Chapter 12

# Molecular Taxonomy: Use of Transcriptional Profiles to Identify Different ALS Subtypes

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Additional information is available at the end of the chapter

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**Abstract**

Advances in diagnostic techniques and high-throughput biotechnologies provide a compelling opportunity to improve the diagnosis and treatment of diseases by developing a "New Taxonomy" that defines diseases on the basis of their underlying molecular and environmental factors rather than on traditional physical signs and symptoms. Oncology represents the first interesting example of how genomic medicine has changed the understanding of diseases and their therapy. However, much work remains to be completed on the molecular characterization and classification of complex and multifactorial diseases, including neurodegenerative disorders. Our research group has recently shown the genomic heterogeneity of sporadic amyotrophic lateral sclerosis (SALS), identifying two divergent subtypes associated with differentially expressed genes and pathways and providing several potential biomarkers and therapeutic targets. This chapter reviews the results emerged from our work, highlighting how molecular characterization of SALS patients may provide a framework for developing a more precise and accurate classification of diseases that could revolutionize the diagnosis, therapy, and clinical decisions of diseases, leading to more individualized treatments and improved outcomes for patients.

**Keywords:** ALS, expression profiling, genomics, molecular taxonomy, pathway analysis, system biology

## 1. Introduction

The current diagnosis and classification of diseases are primarily based on physical signs and symptoms that, despite providing valuable information about clinical course, are often not sufficient to fully characterize the complex and heterogeneous nature of many disorders.

The completion of the human genome sequencing together with advances in high-throughput genomic, proteomic, imaging, and other diagnostic techniques in the past decades has provided a framework for developing a new, more accurate, and refined "molecular taxonomy" of human diseases which implies the use of molecular data (i.e., gene expression, copy number variants, single nucleotide polymorphisms, and haplotype analysis) to classify patients into distinct subgroups with differing diagnostic, prognostic, or therapeutic implications. This new disease classification has profound implications not only providing new insights into studying mechanisms and environmental causes underpinning diseases but also facilitating the development of a more precise diagnosis and individualized treatment for optimal therapeutic efficacy [1–3]. One seminal example of how molecular data may translate into clinical practice is represented by the "drug repositioning "approach. In fact, many drugs that were abandoned at clinical stages because of their low efficacy and/or toxicity in a specific subtype of patients may be re-evaluated for their potential therapeutic role with the consequent possibility to reduce both the time and costs associated with drug discovery and development [4].

Oncology offers multiple examples of how genomic medicine has changed disease understanding and drove targeted therapeutic interventions. Numerous studies, in fact, have demonstrated the power and ability of gene expression profiling, and other molecular approaches, to classify and substratify patients with various types of cancer (e.g., glioblastoma, breast, and colon carcinoma) into selective clinically relevant subtypes characterized by similar clinicopathological features but different biological properties, prognostic biomarkers, and treatment options [5]. Based on these promising results, over the past years, this new molecular reclassification has been extended to other polygenic and multifactorial human disorders, including cardiovascular and rheumatic diseases and multiple sclerosis [6]. However, a lack of progress remains in the understanding of detailed molecular mechanisms of several neurological and neurodegenerative diseases mainly because of the limited access to human brain tissues. Thus, the patient-specific molecular diagnosis of many neurological disorders and the consequent translation of this into tailored clinical trials and specific treatments remain challenging tasks [7].

Recently, by using an unsupervised hierarchical clustering analysis on motor cortex samples of patients with sporadic amyotrophic lateral sclerosis (SALS), our research group has identified two greatly divergent subtypes, each associated with differentially expressed genes and biological pathways [8]. These experiments highlight, for the first time, the genomic heterogeneity of SALS, revealing new clues for defining molecular signatures for this disease that were not put in evidence by considering SALS as a single entity. Moreover, the altered pathways of biological molecules in SALS also provided a number of potential biomarkers and

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therapeutic targets that could be used for developing personalized diagnosis and treatment of amyotrophic lateral sclerosis (ALS) [9, 10].

In this chapter, we will first briefly review the current state of the art in the ALS classification system, showing how the recent advances in technology and genetic discoveries have revolutionized ALS research. Then, we will discuss our data, the experimental setup, and results, highlighting how the molecular characterization of SALS patients may provide a framework for developing a new taxonomy of the disease and establishing the foundation for personalized medicine in ALS.

## 2. Amyotrophic lateral sclerosis: an overview

ALS is a neurodegenerative disease characterized by the progressive muscular paralysis reflecting the degeneration of upper and lower motor neurons which leads to respiratory insufficiency and death after three to five years. ALS is the commonest of the motor unit diseases in Europe and North America and its incidence ranges from 1.7 to 2.3 cases per 100,000 population per year worldwide [11]. Currently, there is no cure or prevention for ALS and Riluzole is the only disease-modifying medication presently approved by the US Food and Drug Administration (FDA) for the treatment of ALS [12]. Riluzole is largely symptomatic and prolongs survival but only with a modest effect. Many clinical trials have been performed but have unfortunately had limited success [13]. Thus, the development of novel treatments and diagnostic research strategies is a goal of increasing urgency.

Accurately understanding the etiopathogenic mechanisms underlying ALS is a crucial step for developing effective diagnostic–therapeutic strategies. Approximately 95% of the cases are isolated or sporadic (SALS), while about 10% are familial (FALS), showing autosomal dominant, recessive, or X-linked inheritance. Although genetic studies in FALS are rendered difficult by the late onset of disease, its incomplete penetrance and the short survival of affected family members, several familial ALS loci, and genes have been identified [14–16], such as *SOD1*, *ALSIN*, *SETX*, *SPG11*, *FUS*, *VAPB*, *ANG*, *TARDBP*, *FIG4*, *OPTN*, *ATXN2*, and *C9ORF72*. The contribution of genetic risk factors also seems to be considerable into the sporadic form of the disease [16]. Despite the identification of several disease-linked mutations, the etiology and pathogenesis of ALS remain largely unknown, supporting the multifactorial and complex nature of this disease, in which multiple genetic variants, each of the small effects, combine with a variety of environmental triggers and risk factors [14, 16–18].

The diagnosis of ALS is primarily based on the clinical observation of symptoms, physical signs, progression, and electrodiagnostic testing, in accordance with the "El Escorial" criteria. These represent a catalog of clinical and diagnostic features, specified by the World Federation of Neurology (www.wfneurology.org), that aim to exclude "ALS-mimic" syndromes (i.e., cervical spondylotic myelopathy, multifocal motor neuropathy, and Kennedy's disease) and permit to classify ALS patients for research studies [19–21]. The broad clinical spectrum of ALS comprehends distinct phenotypes ranging from pure upper motor neuron disease to pure

lower motor neuron disease, with several different intermediate forms (classic, flail arm, flail leg, pyramidal, respiratory, and bulbar), each characterized by different degrees of involvment of Upper Motor Neurons (UMN) and Lower Motor Neurons (LMN), body regions that are affected, degrees of involvement of other systems especially cognition and behavior, and progression rates [22].

Although clinical neurophysiology in ALS plays a fundamental role in both diagnosis and assessment of its severity and progression, the initial symptoms of ALS are often subtle (limb or shoulder weakness and difficulty in walking), leading to a delay in the diagnosis as well as misdiagnosis and, consequently, restricting the possibilities for effective preventive and therapeutic strategies [23]. Thus, the adequate integration of neurophysiological techniques and advanced biological methods is essential in order to obtain a better understanding of disease pathogenesis, support earlier diagnosis, inform about prognosis, and monitor ALS progression in clinical trials.

The advent of high-throughput techniques—microarrays and next-generation sequencing—has shed light on the pathophysiology of complex diseases, including ALS and oriented researchers from a single-molecule analysis toward a "system biology" approach, offering a better understanding of the molecular mechanisms that, interacting with each other, may contribute to ALS pathogenesis [4].

## 3. Transcriptional analysis in ALS

In the last decade, the quantification of the transcriptome has represented one of the most informative research strategies for both discovering and defining mechanisms of pathogenesis in ALS as well as facilitating the discovery of biomarkers or new therapeutic approaches [24, 25]. In this regard, high-throughput genomic technologies, such as DNA microarrays, have been developed to simultaneously screen, on a genome-wide scale, the expression of thousands of genes in parallel in the same experiment, providing a more detailed picture of the ALS-related profile of molecular changes occurring during the disease progression [24, 26–28]. Both ALS post-mortem tissues (e.g., brain, spinal cord, cerebrospinal fluid, and blood) and those taken from animal models have been investigated with this purpose, revealing the involvement of several cellular events into ALS pathobiology, including mitochondrial dysfunction, enhanced apoptosis, glutamate-mediated excitotoxicity, oxidative stress, protein misfolding/aggregation, abnormal calcium metabolism, and altered axonal transport and neuroinflammatory cascades [29, 30]. However, because of the inherent complexity of nervous tissue and the need for post-mortem material, the existing genomic studies of ALS were restricted to a limited number of post-mortem ALS samples (≤11 motor cortex and 14 spinal cord), which did not permit the inclusion of genome changes within a framework of pathways or networks [24, 26–28].

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## 4. Toward a molecular classification of SALS patients: our experience

Recently, our research group has analyzed whole-genome expression profiles of motor cortex samples from control and SALS patients [8]. In particular, using an unsupervised hierarchical clustering, we were able to separate control from SALS patients and subdivide the latter into two different subgroups (SALS1 and SALS2), based on differentially expressed genes and pathways. This molecular stratification of SALS patients has permitted us to reveal a novel etiopathogenic mechanism that was not emerged by considering SALS as a single entity, providing new interesting opportunities for defining molecular signatures for the disease. Moreover, our analysis has revealed a good number of potential therapeutic targets for the treatment of ALS patients [9, 10]. In the following sections, we will review our results, highlighting their potentiality for developing a new molecular taxonomy of ALS disease moving increasingly toward the idea of a personalized medicine for patients.

### 4.1. Data and results

To uncover the entire spectrum of genes and pathways involved in ALS pathology, we have analyzed whole-genome expression profiles of motor cortex samples from control and SALS patients [8]. In particular, we monitored whole genome mRNA expression profiles of genes in motor cortex of control (10) and SALS (31) patients. Unsupervised hierarchical clustering was used to cluster control and SALS patients on the basis of their similarities measured over the most informative genes (9646). Transcriptomic profiles obtained in cortex samples produced a good separation of controls and SALS patients, segregating these latter into two greatly divergent groups: SALS1 and SALS2 (**Figure 1a**). Interestingly, this patient stratification was not related to technical variations (arrays hybridization) or patient demographics (gender, age at onset, age at death, survival time from the date of onset, post-mortem intervals (PMIs)) (**Figure 1a**). By comparing gene expression profiles in SALS patients to controls, we identified a good number of significantly differentially expressed genes: 4485 in SALS1 and 16144 in SALS2. Although some of these genes (1268) were differentially expressed in both pairwise comparisons, the majority of differentially expressed genes were cluster specific (**Figure 1b**). A larger number of genes (21,930) were differentially expressed between SALS1 and SALS2, indicating these clusters were greatly divergent at the genomic level. Moreover, in order to depict genome changes within a framework of pathways, the most informative genes were subjected to pathway analysis by using functional ontologies represented in the MetaCore repository [31]. Statistically significant canonical pathways emerged from our analysis were involved in different cellular processes: apoptosis and survival, cell adhesion, cytoskeleton remodeling and axonal transport, cell cycle, immune response, energy metabolism, and signal transduction (**Figures 2–5**).

### 4.2. Pathway analysis and exploration of potential drug targets in ALS

In the following sections, we will review functional clusters of coregulated genes and pathways emerged in our previous work in light of the main pharmacological targets present, potentially useful for the development of more effective and personalized treatments for

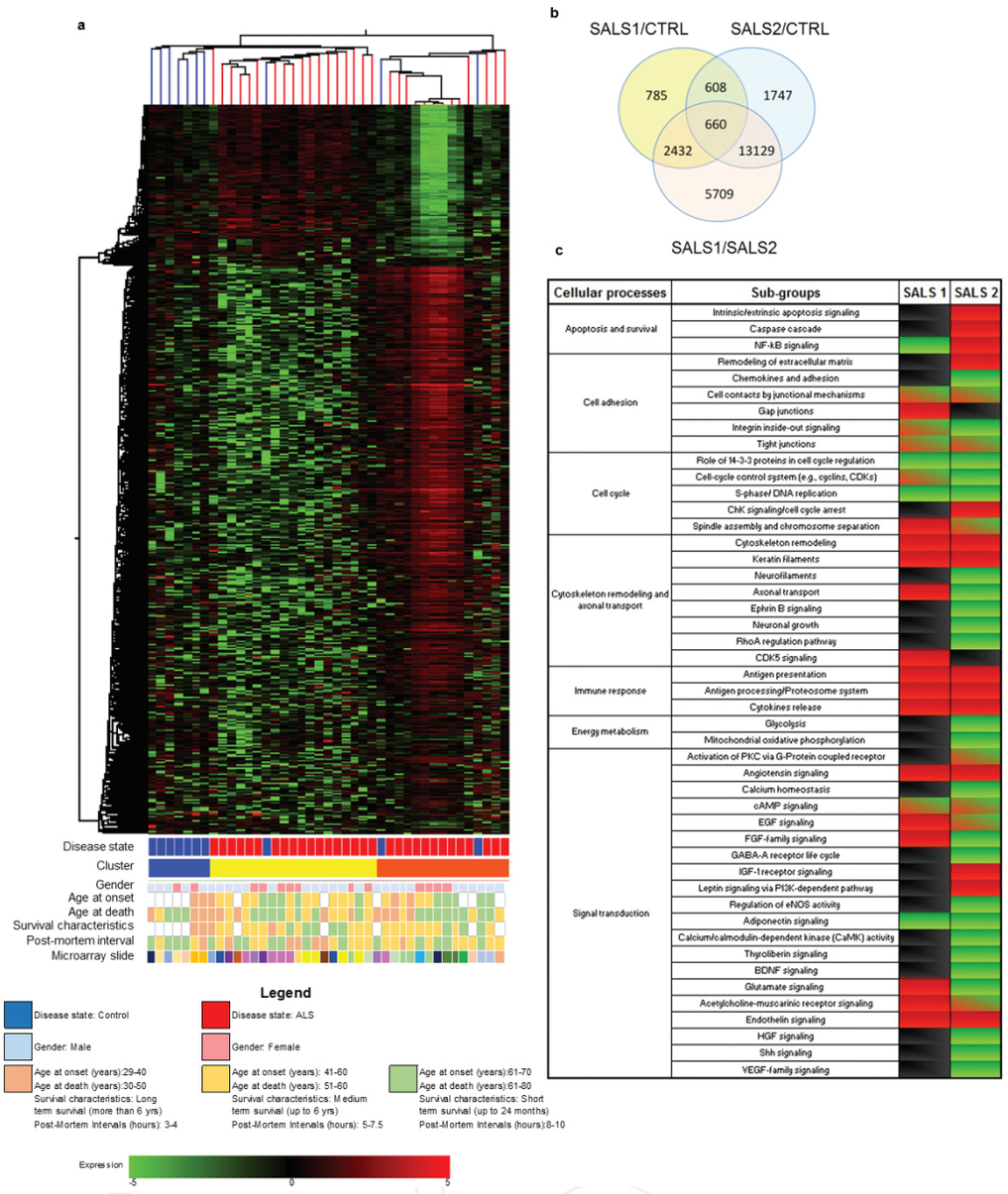


**Figure 1. Panel a:** Unsupervised hierarchical clustering of control and SALS patients and correlation between patient stratification and technical variation (array hybridization) or patient demographic (gender, age at onset, age at death, survival time from date of onset, and PMIs). Unsupervised hierarchical clustering (similarity measure: Pearson centered; linkage rule: average) was used to cluster control and SALS patients on the basis of their similarities measured over the most informative genes expressed in motor cortex (9646 genes with a standard deviation >1.5). Similarly, the same genes were clustered on the basis of their similarities measured over the motor cortex of control and SALS patients. In this two-dimensional presentation, each row represents a single gene and each column a motor cortex from control or SALS patients. In the "microarray slide" bar, samples hybridized on the same array are shown with the same color. As shown in the color bar, red indicates upregulation, green downregulation, and black no change. White squares indicate the n/a values. In the dendrograms shown (left and top), the length and the subdivision of the branches display the relatedness of the expression of the genes (left) and the motor cortex (top). Although SALS patients could be clearly distinguished on the basis of their motor cortex gene expression patterns, no significant association was found between their clinical characteristics and cluster assignment. **Panel b:** Venn diagrams of differentially expressed genes in the motor cortex of control and SALS (clusters 1 and 2) patients. **Panel c:** Pathways differentially deregulated in cluster SALS patients. Red boxes represent cellular processes mainly upregulated, green bars downregulated, red/green bars indicate signal pathways both up- and downregulated, and gray bars indicate no significant change when compared to controls.

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ALS patients [8–10]. In particular, we focused our attention on primary targets of drugs actually undergoing to preclinical or clinical studies for several clinical diseases, including neurodegenerative [8, 9]. As described below and represented in **Figure 1c**, deregulation of identified genes and pathways in SALS patients was cluster specific.

### *4.2.1. Apoptosis and survival*

A shift in the delicate balance between apoptosis- and survival-inducing genes plays a role in most neurodegenerative diseases, including ALS. We identified a number of genes, some encoding for potential drug targets, previously associated with apoptosis and survival that resulted deregulated in the cortex of SALS patients (**Figure 2a**) [8].

SALS2 patients showed increased expression of some genes involved in the triggering of the neuroinflammation and extrinsic apoptotic signaling cascade (Fas-L, FADD, RIPK1, and p38

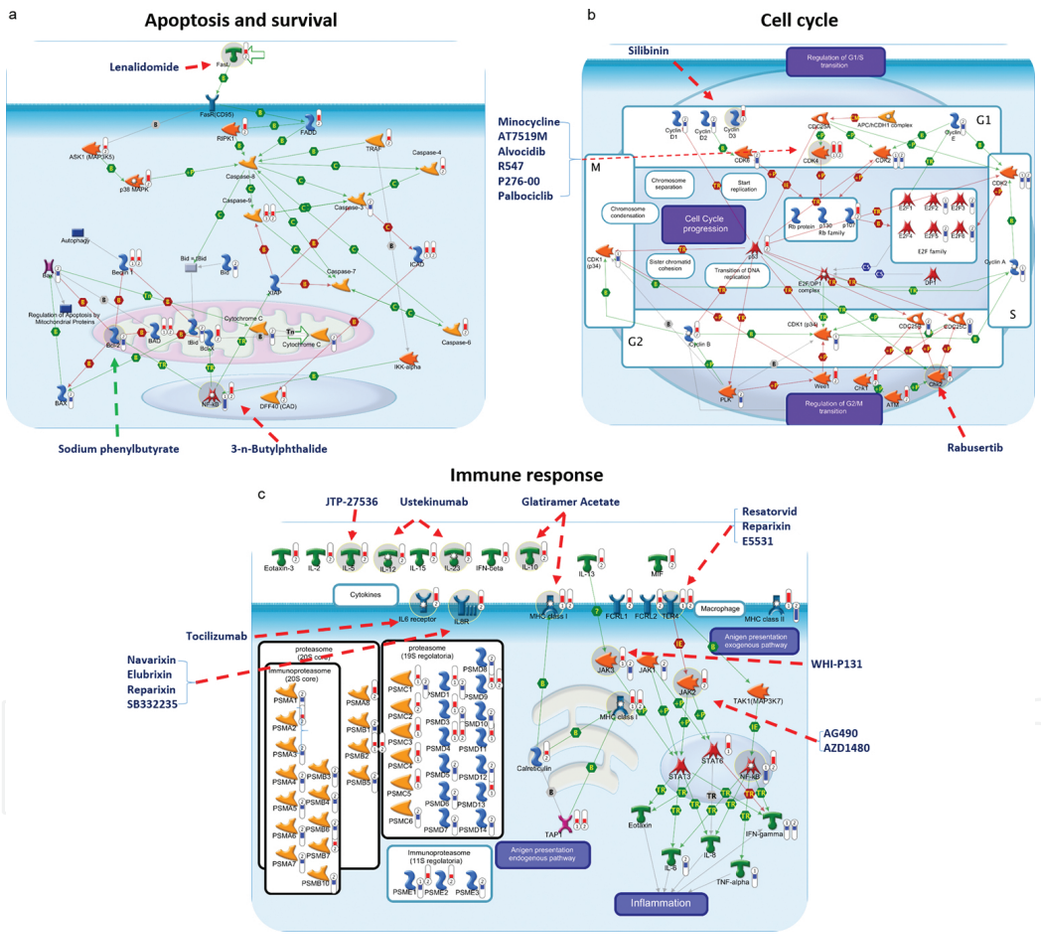


**Figure 2.** MetaCore analysis of altered signaling pathways in the motor cortex of SALS patients. Panel a: Apoptosis and survival. Panel b: Cell cycle. Panel c: Immune response. All maps are drawn from scratch by GeneGo annotators and manually curated and edited. Experimental data are visualized on the maps as blue (for downregulation) and red (upregulation) thermometers indicating expression ratio in the following conditions: 1 (SALS1/control) and 2 (SALS2/control). Some of the differentially expressed genes (highlighted with a yellow circle) are direct or indirect targets of experimental or therapeutic drugs. For each known drug target-pharmacological compound, we connect (dashed line) the drug to the target on which it acts. The line color indicates drug effect on target: red for inhibition and green for activation. Pathway objects and links are described separately in Supplementary Figure 1.

MAPK), accordingly with previous data found in degenerating spinal cord and cerebral cortex motor neurons of ALS mouse models [8]. Among these genes, Fas ligand (FasL or CD95L) may represent a possible target to block or slow the progressive degeneration of the motor neurons in ALS. In fact, preclinical studies have shown that Lenalidomide, a potent immunomodulatory agent that inactivates downstream effector caspases also by reducing the Fas-L expression, extends survival in transgenic mouse models of ALS [9]. Expression of genes encoding proteins (caspase-4, 6, 9, ICAD, and CAD) involved in one of the major processes responsible for the execution phase of cell apoptosis, the caspases signaling, resulted deregulated in SALS2 patients (**Figure 2a**), in accordance with previous findings showing their involvement in motoneuron degeneration in ALS [8].

Deregulated expression of pro- and antiapoptotic regulators (BAD, BID, BCL-2, BAX, and cytochrome c) involved in the mitochondrial intrinsic signaling pathway was also observed in SALS patients, supporting the evidence that the altered functionality of this system contributes to trigger motor neurons degeneration in both ALS patients and animal models [8]. Although the role of these factors in ALS pathogenesis should be further investigated, promising results were obtained from their pharmacological modulation. An example is represented by sodium phenylbutyrate, a programmed cell death inhibitor actually in phase II clinical trials for ALS that exerts its neuroprotective effects by pharmacological induction of BCL-2 with subsequent block both of cytochrome c release and caspase activation, contributing to slow the motor neuron death in ALS mice and patients [9].

The expression of Beclin1, a key autophagy-related gene previously linked to ALS neurodegeneration, was found increased in SALS patients, while that of NF-κB (nuclear factor kappa light chain enhancer of activated B cells) was identified with an opposite regulation in SALS patients (decreased in cluster 1 and increased in cluster 2) [8]. This apparent discrepancy may be explained by the controversial role of NF-κB on neurons. It is, in fact, a protein complex involved in various cellular mechanisms, including immune response and transcription regulation, cytokine production, and cellular responses to oxidative stress as well as processes of synaptic plasticity and memory. Although low levels of NF-κB have been related to loss of neuroprotection, its increased expression has been related to neuroinflammatory processes [32]. Several NF-κB pharmacological inhibitors, including dl-3-*n*-butylphthalide (dl-NBP), have been tested in preclinical studies showing to exert a neuroprotective role in ALS mainly by inhibiting programmed cell death and inflammation, reducing oxidative damage and improving mitochondrial function [9]. Based on these promising results, further studies aimed at defining the role of NF-κB in ALS pathogenesis are needed to elucidate its potentiality as a pharmacological target for personalized treatments of ALS patients.

*4.2.2. Cell cycle*

Progress in cell cycle is driven by oscillations in the activities of cyclin-dependent kinases (CDKs), which are controlled by periodic synthesis and degradation of cyclins as well as by other regulators. Alterations in gene expression and cellular distribution of these and other cell-cycle regulating proteins characterize several human neurodegenerative diseases, including ALS [33–35]. In accordance with this hypothesis, we observed deregulated expres-

sion of genes encoding key regulators of G1, S, M, and G2 phases (**Figure 2b**), some of them may represent an interesting candidate therapeutic target for ALS [9].

Increased expression of CDK4 and its regulatory subunit G1/S-specific cyclin-D3 were observed in SALS patients (**Figure 2b**), supporting the theory that dysregulation of the cyclin system may contribute to ALS neurodegeneration and that cell death may be the result of an unsuccessfully attempt by terminally differentiated neurons to re-entry into the cell cycle [8]. If so, drug inhibitors of the cell cycle might counteract neuronal degeneration in ALS, as suggested by *in vitro* studies on motor neurons. In fact, it has been observed that the pharmacological treatment with the CDK4 inhibitors (AT7519M, Alvocidib, R547, P276-00, Palbociclib, and Minocycline) currently studied for cancer treatment attenuates microgliosis and slows down the neuronal degeneration in a series of CNS disease models, including ALS [9]. Additionally, drugs inhibiting cyclin D3 signaling, including the natural flavonoid silibinin, also decrease CDK4 activity indirectly, with a consequent reduction of oxidative stress and inflammation. Given this, inhibitors of the cyclin–CDK complexes may represent a new promising avenue for ALS therapy and future clinical trials should be truly undertaken to measure their efficacy in ALS patients.

In accordance with previous observations in both SOD1 transgenic mice and ALS patients, in SALS2 patients we found the upregulation of Ataxia-telangiectasia-mutated (ATM), cell-cycle checkpoint kinases (CHK1/CHK2), and the tumor suppressor gene P53, whose increased levels have been associated with the arrest of the cell-cycle progression (**Figure 2b**) [8]. Although the role of CHK2 in ALS pathology remains unclear, our data suggest that an increased activity of this kinase may be the result of a DNA damage induced, for example, by diverse stress conditions, resulting in the alteration of neuronal apoptotic mechanisms. Therefore, pharmacological inhibitors of CHK2 (e.g., Rabusertib) may represent a promising therapeutic approach for delaying the progressive motor neurons degeneration in ALS.

#### *4.2.3. Immune response*

The immune response has been implicated in ALS and may contribute to the pathogenesis of disease or represent a response to damage. Reactive microglia and inflammatory processes have been observed to coincide with ALS onset and disease progression in SOD1 transgenic mice as well as post-mortem examinations of neural tissues in ALS patients show both innate and adaptive immunity activation [36, 37]. Similarly, we found differential expression of an extensive number of immune-related genes in the cortex of SALS patients (**Figure 2c**). While further explorations are needed to clarify the positive and negative effects of this system in ALS pathogenesis, its potential as a drug target is being explored [38].

Toll-like receptor 4 (TLR4) is among the immune-cell-specific genes upregulated in SALS patients, which is a marker of innate immunity response and monocyte/macrophage activation previously observed in reactive glial cells of spinal cords of ALS patients [8]. Several studies have shown that TLR4-signaling inhibition could reduce neuroinflammatory processes associated with ALS, supporting the potential therapeutic role of TLR4 inhibitors (Resatorvid, Reparixin, and E5531) in improving the neurological functionality recovery after brain injury and neuroinflammation [9].

SALS patients sowed differential expression of genes encoding Janus kinase (JAK-1, KAK-2, JAK-3, and Tyk-2) (**Figure 2c**), a family of tyrosine kinases mainly involved in the regulation of gene expression whose dysregulation has been already associated with inflammation and neurodegenerative diseases, including ALS [8]. The pharmacological blockade of JAK-STAT pathway with JAKs inhibitors (AG490, AZD1480, and WHI-P131) has shown neuroprotective effects also for slowing disease progression and increasing survival in an animal model of ALS [9].

In SALS patients, we also observed deregulated expression of several gene encoding proteins involved in antigen processing and presentation. Most of these genes were increased in SALS1 and reduced in SALS2 patients. Proteins involved in antigen presentation include major histocompatibility complex (MHC) class I and class II molecules (HLA-A, HLA-B, and HLA-C), TAP1, and calreticulin. MHC class I antigens were yet observed in the ALS affected tissues and their pharmacological inhibition with the immunomodulatory drug glatiramer acetate (GA) has shown neuroprotective effects in several neurological conditions [9]. This drug, clinically used for the treatment of multiple sclerosis, is currently object of phase II clinical trials for ALS. Proteins involved in antigen processing mainly belong to the proteasome/immunoproteasome system (**Figure 2c**), one of the major intracellular proteolytic mechanisms controlling the degradation of misfolded/abnormal proteins whose accumulation into damaged neurons represents a common hallmark in ALS. The deregulation of the constitutive and inducible proteasome subunits may not only influence the ubiquitin-mediated protein degradation but also lead to the generation of peptides that can be used by MHC I molecules for antigen presentation to the immune system, providing an interesting connection between the immune responses and proteasome function [39]. In this regard, SALS1 patients also showed the increased expression of serpin peptidase inhibitor, clade A, member 3 (SERPINA3), an acute phase reactant protein considered an important link between the immune/inflammatory response and proteasomal turnover [8]. Our findings are consistent with the previous results showing the upregulation of SERPINA3 in several ALS mouse models and human studies.

The upregulation of several cytokines and IFNβ was also observed in SALS2 patients (**Figure 2c**). Enhanced expression of IFNβ was already demonstrated in the spinal cord of SOD1 mice which seems to represent an early response to pathological changes in ALS. The downregulation of the potent anti-inflammatory cytokine interleukin-10 (IL-10) was observed in SALS2 patients (**Figure 2c**), sustaining the neuroprotective role of this factor against motor neuron injury in ALS [8]. It is of interest to note that glatiramer acetate, in addition to its activity as MHC regulator (as discussed above), also increases IL-10 levels suggesting that the multitarget action of this drug may provide an avenue for the treatment of ALS [9]. Although the pathogenic role of cytokines in ALS is still unknown, previous studies have associated their abnormal expression with the clinical status and some of these (IL8R, IL-5, IL-6R, IL-12, and IL-23) have been suggested as potential targets for the pharmacological treatment of ALS patients [9]. In this regard, different interleukin inhibitors, including those targeting the chemokine receptor interleukin 8 receptor (IL8R, also known as CXCR2) (Reparixin, Navarixin, Elubrixin, and SB332235) and IL-6 receptor (IL6R) (i.e., tocilizumab), showed excellent activity

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*in vitro* against inflammatory cascade activation in SALS patients. In addition, the anti-IL-12/IL-23 monoclonal antibody ustekinumab has also shown neuroprotective properties, reversing cognitive decline related to Alzheimer's disease. Based on these evidences, further studies will be needed to elucidate the potential clinical benefits of these and other inflammatory cytokine inhibitors (such as JTP-27536) in the clinical treatment of ALS.

#### *4.2.4. Cell adhesion*

Dysfunction of the cell adhesion system may lead to alterations in cell–cell communication and the formation of multicellular structure, promoting the development of neurodegenerative diseases, such as ALS [40]. Consistently, our analysis has revealed differential expression of numerous genes involved in cell adhesion, mainly in SALS2 patients (**Figure 3a**). These genes encode for 12 integrin receptors (ITGA1, ITGA2, ITGA3, ITGA5, ITGA6, ITGA7, ITGA8, ITGA10, ITGA11, ITGAV, ITGB1, and ITGB4), three extracellular matrix molecules (Collagen IV, Laminin 1, and Fibronectin), seven components of tight junctions (Claudin-1, Claudin-3, Caludin-5, Jam1, Jam2, ZO-1, and ZO-2), and one component of Gap junctions (Connexin 43) [8]. A significant decrease in both protein and mRNA levels of tight junction components has been previously described in ALS patients and animal models. Although such a vast deregulation of integrins has not yet been described, changes in plasma Fibronectin levels were already found in ALS patients and were significantly correlated with the clinical progression of this disorder. A progressive decrease of Collagen IV has also been demonstrated in serum and vascular structures of ALS spinal cord while high levels of Laminin 1, previously observed in ALS spinal anterior horn, may represent a protective measure to aid neuronal survival. Integrins also play a role in the activation of focal adhesion kinase 1 (FAK-1) whose expression was upregulated in SALS patients (**Figure 3a**) as well as in other neurological diseases, such as Alzheimer's [8]. Interestingly, the pharmacological inhibition of FAK-1 signaling cascade by the administration of FAK-1 inhibitors (such as PF562271 and Sulindac) was observed to extend the survival of G93A SOD1 mice [9].

SALS2 patients also show the differential expression of four extracellular matrix metalloproteinases (MMP-1, 2, 9, and 13) together with the metallopeptidase inhibitor TIMP1. MMPs are a family of zinc-dependent endoproteinases that regulate the extracellular matrix structure and play an important role in synaptic remodeling, neuronal regeneration, and remyelination, modulation of blood–brain and blood–cerebrospinal fluid barrier permeability and leukocyte invasion in neuroinflammatory diseases [8]. Although the role of MMPs in ALS pathogenesis is currently unknown, their altered levels may reflect degenerative processes of motor neurons and tissues remodeling. Moreover, deletion of MMP-9 gene has shown to accelerate motor neuron disease and shorten survival in mutant SOD-1 mice. Several studies have sustained the potential role of MMP inhibitors as attractive candidates for ALS therapy and demonstrated the beneficial effects of compounds inhibiting MMP expression, synthesis, and activity in various neurodegenerative conditions [9]. Among these drugs, a particular attention should be addressed to thalidomide, a potent anti-inflammatory drug, which is able to reduce MMP-2 and increase the lifespan of ALS animal models. Although this drug has shown promising results in ALS patients, its clinical use is limited by a variety of side effects. Even so, other

MMPI inhibitors (Marimastat, Tanomastat, Batimastat, ONO4817, Doxycycline, Rebimastat, Pravastatin, S3304, Halofuginone, and Melphalan) in addition to their antineoplastic properties have also gained interest for their therapeutic effects in several neurodegenerative conditions, including Alzheimer's disease [9].

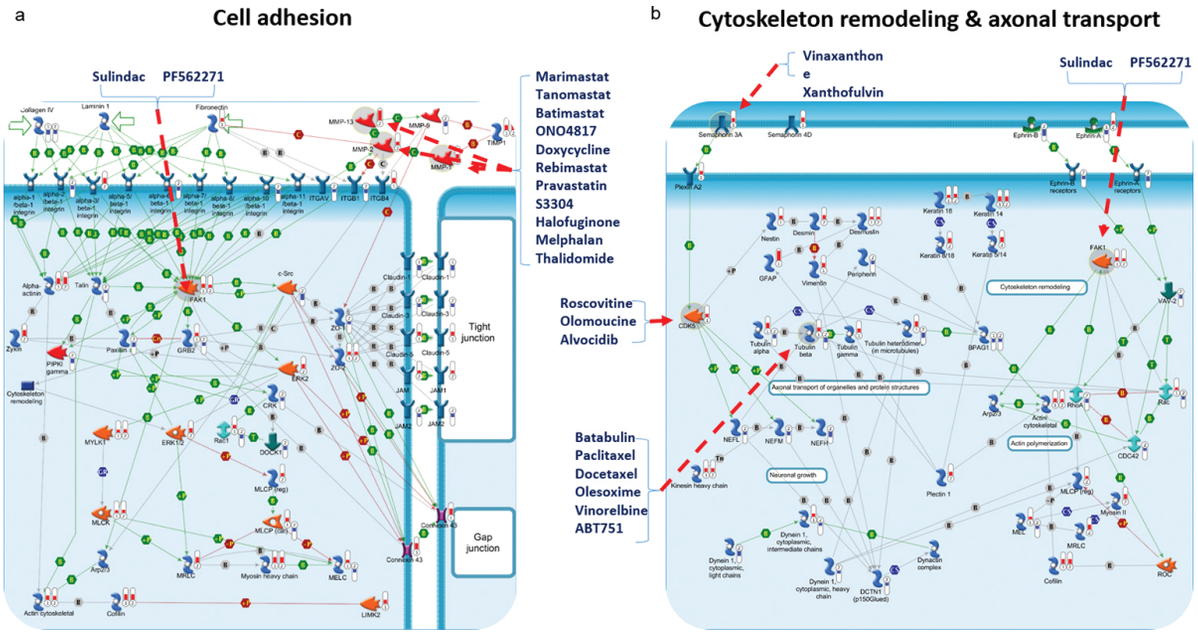


**Figure 3.** MetaCore analysis of altered signaling pathways in the motor cortex of SALS patients. Panel a: Cell adhesion. Panel b: Cytoskeleton remodeling and axonal transport. All maps are drawn from scratch by GeneGo annotators and manually curated and edited. Experimental data are visualized on the maps as blue (for downregulation) and red (upregulation) thermometers indicating expression ratio in the following conditions: 1 (SALS1/control) and 2 (SALS2/control). Some of the differentially expressed genes (highlighted with a yellow circle) are direct or indirect targets of experimental or therapeutic drugs. For each known drug target-pharmacological compound, we connect (dashed line) the drug to the target on which it acts. The line color indicates drug effect on target: red for inhibition and green for activation. Pathway objects and links are described separately in Supplementary Figure 1.

### *4.2.5. Cytoskeleton remodeling and axonal transport*

The cytoskeleton is critical for neuronal maintenance and plasticity, neurite outgrowth, axonal caliber, and transport. Our analysis uncovered differential expression of genes encoding major components of the cytoskeleton in SALS patients, including intermediate filaments proteins (Nestin, GFAP, Desmin, Desmuslin, Vimentin, Peripherin, Keratins 5, 8, 14, and 18), Actin, Tubulin (alpha, beta, and gamma), Myosin, and all three neurofilament subunits (NEFL, NEFM, and NEFH) (**Figure 3b**).

Tubulin beta proteins (TUBBs) belong to the tubulin family of proteins that, together with alpha-tubulin, form and organize structures called microtubules. TUBBs are expressed in neurons which are involved in several processes, including neurogenesis and axon guidance and maintenance/remodeling. Differential expression of genes encoding TUBB1/TUBB3 was observed in SALS patients (**Figure 3b**), supporting previous data that propose tubulin system recovery as a possible mechanism for restoring the altered nerve signal in ALS [8]. In this

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regard, several studies support the ability of taxanes (Paclitaxel and Docetaxel) and other microtubule-targeting drugs (Batabulin, Vinorelbine, and ABT751) to restore lost nerve signals in neurodegenerative conditions, such as Alzheimer's [9]. Although the potential neurotoxicity of these drugs has limited their clinical use, the development of novel compounds may prevent this side effect. Among these novel compounds, olesoxime, a potent trophic factor able to promote neuronal survival and axonal sprouting, has shown prominent neuroprotective properties for motor neurons in both *in vitro* and *in vivo* models.

Despite the controversial role of neurofilaments (NFs) in ALS, their aberrant accumulation in the cell body and proximal axons of motor neurons is a hallmark of ALS [8]. A deletion of NEFL subunits in an ALS mouse model, in fact, is accompanied by an increase of the NEFH and NEFM subunits in the motor neuron cell bodies and their reduction in the axons, slowing the onset and progression of the disease. Increased expression of the NEFH subunit has similar effects, suggesting that NFs may act as buffer for processes that would otherwise be deleterious, for example, offering phosphorylation sites for dysregulated intracellular tyrosine kinases or reducing the axonal burden. Decreasing the axonal burden of NFs may thus protect motor neurons, at least in part, by enhancing axonal transport, a hypothesis supported by the observation of defects in slow axonal transport in presymptomatic ALS animal models.

Consistent with the view that impaired axonal transport may be involved in the degeneration of motor neurons, we observed in SALS2 patients the downregulation of cytoplasmic Dynein intermediate, light, and heavy chains, together with the p150Glued subunit of Dynactin (DCTN1), implicated in retrograde transport of cargoes, such as endosomes (**Figure 3b**). Mutations in the cytoplasmic Dynein heavy-chain gene have previously been found in two mouse models, Legs at odd angles (Loa) and Cramping 1 (Cra1), with late-onset motor neuron degeneration, while mutations of DCTN1 gene are responsible for a lower motor neuron disorder with vocal cord paresis. In addition to this, decreased expression of DCTN1 has been reported in motor neurons of patients with SALS suggesting that abnormalities in Dynactin may play a role in the pathogenesis of ALS [8].

Aberration in axon guidance may also result from differential expression of Semaphorins, Plexins, and Ephrins as well as of their receptors in SALS patients, together with their downstream signaling factors (Cdc42, Rac, and RhoA) (**Figure 3b**). Increased expression of Semaphorin 3A (SEMA3A), a protein involved in the regulation of axon and dendrite growth guidance and neural system development, has previously been associated with deadhesion or repulsion of motor axons away from the neuromuscular junction in terminal Schwann cell of SOD1G93A transgenic mice, probably resulting in axonal denervation and motor neuron degeneration [8]. An altered expression of SEMA3A, in fact, might lead to aberrant outgrowth of corticospinal tract fibers from the cortex, the inappropriate guidance of cranial motor axons and hyperfasciculation or defasciculation of both cranial nerves and MMC and LMC motor axons. Although these changes in SEMA3A expression might be small and may not cause obvious defects during early life, minor changes in motor neuron circuitry due to altered SEMA3A expression may result in motor connections, which are more vulnerable to additional genetic or environmental changes. Although the precise role of SEMA3A in the pathogenesis of ALS is unclear, vinaxanthone and xanthofulvin, two selective SEMA3A inhibitors, have

shown to promote neuronal regeneration, suggesting a potential role of this and other SEMA3A modulatory drugs as protective agents against neurodegenerative conditions, including ALS [9]. Promising results have also been obtained by the pharmacological inhibition of downstream mediators of SEMA3A signaling cascade, such as cell division protein kinase 5 (CDK5), whose expression resulted augmented in SALS1 patients (**Figure 3b**), in accordance with previously published studies [8]. It has been, in fact, demonstrated that the treatment with CDK5 inhibitors, including olomoucine and roscovitine, is able to reduce neuronal death and microglial neurotoxicity as well as reverse axonal transport defects, contributing to reduce ALS neurodegeneration [9]. The promising effects shown by these and other CDK5 inhibitors (e.g., Alvocidib) encourage further studies aimed to evaluate the clinical utility of this drug class in ALS therapy.

Similarly to Semaphorins, Ephrins have a variety of important functions including axonal outgrowth and cytoskeletal structure development, neuronal connectivity, neuronal apoptosis, synaptic maturation, and plasticity. It is, therefore, plausible that variability in such molecules could contribute to the initiation and progression of neurodegenerative diseases. A marked increase of Ephrin A1 has previously been found in motor neurons of SALS patients and SNPs in several Ephrin and Eph receptor genes, including Ephrin B1, have been used to predict susceptibility, survival free, and age at onset of ALS [8]. Our findings in ALS motor cortex support the hypothesis that aberrant expression or function of Ephrins may induce pathological changes in motor neuron circuitry and contribute to ALS pathogenesis.

*4.2.6. Energy metabolism*

Energy metabolism is an extremely complicated series of chemical reactions that breakdown organic matter to harvest energy and involved two main processes: glycolysis and mitochondrial oxidative phosphorylation (**Figure 4**). Among the proteins implicated in the glycolysis process are 6-phosphofructokinase (PFKM and PFKP) and pyruvate kinase (KPYR and PKM2), and MDH1, some enzymes of the rate-limiting step that were previously linked to ALS [8]. These genes were mainly downregulated in SALS2 patients. In the same patients, we observed the coordinated decrease of several gene encoding proteins involved in the oxidative phosphorylation pathway, including those encoding proteins of respiratory complex I (36/46 subunits), II (SDHB and SDHD), III (7/11 subunits), IV (6/13 subunits), and V (10/14 subunits of the catalytic and membrane proton channel of ATP synthase), while a limited but significant number of these genes were increased in SALS1 (**Figure 4**). Our findings are in agreement with previous studies conducted on post-mortem ALS tissues in which an increase of complex I and a deficiency of complex IV are reported. This apparent discrepancy may be explained by the opposite behavior of SALS patients found in the present study (increase of complex I in SALS1 and decrease of complex IV in both clusters). Oxidative phosphorylation represents one of the major cellular energy supply systems and its deregulation has been widely reported to play an important role in ALS pathology [8]. Dysfunctional mitochondria, in fact, exhibit a reduction in bioenergetics efficiency, putting neurons at risk of death when energy demands exceed cellular energy production. Based on these findings, it appears evident that treatments designed to improve respiratory chain function may ameliorate the progression of this

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disorder. In this regard, some drugs that can reverse mitochondrial dysfunction (Acetyl-L-carnitine, Creatine, Minocycline, Olesoxime, Dexpramipexole, and Cyclosporine A) are currently in clinical trials for ALS, showing to be effective and well tolerated [9]. Promising preclinical results for ALS were also reported by other members of this drug class (Uridine, Pyruvate, and Dichloroacetate), supporting the idea that mitochondria-targeted therapies may restore bioenergetics defects observed in SALS2 patients.

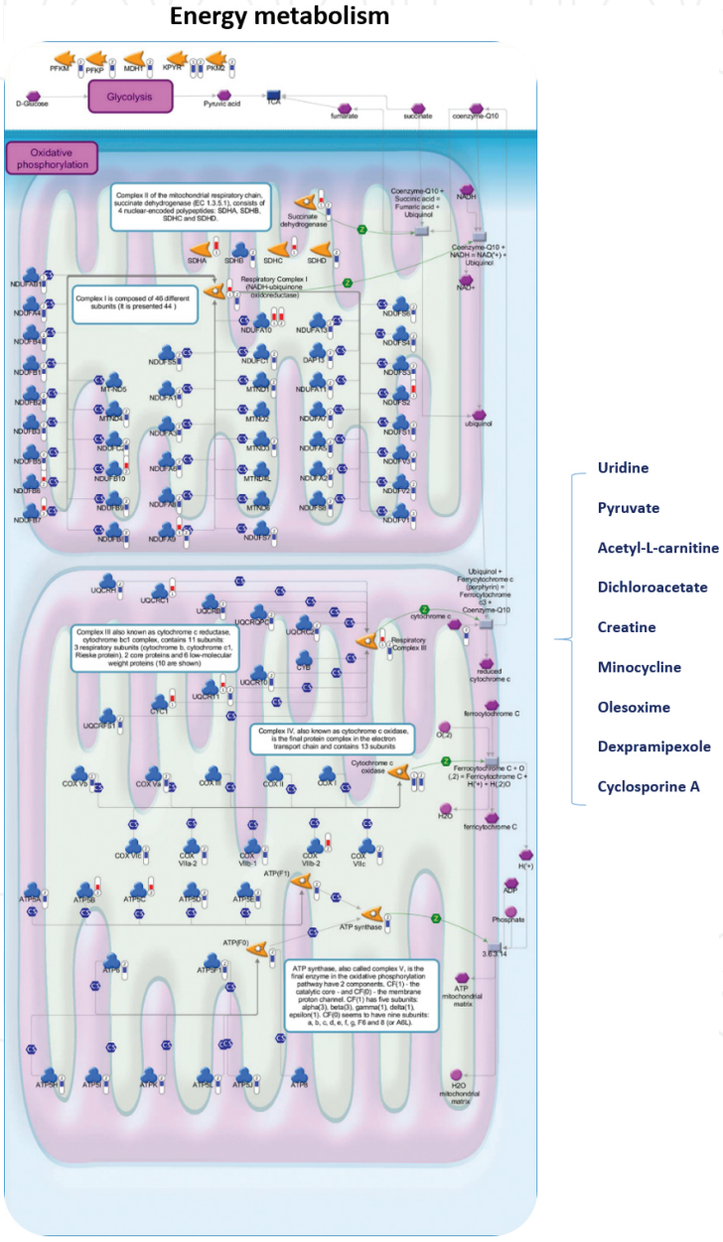


**Figure 4.** MetaCore analysis of altered energy metabolism pathways in the motor cortex of SALS patients. The map is drawn from scratch by GeneGo annotators and manually curated and edited. Experimental data are visualized on the maps as blue (for downregulation) and red (upregulation) thermometers indicating expression ratio in the following conditions: 1 (SALS1/control) and 2 (SALS2/control). Drugs potentially useful for reversing mitochondrial dysfunction are shown on the right side of the map. Pathway objects and links are described separately in Supplementary Figure 1.

#### *4.2.7. Signal transduction*

Neurosignaling involves the largest class of proteins and receptors that transmit chemical signals from the cell surface to their intracellular targets. This section, for the multitude of signaling cascades resulted altered in SALS patients, will be divided into two subsections (neuropeptides and receptors, and neuromodulation and ion homeostasis).

##### *4.2.7.1. Neuropeptides and receptors*

Neurotrophins is a protein family that, among other things, is able to influence the survival and death as well as the development, proliferation, and differentiation of neuronal and non-neuronal cells. The loss of neurotrophins and their receptors and the consequent deleterious effect on neuron health and stability is one of the major pathologic hallmarks of neurodegenerative disorders, including ALS [41]. In accordance with this concept, our results showed the differential expression of genes encoding several neuropeptides and relative receptors, many of them represented potential drug targets, in the motor cortex of SALS patients (**Figure 5a**).

Deregulated expression of diverse adipokines, including Adiponectin, Leptin, and their receptors, was found in SALS patients (**Figure 5a**) [8]. Beyond their peripheral effects on fat metabolism and insulin sensitivity, these proteins and receptors are expressed in the brain which regulate neuronal excitability and exert neurotrophic and neuroprotective effects. Decreased expression of PTCH1 in SALS2 patients is in agreement with the neuroprotective effects of Sonic hedgehog (Shh) signaling observed in ALS mouse models. The same patients also showed downregulated expression of three gene encoding proteins implicated in neurite outgrowth and ALS pathology: Myelin-associated glycoprotein (MAG), Reticulon-4, and its receptor. Anti-MAG antibodies have been yet observed in ALS patients, whereas a number of studies have implicated Reticulon-4 in ALS pathology, demonstrating a protective effect against ALS-like neurodegeneration.

Several trophic factors and their receptors were also differentially expressed in SALS cortex (**Figure 5a**) [8]. Among these, all are the epidermal growth factor receptors (EGFRs) and some of their ligands, according to previous findings in liquor of ALS patients. Evidence suggests that the activation of this signaling pathway may trigger quiescent astrocytes into reactive astrocytes and, consequently, activate the neurodegenerative process. Pharmacological studies showed that EGFR/ErbB2 inhibitors, clinically used in cancer treatment, have recently also gained interest for their neuroprotective properties [9]. Particularly, some of these drugs (Erlotinib, Genistein, and Masoprocol) seem to delay significantly disease onset in ALS animal models. Belonging to the same pharmacological class is Suramin, a drug actually in preclinical phase for several neurological diseases, including Alzheimer's and Parkinson's. Besides inhibiting EGF signaling, Suramin is also able to inhibit the activity of histone deacetylase (HDAC), nitric oxide synthase (NOS), and P2Y2, three proteins already widely associated with ALS pathogenesis [42, 43]. The promising preclinical results obtained with above-mentioned and other EGFR/ErbB2 inhibitors (PKI166, BMS690514, Canertinib, Gefitinib, and PD153035) stimulate their future developments and clinical validations in the hope of translating them into clinical practice.

Decreased levels of Neuregulin were observed in SALS patients, coherently with previous findings observed in cerebral spinal fluid of ALS patients, as well as aberrant Neuregulin signaling was detected in both ALS patients and SOD1 mice [8]. Moreover, altered expression of genes encoding three fibroblast growth factor receptors and seven of their ligands was detected in SALS patients (**Figure 5a**). Reduced expression of FGF9 may be consistent with its role as an autocrine or paracrine survival factor for motoneurons [8]. On the other hand, the release of FGF-1 from motor neurons is expected to happen in response to oxidative stress stimuli, leading to the accumulation of FGFR1 and activation of astrocytes that, in turn, could initiate or promote apoptotic processes in ALS. In support of this theory, studies conducted on FGFR inhibitors (e.g., PD166866) have shown promising effects, by preventing motor neuron apoptosis in ALS mouse models, sustaining the need for additional studies to further investigate the role of this and other inhibitors of FGF signal transduction (Orantinib, Brivanib, Dovitinib, Suramin, and Pentosan polysulfate) in ALS therapy [9].

SALS patients also showed altered expression of genes encoding brain-derived neurotrophic factor (BDNF), one member of the neurotrophin family of growth factors, and their receptors (TrkA, TrkB, TrkC, and NGFR) (**Figure 5a**), according to an extensive amount of data supporting their implication in ALS humans and animal models [8]. In particular, a prolonged TrkB activation may render motor neurons vulnerable to excitotoxic insult, contributing to developing ALS. Therefore, therapeutic strategies aimed to inhibit TrkB signaling may result promising for treating ALS. Although the development of TrkB inhibitors has been difficult, mainly because of the central role of BDNF in cognitive functions, some selective compounds (Cyclotraxin-B and Ana-12) have shown to decrease neurotoxicity without affecting neuronal survival [9].

The expression of vascular endothelial growth factors (VEGF-A, VEGF-B, VEGF-C, and VEGF-D) and their receptors (VEGF-R1, VEGF-R2, Neuropilin 1, and Neuropilin 2) was mainly downregulated in SALS2 patients (**Figure 5a**). There are extensive evidence linking this family of ligands and receptors to ALS pathology [44, 45] and treatment with VEGF was found to strongly protect motor neurons from excitotoxicity and prevent neuronal death in different models of ALS [8]. In addition, some drugs activating VEGF (SB-509 and Celecoxib) have been tested in phase II clinical trials for ALS, showing encouraging results [9].

The downregulation of hepatocyte growth factor (HGF) was observed in SALS2 patients, which is one of the most potent survival-promoting factors for motor neurons with potential therapeutic effects on ALS (**Figure 5a**) [8]. Likewise, decreased expression of thyrotropin-releasing hormone (TRH) receptor in SALS2 patients supports previous studies showing a reduction of this receptor in the spinal cord of ALS patients and may explain the conflicting results obtained by TRH therapy. Masoprocol is among TRHR agonist, which, besides its EGFR inhibitor activity, is also able to exert neuroprotective effects through anti-inflammatory and antioxidant properties as well as by reducing the glutamate neurotoxicity. In addition, Taltirelin, another TRH analog, also exerts neuroprotective effects in several neurodegenerative conditions, contributing to make these attractive candidates for restore damaged or maladaptive neural systems in ALS patients [9].

The expression of several gene encoding G-protein coupled receptors and/or their ligands is resulted altered in SALS patients (**Figure 5a**). Some of these, such as the Muscarinic acetylcholine receptors, the endothelin-1, and its receptor EDNR-B, have been previously associated with ALS pathology and pharmacological approaches, aimed to repair these defective neuromodulatory signaling, are thought to slow motor neuron degeneration in ALS [8]. Several preclinical and clinical studies have corroborated this hypothesis, suggesting that muscarinic agonists (such as Cevimeline, Xanomeline, Bethanechol, and Clozapine) may be able to restore neuronal loss associated with diverse CNS disorders, including ALS. In the same way, evidence has demonstrated that EDNR-B antagonists (such as BQ-788, Bosentan, and IRL-2500) exert neuroprotective and neuroinflammatory properties [9].

Differential expression of leukemia inhibitory factor (LIF) and its receptor emerged from our analysis is consistent with a study proposing LIF as a modifier gene in ALS, while that of Angiotensin II and its type-1 receptor (AGTR1) is in agreement with previous studies showing altered levels of Angiotensin II in liquor of ALS patients [8]. Moreover, angiotensin signaling pathway has been recently proposed as a new potential target in ALS therapy [9]. In fact, agents able to block AGTR1 (ARBs), such as Telmisartan, Olmesartan, and Candesartan, in addition to their clinical usefulness as antihypertensive agents have also shown neuroprotective effects in Alzheimer's and other neuronal diseases. Among these drugs, olmesartan has manifested neurotrophic properties on spinal motor neurons *in vitro* and *in vivo*. This drug class also exerts its neuroprotective actions by significantly enhancing GLT-1 expression, whose loss of function has been widely implicated in the pathogenesis of ALS.

The upregulation of purinergic receptor P2Y2 found in SALS patients (**Figure 5a**) is in agreement with previous studies as well as that of insulin-like growth factor 1 receptor (IGFR-1) that supports the critical role of this signaling cascade in ALS pathogenesis [8]. In this regard, the inhibition of the IGF signaling pathway has been seen to increase lifespan and prevent neurodegeneration in a variety of model organisms [9]. Simvastatin is among IGF receptor inhibitors, which has shown to reduce the excitotoxicity and neuronal death occurring in neurodegenerative diseases, such as Alzheimer's and Parkinson's, suggesting that treatment with this and other IGF receptor inhibitors (such as Masoprocol, BMS-754807, and Linsitinib) may represent a potential strategy for the treatment of ALS.

*4.2.7.2. Ion homeostasis*

A number of genes encoding proteins and/or their receptors involved in the regulation of ion homeostasis were deregulated in the motor cortex of SALS patients and some of them (CACNA1C, GRIAs, and GABRs) may be taken into account as targets in ALS therapy (**Figure 5a**).

Decreased expression of L-type voltage-gated calcium channels (CACNA1C) (**Figure 5a**) is consistent with the presence of immunoglobulins against this L-type voltage-gated calcium channels in ALS patients, which correlate with disease progression and exert neurotoxicity [8]. The downregulation of three subunits of the *N*-methyl-d-aspartate (NMDA) receptor (GRIN1, GRIN2A, and GRIN2D) observed in SALS2 patients (**Figure 5a**) is in agreement with previous studies in animal models and with a large literature indicating that a dysfunction of these

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ligand-gated cation channels may be an underlying molecular mechanism in ALS [8]. Three subunits of the alpha-amino-3-hydroxy-5-methyl-4-isoxazole propionate (AMPA) glutamate receptor were differentially expressed in SALS patients: GRIA1 increased in SALS1 patients, whereas GRIA2 and GRIA3 were reduced in SALS2 (**Figure 5a**). Similar changes in expression of GRIA1 and GRIA2 have been reported in SOD1 mice, whereas in human a defect in the editing of the messenger RNA encoding GRIA2 has been previously reported. AMPA receptors lacking the GRIA2 subunit are permeable to Ca2+ and the entrance of this cation might be responsible for the selective vulnerability of spinal motoneurons in ALS. These results are in accordance with the theory that the receptor-mediated glutamate toxicity, together with alteration in neuronal calcium homeostasis, may induce pathological changes associated with motor neurons in ALS. Therefore, pharmacological strategies aimed to minimize glutamate-mediated neurotoxicity and restore impaired calcium homeostasis could result useful for the treatment of the ALS condition. In this regard, preclinical studies conducted on FPL 64176 and Bay K 8644, two CACNA1C agonists, have highlighted neuroprotective effects exerted by these compounds in ALS [9]. Moreover, the administration of AMPA receptor antagonists has

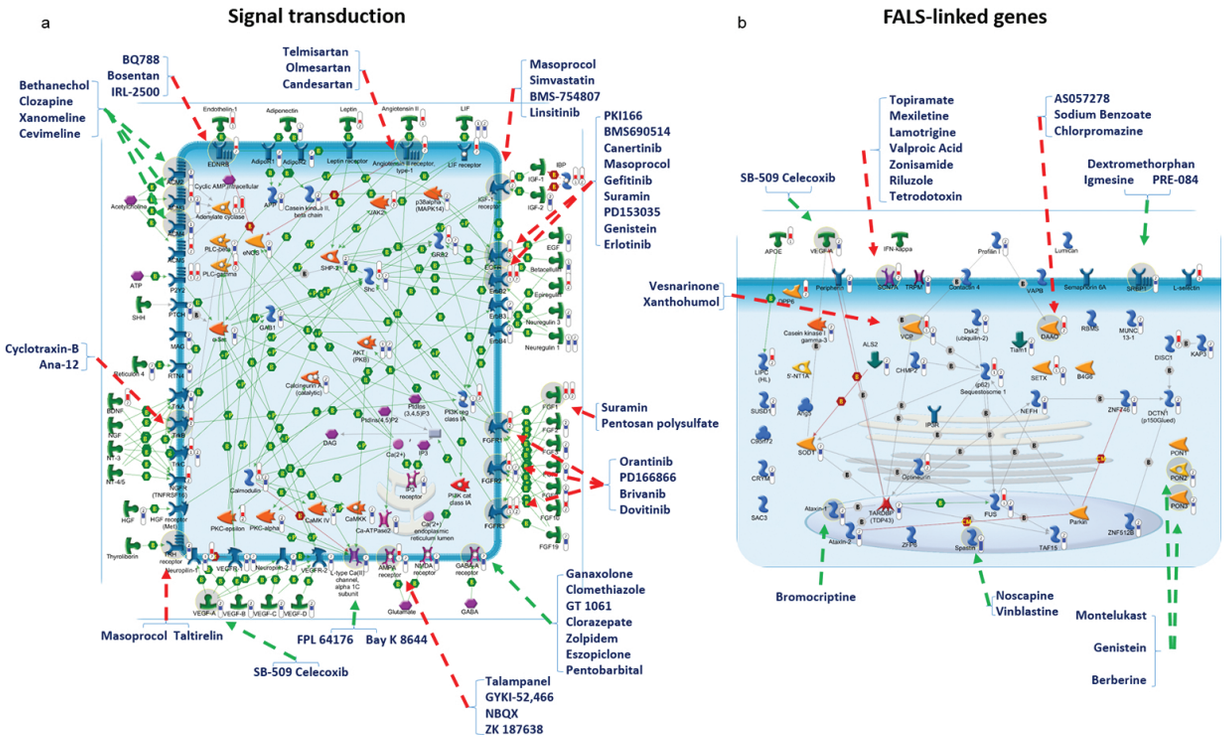


**Figure 5.** MetaCore analysis of altered signaling pathways in the motor cortex of SALS patients. Panel a: Signal transduction. Panel b: FALS-linked genes. All maps are drawn from scratch by GeneGo annotators and manually curated and edited. Experimental data are visualized on the maps as blue (for downregulation) and red (upregulation) thermometers indicating expression ratio in the following conditions: 1 (SALS1/control) and 2 (SALS2/control). Some of the differentially expressed genes (highlighted with a yellow circle) are direct or indirect targets of experimental or therapeutic drugs. For each known drug target-pharmacological compound, we connect (dashed line) the drug to the target on which it acts. The line color indicates drug effect on target: red for inhibition and green for activation. Pathway objects and links are described separately in Supplementary Figure 1.

shown promising effects by re-establishing Ca2+ homeostasis and reducing glutamate excitotoxicity. Among these, ZK 187638, NBQX, and GYKI-52,466 have shown protective effects on motor neurons in ALS animal models, by improving motor functions and prolonging survival. Moreover, also talampanel, another exponent of this drug class, has shown promising results in preclinical stages for ALS, but only a limited efficacy has been observed when tested in ALS patients. This clinical failure may be at least partly explained by the differential expression of GRIA1 and GRIA2 observed in SALS patients (**Figure 5a**). In the light of this, further studies are needed to re-evaluate the potential therapeutic benefits of talampanel for the personalized treatment of ALS patients.

Downregulated expression of six subunits of the gamma-aminobutyric acid type A receptor (GABA-A) was also found in SALS2 patients (**Figure 5a**) [8]. Although impaired GABAergic signaling has been previously observed in the motor cortex of ALS patients, little is known about its receptor composition. The few studies present in the literature confirm the reduced expression of the alpha1 subunit in ALS patients. This may suggest GABA-A signaling agonists as potential therapeutic agents able to reduce or prevent the progressive degeneration of motor neurons occurring in ALS. In accordance with this theory, some GABA-A agonists (Ganaxolone, Clomethiazole, GT 1061, Clorazepate, Zolpidem, Eszopiclone, Pentobarbital, and GT 1061) have shown neuroprotective properties in several neurological conditions by preventing excitotoxicity and neuronal loss [9].

*4.2.8. Genes previously linked to FALS*

Our analysis has revealed differential expression in the cortex of SALS patients of a large number of genes whose mutations have been previously associated with FALS (**Figure 5b**) and available in online databases, such as OMIM, ALSGene, ALS mutation database, and ALSoD [8]. Deregulation of these genes provides a potential common pathogenic link between familiar and sporadic ALS that may lead to the development of new strategies for the treatment of both forms of ALS. VEGF is among these whose protective effects on motor neurons have been previously discussed. Decreased expression of gene encoding VEGF was found in SALS2 patients (**Figure 5b**) as well as in individuals homozygous for a variety of mutations in the VEGF promoter region, suggesting the loss of the neuroprotective role of this factor may contribute to both forms of ALS [8].

Reduced expression of the sigma-1 receptor (SRBP1) and Spastin, an ATPase microtubules-binding protein necessary for normal neurite outgrowth, was also found in SALS2 patients (**Figure 5b**). Mutations in SRBP1 and SPAST (also known as SPG4) genes were proposed as potential genetic risk factors in ALS [46, 47]. These observations support the neuroprotective role of these proteins in maintaining physiological motor neurons functions, including proliferation, survival, and death, and highlight their potential role as a new therapeutic target in both forms of ALS. In this regard, some microtubule-targeting drugs (Noscapine and Vinblastine) and SRBP1 pharmacological ligands (Igmesine, Dextromethorphan, and PRE-084) have shown to exert neuroprotective effects in a variety of neurodegenerative diseases, including ALS [9].

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In SALS2 patients, we also observed the increased expression of voltage-gated sodium channel (SCN7) (**Figure 5b**), according to previous literature data reporting an abnormal increase in the persistent Na+ currents in animal models of ALS [48]. Therefore, pharmacological agents that can restore sodium current, reducing consequently the neuronal hyperexcitability, may represent innovative strategies to treat both familial and sporadic ALS. In this regard, the only approved treatment for ALS, Riluzole, is able to block sodium currents, reducing the motor neuronal hyperexcitability and the firing rate observed in ALS [49]. Other sodium channel blockers (Topiramate, Mexiletine, Lamotrigine, Valproic Acid, Zonisamide, Riluzole, and Tetrodotoxin) have also shown promising results in human and animal models of several neurodegenerative diseases, including ALS [9].

Decreased expression of Ataxin-1 (ATXN1), a protein mainly implicated in mRNA processing by forming an RNA-dependent complex with PolyQ binding protein-1 (PQBP-1), was found in SALS2 patients (**Figure 5b**). ATXN1 normally contains a polyglutamine (polyQ) tract with 22–23 repeats while an augmented number of polyQ repeats (23–34) may increase genetic risk for ALS [50]. Thus, alterations of ATXN-1 functions may be involved in the development of both sporadic and familial ALS and its pharmacological modulation may represent a potential therapeutic strategy against ALS. Bromocriptine is among the agents that prevent mutant ATXN-1 aggregation and the consequent neurotoxicity, which is an FDA-approved drug for the treatment of Parkinson's disease that has also shown promising results in preclinical stages for ALS [9].

SALS patients showed deregulated expression of genes involved in the regulation of the oxidative stress response. Among these, the upregulation of D-amino acid oxidase (DAAO), a flavoenzyme localized in motor neurons that metabolizes D-serine, was found in SALS2 patients (**Figure 5b**), coherently with the observation that altered DAAO activity which leads to an increased accumulation of D-serine in ALS mouse models and patients [51]. Moreover, a mutation in DAAO gene (R199W) has been associated with classical adult onset familial ALS [52]. Drugs inhibiting or controlling the activity of D-serine and DAAO enzymes may thus represent therapeutic strategies for treating both forms of ALS. Recent studies have, in fact, demonstrated that DAO inhibitors (AS057278, Sodium Benzoate, and Chlorpromazine) show promising beneficial effects in several neuronal conditions, including Alzheimer's [9].

Decreased expression of genes encoding for PON2 and PON3, two enzymes involved in the prevention of oxidative damage [53], was also observed in SALS2 patients (**Figure 5b**) and mutations in these two genes have been associated with both familial and sporadic ALS [54]. Decreased expression of PON2 and PON3 was also observed in SALS2 patients (**Figure 5b**), supporting the neuroprotective role exerted by these factors and their modulators into preventing or reducing neurodegeneration in ALS. Among drugs that are able to improve PON-2/PON-3 activity, genistein (previously mentioned for its activity as EGFR/ErbB2 inhibitor) has shown promising results in preclinical studies for ALS and other PON-activating drugs (Montelukast and Berberine) have manifested neuroprotective effects in several animal models of neuronal diseases [9].

Another potential genetic risk factor in FALS patients is gene encoding Valosin-containing protein (VCP), a protein belonging to the ATP-binding cassette superfamily, involved in a

multitude of cellular processes, such as vesicle transport and protein degradation. In accordance with literature studies, *VCP* mutations represent one of the major genetic causes of frontotemporal dementia but, recently, a specific mutation in this gene (p.R191Q) has been detected in some cases of FALS [55]. In our analysis, we observed the differential expression of *VCP* in SALS patients (**Figure 5b**). Moreover, several studies have highlighted the existence of a link between alterations in VCP functionality and the toxic gain of function of full-length TDP-43 [56]. The latter is a DNA/RNA binding protein, involved in several processes, including transcription, pre-mRNA splicing, mRNA stability, and mRNA transport [57]. Because of various types of insults, such as dysregulation in calcium homeostasis and oxidative damage, TDP-43 can migrate from the nucleus to the cytoplasm which can cause sequestration of RNA stress granule as well as a loss or gain of splicing functions. Although several studies have focused their attention on the role of TDP-43 in ALS etiopathogenesis, additional investigations are needed. Recent literature data showed the presence of aberrant nuclear or cytoplasmic TDP-43 inclusions in the nervous tissue of ALS patients [58]. It is thus evident that the pharmacological modulation of VCP may represent a promising strategy for ALS treatment, also through the reduction of TDP-43-mediated motor neuron toxicity. Some VCP inhibitors (Vesnarinone and Xanthohumol) have, in fact, demonstrated how to prevent neuronal death and inflammatory processes occurring in diverse neurological conditions [9].

## 5. Conclusion

ALS is a fatal neurodegenerative disease characterized by the influence of diverse mechanisms that interacting among each other promote the development and progression of the disease. Despite intensive research, ALS is still incurable and the only approved drug, Riluzole, conveys only modest benefits to patients. This clinical failure may be mainly due to the actual classification of ALS diseases, which is mainly based on clinical observation of symptoms and physical signs and does not take into account the complexity and heterogeneity of molecular pathogenic mechanisms underlying ALS.

The advent of high-throughput techniques in the biomedical sciences has provided a framework for developing a new, more accurate, and refined "molecular taxonomy" of human diseases that implies the use of molecular data to classify patients into distinct subgroups with differing diagnostic, prognostic, or therapeutic implications. In our previous work, we used unsupervised hierarchical clustering to identify two subgroups of SALS patients, characterized by their gene expression pattern, and revealed new clues to pathogenesis and potential therapeutic targets.

We argue that, as it is already happening in the genomic cancer field, our genomic analysis in combination with other "omics" data will complement and augment existing phenotypic information, providing a much deeper understanding of etiopathogenic mechanisms, which may have been masked by considering SALS as a single entity, and facilitating the development of a more precise diagnosis and individualized treatments for ALS patients.

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