activation may be a marker of reactive astrogliosis surrounding degenerating motor neurons, we have observed increased expression levels of *EGF* and *EGFR* both in sALS2 patients and SOD1-G93A mouse models, making EGFR signaling pathway and PACAP/EGFR axis as attractive candidates for further pre-clinical studies (Morello et al., 2017c; Aronica et al., 2015; Morello et al., 2015; Morello and Cavallaro, 2015). To this regard, pharmacological inhibition of EGFR signaling cascade has been already successful in ALS preclinical studies by enhancing axon regeneration, and providing a significant delay in the onset of multiple behavioral measures of disease progression (Zhao et al., 2019; Li et al., 2014; Le Pichon et al., 2013; Yang et al., 2011; Koprivica et al., 2005; Trieu and Uckun, 1999).

3.5. Endothelin-1

Another neuropeptide that is able to interfere with EGFR signaling in astrocytes is ET-1 encoded by the *EDN1* gene that, together with its G-protein coupled receptor B (EDNR-B), is abundantly expressed in sALS motor cortex and in reactive astrocytes in the spinal cord of SOD1-G93A mice (Moody et al., 2017; Morello et al., 2017c; Aronica et al., 2015; Morello et al., 2015; Morello and Cavallaro, 2015; Ranno et al., 2014). EDN1 exerts toxic effects on motor neurons by activating several processes implicated in ALS pathogenesis, such as axonal degeneration, alteration of water homeostasis, increased sensitivity to oxidative stress and excitotoxic damage (Ranno et al., 2014; Lederer et al., 2007). Moreover, recent studies have shown that EDN1 overexpression may be a direct consequence of reduced C9ORF72 levels, one of the most common known cause of ALS (Fomin et al., 2018; Kotni et al., 2016). Consistently with these data, we have demonstrated that pharmacological treatments aimed at lowering EDN1 levels or antagonizing its effects may represent interesting therapeutic strategies in ALS, by protecting motor neurons from oxidative stress, inflammation and axonal damage both in ALS animal models and in mixed spinal cord cultures enriched with reactive astrocytes (D'Antoni et al., 2017; Ranno et al., 2014).

3.6. Fibroblast growth factors

FGFs constitute a family of multifunctional proteins expressed in the brain where mediate diverse physiologic functions, including cell differentiation, migration and survival, playing an important role in brain development and neuroprotection (Henriques et al., 2010). The aberrant activity of FGFs and their receptors, either through gain or loss of function, has been involved in different neuropathological conditions, including ALS (Cassina et al., 2005; Vargas et al., 2005). Differential expression of multiple genes encoding FGFs and their receptors FGFRs has been reported in sALS patients and in SOD1-G93A transgenic mice, starting at a pre-symptomatic stage and progressing with the spread of disease (Morello et al., 2017c; Morello and Cavallaro, 2015; Morello et al., 2015). If, on the one hand, disruption of FGFs signaling induces demyelination and axonal damage in ALS, on the other one, SOD1 aberrant function seems to mediate oxidative-stress-induced damage by

inducing the release of FGF-1 from motor neurons that, in turn, activates spinal cord astrocytes and initiates motor neuron apoptosis in ALS (Cassina et al., 2005). Of note, the selective inhibition of FGFRs has shown promising results in ALS preclinical studies demonstrating to prevent motor neuron death by reducing astrocyte activation and oxidative damage. Similarly, molecular and pharmacological modulation of FGFs signaling may represent a therapeutic approach for ALS due to effects not only on neurogenesis, but also on synaptic formation, neuron-glia interactions and inflammation (Woodbury and Ikezu, 2014; Li et al., 2010; Ohta et al., 2006).

3.7. Insulin-like growth factor 1

In addition to the FGFs signaling system, also IGFs are part of a complex system used by cells to communicate with their physiologic environment and regulate normal physiology as well as a number of pathological states. It has been reported that the production of IGF-1 is impaired in skeletal muscle fibers of ALS patients (Lunetta et al., 2012). Administration of IGF-1 is protective in the transgenic rodent model of ALS (Kaspar et al., 2003), suggesting the possibility to use IGFs signaling as a potential strategy for the treatment of ALS. While intraparenchymal spinal cord delivery of IGF in SOD1-G93A mice has shown higher expression of IGF-1 accompanied only by partial rescue of pathological features, a stereotaxic injection into the deep cerebellar nuclei significantly extends mice lifespan (Dodge et al., 2008; Lepore et al., 2007). Recently, the injection of self-complementary adeno-associated viral vector 9 (scAAV9), a more efficient transducing agent for IGF-1, has extended survival, and motor performance of SOD1-G93A mice when injected either intramuscularly or intravenously (Lin et al., 2018; Wang et al., 2018). Despite these premises, the safety and efficacy identified in animal studies was not translated to human trials. In fact, two randomized double-blind placebo-controlled clinical trials administering recombinant human IGF to patients with ALS have demonstrated limited or no effect on disease progression, while a phase II clinical trial is under investigation but results are not available yet (Sakowski et al., 2009; Borasio et al., 1998). The differential expression of IGF and its receptors between the two subgroups of sALS patients, as demonstrated previously (Aronica et al., 2015), may contribute to explain this clinical failure, suggesting that further clinical investigations should take into consideration genomic heterogeneity of ALS for a more accurate enrollment of patients into clinical trials.

3.8. Vascular endothelial growth factor

Trophic factors possess a short time frame for protection of motor neurons once the noxious process is triggered, and this is probably due to the rate at which motor neurons die during the time course of the disease. In murine models of familial ALS, the administration of VEGF before the beginning of symptoms provides a significantly better protection, as proven by delayed symptoms progression and increased lifespan, as compared to that obtained when the trophic factors are administered at the symptoms onset (Storkebaum et al., 2005; Azzouz et al., 2004). VEGF is part of the system that restores the oxygen supply to tissues when blood circulation is inadequate such as in hypoxic conditions. VEGF's normal function is to create new blood vessels during embryonic development, after injury, in muscle following exercise, and new vessels to bypass blocked vessels. Decreased expression levels of *VEGF-A* are found in a specific subgroup of sALS patients (sALS2), enforcing the view that reduced VEGF signaling may play a role in the pathogenesis of ALS (Aronica et al., 2015). Activation of VEGF receptor 2 triggers the phosphorylation of intracellular pathways driven by phosphatidyl-inositol-3-kinase (PI3-K), Akt, phospholipase C-β, and mitogen-activated protein kinase that promote the inhibition of pro-apoptotic factors like Bad and caspases 9 and 3. The activation of these intracellular signaling pathways has been extensively studied in the CNS, and VEGF-dependent activation of PI3-K/Akt is sufficient to prevent motor neuronal death in familial models of ALS *in vitro* (Tolosa et al., 2008; Li et al., 2003). In addition, some drugs activating VEGF (i.e., SB-509, Celecoxib) have been tested in phase II clinical trials for ALS, showing encouraging results (Pronto-Laborinho et al., 2014; Cudkowicz et al., 2006).

3.9. Granulocyte-colony stimulating factor

G-CSF, a cytokine/hormone produced by the endothelium, macrophages, and immune cells, stimulates the bone marrow to produce granulocytes and hematopoietic stem cells, and release them into the bloodstream stem. Protective properties with reduction of disease progression and increased survival and rescue of motor neurons are demonstrated in SOD1-G93A mice when delivered continuously of G-CSF (Pitzer et al., 2008). Similar results are also obtained with subcutaneous injection of a more stable analog of G-CSF, pegfilgrastim (Rando et al., 2017). It is interesting to note that also another secreted cytokine belonging to the same family, the colony stimulating factor 1 (CSF1), has been associated with inflammation in the central and peripheral nervous system in ALS, particularly by inducing microglial cell proliferation and neuronal damage in disease (Rando et al., 2017). Differential expression of CSF1 is found in the motor cortex of sALS patients as well as in the spinal cord of SOD1-G93A mice (Morello et al., 2017c; Aronica et al., 2015; Morello et al., 2015; Morello and Cavallaro, 2015). Pharmacological administration of selective inhibitors of CSF1-CSF1R signaling has demonstrated neuroprotective and anti-inflammatory effects in ALS, by reducing microglial cell proliferation and protecting skeletal muscle from denervation, consequently slowing disease progression, attenuating motor neuron cell death and extending survival of SOD1-G93A mice (Chitu et al., 2016; Martínez-Muriana et al., 2016).

3.10. Interactions with glutamate

Due to their wide spectrum of functions in the CNS, neurotrophic factors have been exploited for decades as therapeutic hypothesis in ALS. However, some intrinsic drawbacks have emerged between preclinical success and clinical failure of trophic factors in ALS therapy, perhaps due to routes of administration, CNS penetrance, safe dosages, treatment start time or long-term efficacy, and need of synergistic drug association. Notwithstanding these limitations, trophic factors remain essential for motor neuron maintenance and survival and are still considered as potential candidate molecules for the treatment of patients with ALS. To this regard, the use of new delivery systems to improve bioavailability, including prodrugs, nanocarriers and small molecules that can mimic the effects of these molecules, may overcome NTFs limits and represent a useful therapeutic strategy for ALS patients.

In addition to their trophic roles, neurotrophic factors are known to interact with glutamate and modify glutamate signaling directly, by modulating the expression of glutamate receptor subunits and calcium-regulating proteins, or also indirectly, by controlling the production of antioxidant enzymes, energy-regulating proteins, and anti-apoptotic or autophagy protein family members. Physiological glutamate has been known for several years to regulate neurogenesis, neurite outgrowth, synaptogenesis, and neuron survival in the developing and adult mammalian nervous system, other than possessing its canonical role as neurotransmitter at the synapses. The trophic effect of glutamate receptor activation is developmental stage-dependent, and determinant to the selective survival of neurons that have to make proper connections. During this sensitive developmental stage, any interference with the glutamate receptor functioning may generate widespread neuronal loss. This often involves neuron-glia interactions also through glutamate-induced release of trophic factors from glia. For instance, glutamate can stimulate the production of BDNF, which, in turn, modifies neuronal glutamate sensitivity, calcium homeostasis, and plasticity. However, depending on the strength of the stimulus, glutamate receptors can

mediate biphasic effects, with excessive stimulation becoming neurotoxic. In particular, under conditions of oxidative and metabolic stress, excessive activation of glutamate receptors may contribute to neuronal dysfunction and degeneration in diseases ranging from stroke to Alzheimer's disease, to psychiatric disorders (Martínez-Muriana et al., 2016). Attention must therefore be paid to these features, when therapeutic manipulation of excitatory amino acid receptors is considered in the clinical setting.

4. Omics-based exploration and functional validation of histamine signaling as therapeutic target in ALS

Not only neuropeptides and neurotrophic factors, but also several other neurotransmitters and neuromodulators can interfere with the glutamatergic signaling in the nervous system. Among these, histamine (HA) modulates glutamate receptor activity, increases the excitatory post-synaptic potentials, and facilitates the induction of long-term potentiation in the hippocampus, also favoring the direct release of glutamate evoked for instance by depolarization in hippocampal synaptosomes and in cultured astrocytes (Kárpáti et al., 2018; Rodriguez et al., 1997).

Since its discovery back in 1910 and further acceptance as a neurotransmitter in 1984, HA has indeed gained always more attention in health and disease, although its role in CNS dysfunctions still remains under investigation. Histaminergic neurons are located in the tuberomamillary nucleus of the posterior hypothalamus from where they project their axons all over the CNS, thus granting to HA a well-distinct and important pleiotropic role. As a neuroimmune modulator acting both *in vivo* and *in vitro*, of course HA has a central role in several neurological functions, for instance regulating the sleep-wake cycle, nociception, motor circuits, satiety signaling, energy balance, learning, and memory (Cacabelos et al., 2016; Fukui et al., 2016; Wada et al., 1991). HA signaling in neuronal and non-neuronal cells including glia and mast cells, is mediated by different classes of proteins abundantly distributed throughout the CNS that are: i) G protein-coupled receptors named H1R, H2R, H3R, H4R; ii) intracellular and extracellular enzymes accountable for HA synthesis (histidine decarboxylase, HDC) and degradation (histamine *N*-methyltransferase, HNMT, and diamine oxidase, DAO or AOC); iii) transporters among which primarily the VMAT2 that is responsible for HA vesicular uptake in the CNS (Passani and Blandina, 2011; Haas and Panula, 2003; Peter et al., 1995). These receptors, enzymes and transporters are thus the fundamental partners in the intercellular communication system mediated by HA in the CNS and involved in several neuropathological conditions (Hu and Chen, 2017).

Recent studies by our group have shown that HA behaves as a bioactive molecule also in ALS (Volonté et al., 2019a, b; Volonté et al., 2015). First of all, H1R-H4R, HDC, HNMT and DAO proteins are abundantly expressed in primary microglia (Apolloni et al., 2017) and motor neurons (Apolloni et al., 2019a) isolated from SOD1-G93A mice. Of note, HA receptors and enzymes are expressed also in microglia and motor neurons localized in lumbar spinal cord of SOD1-G93A mice at symptomatic phase of the disease and, most importantly, they are dysregulated during disease progression. For example, H1R is down regulated in lumbar spinal cord at pre-symptomatic and symptomatic phases, but up regulated in cortex and hypothalamus at symptomatic and end stage of the disease. H2R is down regulated in spinal cord at end stage and in cortex at symptomatic phase. H3R is increased of about two-fold in the hypothalamus at end stage, while it's not apparently modulated as a function of disease progression in spinal cord and in motor cortex. H4R is increased in spinal cord at symptomatic phase, while in cortex at pre-symptomatic phase. HDC in spinal cord is significantly down regulated at pre-symptomatic phase, but up regulated at end stage of disease, and over expressed in cortex and hypothalamus at symptomatic phase. HNMT is augmented in both spinal cord and cortex at symptomatic phase, and in hypothalamus at symptomatic and end stage of disease. Finally, DAO is up-regulated in lumbar spinal cord and hypothalamus at symptomatic and end stage, but it's not affected during the disease in cortex (Apolloni et al., 2017).

In addition to the protein expression data, whole-genome expression profiles of motor cortex and spinal cord from healthy subjects and sporadic patients have demonstrated that numerous genes involved in HA receptors, metabolism, transport, secretion and signal transduction, are deregulated in the two transcriptome-based subgroups of sALS1 and sALS2 patients (Table 1) (Apolloni et al., 2019a, 2017), as segregated by unsupervised hierarchical clustering (Aronica et al., 2015). In particular, the cortical mRNA expression of H1R, HDC, HNMT and DAO is increased in sALS2, while H2R and H3R are selectively reduced. H4R is differently modulated in sALS1 (down-regulated) versus sALS2 (up-regulated), with respect to healthy individuals. However, spinal cord analysis has demonstrated that H1R is reduced and H3R is increased in sALS1 and sALS2 patients (Apolloni et al., 2017). Despite some peculiarities and exceptions, the HA axis in both sALS1 and sALS2 subgroups of patients demonstrates a substantial up-regulation of H1R in cortex (similarly to what reported in SOD1-G93A mice) and down-regulation in spinal cord (as found in pre-symptomatic and symptomatic SOD1-G93A mice), in addition to the increased expression of H3R in spinal cord. These results clearly indicate that the histaminergic system is affected during ALS, within relevant tissue- and disease phase-specific deregulations of expression. Furthermore, the vast majority of HA-related genes that appear deregulated in SOD1-G93A mice at terminal stage are also deregulated in at least one of sALS patient subgroups (e.g. HRH1, HRH3). This evidence offers a good rationale for the selection and prioritization of HA genes as potential biomarkers and targets for patient-oriented ALS care.

Under this perspective, a large-scale meta-analysis of genome-wide association studies performed on patients affected by sALS has moreover identified that the Ile105 polymorphism on the Thr105Ile allele in the *HNMT* gene (generating about 60 % decrease of HA-degrading activity), causes a trend in delaying the onset of ALS symptom by about three years. This suggests that the Thr105Ile allele in the *HNMT* gene could potentially become an important therapeutic target and protective modifier for the treatment of ALS (Chen et al., 2018).

By combining gene expression profiles, copy number variants and single nucleotide polymorphisms of ALS patients, further studies have then adopted a multiomics approach for integrating transcriptomic and genomic data with the ALS-linked pathogenic variants obtained from the ALSdb database. This approach has allowed to capture HA pathway associations in ALS (Apolloni et al., 2019a). The genes coding for HA receptors, enzymes and transporters have shown numerous pathological variants. In particular, genome-wide analysis of multiple genomic aberrations occurring in sALS patients has identified some HA-related genes that are copy number variants-affected (*H2R* and *DAO* show amplification, while *H3R* duplication), and that also show a positive correlation with transcriptomic changes in one or both sALS patient subgroups. Among these, we find for instance the genes encoding *ADCYAP1*, *CCKBR* and *H3R*. Moreover, single nucleotide polymorphisms occur in *H1R*, *H2R*, *H3R*, *H4R*, *HDC*, *HNMT* and *DAO* genes (Apolloni et al., 2019a). In particular, the histamine H3R is the only to present genetic, transcription as well as protein variations correlated with ALS (Fig. 1). The genome profiling from sALS patients' data indeed indicates that the *HR3R* gene associates with CNV duplication regions and missense single nucleotide variants (SNV). Moreover, the profiling from post-mortem spinal cords from the two molecularly distinct sALS patients subgroups, proves that the transcription of *HR3R* is increased between 3.2- and 3.3-fold in comparison with individual controls. Finally, protein expression data demonstrates that the receptor is increased at least 2-fold in the hypothalamus of SOD1-G93A mice at end stage of disease. In other words, a clear homogeneity exists between the HA-related HR3R gene driven by protein, gene expression, CNV, and single nucleotide polymorphisms data. This knowledge validates the hypothesis that HA-related genes might indeed represent candidate

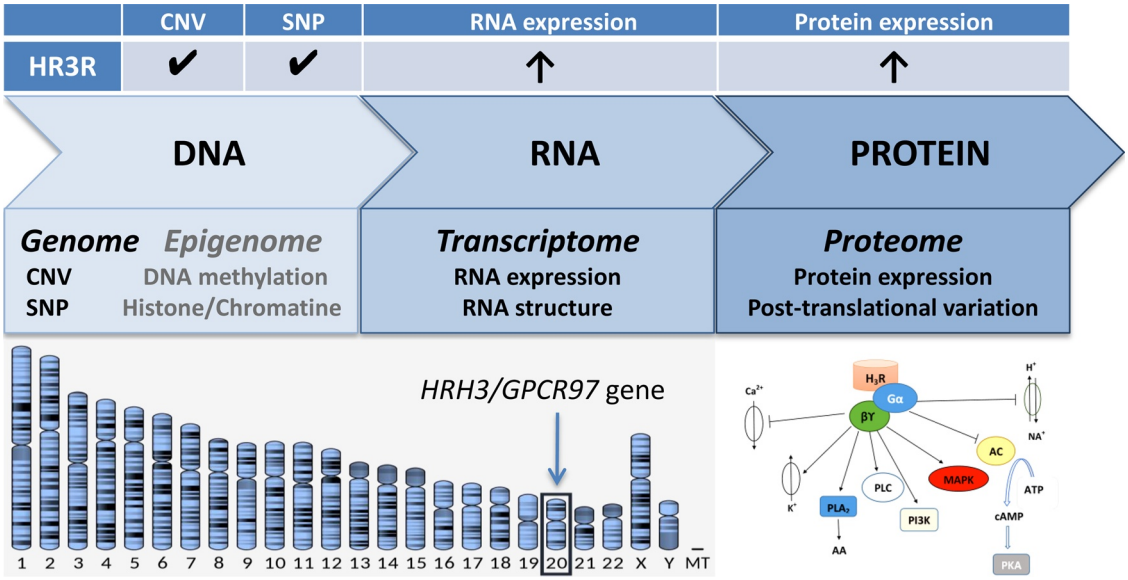

Fig. 1. Multiomic involvement of histamine H3 receptor in ALS. The picture shows that the histamine H3 receptor presents genetic, transcription and protein variations correlated with ALS. In particular from sALS patients' data, the genome profiling indicates that the HR3R gene associates with copy number variants (CNV) duplication regions and missense single nucleotide variants (SNV). Moreover, the profiling from post-mortem spinal cords from two molecularly distinct sALS patients subgroups, shows that the transcription of HR3R is increased between 3.2- and 3.3-fold in comparison with control healthy subject. Finally, protein expression data demonstrates that the receptor is increased at least 2-fold in the hypothalamus of SOD1-G93A mice at end stage of disease. The data are reported from references Apolloni et al. (2019a) (2019b), (2017).

drivers in ALS pathogenesis, and that the HA axis might become a potential target for therapy.

In support of this, current research validates this hypothesis by demonstrating that chronic administrations of the HA precursor histidine to SOD1-G93A mice from disease onset up to end stage of disease increases the histamine content in spinal cord, most importantly giving raise to general ameliorative effects. In particular, histidine improves behavioral features of ALS, retards disease progression, recovers motor performance, increases life-span, reduces motor neuron loss and neuroinflammation in spinal cord, finally ameliorating neuromuscular junction integrity and muscle atrophy (Apolloni et al., 2019a). Besides the established neuroprotective effects described in motor neurons and anti-inflammatory actions in microglia, the heat shock and autophagy responses also contribute to the rescue of motor neurons and spine density loss occurring in the motor cortex of HA-treated ALS mice (Apolloni et al., 2019b). By proposing that the histaminergic modulation can indeed interfere at different levels and within different time frames with the ALS course, we trust that HA might assume a new translational benefit in the development of more effective therapeutics against the disease (Apolloni et al., 2016a, b).

5. Systems pharmacology: a new paradigm for drug development and personalized medicine in ALS

Given the multifactorial etiopathogenesis of ALS, it is not surprising that the conventional 'one target-one drug' paradigm fails to provide effective treatments against the disease. Indeed, complex diseases like ALS, are rarely caused by a single gene abnormality, but rather by the perturbation of multiple series of intracellular and intercellular interactions between molecular entities, including protein-protein binding, gene co-expression, RNA coupling, and many other types of molecular functional association (Benchekroun and Maramai, 2019; Ramsay et al., 2018, 2016; Geldenhuys and Van der Schyf, 2013). On this basis, it would be advisable to shift the ALS drug discovery research towards new alternative approaches aimed at the simultaneous targeting of multiple proteins (and therefore etiologies) involved in disease onset and progression, and at the identification of synergistic drug effects, thus offering the possibility to reach dysfunctional processes that may be impractical to resolve with a single drug therapy. This approach, known as polypharmacology, will not only facilitate the discovery of new and interesting drug-target associations, but also will provide a comprehensive understanding of the drug's mechanisms of action and off-target effects, thus offering new hope for the design of more effective and safer treatments for patients (Bolognesi and Cavalli, 2016; Villaveces et al., 2015). Moreover, the application of network-based approaches, particularly protein-protein interaction networks, represents a useful tool in the drug-discovery pipeline, because each protein-target is not working alone, but in a framework containing its connectivity with other proteins, allowing for a comprehensive understanding of the molecular basis of the disease and identification of important alternative targets for its treatment, by using multi-omics datasets (Casas et al., 2019; Murakami et al., 2017; Athanasios et al., 2016; Chakraborty et al., 2014). Despite in the last years a diverse range of works have been reported in the literature highlighting the power of multi-target approaches and network pharmacology for neurodegenerative diseases, including Charcot-Marie-Tooth disease type I and Alzheimer's disease, the application of polypharmacology to ALS has been minimal (Morgan et al., 2018; Vaz et al., 2017; Ramsay et al., 2016; Hughes et al., 2016; Liu et al., 2014; Chumakov et al., 2014). To provide an example of how the use of the molecular interactome could substantially support drug discovery for personalized medicine in ALS, we have built two drug-target networks genes encoding neurotrophins and HA signaling mediators that we have found differentially deregulated in the two sALS patient subgroups sALS1 and sALS2, together with their pharmacological modulators (Figs. 2, 3). In particular, we have downloaded 373 human genes related to neurotrophins (CNTF, BDNF, PACAP, EGF, ET-1, GFG, IGF-1, VEGF, G-CSF) and histamine signaling mediators from the Gene Ontology database AmiGO (http://amigo.geneontology.org/amigo), and then evaluated their expression in the two sALS patient subgroups. Next, we have investigated drug-gene interactions and potential druggability of these neurotrophins/HA-related differentially expressed genes, by using the Drug Gene Interaction Database (DGIdb, http://dgidb.org). Finally, the two sALS clusters-associated drug-target networks were visualized and analyzed by Cytoscape software (v3.2.0, http://www.cytoscape.org/), in order to identify the most highly-interconnected drugs/targets, defined as "hub nods"

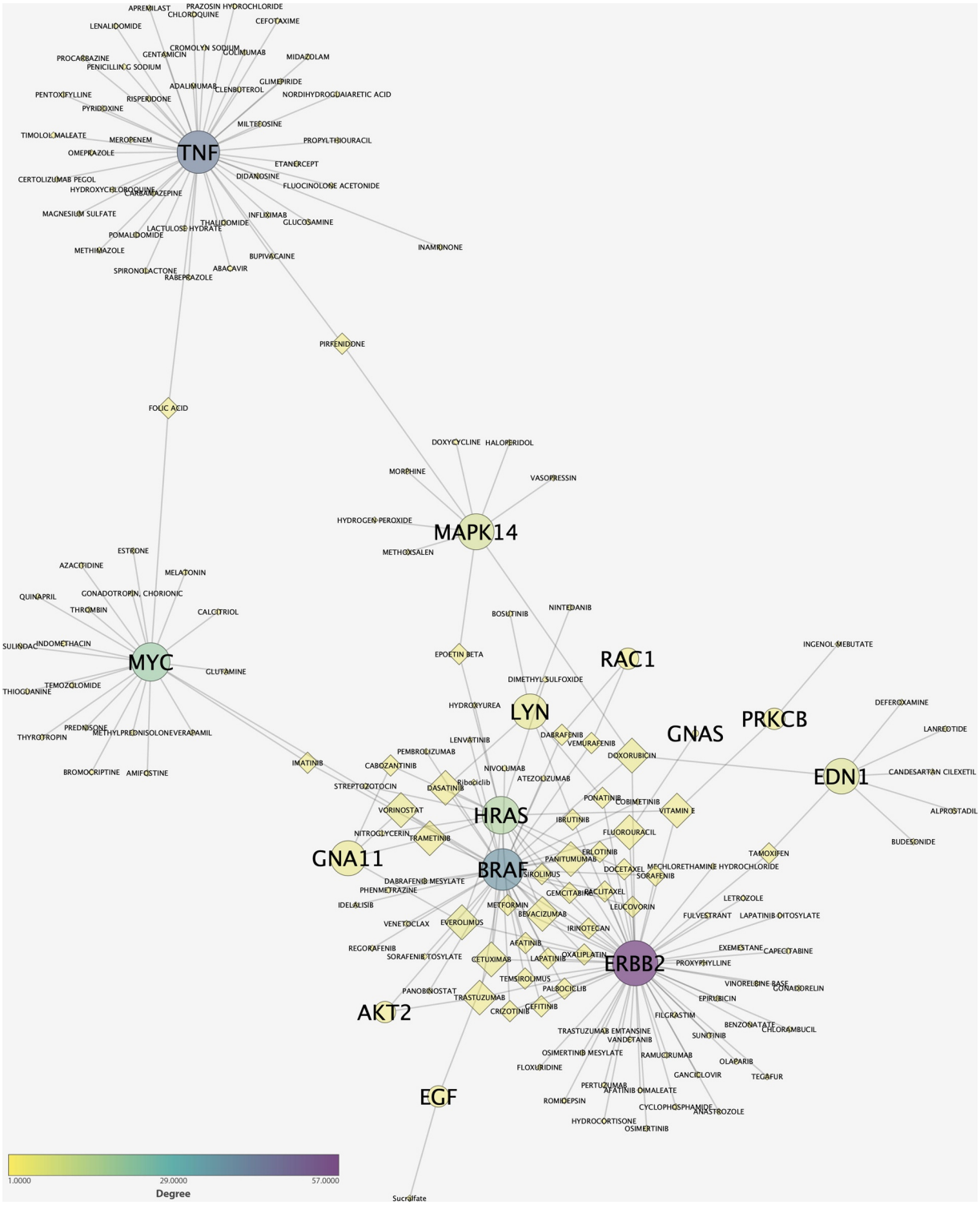

Fig. 2. Drug–Gene network in sALS1. The DG-network is generated between the neurotrophins and histamine related target genes in sALS1 and their known pharmacological modulators. Circles and rhombus correspond to target genes and drugs, respectively. Each node (target/drug) is colored along a color gradient on the basis of its degree of connectivity (the number of connections with other nodes) and the edges represent interactions between drugs and targets as well as between two genes. *ERBB2* and everolimus are respectively the hub protein and drug in the network. Network properties for the 5 most connected "hub nodes" (drugs and targets) are detailed in Table 2.

(Figs. 2, 3). Personalized drug-target interactions not only provide a comprehensive mapping of the different involvement of neurotrophins and HA signaling cascades in two sALS subgroups, but also identify selective candidate driver genes and drugs, thus providing guidance for precision medicine in the future. For example, both networks have highlighted a significant role of EGFR/ERBB signaling in ALS pathogenesis, supporting its relative pharmacological modulators as promising therapeutic approaches. In particular, *ERBB2* and one of its modulators, everolimus, are the most interconnected target/drug in the sALS1-related network and among the top five hub nods in the sALS2-

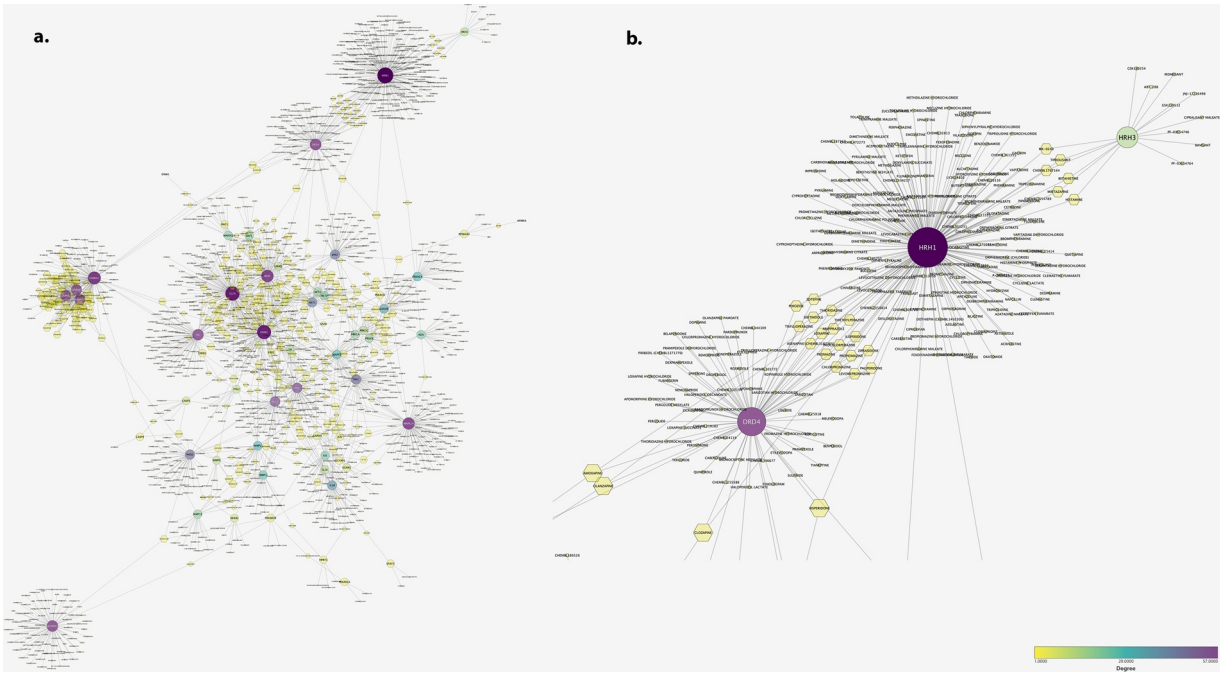

Fig. 3. Drug–Gene network in sALS2. The DG-network (**a**) is generated between the neurotrophins and histamine related target genes in sALS2 and their known pharmacological modulators. Circles and rhombus correspond to target genes and drugs, respectively. Each node (target/drug) is colored along a color gradient on the basis of its degree of connectivity (the number of connections with other nodes) and the edges represent interactions between drugs and targets as well as between two genes. (**b**) *HRH1* and paclitaxel are respectively the hub protein and drug in the network. Network properties for the 5 most connected "hub nodes" (drugs and targets) are detailed in Table 2.

Table 2
The 5 top nodes (Targets/Drugs) of sALS1 and sALS2-related networks based on degree values.

Target	Degree	Drug	Degree
sALS1			
ERBB2	57	EVEROLIMUS	5
TNF	43	DOXORUBICIN	4
BRAF	38	VORINOSTAT	4
MYC	21	CETUXIMAB	4
HRAS	17	TRASTUZUMAB	3
sALS2			
HRH1	186	PACLITAXEL	9
EGFR	146	GSK-690693	8
ERBB2	139	EVEROLIMUS	7
GABRA1	113	DOXORUBICIN	7
BRAF	100	ENZASTAURIN	6

related network (Figs. 2, 3, Table 2). Despite not tested yet in ALS, the rapamycin analogue everolimus has demonstrated to reduce degeneration of neurons and ameliorate early declines in motor performance in preclinical models of multiple neurodegenerative diseases including Huntington's and Alzheimer's diseases, suggesting its repositioning in ALS (Cassano et al., 2019; Talboom et al., 2015; Bové et al., 2011; Fox et al., 2010). In addition, the sALS1-related network has also revealed the selective alteration of tumor necrosis factor-α (TNF), suggesting that its pharmacological modulation may represent a therapeutic strategy to block or slow disease progression in specific subgroups of ALS patients (Fig. 2, Table 2). To this regard, lenalidomide is a potent immunomodulatory agent that, by reducing the TNF expression, inactivates downstream effector caspases, extending survival in transgenic mouse models of ALS (Neymotin et al., 2009; Kiaei et al., 2006). On the other hand, the complex sALS2-related drug-target network has highlighted several targets involved in inflammatory, apoptotic, and survival gene signaling (Fig. 3**a** and **b**). In particular, we have observed a significant and selective contribution of GABAergic and histaminergic signaling with *HRH1* as the most interconnected target in the network (Fig. 3**a** and **b**, Table 2). In the context of a multi-targeted therapy for ALS, an interesting example is represented by clozapine, an HRH1 antagonist that induces changes in GABA release in brain regions and also modulates DRD4, exerting neuroprotective effects in a variety of neurological disorders (Turner et al., 2003; Factor and Friedman, 1997) (Fig. 3**a** and **b**, Tables 1 and 2). Moreover, sALS2-related network analysis has supported the potential repurposing of multiple microtubule-binding anticancer drugs (i.e., paclitaxel, docetaxel) for the genomics-driven therapy in ALS (Fig. 3**a** and **b**, Table 2). Despite the clinical use of these drugs is often limited by their neurotoxicity, they were shown to restore lost nerve signals in neurodegenerative diseases like Alzheimer's and their safe analogues have shown remarkable neuroprotective properties for motor neurons both in cell culture and in rodents, by stimulating neuronal survival and axonal sprouting (Sunyach et al., 2012; Shemesh and Spira, 2011).

6. Conclusions

While ALS has been known for over 200 hundred years, we are still in an initial stage for its comprehension, with most research carried out in the last thirty years or so, thanks also to the advent of high-throughput omics data analysis for the identification of molecular alterations, candidate gene drivers and their impact on the outcome of the disease. In the last years, our research group has highlighted for the first time the existence of a biological and molecular heterogeneity in ALS postmortem cortex samples. Although the use of postmortem brain tissues does not allow to clarify whether investigated signaling cascades are a cause or consequence of the disease process, it represents a vanishingly rare resource for investigating molecular mechanisms, underlying neurological disorders, and providing essential features that cannot be obtained by using other approaches or living patients.

With this in mind, the most important goal for the next decade of ALS research should now include the (i) understanding of the origins of motor neuron death as hallmark of the disease; (ii) recognition of the

overall contribution of genes, protein targets and mechanisms involved in the pathogenesis and progression of the disease; (iii) validation of new biomarkers to help early and precise diagnosis; (iv) rational and effective patient stratification and system therapy efforts; (v) not least, repurposing or discovering new treatments including drugs, antibodies, stem cell and gene therapies. Overall, as highlighted by the several examples addressed in the present work and regarding the power of neurotrophic factors and histaminergic signaling, the potential of network pharmacology and molecular subtyping in ALS may not only provide new important clues to the causes of the disease but, mostly, provide a clear understanding of disease prognosis and progression and thus guide personalized treatments.

reference

Chumakov et al. (2014)

Declaration of Competing Interest

None

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