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Aberrations in the cross-talks among redox, nuclear factor-κB and Wnt/catenin pathway signaling underpin Myalgic Encephalomyelitis and chronic fatigue syndrome: a review and new hypothesis based on results of network, enrichment and annotation analyses.

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Abstract

There is evidence that chronic fatigue spectrum disorders (CFAS-D) including Myalgic Encephalomyelitis (ME), chronic fatigue syndrome (CFS) and chronic fatigue with physiosomatic symptoms including when due to comorbid medical disease are characterized by neuroimmune and neuro-oxidative biomarkers. The present study was performed to delineate the protein-protein interaction (PPI) network of CFAS-D and to discover the pathways, molecular patterns and domains enriched in their PPI network. We performed network, enrichment and annotation analysis using differentially expressed proteins and metabolics, which we established in CFAS-D patients. PPI network analysis revealed that the backbone of the highly connective CFAS-D network comprises *NFKB1, CTNNB1, ALB,* peroxides, *NOS2, TNF*, and *IL6*, and that the network comprises interconnected immune-oxidative-nitrosative and Wnt/catenin subnetworks. MultiOmics enrichment analysis shows that the CFAS-D network is highly significantly associated with cellular (antioxidant) detoxification, hydrogen peroxide metabolic process, peroxidase and oxidoreductase activity, IL10 anti-inflammatory signaling, and neurodegenerative, canonical Wnt, the catenin complex, cadherin domains, cell-cell junctions and TLR2/4 pathways; and the transcription factors NF-κB and RELA. The top-10 DOID annotations of the CFAS-D network include four intestinal, three immune system disorders, cancer and infectious disease. Custom GO term annotation analysis revealed that the CFAS-D network is associated with a response to a toxic substance, lipopolysaccharides, bacterium or virus. In conclusion, CFAS-D may be triggered by a variety of stimuli and their effects are mediated by aberrations in the cross-talks between redox, NF-κB, and Wnt/catenin signaling pathways leading to dysfunctions in multicellular organismal homeostatic processes.

Key words: chronic fatigue syndrome, Myalgic Encephalomyelitis, inflammation, neuro-immune, oxidative and nitrosative stress, antioxidants

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Introduction

Contemporary research of chronic fatigue spectrum disorders (CFAS-D) including Myalgic Encephalomyelitis (ME), chronic fatigue syndrome (ME/CFS) and chronic fatigue (CF) and associated psychosomatic symptoms is plagued by a cacophony of controversies. These different approaches, sometimes even competing, comprise folk psychology (the culprit of CFAS-D is a psychological problem) and the medical approach (the culprit is one specific virus or different viruses or bacteria). The dominant view, especially in Europe, is that of the cognitive-behavioral and the biopsychosocial schools [1]. This view entails that CFAS-D, even when due to medical disease (e.g. cancer), are the consequence of psychosocial and biological factors and negative cognitions [1-4]. The Wessely model, for example, conceptualizes that the effects of a trigger factor, which may be a virus, are mediated by boom and bust activity and bedrest and the Vercoulen model considers that CFAS-D symptoms are aggravated by causal attributions and reduced physical activity [1]. Nevertheless, it appears that the label “biopsychosocial” is more window dressing than the actual approach because, in fact, folk psychology statements abounds in their publications as for example “it is in the mind”, “they think themselves ill” and “it is a disorder of perception whereby patients think those symptoms are the consequence of a virus” [review: 2]. Nevertheless, this folk psychology approach is embraced by the NHS, the Lancet and national health case system all over Europe (e.g.. UK, France, Sweden, Benelux).

It is incomprehensible that expert committees make consensus diagnostic criteria of ME/CFS (including the 1994 International Research Case Definitions and the 2003 Canadian Consensus criteria), whereas, in fact, these new diagnostic classes were never validated, for example by using machine learning techniques, and there is no evidence that the case definitions have reliability and replicability validity [2,4,5]. One diagnostic criterion of both abovementioned

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case definitions is that ME and CFS are not secondarily to medical disorders [6]. However, even CFS and ME, which are not secondary to any medical disorder, show a high comorbidity with symptoms of irritable bowel syndrome and depression [7], while CFAS-D symptoms are hallmarks of autoimmune, infectious, immune system and psychiatric disorders, including major depression and schizophrenia, and many more medical conditions [1,3,7-10]. CFAS-D symptoms are reflective manifestation of the symptomatome of for example COVID-19, chronic kidney disease with hemodialysis, schizophrenia, and rheumatoid arthritis [9,10-14], suggesting that CFAS-D symptoms are part of the core of these medical disorders and not the result of some "it is in the mind" process, if there would exist such a process.

It was not until 2010-2013, when Maes et al. [1,3,15] launched a new biomedical model, that more targeted medical research into CFAS-D began. These biomedical models of Maes et al. conceptualized CFAS-D as medical conditions whereby trigger factors such as immune activation, bacterial and viral infections, and a reduced protectome due to, for example, reduced antioxidant levels, may cause multiple adverse outcome pathways (AOPs) leading to the phenome of CFAS-D. The latter comprises CF symptoms, neurocognitive impairments, and depressive, sleep, gastro-intestinal and autonomic symptoms, hyperesthesia and fibromyalgia-like symptoms, a flu-like malaise including post-exertional malaise [1,3,15]. Furthermore, we conceptualized psychosomatic symptoms accompanying CFAS-D as being the consequence of different AOPs and, therefore, re-labelled those symptoms as "physiosomatic" symptoms [16,17].

The key AOPs of CFAS-D (including those that are secondary to medical disorders such as COVID-19, chronic kidney disease, rheumatoid arthritis, schizophrenia, and major depression which we established in our studies between 2001-2021, are: a) oxidative and nitrosative stress (O&NS) as assessed by increased hydroperoxides (H_2O_2) and inducible nitric oxide (NOS)

synthase, and reduced antioxidant balances, including coenzyme Q10, zinc, dehydroepiandrosterone (DHEA), glutathione peroxidase (GPX)1, and albumin (ALB); b) a multitude of immune disorders as indicated by an altered expression of CD (cluster of differentiation) markers on peripheral blood mononuclear cells (CD3, CD8, CD19, CD38, CD69, and HLA-DR), increased levels of interleukin (IL)-1α and IL-1β, IL-6, IL-10, tumor necrosis factor (TNF)-α and TNF receptors (TNFRs), soluble IL-1R antagonist (sIL-1RA), colony stimulating factor-2 (CSF2), high mobility group box protein 1 (HMGB1), neopterin, C-reactive protein (CRP), haptoglobin (HP), C–C motif chemokine ligand (CCL)2, CCL4, CCL11, cyclo-oxygenase-2 (PTGS2), soluble Toll Like Receptor (TLR)4, lysozyme (LYZ), and elastase (ELANE); c) increased levels of nuclear-factor (NF)-κB, a transcriptional factor; d) changes in cell-cell-junction and Wnt pathway proteins including catenin-β (CTNNB1), claudin-5 (CLDN5), occludin (OCLN), Dickkopf Wnt Signaling Pathway Inhibitor 1 (DKK1), and R-spondin-1 (RSPO1), e) altered levels of endorphin-opioid molecules; and f) induction of the tryptophan catabolite (TRYCAT) pathway with increased levels some neurotoxic TRYCATs, including 3-OH-kynurenine.

In addition, we also discovered that ME/CFS is characterized by increased IgA/IgM responses to Gram-negative bacteria indicating increased bacterial or lipopolysaccharide translocation, and IgM-mediated autoimmune responses to a number of neoantigens including malondialdehyde (MDA), azelaic acid and nitrosylated proteins, indicating increased nistrosylation [18,19].

All in all, these findings show that multiple differentially expressed proteins (DEPs) and metabolic pathways are involved in the pathophysiology of CFAS-D. However, no research has delineated the protein-protein interaction (PPI) and metabolic-protein interactions (MPI) networks

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of CFAS-D and the biological processes, molecular functions and complexes, cellular components, pathways, transcriptional regulatory relationships, protein domains and human disease annotations which are associated with the PPI and MPI networks of CFAS-D.

Hence, we have conducted network, enrichment and annotation analyses in order to delineate the hotspots in the CFAS-D network and the top functions and paths enriched in the networks. This is important because the most influential genes, metabolics, and pathways may constitute new drug targets to treat CFAS-D. Moreover, such analyses may disclose the putative trigger factors of the CFAS-D interactome and its associations with comorbid medical disorders. As such, these enrichment and annotation analyses may help to explain the strong comorbidity of CFAS-D with immune, infectious, and neuro-psychiatric disorders and the possible shared pathophysiological core which may underpin CFAS-D.

Methods

Selection of seed proteins and metabolic markers

"This study is a secondary data analysis on existing data using open, deidentified and non-coded data sets and, therefore, this is non-human subjects research which is not subject to Institutional Review Board approval" [20]. In case-control studies, we previously have identified metabolic pathways and differentially expressed proteins (DEPs) in CFAS-D including when due to comorbid medical disease. Almost all biomarkers included in this study were extracted from our studies on ME/CFS and CF-like symptoms with a duration > 6 months in comorbid disorders including major chronic kidney disease with hemodialysis, depression, schizophrenia, and rheumatoid arthritis (**Electronic Supplementary File (ESF), 1, References**). One study was performed on patients with CF-like symptoms due to acute COVID-19 infection. We were able to

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include a) 42 DEPs, namely ACE2 (angiotensin converting enzyme 2), AGER (advanced glycosylation end-product specific receptor), AGRN (agrin), ALB, CCL2, CCL4, CCL11, CD3D, CD8A, CD19, CD38, CD69, HLA-DR, CKM (creatine phosphokinase), CLDN5, CSF2, CRP, CTNNB1, DKK1, ELANE, GPX1, HbA1 (hemoglobin), HP, HMGB1, IL1A (interleukin-1α), IL1B (interleukin-1β), IL1RN (IL-1RA), IL6, IL10, LYZ, NFKB1 (NF-κB), NOS2, OCLN, OPRK1 (opioid receptor Kappa 1), OPRM1 (opioid receptor Mu 1), POMC (proopiomelanocortin as precursor of β-endorphins), PTGS2, RSPO1, TLR4, TNF (TNF-α), TNFRSF1A (TNFR60) and TNFRSF1B (TNFR80); and b) 12 metabolic KEGG (Kyoto Encyclopedia of Genes and Genomes; https://genome.jp/kegg/) pathways, namely C01290 (lactosylceramide), C02470 (xanthurenate), C10164 (picolinic acid), C03227 (3-OH-L-kynurenine, 3OHK), C11378 (coenzyme Q10), C00038 (zinc), C00070 (copper), C06428 (eicosapentaenoic acid; EPA), C00027 (hydrogen peroxides), C19440 (malondialdehyde, MDA), C004555 (DHEA), and C05926 (neopterin).

PPI network construction, and enrichment and annotation analyses.

The network, enrichment and annotation analysis were conducted as reviewed previously [20]. In brief, we constructed two network subtypes, the first was constructed using the abovementioned DEPs whereby the physical interactions between the DEPs were visualized using STRING version 11.0 (https://string-db.org) and Cytoscape (https://cytoscape.org). The second was constructed using OmicsNet (www.omicsnet.ca) using the abovementioned metabolites and examining the MPI based on KEGG reactions. Consequently, the genes interacting with the metabolics in the different subnetworks were used in STRING to

build a giant network based on our seed genes enlarged with the MPI-derived DEPs, which was, consequently, analyzed in OmicsNet to construct a composite network consisting of metabolites and DEPs first entering MPIs and then the PPIs (analyzed with IntAct Molecular Interaction Database: https://www.ebi.ac.uk/intact/) an with a targeted incorporation of TF-protein interactions (TFPI) (analyzed using TTRUST (www.grnpedia.org/trrust).

Network features were computed using STRING and the Cytoscape plugin Network Analyzer and comprise number of nodes, number of edges and expected number of edges, average node degree, network diameter and radius, characteristic path length, and network density and heterogeneity. The top hubs (high degree) and bottlenecks (high betweenness centrality) were computed and used to delineate the backbone of the network, i.e. the top7 hubs and the top-2 non-hub bottlenecks. We used Markov Clustering (MCL) employing STRING to discover communalities of interconnected nodes, which display similar attributes and/or functions.

We examined the different networks and MCL subnetworks for their enrichment scores and annotated terms and these analyses were also performed on the downregulate seed genes and the hotspots of the enlarged network. Enrichment/annotation analyses were performed using STRING, Enrichr (https:/maayanlab.cloud/Enrichr/), OmicsNet, MetaScape (https://metascape.org), inBio Discover (https://inbio-discover.com), and the R package ClusterProfiler. Heatmaps were produced using Appyter and MetaScape. Functional enrichments were established using gene Ontology (GO) biological processes, GO molecular functions and GO cellular components, STRING local network clusters, KEGG pathways, Reactome pathways (the European Bio-Informatics Institute Pathway Database; https://reactome.org), PANTHER biological processes (PANTHER - Gene List Analysis (pantherdb.org), TTRUST transcriptional

regulatory relationships, InterPro domains (InterPro (ebi.ac.uk), Wiki pathways (WikiPathways - WikiPathways), and DOID annotations of human diseases (Disease Ontology - Institute for Genome Sciences @ University of Maryland (disease-ontology.org). We also performed Molecular Complex Detection (MCODE) (using Metascape) to delineate small molecular complexes.

Results

The DEP PPI network topography of CFS

Figure 1 shows the first order protein network of CFAS-D. This network comprises 91 nodes and 938 edges, exceeding the expected number of edges (n=323) with a p-enrichment value of 1.0E-16. The network has an average local clustering coefficient of 0.683 and average node degree of 20.6, with a network diameter of 4, radius of 2, a characteristic path length = 1.978, network density = 0.229, and heterogeneity = 0.682. The top-7 hubs (highest degree) were in descending order of importance: TNF (degree=63), IL6 (61), IL1B (58), ALB (54), TLR4 (47), IL10 (47) and CTNNB1 (46). The top-two non-hub bottlenecks were: EGFR (0.066) and TLR2 (0.0188). EGFR and TLR were the top 5 and 9 in the bottleneck list (top 3 in descending order was ALB, CTNNB1 and TNF). The network of the seed proteins showed 42 nodes and 311 edges, thus, exceeding the expected number of edges (n=70) with a p-enrichment value of 1.0E-16. The network has an average local clustering coefficient of 0.74 and an average node degree of 14.8.

MCL cluster analysis (inflation parameter of 2) resulted in two protein subnetworks as shown in Figure 1. The first subnetwork contains predominantly immune genes, EGFR, ALB, AGER, HP, GPX1, CD19, etc., and the second subnetwork contains Wnt and cell-cell junction associated genes including CTNNB1, AGRN, RSPO1, and DKK1. The major connectors

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(switches) of both subnetworks were CTNNB1 (belongs to subnetwork 2) and ALB and IL6 (nodes in subnetwork 1). CTNNB1 showed relevant interactions (confidence level > 0.4) with DKK1 and RSPO1 and with 11 subnetwork 1 seed genes (e.g. NFKB1, ALB, OCLN, TLR4, HMGB1). ALB showed significant interactions with 25 other subnetwork 1 genes, and with CTNNB1, AGRN and DKK1. IL6 showed interactions with CTNNB1, DKK1, and AGRN and 28 other subnetwork 1 seed nodes. In the first-order non-seed genes we found that EGFR was connected with 3 subnetwork 2 seed genes (GRN, CTNNB1, and DKK1) and with 19 seed genes in subnetwork 1.

MultiOmics Analysis including the oxidative stress-associated metabolites.

In order to construct a second giant network including proteins interconnecting with the metabolites, we entered the latter in OmicsNet analysis and examined the MPIs (using KEGG and IntAct and using only the first order MPIs). We found 5 subnetworks: one centered around hydroperoxides (46 nodes), another around EPA (6 nodes), 6 around 3OHK, 4 around NO, and 3 around DHEA (albeit some overlapping). The 59 nodes coupled with the nodes from the first network (see Figure 2) were consequently examined using STRING and Network Analyzer. **Figure 2** shows the network based on this combination of DEPs. This network comprises 147 nodes and 1407 edges, exceeding the expected number of edges (n=413) with a p-enrichment value of 1.0E-16. The network has an average local clustering coefficient of 0.624 and average node degree of 19.1, with a network diameter of 5, radius of 3, characteristic path length = 2.278, network density = 0.131, and heterogeneity = 0.799. The top-7 hubs (highest degree) were in descending order of importance: TNF (degree=82), IL6 (79), ALB (75), IL1B (74), CAT (59; catalase), IL10 (57) and TLR4 (57). The top-5 bottlenecks were CAT (0.1534), ALB (0.1212), CTNNB1 (0.0918), TNF (0.08867, and IL-6 (0.06755). The first non-hub bottlenecks were EGFR (0.04909) and NOS2 (0.04572).

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Figure 2 shows the results of MCL cluster analysis (inflation parameter of 1.7) displaying two significant protein subnetworks, a first comprising immune and nitro-oxidative stress genes (see Figure 6; this cluster is now re-named the immune-inflammatory, oxidative and nitrosative cluster, IO&NS), and a second Wnt/catenin cluster. There were also some communities with only few nodes, for example one centered around KYNA (kynureninase) and KMO (kynurenine 3-monooxygenase).

Figure 3 shows the results of OmicsNet analysis which included all IO&NS/Wnt genes of the network presented in Figure 2 with integration of the metabolics and NFKB (as transcriptional factor). This network was constructed using three interaction types, namely MPI (first rank), PPI (second rank) and TFPI (third rank) and included 574 nodes and 694 edges. The top hubs (including non-seeds) in this network were NOS2 (177), NOS3 (63), SOD2 (57), PRDX6 (47), peroxides (45), SOD1 (41), KYNU (32), and NFKB1 (29). We also examined the hubs of the network built with PPIs entered in first order and MPI in second order (2593 nodes and 4063) and found that the top-3 hubs were in descending order of importance: NFKB1 (307), CTNNB1 (153), and ALB (152). As such, the common backbone of the different networks (top-3 of each network) in our study consists of NFKB1, CTNNB1, ALB, peroxides, NOS2, TNF, and IL6.

Table 1 shows the results of PANTHER functional explorer analyses. The top PANTHER molecular functions which were enriched in this network revolved around protein binding, peroxidase and oxidoreductase activity; and the top PANTHER cellular components were the cytosol, protein-containing complex, cytoplasm and the nucleus.

Enrichment and annotation analysis in all IO&NS/Wnt genes of CFAS-D.

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Table 2 displays the results of MCODE analysis using KEGG, WikiPaths, GO biological and molecular, REACTOME and PANTHER performed on the IO&NS/Wnt genes. We found five significant molecular complexes, the first represents cytokine/IL10 signaling and a response to LPS; the second comprises cellular oxidant detoxification and response to a toxic substance; the third amine oxidase reactions, hydrogen peroxide metabolic process and degradation of beta catenin; the fourth peroxisomal protein import and a carboxylic acid catabolic response; and the fifth was the same as MCODE2 in Table 2.

Table 3 shows the most important GO biological processes, InterPro domains, and KEGG pathways enriched in the IO&NS/Wnt network. GO biological process enrichment analyses showed that this network was significantly associated with oxidoreductase, antioxidant and peroxidase activity and beta-catenin binding. InterPro enrichment analyses showed that haem peroxidase and glutathione peroxidase were the top terms. KEGG path analysis showed that the network was highly associated with the Wnt signaling and NF-κB pathway, tryptophan metabolism and illnesses including tuberculosis and Chagas disease.

Figure 4 shows the enriched ontology clusters (using MetaScape) in the network with cellular oxidant detoxification, IL10 signaling and a response to LPS as the top terms. **Figure 5** shows a heatmap (made using Enrichr and Appyter) with the top KEGG pathways that were over-represented in the IO&NS/Wnt network, namely pathways of neurodegeneration, Wnt and NF-κB signaling and peroxisome. **Figure 6** shows a heatmap with the PANTHER 2016 pathways which were over-represented in the network, namely Wnt, apoptosis, TLR and cadherin pathways and the Alzheimer disease presenilin pathway. TTRUST analysis showed that NF-κB (pFDR=6.58E-23) and RELA (pFDR=8.691E-21) were the most important transcriptional factors of the network followed at a large distance by SP1 (pFDR=7.079E-13).

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Table 4 shows the top-10 DOID annotations of an extended IO&NS/Wnt network (inBio Discover) including four intestinal disorders as the top 4 annotations, immune disorders and cancer.

Electronic Supplementary File (ESF) 1 and ESF 2 show the results of enrichment and annotation analysis performed on the DEPs (or selected DEPs) presented in Figure 1.

Discussion

The networks and subnetworks of CFAS-D

The first major finding of this study is that the PPI network of the DEPs and metabolics of CFAS-D show high connectivity and comprises two subnetworks, a first centered around IO&NS genes and a second around Wnt/catenin genes. The backbone of the master network comprises DEPs/metabolics including NFKB1, CTNNB1, ALB, peroxides, NOS2, TNF, and IL6, while in the giant network many redox-related enzymes were predicted to be hubs, including NOS3 (endothelial NO synthase producing NO relaxing smooth muscle relaxation), SOD2 (mitochondrial superoxide dismutase 2) and PRDX6 (peroxiredoxin 6; catalyzes the reduction of hydrogen peroxides). It is important to note that without the delineation of the MPIs, one would have concluded that especially pro-inflammatory cytokine genes are the dominant forces in this network, whereas, in fact, the IO&NS subnetwork is more dominated by redox genes, NF-κB and IL-10.

CTNNB1, which belongs to the Wnt subnetwork was another hotspot and additionally showed many interactions with genes in both subnetworks indicating that this gene is a relevant switch linking both subnetworks. Other relevant switches belonging to the immune subnetwork were ALB and IL6 which showed many interactions with cluster 1 genes, but also with CTNNB1,

DKK1 and AGRN. Hotspots and switches are considered to be new drugs targets because they govern and control the network and/or link the subnetworks [20]. All in all, we may conclude that dysfunctions in the IO&NS and Wnt subnetworks underpin the pathophysiology of CFAS-D.

Terms over-represented in the IO&NS MultiOmics subnetwork

The second major finding of this study is that the top relevant functions and pathways in the first network revolve primarily around redox mechanisms, namely cellular oxidant detoxification (and thus cellular detoxification and a cellular response to toxic substance), the hydrogen peroxide metabolic process, antioxidant activity, amine oxidase reactions, oxidoreductase activity, peroxidase activity, and haem and glutathione peroxidase. These findings indicate that increased oxidative stress (hydrogen peroxides) probably as a response to a toxic substance coupled with reduced antioxidant defenses (peroxidase, oxidase, oxidoreductase, ALB) are the major pathways in CFAS-D. Indeed, many of the seed DEPs and metabolics show antioxidant properties including ALB, GPX1, HP, CoQ10, DHEA, zinc and EPA, and pro-oxidant properties including NFKB1, NOS2, PTGS2, and hydroperoxides. Moreover, the giant network based on seed DEPs and MPI-derived genes indicates that many redox genes are expected to participate in the CFAS-D network, including CAT, GPX2-9, SOD1, SOD2, LPO, LOX, MPO, NOS1, NOS3, and PRDX6.

Such findings agree with the evidence that CFAS-D is characterized by reduced antioxidant defenses, and oxidative damage to lipids, proteins, DNA and mitochondria as reviewed in [1,3,7,15]. Such damage in ME/CFS is accompanied by formation of immunogenic oxidative modified neoepitopes which in turn may cause an IgM-mediated immune response against neoepitopes like malondialdehyde and azelaic acid [18]. The participation of NOS2 in the CFAS-

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D network is in agreement with our reports that ME/CFS is accompanied by increased nitrosative stress, which is a consequence of increased NO and superoxide production resulting in hypernitrosylation [18]. In addition, also more recent papers indicate that increased nitro-oxidative stress and reduced antioxidant defenses contribute to idiopathic chronic fatigue, including increased reactive oxygen species, malondialdehyde, and F2-isoprotan, and lowered catalase and glutathione levels [21]. In patients with CFAS-D, baseline thiobarbituric acid–reactive substances (TBARS, an indicant of lipid peroxidation) are associated with exercise-induced pain [22]. A recent review describes how oxidative stress may cause membranopathies, channelopathies and mitochondrial dysfunctions which in turn may interfere with intracellular energy processes and imbalances in signal conversion system [23]. There are many reports showing that antioxidants may improve CFAS-D in animal models, including carvedilol, melatonin, Withania somnifera, quercetin and Hypericum perforatum L [24], curcumin [25], Sarcodon imbricatus [26] and many more.

The results of our network and enrichment analyses show that NFKB1 was not only one of the most important hotspots in the CFAS-D network, but also that NF-κB (p50-p52 unit) and RELA (NF-κB p65 unit or transcription factor p65) were the most important transcription factors controlling the network and that the NF-κB signaling pathway was one of the most important paths enriched in the network. NF-κB is a major transcriptional factor involved in the response to a vast array of stimuli including bacterial or viral antigens, cytokines, free radicals, oxidized epitopes, and glutamate, and is a transcriptional inducer of many genes including cellular adhesion molecules, pro-inflammatory cytokines, chemokines, and growth, apoptosis and coagulation factors, and antioxidants and pro-oxidants [27-29]. RELA is involved in the activation of NF-κB and stimulates NF-κB translocation to the nucleoplasm and by forming a RELA-NF-κB complex

activates target gene expression [30]. NF-κB p50, which is associated with RELA, and NF-κB p52 are major components of the canonical and noncanonical NF-κB signaling pathways which both lead to target gene activation [29]. Both transcription factors are known to regulate different pathways that we observed to participate in the network including the MAPK pathway [31] and ROS and Wnt/catenin pathways.

There are many intersections between reactive oxygen species (ROS) and NF-κB signaling. Indeed, ROS may modulate the NF-κB response leading to transcriptional activation of NF-κB-target antioxidant genes (e.g. *SOD, HO1, GPX1, TRX1* and *TRX2*), which reduce ROS production, thereby promoting cell survival [29], and NF-κB-target pro-ROS genes including *NOX2, COX2,* and *NOS2* [29,32]. It should be stressed that the effects of ROS on NF-κB are more than complex with stimulatory effects in the cytoplasm and inhibitory effects in the nucleus [33], while ROS may also oxidize NF-κB p50 leading to decreased DNA binding capacity [34].

All in all, the results of our network and enrichment analysis and Maes et al. [35,36] indicate that the complex cross-talks between NF-κB and ROS signaling are involved in the pathophysiology of CFAS-D. Moreover, recent studies show that increased NF-κB expression may be associated with central fatigue by modulating central nervous system genes and regulating immune-inflammatory processes, synaptic plasticity, and memory and exerting neurotoxic effects [35-37].

Our MCODE analysis revealed that the IL-10 anti-inflammatory signaling pathway, regulation of cytokine production, signaling by interleukins and a response to LPS was a relevant molecular complex in the IO&NS subnetwork of CFAS-D. These findings agree with those of our previous studies showing increased IgA/IgM response to LPS of 6 different Gram-negative bacteria in CFAS-D, indicating increased bacterial translocation [19]. Previously, we have argued

that part of the effects of translocated LPS on cytokine production could be explained by induction of the TLR signaling pathway which also signals to NF-κB which in turn may activate cytokine genes [38,39].

Our enrichment analysis also showed an association between the IO&NS subnetwork and the lactoferrin (LTF) danger signal response pathway. LTF, an iron-binding glycoprotein, activates NF-κB via the TLR2/4 complexes and additionally RAGE and TREM-1 receptors [40]. LTF functions as a first line defense against injuries, either pathogenic or non-pathogenic and controls cell homeostasis [40]. Furthermore, LTF sequesters ROS (thereby attenuating tissue damage due to excessive IO&NS) and maintains intestinal integrity during endotoxemia [40]. Our enrichment analysis shows that the pro-inflammatory TNF-related weak inducer of apoptosis (TWEAK) signaling pathway is another possible link between tissue injury and upregulation of NFKB1-associated DEPs/genes by activating the NF-κB pathways [41].

The NF-κB signaling pathway not only regulates the transcription of pro-inflammatory genes including IFNG, IL1 and TNF, but also IL10, which has negative immune-regulatory effects, which protect against an overzealous inflammatory response [42]. IL10 has negative immune-regulatory activities by inhibiting the production of M1 macrophage and T helper (Th)-1 cytokines, dendritic cells stimulating CD4+ T cells, and Th-2 responses, and attenuates CD8+, M1 macrophage, Th-1 and Th-2 associated immunopathology [43,44]. It is important to note that our enrichment analysis stresses the importance of anti-inflammatory IL10 signaling as a response to LPS and regulation of cytokine signaling, suggesting that a predominant IL10 phenotype may occur in CFAS-D. Presuming that the latter are frequently triggered by a low/moderate pathogen (or LPS) virulence [1], we may expect that IL10 suppresses the ongoing immune response thereby contributing to long-term escape of pathogens from immune control and causing persistent or

recurrent infections [1,43]. Therefore, immunosuppression which is another hallmark of CFAS-D may not only be explained by T cell exhaustion through increased pro-inflammatory cytokine production [45] but also by increased IL10.

Finally, our network and enrichment analyses showed that the TRYCAT pathway may be involved in CFAS-D, although it is not a key component but rather a spin-off of the IO&NS response. Previously, it was reported that this pathway is highly strongly associated with somatization disorder, a psychiatric disease accompanied by physiosomatic symptoms but not necessarily by fatigue [46]. The TRYCAT pathway acts as a redox-regulator and is one of the major antioxidant systems, although some TRYCATs have pro-oxidant and neurotoxic activities [47]. Thus 3OHK, one of the metabolics in our MPI network, is one of the neurotoxic TRYCATS produced during activation of this pathway as a consequence of IO&NS activation. KYNU (kynurenine hydroxylase) is as IDO an oxygen-consuming enzyme which catabolizes kynurenine into 3OHK (STRING).

Terms over-represented in the Wnt MultiOmics subnetwork

Our enrichment analyses revealed that the CFAS-D network was highly significantly associated with two major interrelated functions/pathways/domains namely the canonical Wnt/β-catenin pathway, T-cell factor (TCF) dependent signaling in response to Wnt, degradation of β-catenin by the destruction complex, the catenin complex and DIX domain, and adherens junctions, cadherin prodomain, and cell-cell junctions. The DIX domains and axin, GSK-3 and Dishevelled (Dvl) are key players in the β-catenin destruction complex thereby determining the interaction of β-catenin with the transcription factor TCF and, thus, the expression of the Wnt target genes [48].

The β-catenin/E-cadherin complex is the key component of adherens junctions (AJs), which stabilizes cell-cell junctions, provides cell-cell adhesion and bind catenin with the actin cytoskeleton [9,49]. Moreover, aberrations in the AJs lead to a breakdown of the tight junctions (TJs), another component of the paracellular route [50]. β-catenin and occludin are key factors in both the AJs and TJs (paracellular pathway) including those of the blood-brain barrier (BBB) and gut barriers, whereas claudin-5 is more specific to the BBB and E-cadherin to the gut barrier [9].

As a consequence, dysfunctions in AJs and TJs may cause aberrations in cell-cell adhesion and the actin cytoskeleton [51]. Furthermore, degradation of the E-cadherin-β-catenin complexes may cause aberrations in intracellular signaling, the actin cytoskeleton and the Wnt/β-catenin signaling pathway, a key component in cell homeostasis [52]. Previously, we reported that increased levels of zonulin (prehaptoglobin-2) are strongly associated with CFAS-D in schizophrenia and explain, at least in part, the aberrations in TJs and AJs and consequent breakdown in the paracellular route [9]. It should be added that aberrations in the paracellular route of the gut barrier are associated with increased bacterial or LPS translocation (leaky gut) and thus activation of the IO&NS pathways [9]. Moreover, increased ROS and NO production and pro-inflammatory pathways (TNF, IL6, IFNG) may damage the epithelial and endothelial TJs [53]. Furthermore, hydrogen peroxides may redistribute β-catenin and E-cadherin from the paracellular route into the cell thereby affecting the TJs and deplete occludin and damage the cytoskeleton as well [53]. It should be added that virulence factors of many pathogenenic bacteria may exploit the Wnt/catenin pathway to alter the anti-bacterial immune response, including *Pseudomonas aeruginosa*, one of the bacteria involved in CFAS-D [19,54].

Importantly, our MCODE analysis revealed another highly significant molecular complex in the CFAS-D network comprising the hydrogen peroxide metabolic process, amine oxidase

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reactions and the β-catenin degradation pathway, indicating that interactions between those factors are involved. Importantly, dysregulated Wnt/catenin signaling may cause oxidative stress in pregnant mice with CFS [55]. Nucleoredoxin (NRX), a thioredoxin-like protein, strongly inhibits Wnt/catenin binding and the expression of early genes, whereas H_2O_2 treatment stabilizes β-catenin and increases expression of Wnt genes [56]. Increased ROS promotes intrinsic apoptosis, and the consequent caspase 3 expression inhibits Wnt signaling in association with cleavage of E-cadherin [57]. H_2O_2 not only inhibits Wnt/b-catenin signaling but may also increase Wnt/catenin signaling [58]. In fact, peroxides have a biphasic effect on Wnt signaling with an increase 20 minutes after activation and reduced signaling some hours later [57]. Finally, β-catenin is a key regulator of the homeostatic cell response, which helps to repair the damage due to nitro-oxidative stress [59].

Furthermore, the NF-κB and Wnt pathways shows multiple cross-talks and negatively or positively regulate each other, thereby forming a mutual regulatory network [60]. Wnt modulates the production of inflammatory cytokines and NF-κK signaling and bridges innate and adaptive immune pathways [61]. On the other hand, increased levels of IL-6 and TNF-α may maintain increased Wnt signaling associated with low β-catenin, Dishevelled and axin levels [62]. It should be stressed that our network analysis showed that the key seed DEPs of both IO&NS (NFKB1) and Wnt (CTNBB1) pathways have significant physical interactions based on co-expression, experimental and biochemical data (STRING) thereby also functioning as an anchor linking the IO&NS and Wnt subnetworks. All in all, it appears that the pathophysiology of CFAS-D may be characterized by aberrations in the intertwined cross-talks between ROS, NF-κB and Wnt/catenin signaling.

Previously, we have explained how IO&NS and NF-κB and their associated pathways may explain the central and peripheral symptoms of CFAS-D [3,15,16,23,36,63]. The current study shows that alterations in the Wnt pathway may contribute to these symptoms because this pathway regulates the homeostasis in self-renewing tissues and has additionally organ-specific effects. For example, the Wnt/catenin pathway is involved in BBB integrity, synapse assembly, synapse functions, synaptic plasticity, neurogenesis, white matter lesion remyelination, dopamine neuron neuronal survival protection and regeneration, whereas aberrations in the Wnt/catenin pathway are associated with synaptic loss, BBB breakdown and neurodegenerative disease including Alzheimer's and Parkinson's disease [64-68].

The Wnt/catenin pathway is also involved in a) pain and neuropathic pain with Wnt inhibition improving pain [67,70]; b) skeletal muscle dynamics, the neuromuscular synapse, and musculoskeletal functions including the electrophysiologic properties of muscle cells [71,72]; c) intestinal functions including epithelial homeostasis and integrity, the physiological proliferation of the transit-amplifying cells and differentiation of Paneth, goblet, and enteroendocrine cells, and the maintenance of mucosa and barrier functions [73-76]. The Wnt/catenin pathway also plays a key role in autoimmunity as observed in rheumatoid arthritis [77,78] and in the response to bacterial infections and inflammation [79,80].

Conclusions

Figure 8 summarizes the pathways and molecular complexes or functions that accompany CFAS-D. Aberration in the cross-talks among redox, NF-κB, and Wnt signaling may be key pathways, which are associated with dysfunctions in multicellular organismal homeostatic processes including in cell-junction organization. Disorders in the intertwined interactions between

these systems may explain the broad spectrum of organs and dysfunctions that participate in CFAS-D (brain, musculoskeletal system, immune system, gastro-intestinal system). An important spin-off is increased IL10 production, which may contribute to immunosuppression and recurrent or protracted infections, and also increased TRYCAT production may aggravate the neurotoxic effects of oxidative stress. Increased translocation of Gram-negative bacteria with increased LPS load is probably a major trigger factor but also other bacterial infections, toxoplasmosis, viral infections (e.g. cytomegalovirus), cancer, and gastro-intestinal, autoimmune, immune-inflammatory, neuroinflammatory and neurodegenerative disorders appear to be associated with those pathways via activation of TLR/LTF/TWEAK signaling.

Future research should scrutinize the specific role of the NF-κB, ROS and Wnt axis in CFAS-D. The cross-talks between these three pathways may also constitute new drug targets to treat CFAS-D. Given that the Wnt pathway shows many complex, negative and positive feedback loops interacting with redox systems and NK-κB signaling, manipulations of Wnt signaling and β-catenin (despite being a hub and master switch) appears to be very challenging. It may be more promising to simultaneously target the crosstalk among redox and NF-κB pathways. New knowledge on the precise aberrations in the Wnt pathway in association with ROS and NF-κB signaling may lead to even more effective treatments by specifically targeting β-catenin, the β-catenin destruction complex or the TCF transcription factor.

Our new model shows that disorders in cross-talks among these three key pathways mediate the effects of a variety of trigger factors in the onset of CFAS-D. These findings also support the theory that once CFAS-D is present the abovementioned pathways may increase morbidity and even mortality of IO&NS-associated medical disorders through the detrimental effects of disorders in redox, NF-κB and Wnt axis [81,82]. The model also explains that the acute

phase of inflammatory conditions may lead to CFAS-D via disorders in this axis, oxidative damage and the neurotoxic effects of LPS, pro-inflammatory cytokines and TRYCATs. Since most biomarkers included in this study were extracted from studies on ME/CFS and CF-like symptoms in comorbid disorders with duration > 6 months, we may conclude that after resolution of acute inflammation, CAFS-D symptoms are maintained by continued aberrations in the redox, NF-κB, and Wnt axis, increased IL-10 production and increasing oxidative damage including secondary autoimmune responses and nitrosylation.

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Conflict of interest

The authors have no conflict of interest with any commercial or other association in connection with the submitted article.

Author's contributions.

All the contributing authors have participated in the manuscript. MM designed the study and performed the network, enrichment and annotation analyses. All authors contributed to interpretation of the data and writing of the manuscript.

Compliance with ethical standards.

The study was conducted according to international ethics and privacy laws.

IRB statement.

This study is a secondary data analysis on existing data using open, deidentified and non-coded data sets and, therefore, this is non-human subjects research, which is not subject to IRB approval.

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Table 1. PANTHER molecular function and components terms associated with chronic fatigue spectrum disorders

PANTHER molecular functions	total	expected	hits	P	pFDR
Protein binding	9580	334	461	9.74E-33	1.89E-30
Peroxidase activity	31	1.08	21	5.38E-24	5.22E-22
Oxidoreductase activity	590	20.6	75	7.24E-23	4.68E-21
RNA binding	1510	52.6	107	4.94E-13	2.39E-11
Nuclear receptor activity	45	1.57	14	2.04E-10	7.93E-09
Signaling receptor binding	413	14.4	38	5.05E-08	1.63E-06
Double-stranded DNA binding	128	4.46	18	4.71E-07	1.30E-05
Antioxidant activity	26	0.906	8	1.86E-06	4.50E-05
PANTHER cellular components	total	expected	hits	P	pFDR
Cytosol	5030	157	330	1.11E-53	5.88E-52
Protein-containing complex	656	20.6	85	1.34E-29	3.54E-28
Cytoplasm	6540	205	334	3.10E-29	5.48E-28
Nucleus	6480	203	309	2.33E-20	3.08E-19
Mitochondrion	1510	47.2	94	6.79E-11	7.19E-10
Peroxisome	119	3.73	21	1.19E-10	1.05E-09
Protein-DNA complex	44	1.38	12	6.72E-09	5.09E-08

FDR: false discovery rate.

Table 2. Results of Molecular Complex Detection (MCODE) analysis performed on the differently expressed proteins (DEPs) of chronic fatigue spectrum disorders

MCODE Components	GO ID	Biological term	Log10 (p)
All DEPs, MCODE_ALL	GO:0098869	Cellular oxidant detoxification	-33.0
	GO:1990748	Cellular detoxification	-31.5
	GO:0097237	Cellular response to toxic substance	-30.8
All DEPs, MCODE1	R-HSA-449147	Signaling by Interleukins	-31.9
	R-HSA-6783783	Interleukin-10 signaling	-30.0
	GO:0032496	Response to lipopolysaccharide	-29.7
All DEPs, MCODE2		Same as MCODE_ALL	
All DEPs, MCODE3	R-HSA-140179	Amine Oxidase reactions	-9.0
	GO:0042743	hydrogen peroxide metabolic process	-7.2
	M31	PID BETA CATENIN DEG PATHWAY	-6.7
All DEPs, MCODE4	R-HSA-9033241	Peroxisomal protein import	-16.6
	R-HSA-9609507	Protein localization	-13.6
	GO:0046395	Carboxylic acid catabolic process	-10.2

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Table 3. GO biological process terms, InterPro domains and KEGG pathways associated with chronic fatigue spectrum disorders

Path ID	Enrichment GO biological process	Observed	background	Strength	pFDR
GO:0016491	Oxidoreductase activity	49	726	0.95	1.29E-28
GO:0016209	Antioxidant activity	22	74	1.6	1.35E-23
GO:0004601	Peroxidase activity	17	41	1.74	6.98E-20
GO:0008013	Beta-catenin binding	16	86	1.39	4.55E-14
GO:0005515	Protein binding	102	7026	0.29	1.96E-13
GO:0042802	Identical protein binding	51	1896	0.55	1.96E-13
GO:0016641	Oxidoreductase activity, acting on the CH-NH2 group of donors, oxygen as acceptor	10	16	1.92	1.22E-12
GO:0050660	Flavin adenine dinucleotide binding	14	82	1.36	7.21E-12
GO:0020037	Heme binding	15	134	1.17	1.75E-10
GO:0003824	Catalytic activity	80	5486	0.29	8.35E-09
GO:0004602	Glutathione peroxidase activity	8	20	1.73	8.35E-09
Path ID	**Enrichment InterPro**	**Observed**	**background**	**Strength**	**pFDR**
IPR010255	Haem peroxidase superfamily	8	10	2.03	9.50E-09
IPR019791	Haem peroxidase, animal-type	8	10	2.03	9.50E-09
IPR037120	Haem peroxidase domain superfamily, animal type	8	10	2.03	9.50E-09
IPR000889	Glutathione peroxidase	7	8	2.07	4.98E-08
IPR029760	Glutathione peroxidase conserved site	7	8	2.07	4.98E-08
IPR002937	Amine oxidase	7	10	1.97	9.91E-08
IPR036188	FAD/NAD(P)-binding domain superfamily	10	56	1.38	2.41E-07
IPR029759	Glutathione peroxidase active site	6	7	2.06	9.34E-07
Path ID	**Enrichment KEGG**	**Observed**	**background**	**Strength**	**pFDR**
hsa04310	Wnt signaling pathway	18	154	1.19	3.25E-13
hsa05152	Tuberculosis	18	168	1.15	6.53E-13
hsa04064	NF-kappa B signaling pathway	15	101	1.3	1.30E-12
hsa00380	Tryptophan metabolism	11	41	1.55	1.43E-11

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hsa04146	Peroxisome	13	79	1.34	1.43E-11
hsa05142	Chagas disease	14	99	1.27	1.43E-11
hsa05163	Human cytomegalovirus infection	18	218	1.04	1.43E-11
hsa01100	Metabolic pathways	40	1447	0.57	2.64E-11
hsa04520	Adherens junction	12	67	1.38	3.16E-11
hsa05010	Alzheimer disease	21	355	0.9	3.21E-11

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Table 4. Results of inBio Discover annotation analysis with the top-10 and custom-made DOID annotations associated with chronic fatigue spectrum disorders

DOID ID	**Disease**	**Size**	**Overlap**	**Enrichment**	**p-value**
DOID:77	Gastrointestinal system disease	2.3k	133/419	2.81	6.1E-30
DOID:5295	Intestinal disease	1.0k	82/419	3.86	1.3E-26
DOID:0050589	Inflammatory bowel disease	306	46/419	7.18	4.4E-26
DOID:00600180	Colitis	237	41/419	8.26	9.1E-26
DOID:2914	Immune system disease	1.9k	111/419	2.79	3.1E-24
DOID:612	Primary immunodeficiency disease	1.3k	87/419	3.12	6.4E-22
DOID:7	Disease of anatomical entity	7.3k	248/419	1.62	1.5E-21
DOID:417	Autoimmune disease	1.1k	75/419	3.36	1.2E-20
DOID:0050686	Organ system cancer	3.9k	161/419	1.99	2.8E-20
DOID:162	Cancer	4.2k	168/419	1.93	8.0E-20
DOID ID	**Selected annotations**	**Size**	**Overlap**	**Enrichment**	**p-value**
DOID:0097237	Cellular response to a toxic substance	77	23/419	14.26	1.2E-20
DOID:0042221	Response to chemical	2.4k	111/419	2.20	2.5E-16
DOID:0040085	Bacterial sepsis	13	9/419	33.05	4.7E-13
DOID:934	Viral infectious disease	664	43/419	3.23	4.3E-12
DOID:104	Bacterial infectious disease	268	27/419	4.81	1.7E-11
DOID:0042742	Response to bacterium	251	23/419	4.37	3.6E-9

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DOID:0071219	Cellular response to a molecule of bacterial origin	80	13/419	7.76	1.1E-8
DOID:0050338	Primary bacterial infectious disease	219	20/419	4.36	4.08E-8
GO:0002237	Response to a molecule of bacterial origin	107	13/419	5.80	3.8E-7
DOID:0050339	Commensal bacterial infectious disease	46	9/419	9.34	4.0E-7

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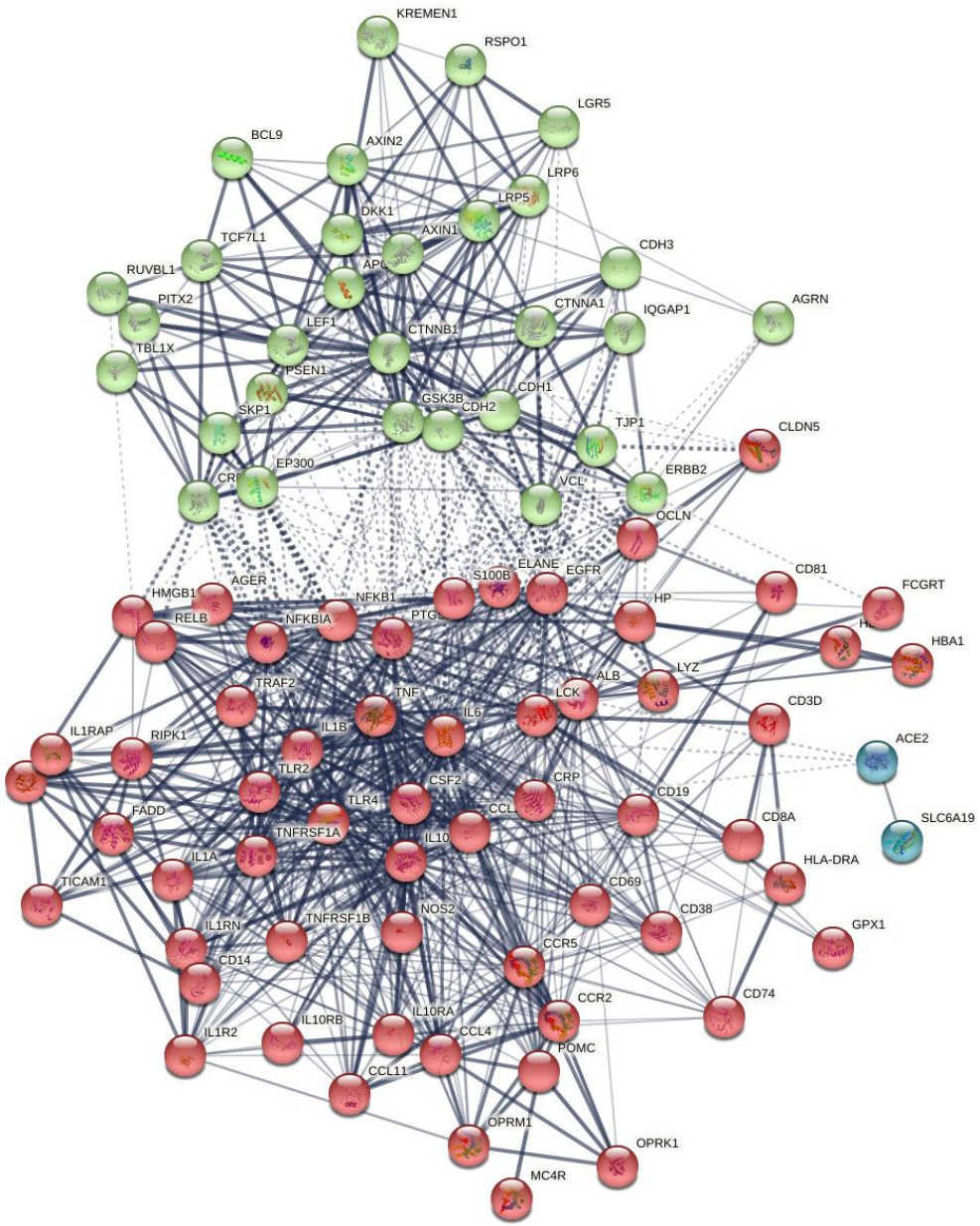

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Figure 1. First order protein-protein interaction network of chronic fatigue spectrum disorders. MCL cluster analysis found two subnetworks: 1) a first immune subnetwork (red color) was centered around NFKB1, TNF, IL6, IL10, IL1, TLR4, etc., and 2) a second Wnt/catenin subnetwork (green nodes) centered around CTNNB1

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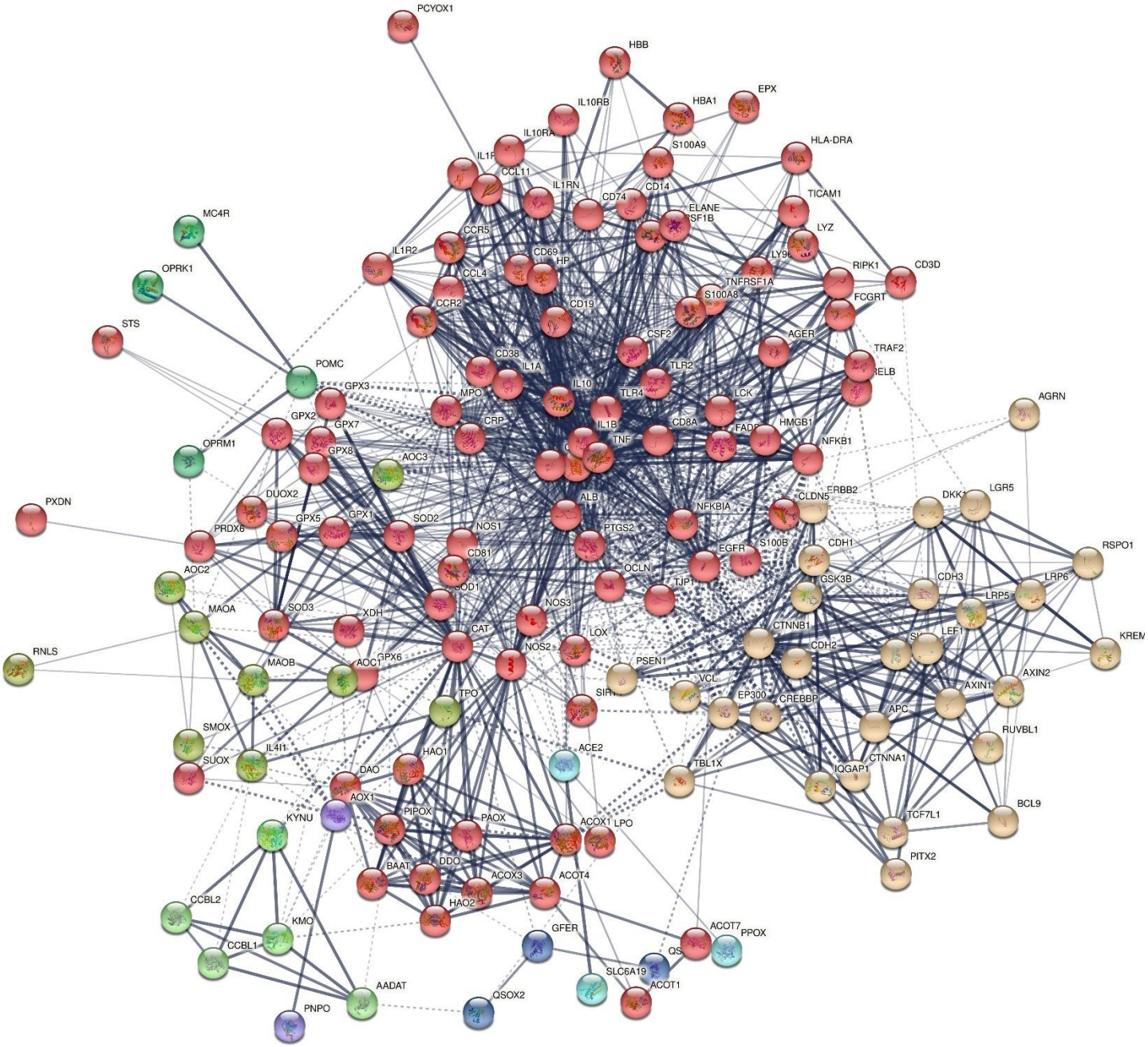

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Figure 2. First order protein network of chronic fatigue spectrum disorders. MCL cluster analysis found two major subnetworks: 1) a first immune, oxidative and nitrosative subnetwork (IO&NS; red color) was centered around NOS2, NFKB1, IL10, etc, and 2) a second Wnt/catenin subnetwork (yellow nodes) was centered around CTNNB1. The tryptophan catabolite pathway (KYNU and KMO) and opioid (OPRK1 and OPRM1) genes appear to be spin-offs of the IO&NS subnetworks

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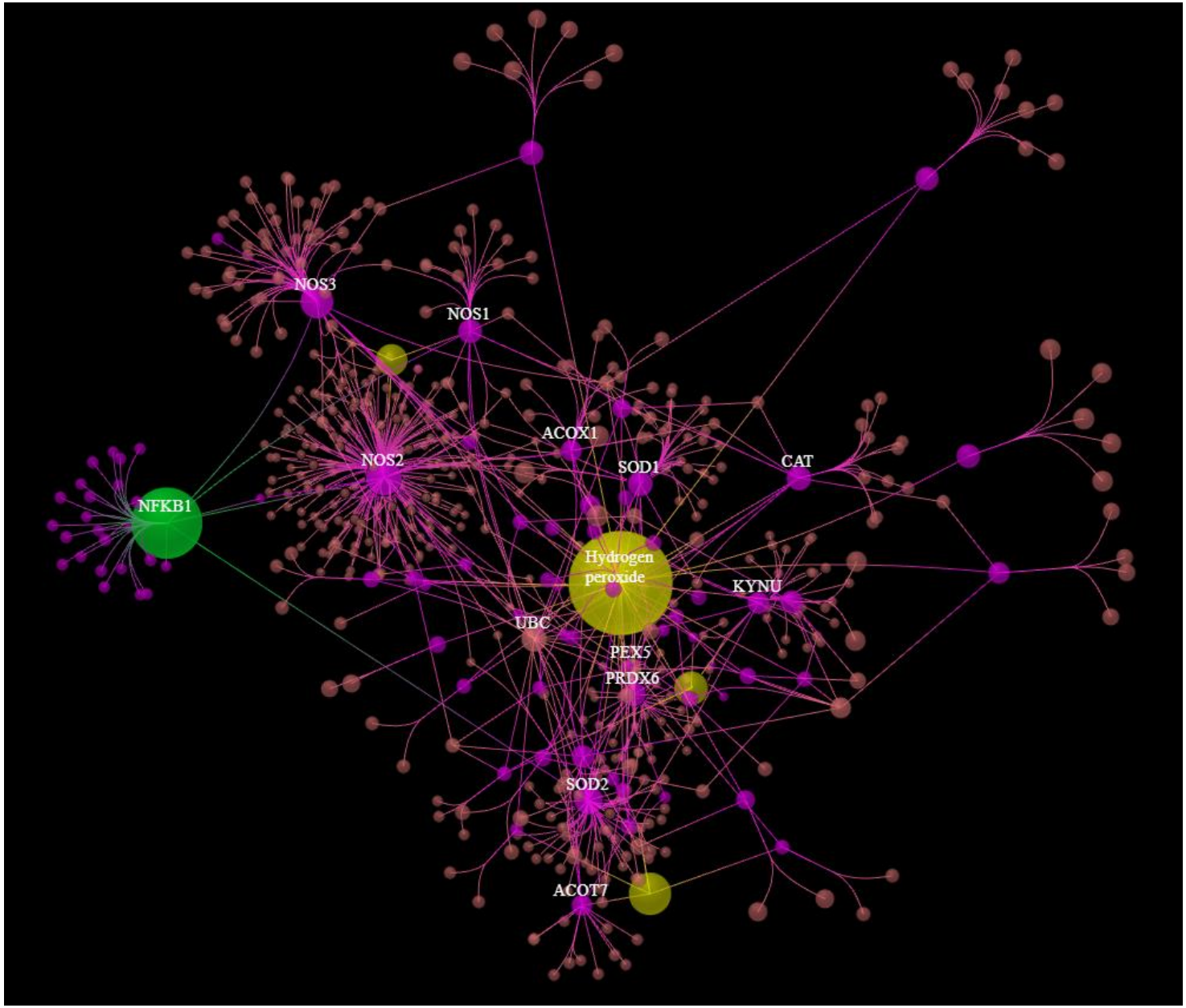

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Figure 3. Results of OmicsNet analysis which included all genes of the network with integration of the metabolics and NFKB1. Metabolics are shown in yellow color, and NFKB1 (as transcriptional factor) in green. 3HK: 3-hydroxy-kynurenine; NO: nitric oxide; EPA: eicosapentaenoic acid

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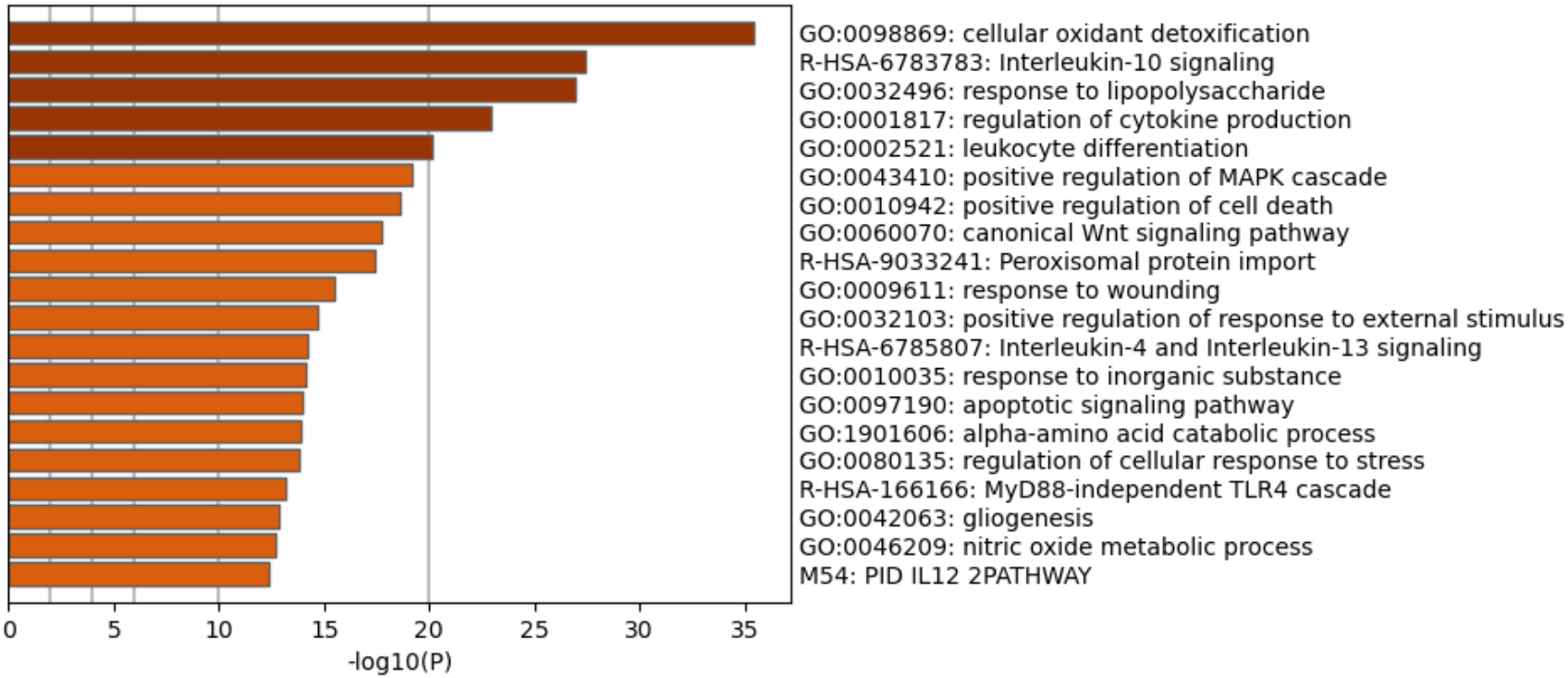

Figure 4. Heatmap of enriched ontology clusters showing the top-20 functions that were overexpressed in the network of patients with chronic fatigue spectrum disorders (accumulative hypergeometric p-values)

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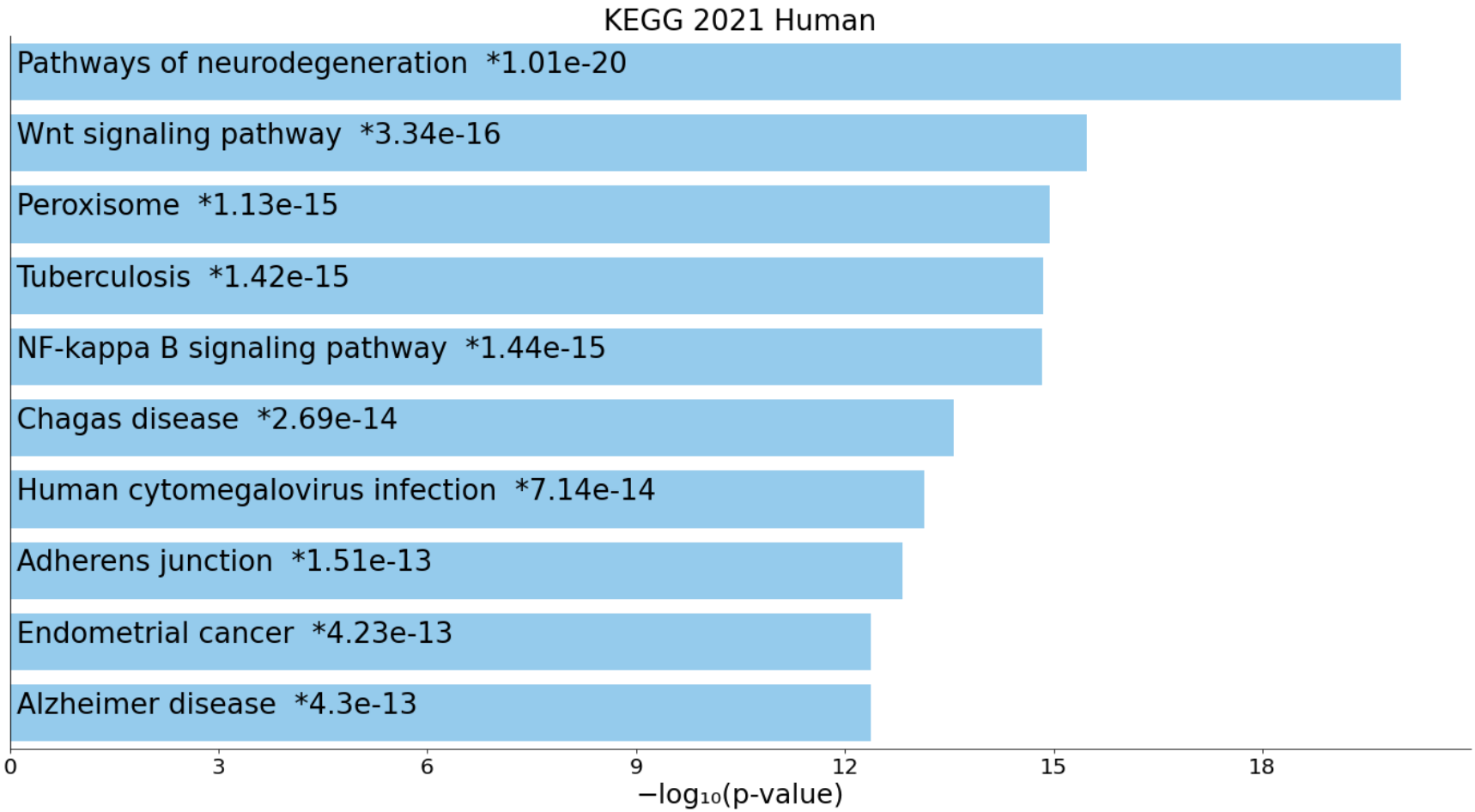

Figure 5. Heatmap (top-10) of enriched KEGG terms accumulated in the differently expressed proteins of the enlarged MultiOmics network of chronic fatigue spectrum disorders

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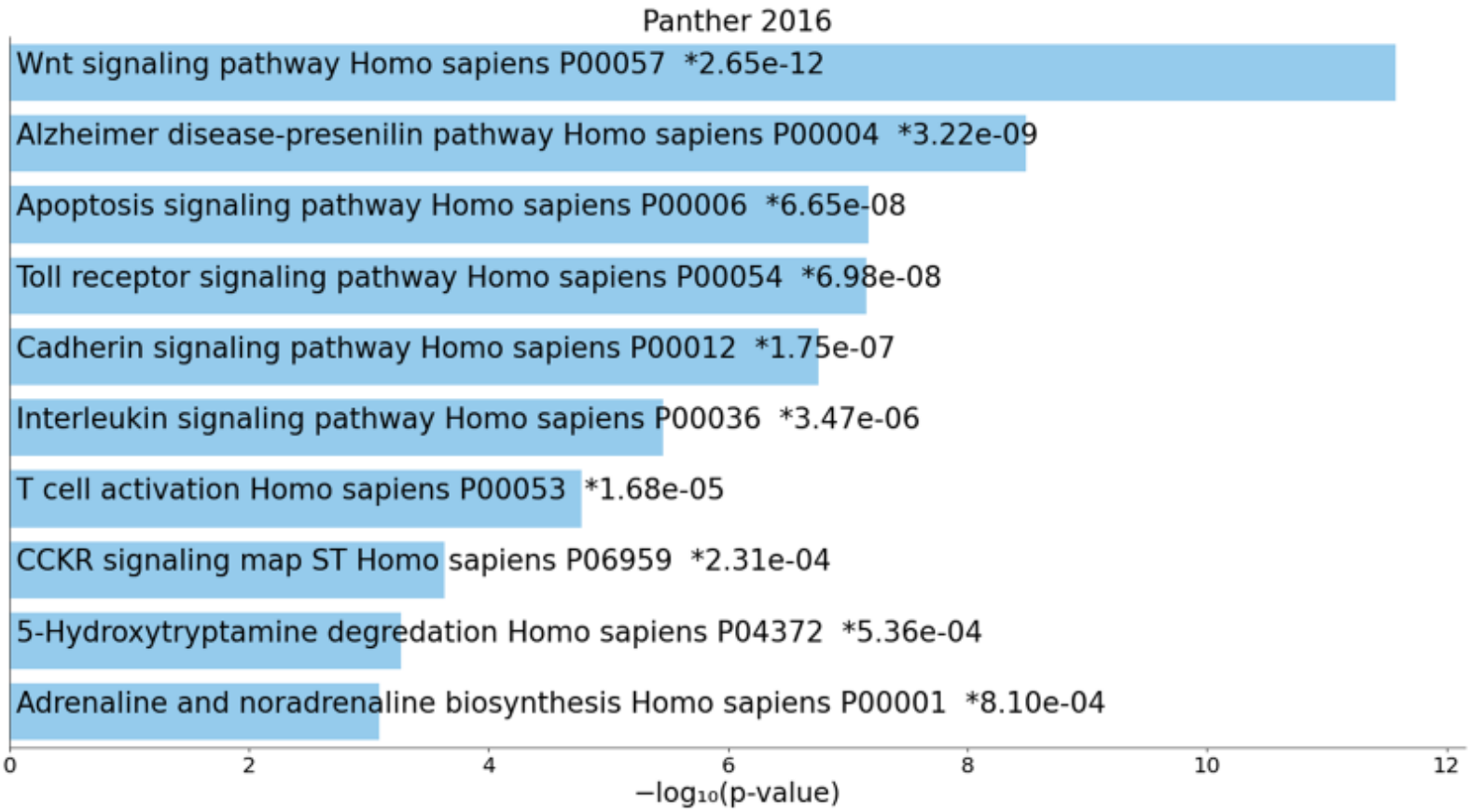

Figure 6. Heatmap (top-10) of enriched PANTHER terms accumulated in the differently expressed proteins of the enlarged MultiOmics network of chronic fatigue spectrum disorders

ELECTRONIC SUPPLEMENTARY FILE (ESF) 1

Aberrations in the cross-talks among redox, nuclear factor-κB and Wnt/catenin pathway signaling underpin Myalgic Encephalomyelitis and chronic fatigue syndrome: a review and new hypothesis based on results of network, enrichment and annotation analyses.

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Enrichment analysis in all DEPs of CFAS-D.

ESF 1, Table 1 displays the results of MCODE analysis employing KEGG, WikiPaths, GO biological and molecular, REACTOME and PANTHER performed on the first-order network built using all seed genes. We observed three significant molecular complexes: the first represents IL10 signaling and signaling by interleukins; the second reflects canonical Wnt signaling and cell-cell signaling by Wnt; and the third represents TCF-dependent signaling in response to Wnt and Wnt signaling.

ESF 2, Figure 1 shows the enriched ontology term clusters in the first order network build using all genes (the network is constructed and visualized using MetaScape and Cytoscape, v3.1.2). This figure shows that the immune and Wnt subnetworks are strongly intertwined as well as the MAPK cascade and that these pathways are interconnected with multicellular organismal homeostasis.

ESF 2, Figure 2 shows the top-20 terms which were over-represented in the first order network built using all genes. This bar graph shows that besides cytokine (especially IL10) and Wnt signaling also positive regulation of the MAPK cascade is a significant path.

ESF 1, Table 2 shows the GO biological process and WikiPathways associated with CFAS-D. The top GO annotation terms were: the cell surface receptor signaling pathway, positive regulation of response to stimulus, and positive regulation of cell communication. The top WikiPathway names associated with CFAS-D were Alzheimer's disease, TNF-related weak inducer of apoptosis (TWEAK) signaling pathway, LTF danger signal response pathway, TLR signaling pathway, and miRNAs involvement in the immune response in sepsis

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Enrichment analysis on the immune subnetwork genes of CFASD.

ESF 2, Figure 3 shows the enriched ontology term clusters in the immune subnetwork of CFAS-D and that cytokine (in particular IL10, but also IL4 and IL13) signaling, a response to LPS or an external stimulus were strongly interacting pathways.

ESF 2, Figure 4 shows the top WikiPaths which were over-represented in the immune subnetwork, namely the LTF danger signal response pathway and the TLR pathway. **ESF 2, Figure 5** shows a bar graph with the top-10 BioCarta terms which are over-represented in the immune subnetwork, namely NFKB pathway, anti-inflammatory IL10, IL1R, TNFR1 and ceramide signaling. **ESF 2, Figure 6** shows the top-10 KEGG annotations including Chagas disease, tuberculosis, CMV infection, toxoplasmosis, and viral protein interactions with cytokine and cytokine receptor.

Enrichment analysis on the Wnt subnetwork genes of CFAS-D.

ESF 2, Figure 7 and 8 shows the enriched ontology term clusters in the Wnt subnetwork of CFAS-D. Besides Wnt/catenin-associated paths, these also include diseases of signal transduction, signaling by Wnt in cancer, PID PS1 pathway and cell-junction organization. **ESF 2, Figure 9** shows the WikiPathways which were statistically over-represented in the Wnt/catenin subnetwork, including involvement of this pathway in colorectal cancer, leukemia, endometrial and breast cancer, ect. The bar graph shown in **ESF 2, Figure 10** shows the top-10 InterPro domains which were enriched in the Wnt subnetwork, including the cadherin prodomain and cytoplasmic domain, and the DIX domain. **ESF 2, Figure 11** shows a bar graph with top-10 enriched GO cellular components, including

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the catenin complex and adherens and cell-cell junctions. TTRUST enrichment analysis showed that NFKB1 (pFDR=2.911E-25) and RELA (pFDR=1.014E-22) were the two most important transcriptional factors in this network.

Annotation analyses and functional categorization of the PPI network and selected genes

ESF 2, Figures 12 and 13 depict the hierarchical structure of GO terms and the hubs and master regulatory transcription factor (ESF 2, Figure 12), downregulated seed genes (**ESF 2, Figure 13A**), seed genes of the Wnt/catenin pathway subnetwork (**ESF 2, Figure 13B**), and the major hotspots in the STRING enlarged networks (**ESF 2, Figure 13C**). Thus, ESF 2, Figure 12 shows that the GO functions which are regulated by NFKB1, CTNNB1, TNF and IL6. ESF 2, Figure 13A shows that the downregulated genes are associated with cellular oxidant detoxification and detoxification in general. ESF 2, Figure 13B shows that the seed genes of the Wnt subnetwork are associated with synapse organization and cell-cell signaling, whereas the major hotspots of the enlarged network (ESF 2, Figure 13C) are associated with a variety of processes including smooth muscle cell proliferation, regulation of DNA metabolic and apoptotic processes, and response to lipids, steroid hormones.

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ESF 1, Table 1. Results of Molecular Complex Detection (MCODE) analysis performed on the differently expressed proteins (DEPs) of chronic fatigue spectrum disorders.

MCODE Components	GO ID	Biological term	Log10 (p)
All DEPs, MCODE1	R-HSA-6783783	Interleukin-10 signaling	-32.5
	R-HSA-449147	Signaling by Interleukins	-31.9
	R-HSA-1280215	Cytokine signaling in immune system	-30.2
All DEPs, MCODE2	GO:0060070	Canonical Wnt signaling pathway	-12.4
	GO:0016055	Wnt signaling pathway	-10.9
	GO:0198738	Cell-cell signaling by Wnt	-10.9
All DEPs, MCODE3	R-HSA-201681	TCF dependent signaling in response to Wnt	-5.3
	GO:0060070	Canonical Wnt signaling pathway	-4.9
	R-HSA-195721	Signaling by WNT	-4.8

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ESF 1, Table 2. Go Biological Process and WikiPathway terms associated with the network of chronic fatigue spectrum disorders (CFAS-D).

Path ID	GO biological process names associated with CFAS-D	Observed	background	Strength	pFDR
GO:0007166	Cell surface receptor signaling pathway	63	2325	0.77	1.23E-32
GO:0048584	Positive regulation of response to stimulus	60	2257	0.76	4.05E-30
GO:0048583	Regulation of response to stimulus	72	4114	0.58	3.43E-28
GO:0010647	Positive regulation of cell communication	52	1823	0.79	3.87E-26
GO:0023056	Positive regulation of signaling	52	1831	0.79	3.87E-26
GO:0009967	Positive regulation of signal transduction	50	1654	0.81	4.58E-26
GO:0042221	Response to chemical	71	4333	0.55	5.73E-26
GO:0009893	Positive regulation of metabolic process	68	3893	0.57	9.50E-26
GO:0070887	Cellular response to chemical stimulus	61	2919	0.65	1.05E-25
GO:0048522	positive regulation of cellular process	77	5579	0.47	2.79E-25
Path ID	**WikiPathway names associated with CFAS-D**	**Observed**	**background**	**Strength**	**pFDR**
WP5124	Alzheimers disease	20	255	1.23	1.94E-15
WP2036	TNF-related weak inducer of apoptosis (TWEAK) signaling pathway	11	42	1.75	4.25E-13
WP5039	SARS-CoV-2 innate immunity evasion and cell-specific immune response	12	66	1.59	6.76E-13
WP4478	LTF danger signal response pathway	9	19	2.01	1.46E-12
WP75	Toll-like receptor signaling pathway	13	103	1.43	2.01E-12
WP4329	miRNAs involvement in the immune response in sepsis	10	37	1.76	3.05E-12
WP4155	Endometrial cancer	11	63	1.57	8.07E-12
WP3658	Wnt/beta-catenin signaling pathway in leukemia	9	26	1.87	8.09E-12
WP231	TNF-alpha signaling pathway	12	92	1.45	9.77E-12
WP4258	lncRNA in canonical Wnt signaling and colorectal cancer	12	93	1.44	9.99E-12

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ELECTRONIC SUPPLEMENTARY FILE (ESF) 2

Aberrations in the cross-talks among redox, nuclear factor-κB and Wnt/catenin pathway signaling underpin Myalgic Encephalomyelitis and chronic fatigue syndrome: a review and new hypothesis based on results of network, enrichment and annotation analyses.

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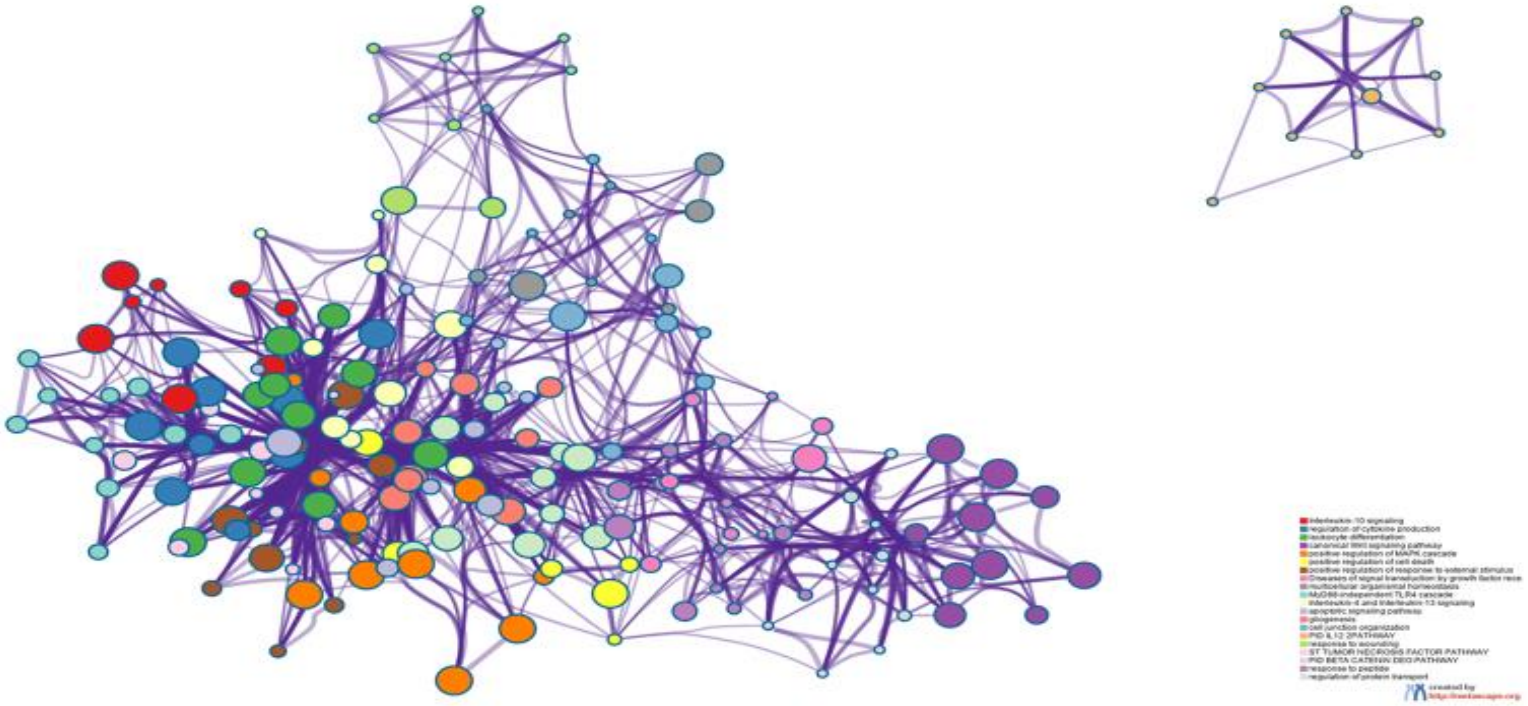

ESF 2, Figure 1. Enriched ontology term clusters in the chronic fatigue spectrum disorders network. Terms are represented by a circle node, with the colors representing cluster identity and their size reflecting the number of input genes. One term is used to describe the clusters (colored labels). The thickness of the (bundled) edges linking the terms reflects the similarity score (threshold is > 0,3). The network is constructed and visualized using MetaScape and Cytoscape (v3.1.2)

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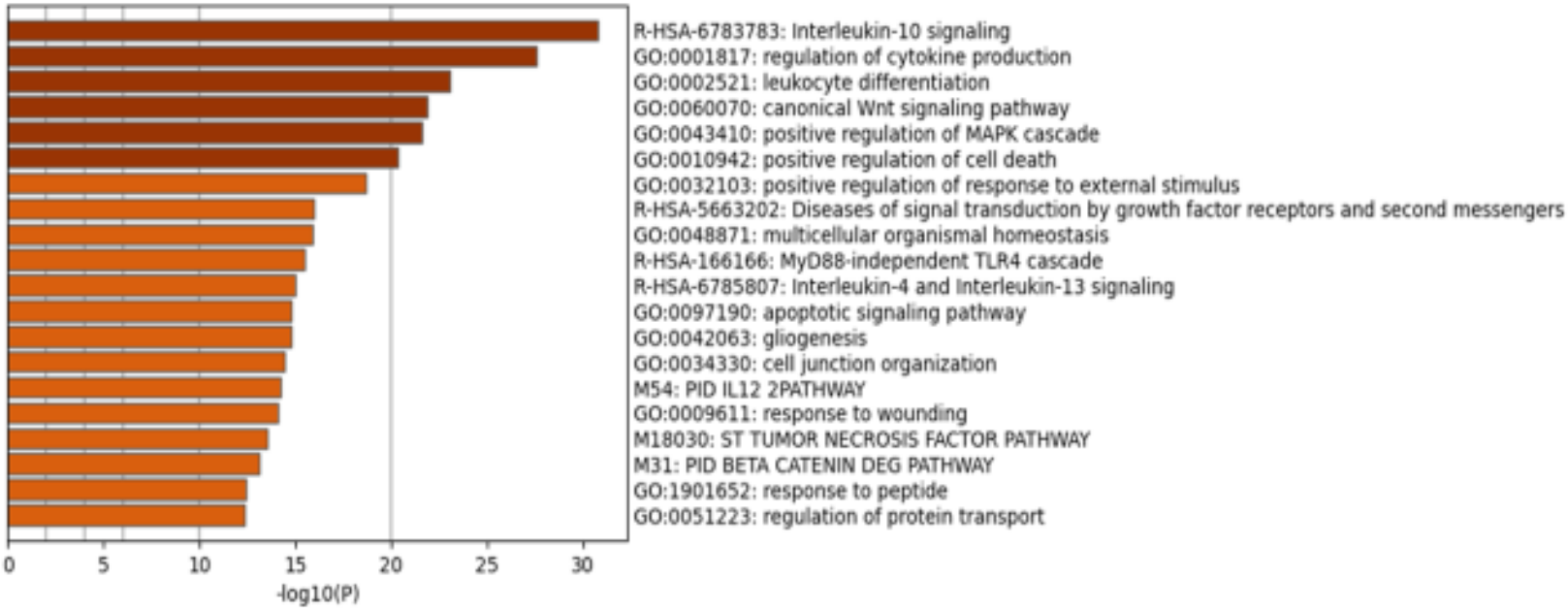

ESF 2, Figure 2. Heatmap of enriched ontology clusters showing the top-20 functions that were overexpressed in the expanded network of patients with chronic fatigue spectrum disorders (accumulative hypergeometric p-values)

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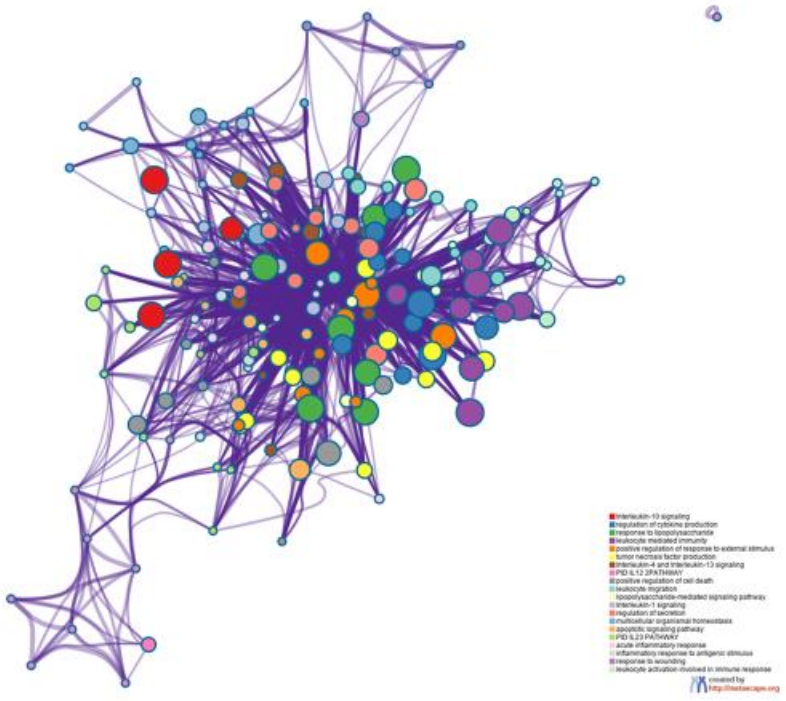

ESF 2, Figure 3. Enriched ontology term clusters in the immune subnetwork of chronic fatigue spectrum disorders.
Terms are represented by a circle node, with the colors representing cluster identity and their size reflecting the number of input genes. One term is used to describe the clusters (colored labels). The thickness of the (bundled) edges linking the terms reflects the similarity score (threshold is > 0.3). The network is constructed and visualized using MetaScape and Cytoscape (v3.1.2)

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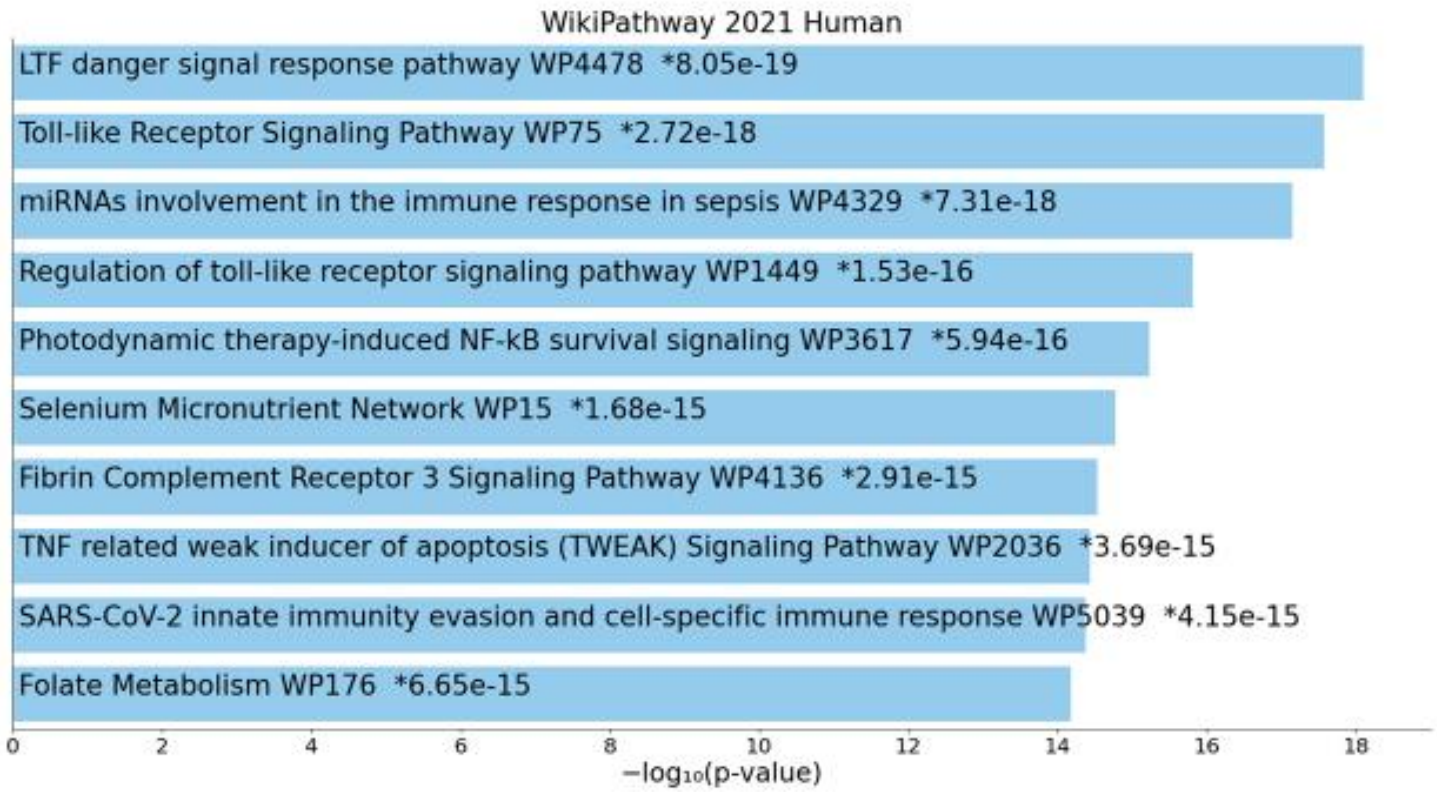

ESF 2, Figure 4. Heatmap (top-10) of the enriched WikiPathway terms accumulated in the differently expressed proteins of the immune subnetwork of chronic fatigue spectrum disorders

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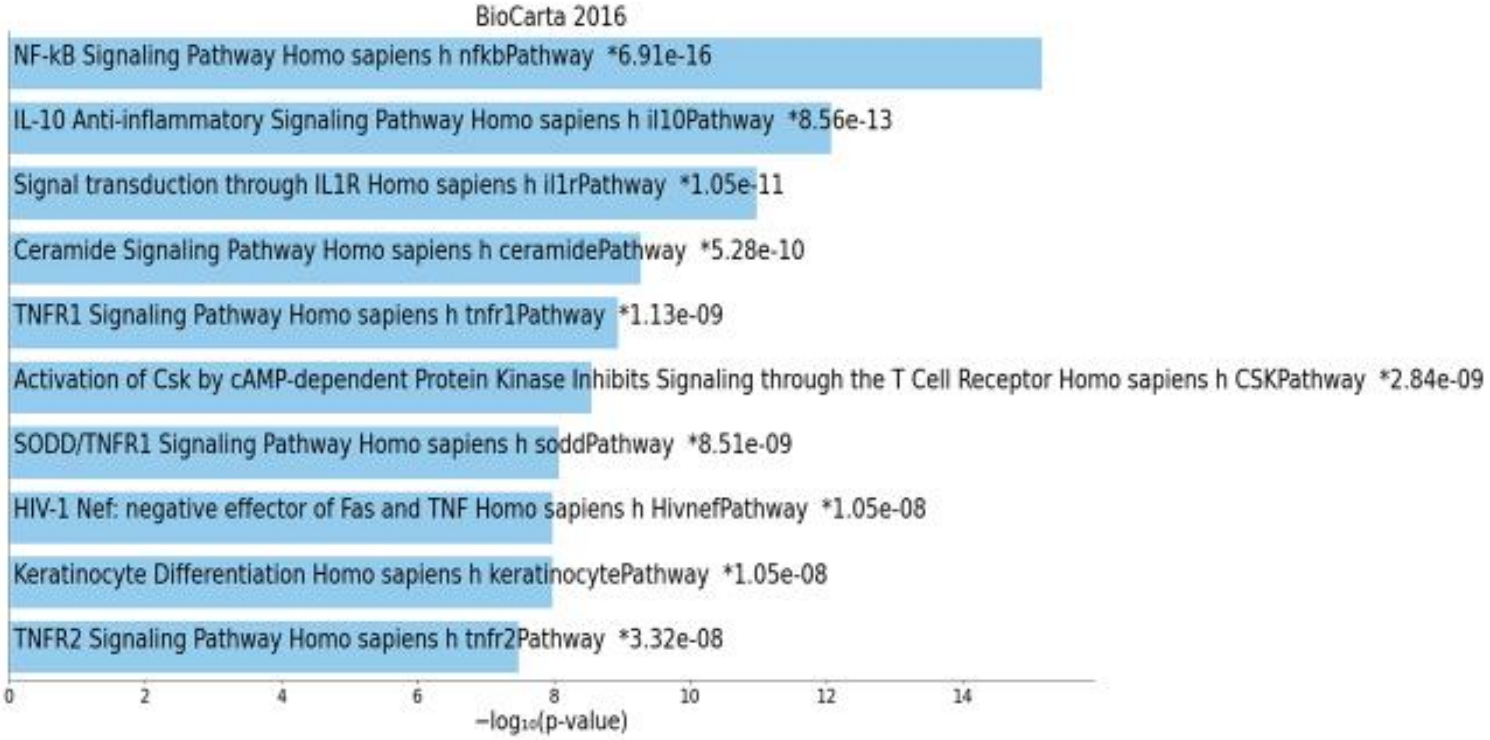

ESF 2, Figure 5. Heatmap (top-10) of the BioCarta terms accumulated in the differently expressed proteins of the expanded immune subnetwork of chronic fatigue spectrum disorders

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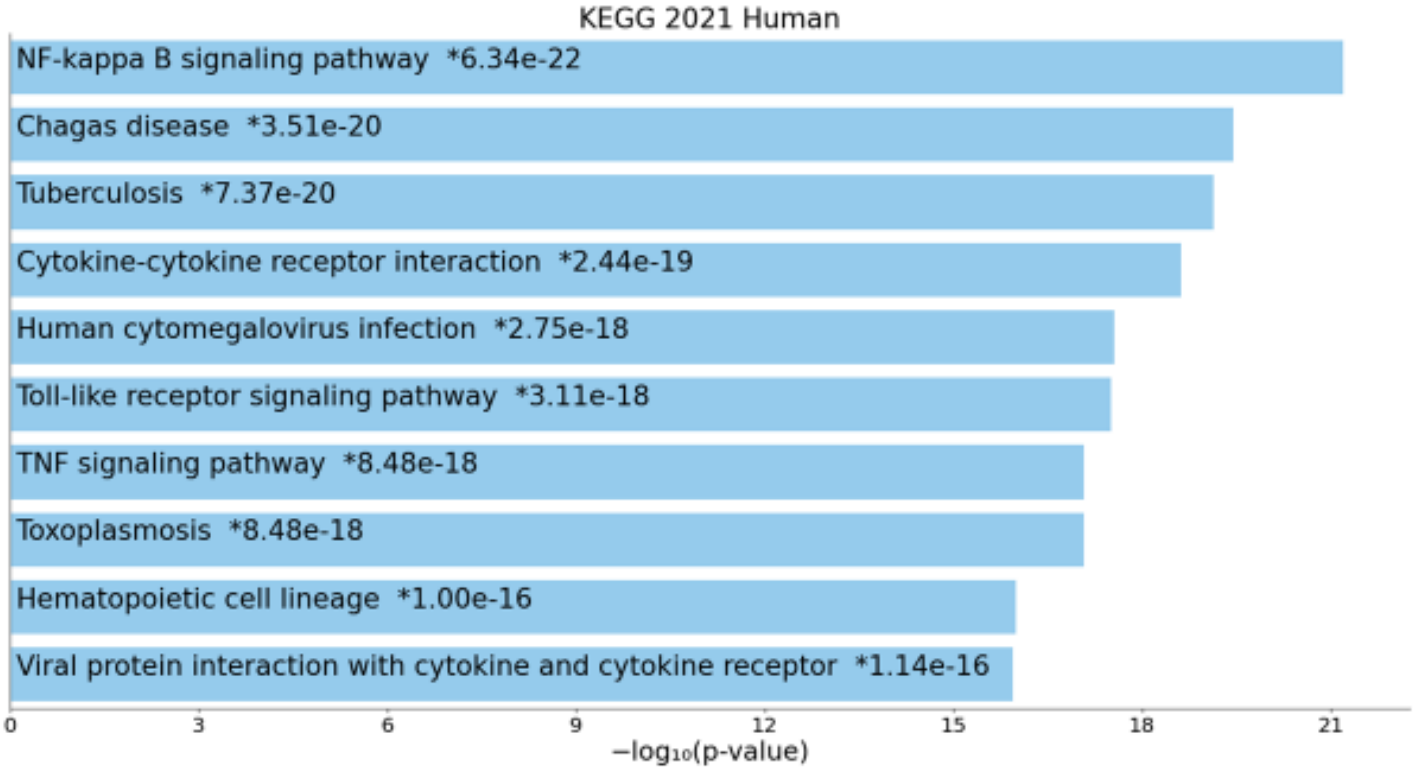

Figure 6. Heatmap (top-10) of the KEGG pathways accumulated in the differently expressed proteins of the expanded immune subnetwork of chronic fatigue spectrum disorders

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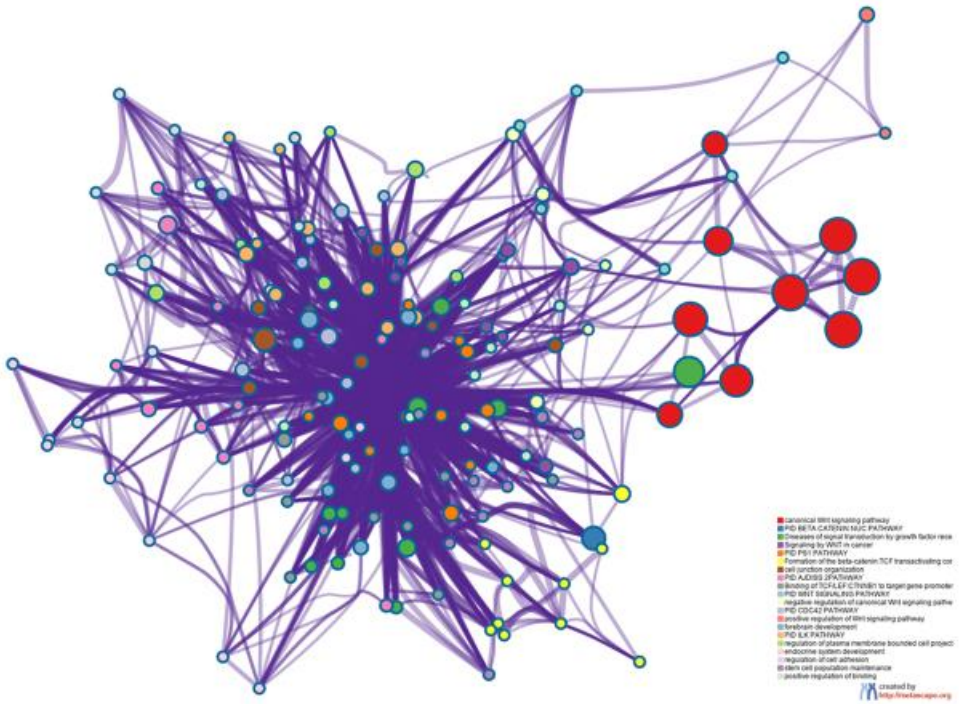

ESF 2, Figure 7. Enriched ontology term clusters in the Wnt/catenin subnetwork of chronic fatigue spectrum disorders. Terms are represented by a circle node, with the colors representing cluster identity and their size reflecting the number of input genes. One term is used to describe the clusters (colored labels). The thickness of the (bundled) edges linking the terms reflects the similarity score (threshold is > 0.3). The network is constructed and visualized using MetaScape and Cytoscape (v3.1.2).

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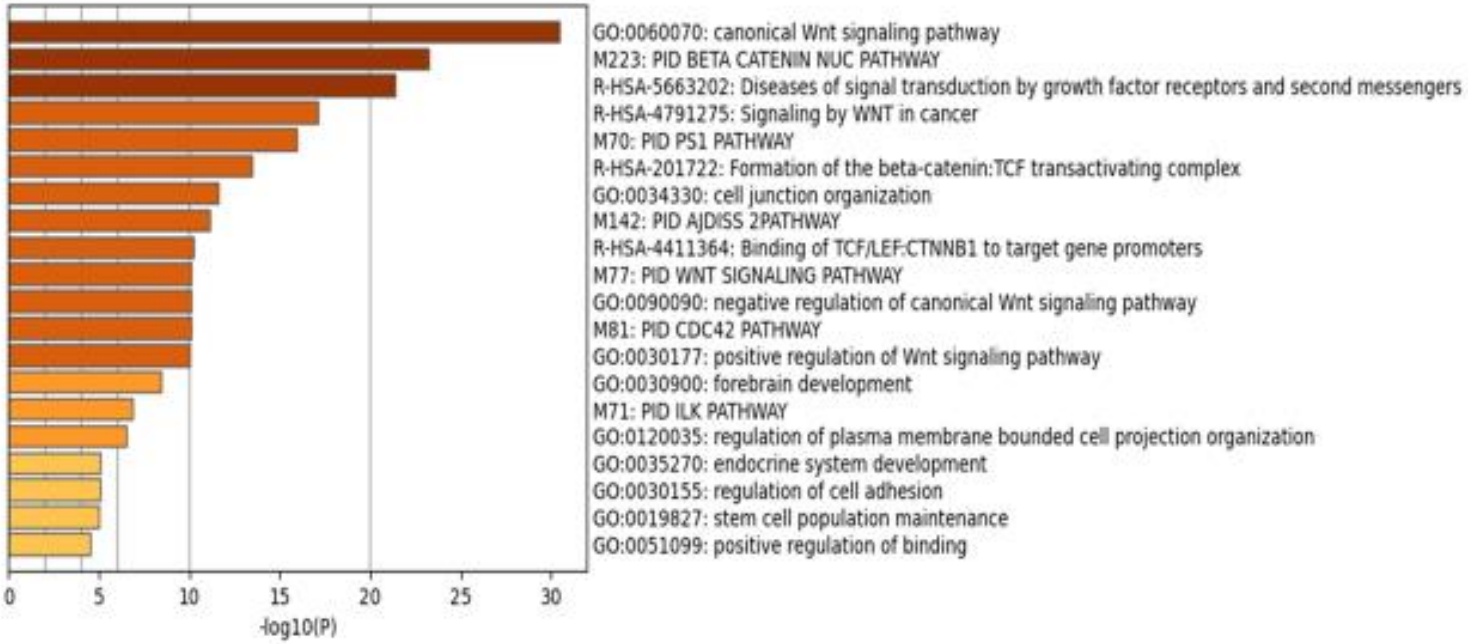

ESF 2, Figure 8. Heatmap of enriched ontology clusters showing the top-20 functions that were overexpressed in the Wnt/catenin subnetwork of patients with chronic fatigue spectrum disorders (accumulative hypergeometric p-values)

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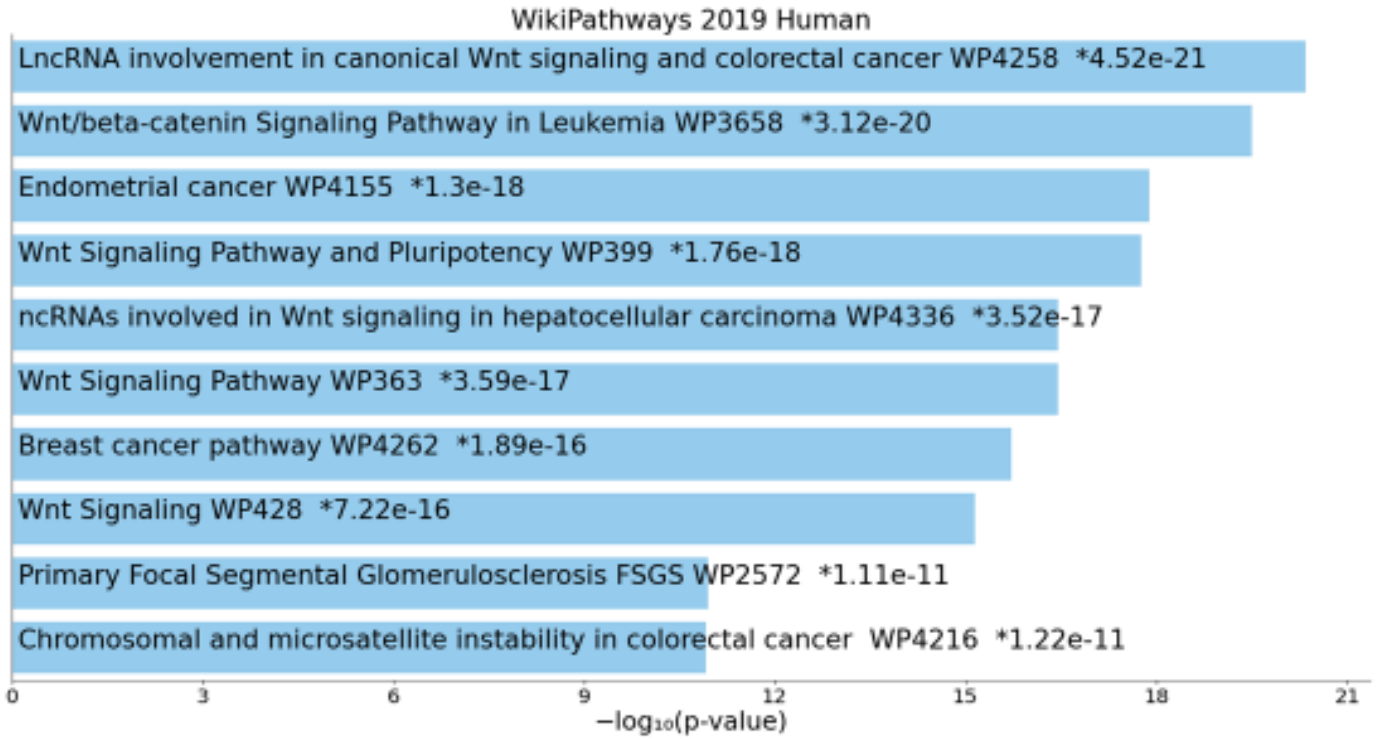

ESF 2, Figure 9. Heatmap (top-10) of the WikiPathways accumulated in the differently expressed proteins of the expanded Wnt subnetwork of chronic fatigue spectrum disorders

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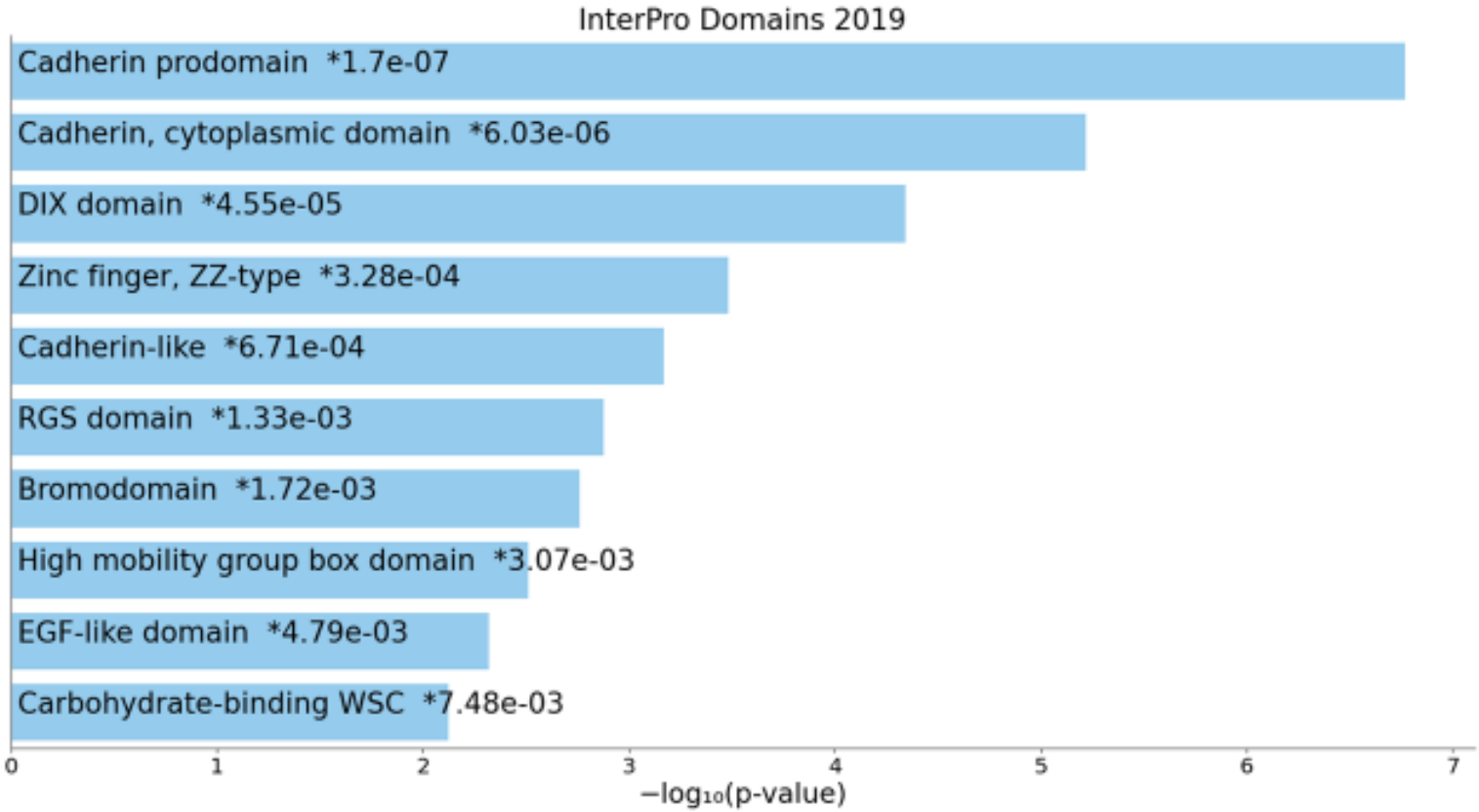

ESF 2, Figure 10. Heatmap (top-10) of enriched InterPro domain terms accumulated in the differently expressed proteins of the Wnt/catenin subnetwork of chronic fatigue spectrum disorders

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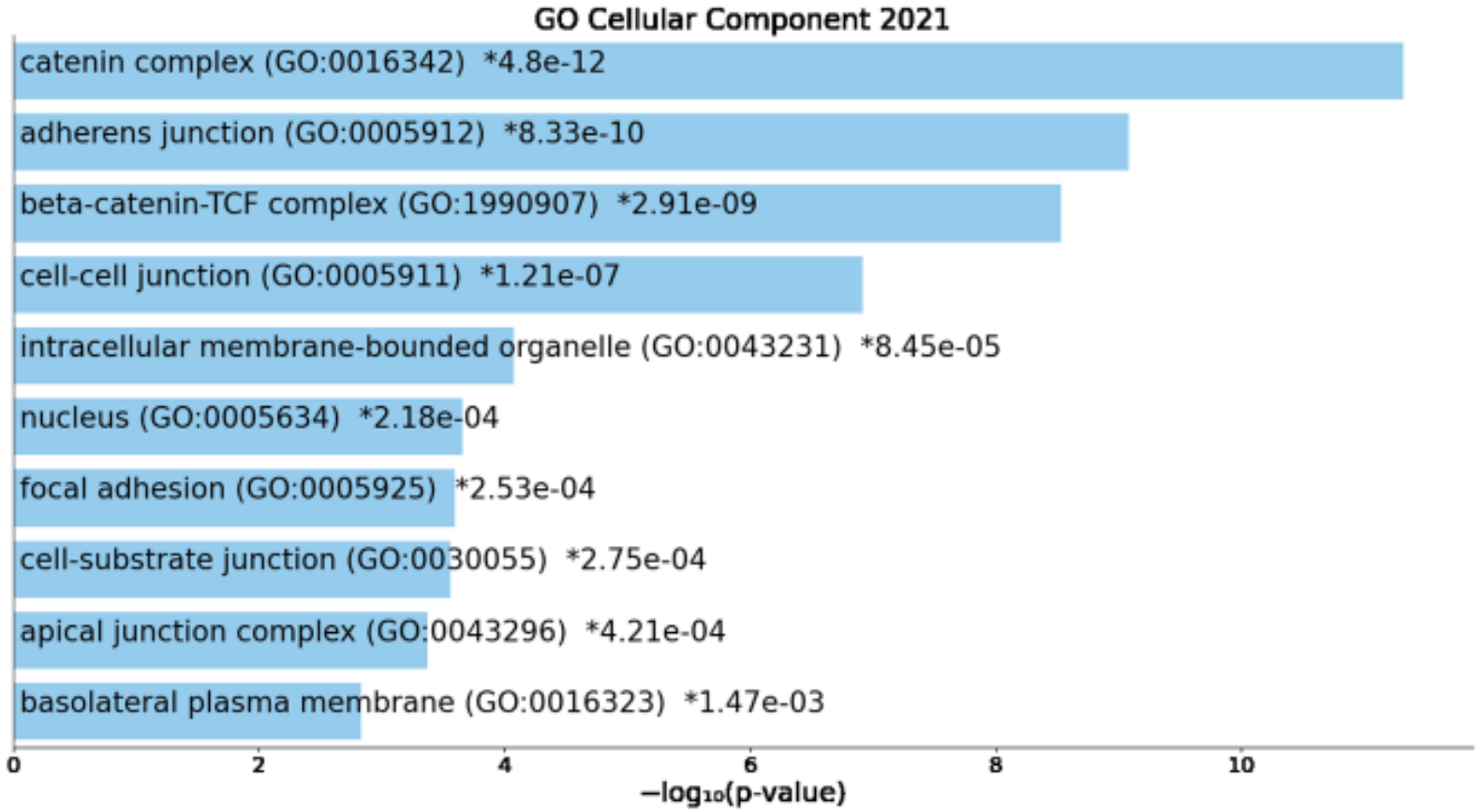

ESF 2, Figure 11. Heatmap (top-10) of enriched cellular component terms accumulated in the differently expressed proteins of the Wnt/catenin subnetwork of chronic fatigue spectrum disorders

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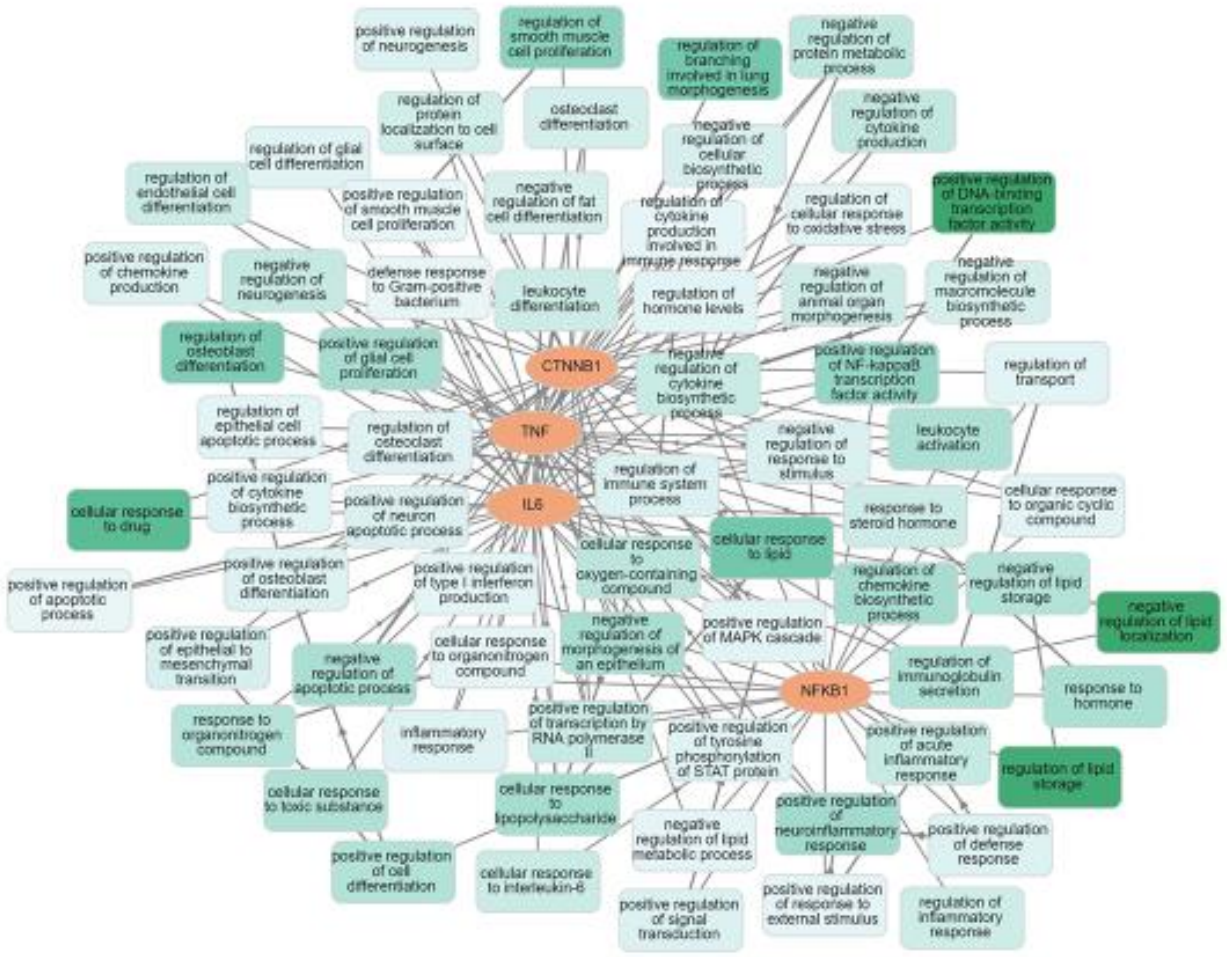

ESF 2, Figure 12. Results of GOnet annotation visualization in chronic fatigue spectrum disorders depicting the hierarchical structure of GO terms and the hubs and master regulatory transcription factor

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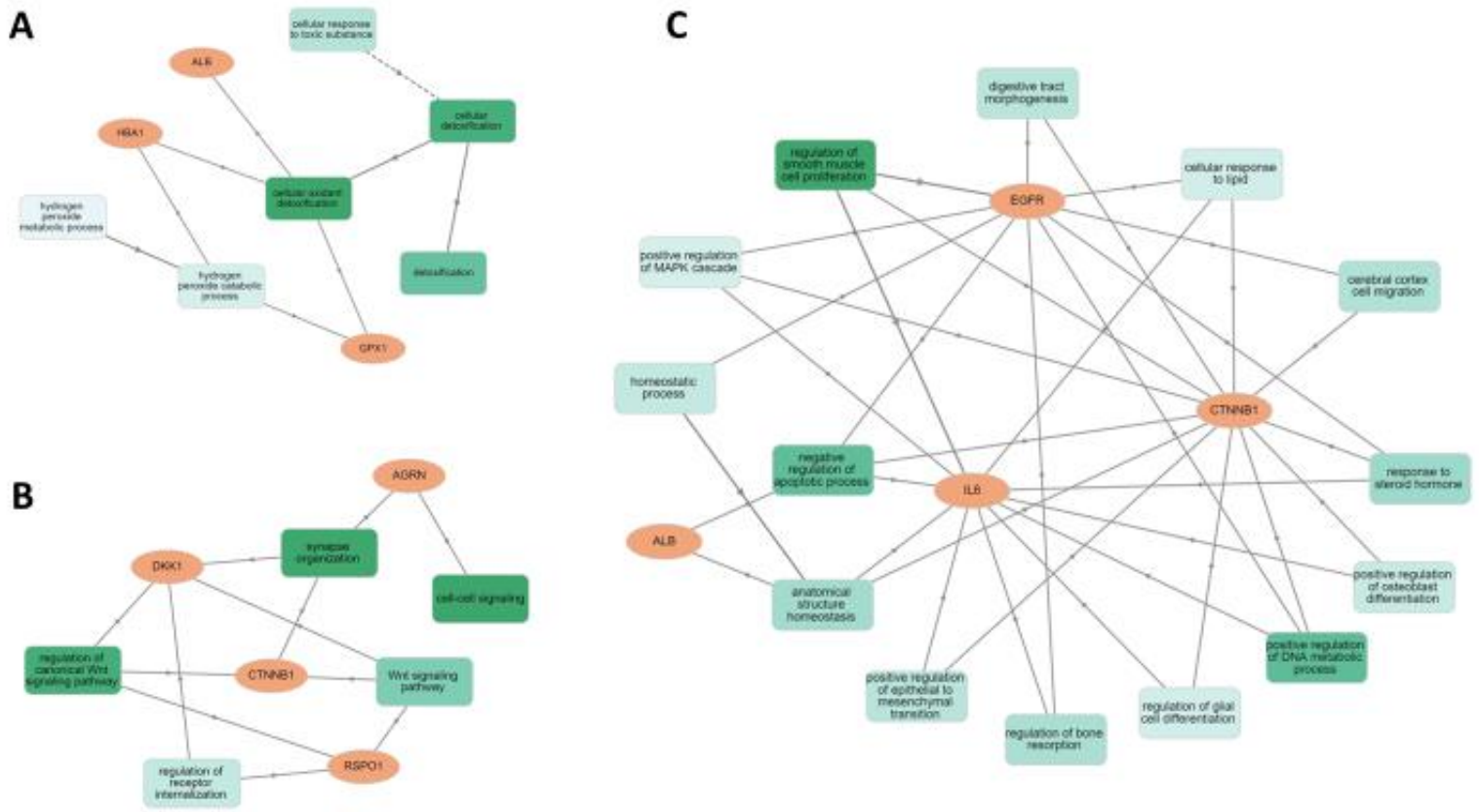

ESF 2, Figure 13. Results of GOnet annotation visualization in chronic fatigue spectrum disorders depicting the hierarchical structure of GO terms and A: downregulated seed genes; B: seed genes of the Wnt/catenin pathways; and C: the hotspots in STRING enlarged networks

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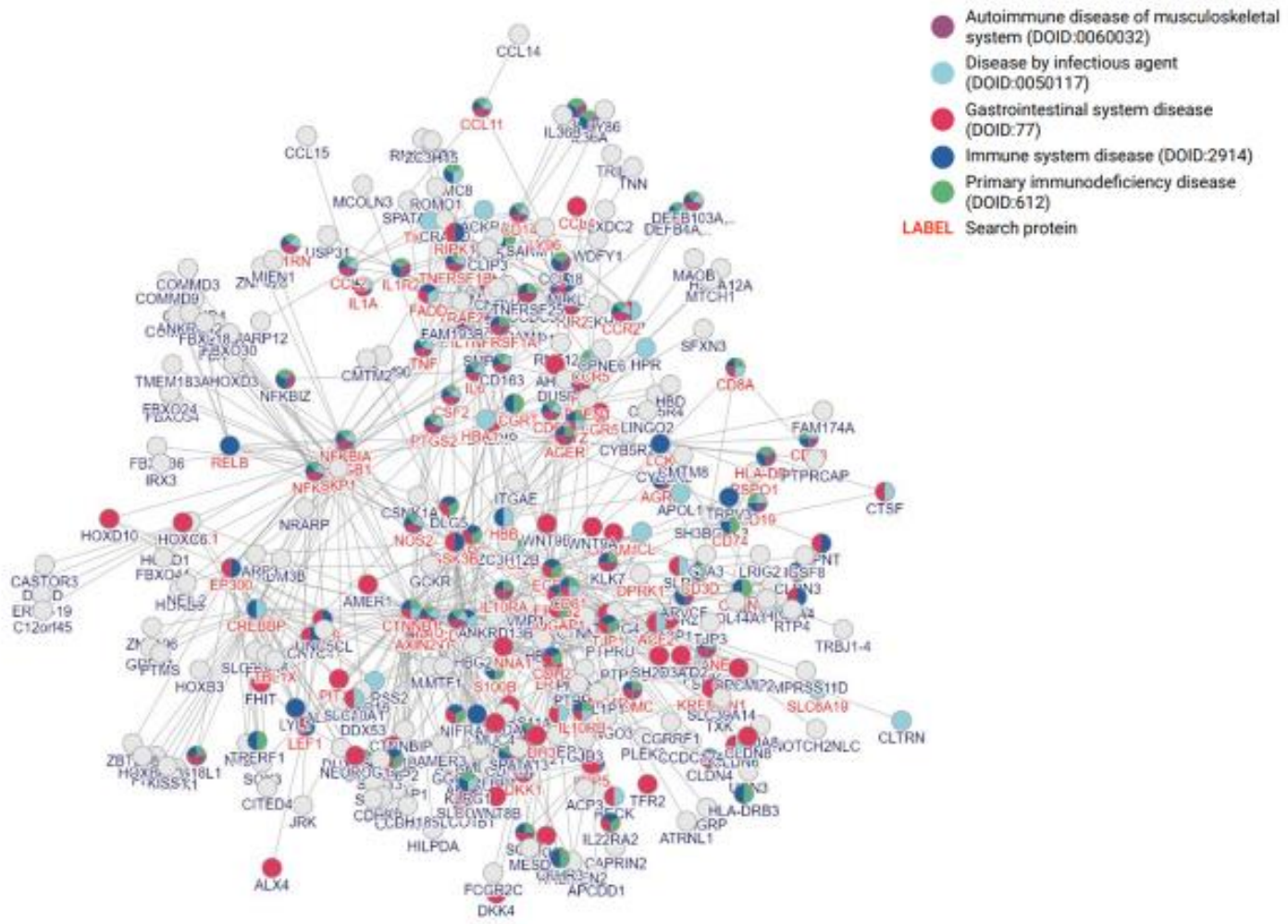

ESF 2, Figure 14. An extended network constructed with inBio Discover showing the top Disease Ontology (DOID) annotations of chronic fatigue spectrum disorders

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