

1 Functional characterization of SARS-CoV-2 infection suggests a complex inflammatory
2 response and metabolic alterations

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25 **Abstract**

26 Covid-19, caused by the SARS-CoV-2 virus, has reached the category of a worldwide pandemic.
 27 Even though intensive efforts, no effective treatments or a vaccine are available. Molecular
 28 characterization of the transcriptional response in Covid-19 patients could be helpful to
 29 identify therapeutic targets. In this study, RNAseq data from peripheral blood mononuclear
 30 cell samples from Covid-19 patients and healthy controls was analyzed from a functional point
 31 of view using probabilistic graphical models. Two networks were built: one based on genes
 32 differentially expressed between healthy and infected individuals and another one based on
 33 the 2,000 most variable genes in terms of expression in order to make a functional
 34 characterization. In the network based on differentially expressed genes, two inflammatory
 35 response nodes with different tendencies were identified, one related to cytokines and
 36 chemokines, and another one related to bacterial infections. In addition, differences in
 37 metabolism, which were studied in depth using Flux Balance Analysis, were identified. SARS-
 38 CoV2- infection caused alterations in glutamate, methionine and cysteine, and
 39 tetrahydrobiopterin metabolism. In the network based on 2,000 most variable genes, also two
 40 inflammatory nodes with different tendencies between healthy individuals and patients were
 41 identified. Similar to the other network, one was related to cytokines and chemokines.
 42 However, the other one, lower in Covid-19 patients, was related to allergic processes and self-
 43 regulation of the immune response. Also, we identified a decrease in T cell node activity and
 44 an increase in cell division node activity. In the current absence of treatments for these
 45 patients, functional characterization of the transcriptional response to SARS-CoV-2 infection
 46 could be helpful to define targetable processes. Therefore, these results may be relevant to
 47 propose new treatments.

48 Introduction

49 The emerging coronavirus SARS-CoV-2 has rapidly expanded from its origin in Wuhan, China,
50 to become a worldwide pandemic only after four months since its first identification. At
51 September 20th of 2020, 30,675,675 cases and 954,417 deaths have been reported worldwide,
52 according to the World Health Organization [1].

53 The most common symptoms are fever, cough, fatigue, shortness of breath, accompanied by
54 elevated inflammatory biomarkers and pulmonary infiltrates. However, during the SARS-CoV-2
55 infection, a fraction of patients will develop severe pneumonia, pulmonary oedema, severe
56 acute respiratory syndrome (SARS) or multiple organ failure, ending in death [2]. These severe
57 symptoms are associated with systemic inflammation related to an overproduction of
58 macrophagic cytokines. Different treatments focused on these inflammatory processes are
59 being investigated [3].

60 Recently, Xiong et al. analyzed the transcriptional response in samples from peripheral blood
61 mononuclear cells (PBMCs) from patients diagnosed with SARS-CoV-2 and compared them
62 with healthy controls. Based on the results, the authors suggested that patient's lymphopenia
63 may be caused by an activation of apoptosis in lymphocytes, and also that SARS-Cov-2 induced
64 excessive cytokine production, which correlates with lung tissue injury [4]. However, these
65 conclusions were based only in the functional enrichment analysis of differentially expressed
66 genes.

67 Probabilistic graphical models (PGMs) have demonstrated their utility in analyzing gene
68 expression data by identifying relevant biological processes [5, 6]. These models allow making
69 associations between genes according to their expression patterns across a series.

70 Interestingly, the PGM networks have functional structure, allowing study expression data
71 from a functional point of view. The main advantage of this type of models is that they offer an
72 integrated view about what biological processes are involved in a disease, instead of the
73 classical gene-based analysis which offers a list of differential genes without a context. Thus,
74 we set out to re-analyze Xiong et al. data using PGMs, aiming for a deeper understanding of
75 biological processes involved in SARS-CoV-2 pathogenesis.

76 **Results**

77 **Processing of RNA sequencing data**

78 After alignment of raw files, 13,398 expressed genes were identified. After applying the quality
79 criteria of a detectable reading in at least 50% of the samples, 13,182 genes were used for the
80 subsequent analyses.

81 **Analysis of differential genes between healthy controls and patients**

82 Using CuffDiff, 1,569 differentially expressed genes were determined between SARS-CoV-2
83 patients and healthy controls. After applying the quality criteria of detectable measurements
84 in at least 50% of the samples, 1,234 genes remained as differential ones. These genes were
85 mostly related to inflammatory response, innate immune response, T cells, lysosomes,
86 apoptotic processes and angiogenesis, among others.

87 A PGM was used to organize these genes according to their biological functions. The resulting
88 network was composed by eight functional nodes: metabolism, lysosomes, T cells, two nodes
89 related to inflammatory response, two nodes related to response to virus, and one node with
90 no overrepresented function (Fig 1, S1 File).

91 Inflammatory response A, lysosome ad metabolism functional nodes activities were
92 significantly differential between healthy controls and patients. Strikingly, one of the nodes of
93 inflammatory response had a higher functional node activity in healthy controls than in
94 patients, and the other node of inflammatory response had a functional node activity higher in
95 patients than healthy controls. The same tendencies were shown in response to virus nodes.
96 Lysosome and metabolism had a higher functional node activity in patients than in controls.
97 Finally, T cell functional node activity was higher in healthy individuals than in patients (Fig 2).

98 *Metabolism functional node*

99 Metabolism node was composed of 102 genes, 19 of them related with metabolism pathways.
100 This node contained the genes PKM (pyruvate kinase) and PDHB (pyruvate dehydrogenase),
101 both implicated on glycolysis, and several ATPases from the mitochondrial complex,
102 responsible for H⁺ transporting. There were also genes related to drug metabolism such as
103 PAPSS1 or CES1.

104 *Inflammatory response A functional node*

105 This node included 236 genes, of which 18 were implicated in an inflammatory response. This
106 node mostly comprised chemokines such as CXCR2, CCR3, CXCR1, CXCL1 or CXCL8, widely
107 associated with SARS-CoV-2 infection.

108 *Inflammatory response B functional node*

109 This node included 129 genes, of which 17 were involved in inflammatory response. This
110 functional node included three toll-like receptors, TLR2, TLR5 and TLR4. Among other
111 functions, TLR2 promotes apoptosis in response to bacterial lipoproteins. TLR5 protein
112 recognizes bacterial flagellin, the principal component of bacterial flagella. Additionally, TLR4
113 has been implicated in signal transduction events induced by lipopolysaccharide found in most
114 gram negative bacteria. This node also contained AOA1 and CD14, both genes implicated in
115 response to bacterial lipopolysaccharides as well. Finally, this functional node included VNN1
116 which plays a suppressive role in influenza virus replication in human alveolar epithelial cells
117 [7]. Therefore, this node is mostly related to the response to bacterial infections.

118 *Lysosome functional node*

119 Lysosome node included 112 genes, of which 12 were related to lysosomal processes. Most of
120 these genes are lysosomal enzymes such as CTSH, NAGA or PLA2G15, but this node also
121 included HPSE, which is the gene that encodes an enzyme that cleaves heparan sulfate
122 proteoglycans to allow cell movement through remodeling of the extracellular matrix, or
123 DRAM1, which encodes a lysosomal membrane protein that is required for the induction of
124 autophagy.

125 **Metabolic modeling**

126 FBA was performed to characterize in depth metabolic alterations caused by SARS-CoV-2
127 infection (S2 File). Glutamate metabolism, methionine and cysteine metabolism, and
128 tetrahydrobiopterin metabolism flux activities were differential between healthy controls and
129 Covid-19 patients (Fig 3). In addition, several tendencies in other metabolic pathways such as
130 TCA cycle and steroid metabolism that need to be confirmed were shown (S1 Figure).

131 **Functional characterization based on the 2,000 most variable genes**

132 We obtained an alternative PGM network, now based on the 2,000 most variable genes
133 according to their SD and functionally characterized. The resulting network was divided into
134 nine functional nodes: apoptosis, oxygen binding, blood coagulation, response to the virus, T
135 cell, cell division, and three nodes related to an inflammatory response (Fig 4, S3 File).

Cell division and inflammatory response B functional node activities were differential between controls and patients. On the one hand, patients had a higher activity of response to virus, cell division, and one of the inflammatory functional nodes. On the other hand, healthy individuals had a higher activity of T cell and two out of the three functional nodes related to inflammatory processes (Fig 5).

Inflammatory response A functional node

This node is composed of 77 genes, of which 28 were related to an inflammatory response. Most of these genes were cytokines, chemokines and toll-like receptors, whose function is the modulation of the inflammatory response. This functional node included toll-like receptors TLR6, TLR8, TLR5, TLR1, and TLR4.

Inflammatory response B functional node

This node was formed by 69 genes, 6 of them related to the inflammatory response. These six genes were CCR3, CCL4L2, TNFRSF18, NCR3, CCL5, and MS4A2. CCL5 and CCL4L2 are chemokine ligands, and CCR3 and MS4A2 are implicated in an allergic response.

Inflammatory response C functional node

This functional node had 77 genes, 6 of them related to inflammatory processes. These genes were CCL4, CXCR2, FPR2, IL1RAP, CXCL8, and ORM1, mostly of them chemokines.

T cell functional node

This functional node was composed of 210 genes, 6 of them related to T cells, more concretely with T cell receptors, including GATA3 gene, which plays a vital role in nasopharyngeal virus detection.

Cell division functional node

This node was composed of 141 genes, 5 of them involved in cell division. These genes were CDK1, CENPW, CCNB1, UBE2C, and CCNB2, mostly related to M-phase promoting factor complex and microtubules.

Response to virus functional node

This node had 130 genes, nine related to response to virus ontology. This node contained two genes whose proteins are induced by interferon, IFI44L and IFITM3.

Discussion

SARS-CoV-2 infection has reached the category of a pandemic. Tremendous efforts have been made to find a suitable vaccine and to determine effective treatments but till the date there are neither of them [8].

We have re-analyzed the work of Xiong et al. [4] with a different functional inference approach. Coincidences between both results were expected. Xiong et al. described an up-regulation of genes related to cell cycle and cytokines in SARS-CoV-2 patients, which agreed with the higher functional node activity that we observed in cell division node and one of the inflammatory nodes, mostly composed by cytokines and chemokines. They also described a reduction of immune cells in blood patient samples, which may be related to the lower activity of T cell node in patients than in healthy controls.

Additionally, our analysis offered complementary information. For instance, in the network that characterizes differences between healthy individuals and SARS-CoV-2 patients based on the 1,569 differential genes identified by CuffDiff, two functional nodes related to inflammatory response were identified. Strikingly, inflammatory response A functional node activity was higher in patients than in healthy controls. This node was composed by cytokines and chemokines. However, inflammatory response B node activity, that is related to response to bacterial infections, was higher in healthy controls than in patients. SARS-CoV-2 coexist with a bacterial co-infection of *Mycoplasma pneumoniae* so the study of those genes related to the presence of a bacterial infection in these patients may be relevant [9]. Metabolism node showed also a higher functional node activity in Covid-19 patients than in healthy controls. The increase in glycolysis reactions implies an increase in Krebs cycle reactions as well and therefore in ATP production, essential for the virus replication [10, 11]. The differences on the metabolism functional node suggested that a deeper analysis of metabolism, as Flux Balance Analysis, could supply more detail information. Glutamate metabolism showed differences between controls and Covid-19 patients. Interestingly, an alteration in glutamate metabolism caused by another RNA virus, the HIV-1, has been previously described [11]. Moreover, it has been previously suggested that methionine plays a relevant role in viral replication of other coronaviruses [12]. No alterations in tetrahydrobiopterin metabolism have been previously described related to SARS-CoV-2 infection. However, it is remarkable that tetrahydrobiopterin is a NO synthase cofactor which is involved in immune regulation and inflammation processes. It has been described that a blockade of tetrahydrobiopterin synthesis annuls T-cell mediated autoimmunity and allergic inflammation. On contrast, higher levels of tetrahydrobiopterin

198 increase CD4 and CD8 responses [13]. It has also been described that acute inflammatory
199 stimulation increases levels of plasma BH4, in parallel with increased IL-6 [14], which it has
200 been widely associated with SARS-CoV-2 infection and severity [15, 16]. Recent articles where
201 plasma samples from Covid-19 patients were analyzed by metabolomics have shown
202 differences in metabolism caused by SARS-CoV-2 infection, especially in steroid, aminoacid and
203 mitochondrial metabolism [17].

204 Lysosomes have been previously associated with coronaviruses. In 1984, a study described
205 virus-containing electron-dense bodies in lysosomes of coronavirus-infected cells as a defense
206 mechanism [18]. Moreover, a study done in murine hepatitis virus, a prototype to study
207 coronaviruses, established that the virus depends on the lysosomal traffic for a proteolytic
208 cleavage site in the S protein, necessary for the intracellular fusion and entry [19]. In addition,
209 this node contains the HPSE genes which it has been previously associated with viral infection
210 and its activation is associated with a production of pro-inflammatory factors [20].

211 On the other hand, in the network obtained for the 2,000 most variable genes, two functional
212 nodes related to inflammatory response (inflammatory response A and inflammatory response
213 B) were also identified. Inflammatory response A functional node was again mostly composed
214 by cytokines and chemokines. Inflammatory response B functional node had a lower activity in
215 SARS-CoV-2 patients. Interestingly, the inflammatory response B node was composed of genes
216 related to allergic response and regulation of immunological self-tolerance. This fact may be
217 related to the severe acute respiratory syndrome, associated with a dysregulation of the
218 immune response [2].

219 In this inflammatory response B node is included CCR3, a chemokine highly expressed in
220 eosinophils and basophils, and is also detected in TH1 and TH2 cells, as well in airway epithelial
221 cells [21, 22]. This receptor may contribute to the accumulation and activation of inflammatory
222 cells in allergic airway and it is also known to be an entry co-receptor for HIV-1. MS4A2 is also
223 implicated in allergic processes [23]. Therefore, this node seems to be more related to the self-
224 control of the inflammatory response instead of the other inflammatory functional nodes,
225 more related to chemokines and cytokines.

226 Cell division functional node had a significantly higher activity in Covid-19 patients than in
227 controls. This node is mainly composed by genes related to M-phase and mitosis process,
228 which may be related to viral infection. An accumulation of G2/M phase cells in other
229 coronaviruses has been previously described in order to promote favorable conditions for viral
230 replication [24].

231 As expected, functional nodes related to response to the virus were relevant in both networks.
 232 In the case of the network built based on the 2,000 most variable genes, this functional node
 233 was mainly related to interferon response. Remarkably, this node included IFITM3 gene, which
 234 codifying sequence is associated with immunity to other well-known viruses such as influenza
 235 A or dengue virus [25, 26]. IFITM3 protein has been described as related to the entry of MERS-
 236 CoV and SARS-CoV[27]. The first response to a viral infection of the immune system is
 237 mediated by interferons so it seems logical that these genes were overexpressed in patients
 238 infected by SARS-CoV-2. Additionally, interferon-mediated response has been associated with
 239 severe cases of Covid-19, so a study of the genes included in this functional node in a large
 240 cohort with different grades of severity of Covid-19 may be interesting [28].

241 In addition, in the T cell functional node appeared GATA3 gene which has been previously
 242 related to nasopharyngeal virus infections [29]. Since SARS-CoV-2 presents mainly respiratory
 243 tropism, GATA3 may play an essential role.

244 Our study had some limitations. Probably the most important one was that the reduced
 245 number of samples limited the statistical power and the information that could be obtained by
 246 functional analyses. A larger number of samples will be useful to deepen into the molecular
 247 characterization of this disease. Also, a study based on a larger cohort stratified according the
 248 severity of the disease could be of much interest as it may help define how functional modules
 249 vary in relation to the virulence of the infection.

250 In this study, some previously not described relevant processes in SARS-CoV-2 pathogenesis
 251 such as bacterial inflammatory response processes, tetrahydrobiopterin metabolism or allergic
 252 processes, were proposed. In the absence of treatments for these patients, molecular
 253 characterization of the disease could be helpful to improve the understanding of the
 254 mechanisms of the disease and to define targetable processes. The application of these type of
 255 analyses in larger cohorts may be useful not just to determine therapeutic targets but also to
 256 define predictors of immune response to infection. Therefore, these results may be relevant
 257 to propose new therapeutic treatments in the future.

258 **Materials and Methods**

259 **Patient cohort**

260 Three samples from peripheral blood mononuclear cells (PBMCs) from three patients infected
261 with SARS-CoV-2 and three samples from healthy controls were analyzed. These samples are
262 all from the work of Xiong et al. [4] and raw data can be downloaded from SRA database.

263 **Processing of RNA sequencing data**

264 Before processing fragments per kilobase of exon model per million of reads (FPKM) data, we
265 checked their quality using FastQC (v0.11.9, Brabham, UK). Reads longer than 100 nt showed
266 the presence of Illumina adapter sequences which were removed by trimming using Prinseq
267 [30] so all samples were matched to 2x100 format. Then, reads were mapped against the
268 human genome (GRCh38.96) using TopHat, using an estimated paired-end inner size of 25 and
269 finally FPKM data were obtained using CuffDiff. All these programs were accessed using the
270 integrated GPRO suite (Biotechvana, Valencia, Spain) [31].

271 After FPKM processing, Perseus v1.6.5 software was used to filter RNAseq data [32]. Log2 was
272 calculated and only those genes with at least 50% of the detectable readings were used for the
273 subsequent analyses.

274 **Probabilistic graphical models**

275 2,000 most variable genes were selected according to their standard deviation (SD) of
276 expression across the series and used to build a PGM network. RNAseq expression data was
277 used without other a priori information.

278 The resulting network was divided into functional nodes by gene ontology analyses. These
279 gene ontology analyses were performed in DAVID webtool v8 using "Homo sapiens" as
280 background and KEGG, Biocarta and GOTERM-FAT as categories [33].

281 The same analysis pipeline was used to characterize the differential genes defined by CuffDiff,
282 i.e. a network was built using the genes defined as significantly differential between healthy
283 controls and patients.

284 These analyses were done using *graphD* package [34] and R v3.2.5. Network visualization was
285 done in Cytoscape [35]. PGMs were built in two steps, first, the spanning tree with the
286 maximum likelihood was found, and then, the edges were refined based on the minimization
287 of the Bayesian Information Criterion (BIC) [36].

288 **Statistical analysis**

289 Functional node activities were calculated as previously described [6]. Briefly, the mean
290 expression of those genes of each node related to the overrepresented function in this node
291 was calculated. Then, functional node activities were compared between healthy individuals
292 and patients using a T-test.

293 **Flux Balance Analysis and metabolic models**

294 Flux Balance Analysis (FBA) allows metabolic modeling from gene expression data. It is widely
295 used in microbiology and cancer [37]. The complete human metabolic reconstruction Recon 3D
296 was used to perform these analyses. It contains 10,600 reactions, 5,835 metabolites and 5,939
297 Gene-Protein-Reaction rules (GPRs), which contain information in the form of Boolean
298 expressions about which genes are involved in each metabolic reaction. GPRs were solved
299 using a modification of Barker et al. algorithm [38, 39], solving “AND” expressions as the
300 minimum and “OR” expressions as the sum. Then, the obtained values were introduced as the
301 reaction bounds by a modified E-flux algorithm based on the Max-min function [39, 40].
302 Finally, FBA was solved using COBRA Toolbox library v2.0 [41] and MATLAB.

303 The 10,600 metabolic reactions are grouped into 103 metabolic pathways or subsystems. In
304 order to compare metabolic activity between controls and Covid-19 patients, flux activities
305 were calculated as previously described as the sum of fluxes of the reactions contained in a
306 concrete metabolic pathway [5, 42]. To compare flux activities between control and patients a
307 T-test was used.

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466 **Figure legends**

467 **Fig 1: Probabilistic graphical model based on the expression of 1,234 differential genes**
468 **between healthy individuals and patients.**

469 **Fig 2: Functional node activities from the network based on the expression of the 1,234**
470 **genes defined as significantly differential by CuffDiff.** In the Y axis the activity of the
471 functional node in arbitrary units, understanding as the mean expression of those genes in
472 each node that were related to the overrepresented function in the node. In the x axis, healthy
473 controls and Covid-19 patients. **, ≤ 0.01 ; * ≤ 0.05 .

474 **Fig 3: Differential flux activities between healthy controls and patients.** a.u. = arbitrary units.
475 * $p < 0.05$.

476 **Fig 4: Probabilistic graphical model based on the expression of the 2,000 most variable**
477 **genes.**

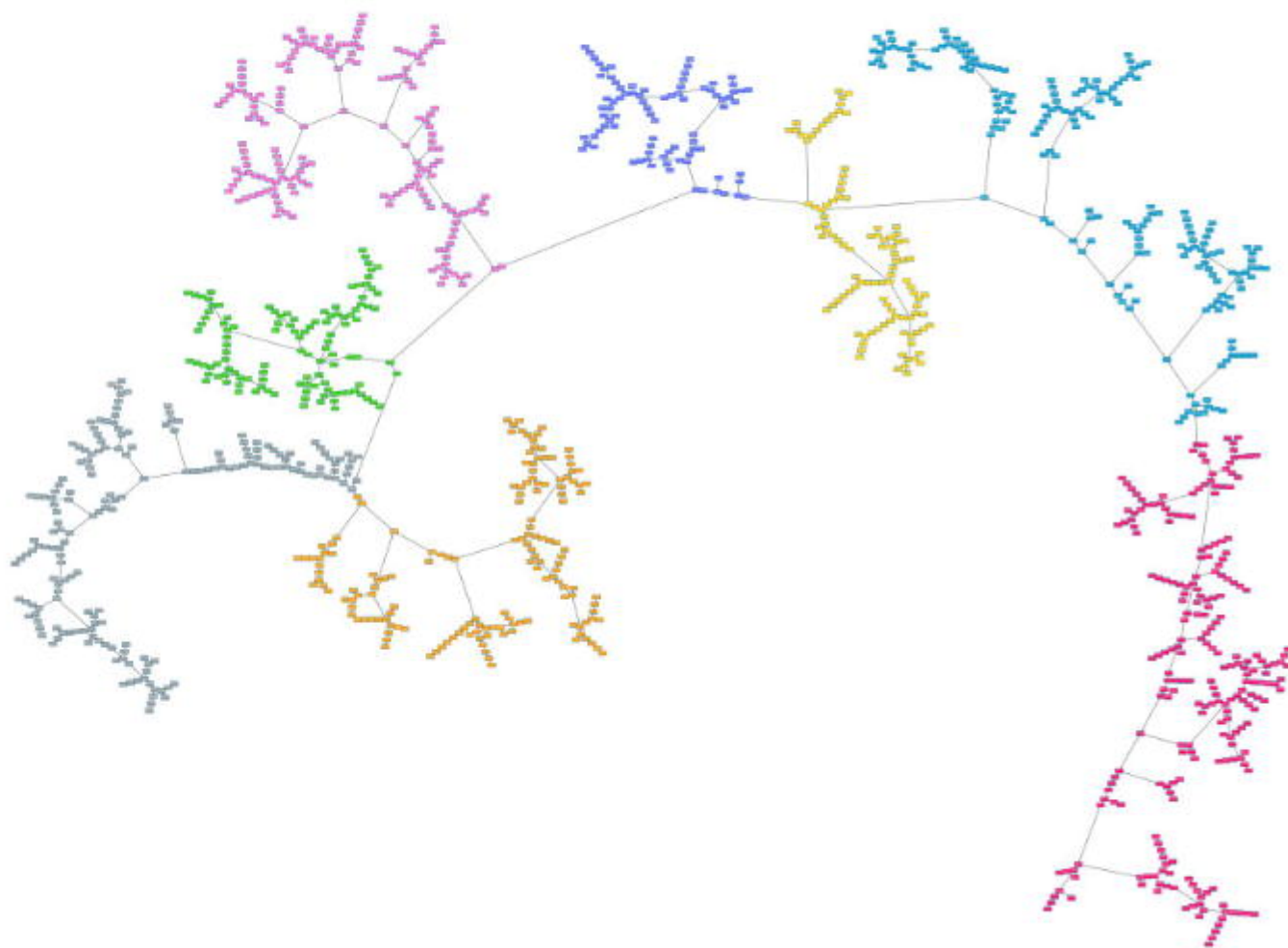
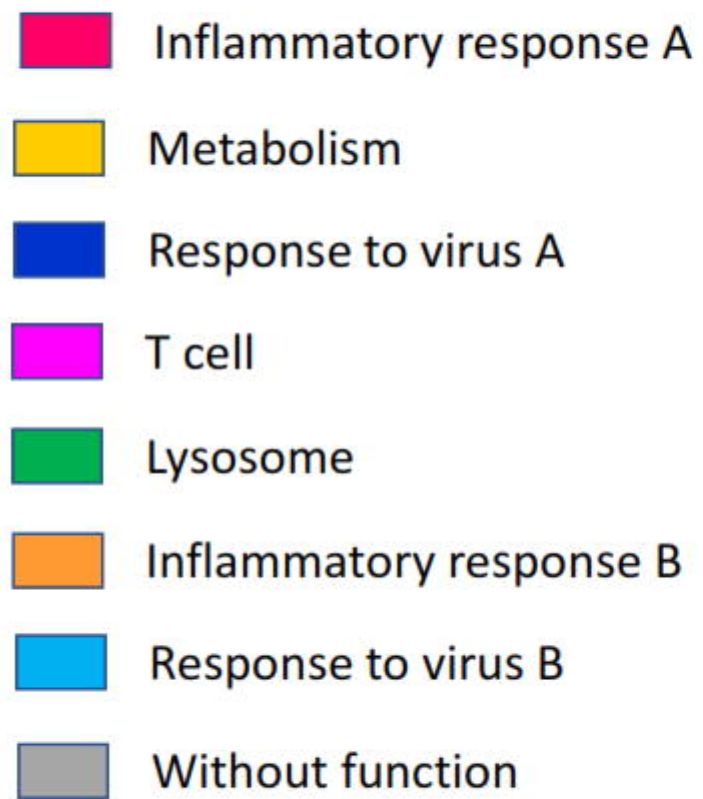
478 **Fig 5: Functional node activities from the network based on the expression of the 2,000 most**
479 **variable genes.** In the Y axis the activity of the functional node in arbitrary units,
480 understanding as the mean expression of those genes in each node that were related to the
481 overrepresented function. In the X axis, healthy controls and Covid-19 patients. ***, ≤ 0.001 ;
482 **, ≤ 0.01 ; * ≤ 0.05 .

483 **Supporting information**

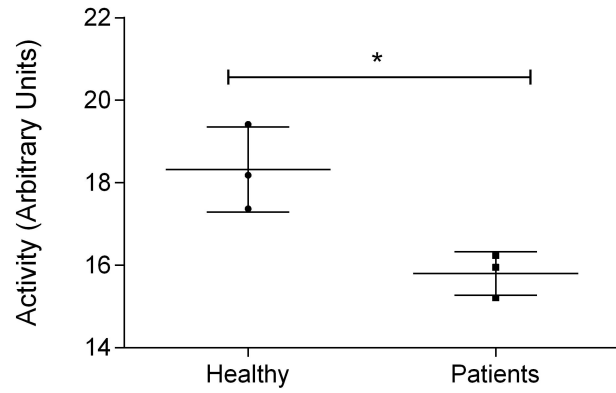
484 S1 File: Genes included in the probabilistic graphical model based on the expression of 1,234
485 differential genes between healthy individuals and patients.

486 S2 File: Genes included the probabilistic graphical model based on the expression of the 2,000
487 most variable genes.

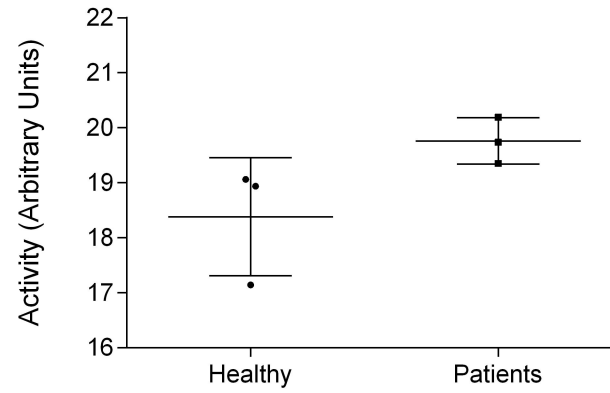
488 S3 File: Flux Balance Analysis results.



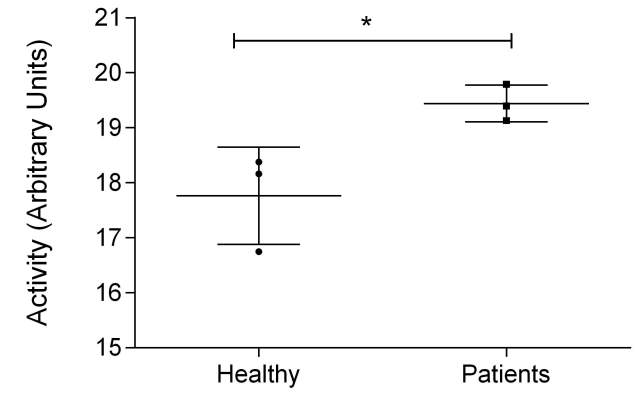
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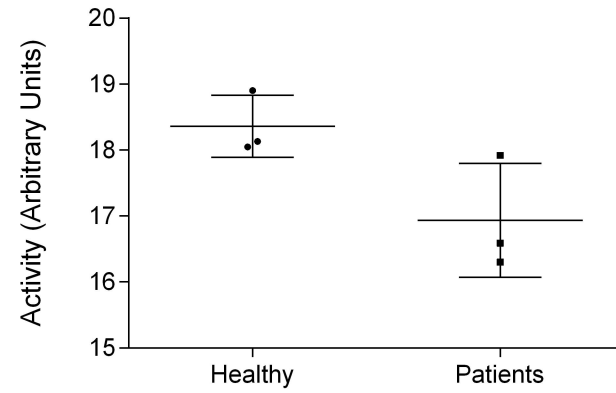
Inflammatory response B



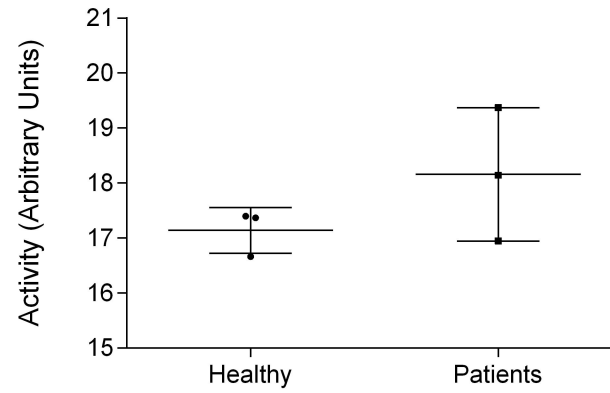
Lysosome



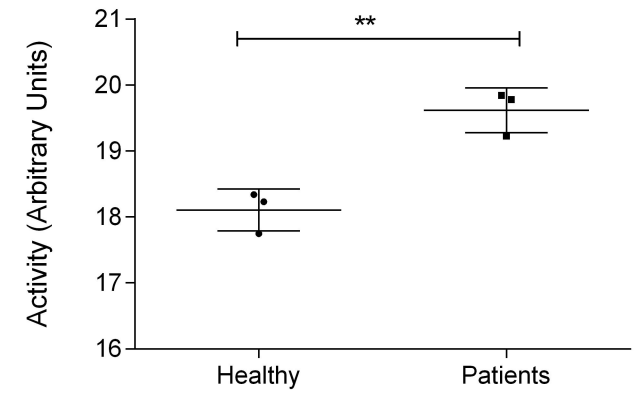
T cell



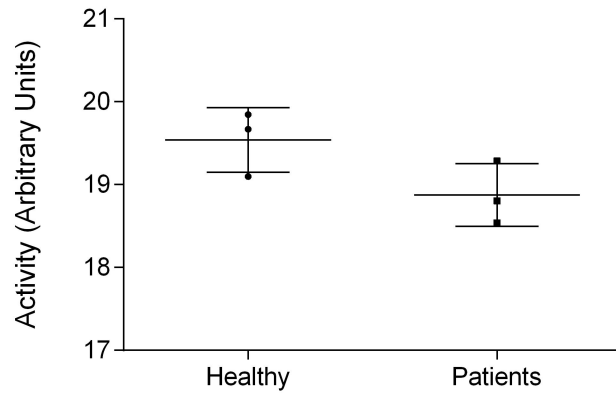
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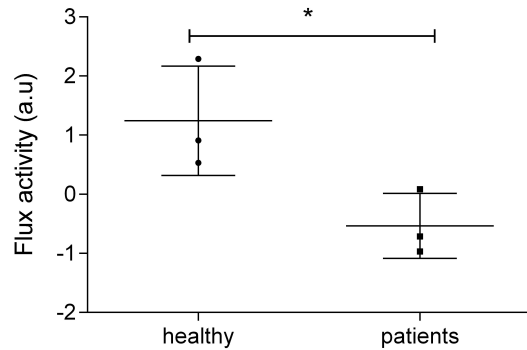
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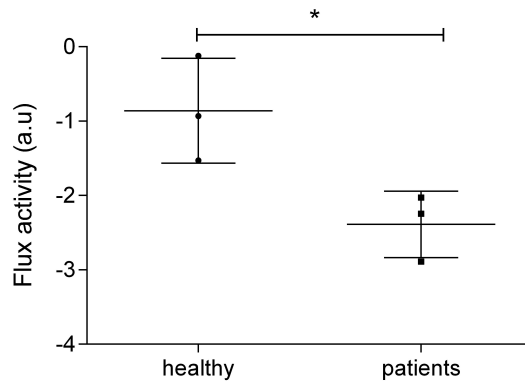
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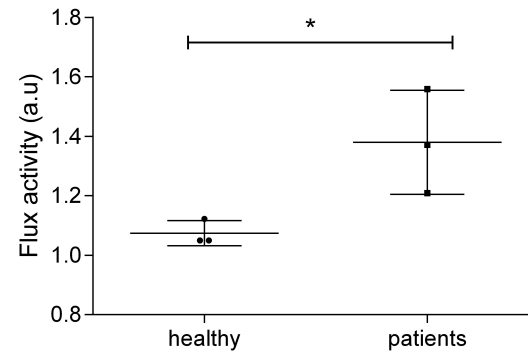
Glutamate metabolism



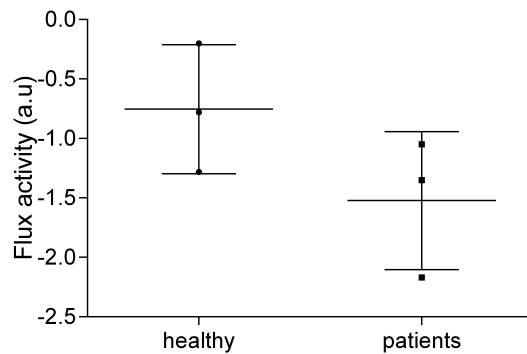
Methionine metabolism



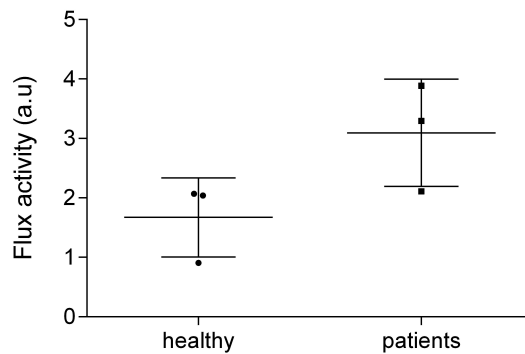
Tetrahydrobiopterin metabolism

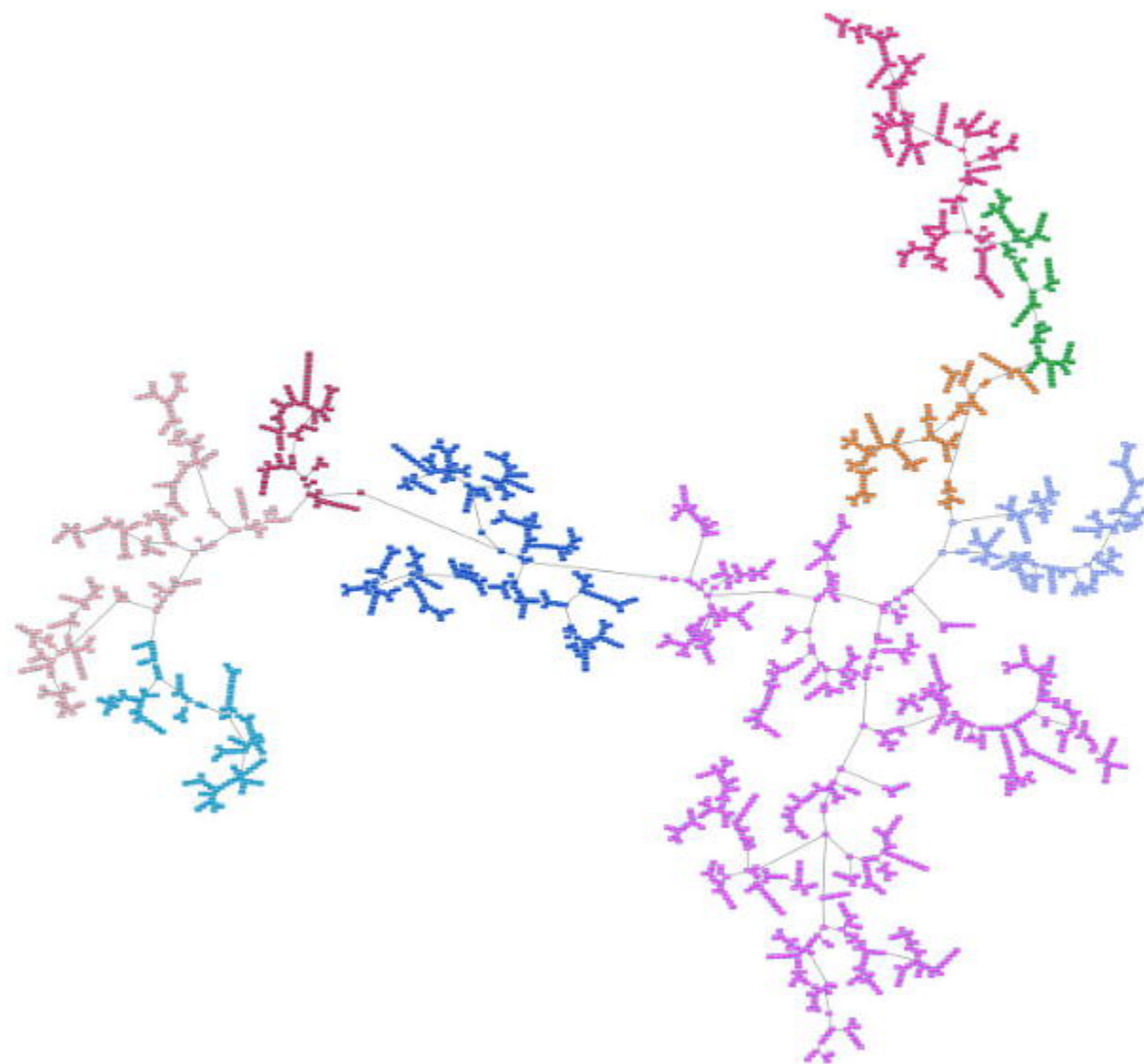
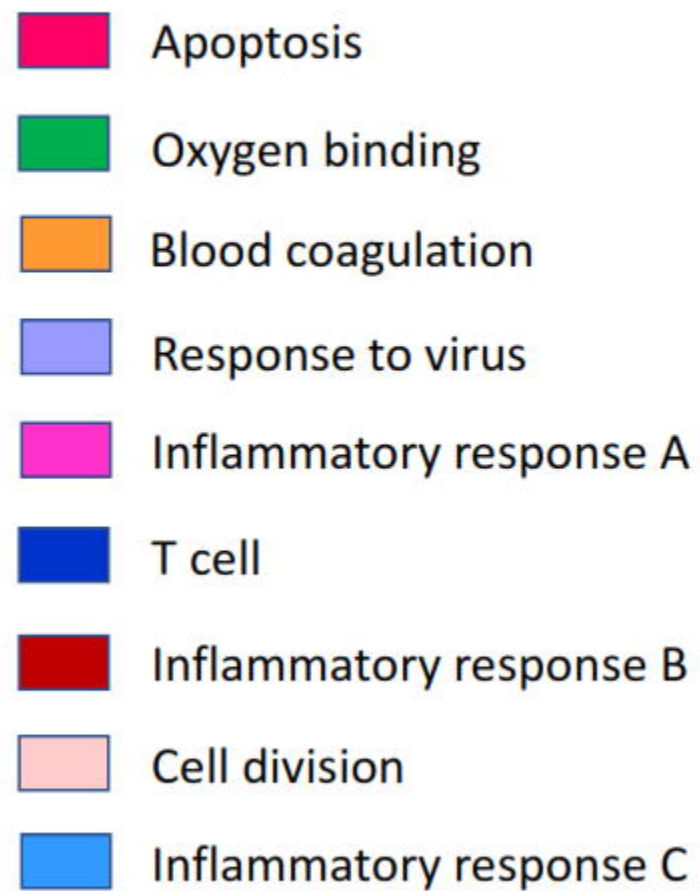


TCA

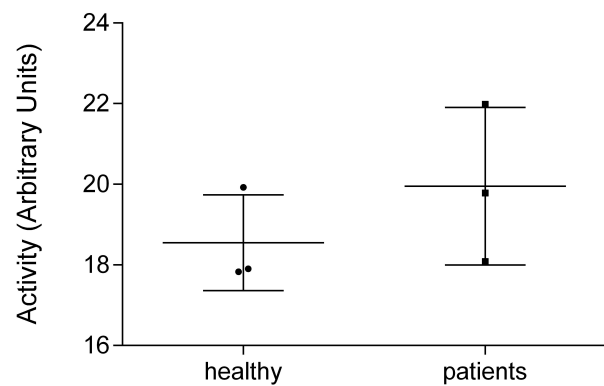


Steroid metabolism

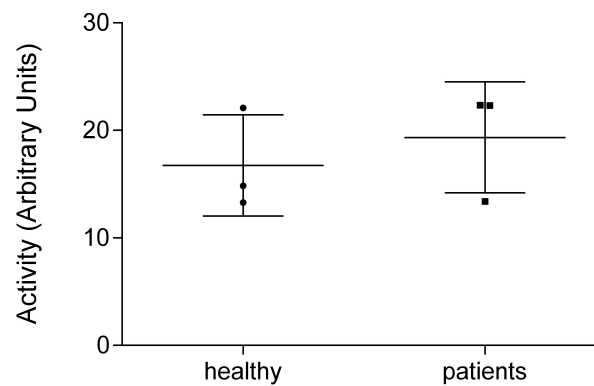




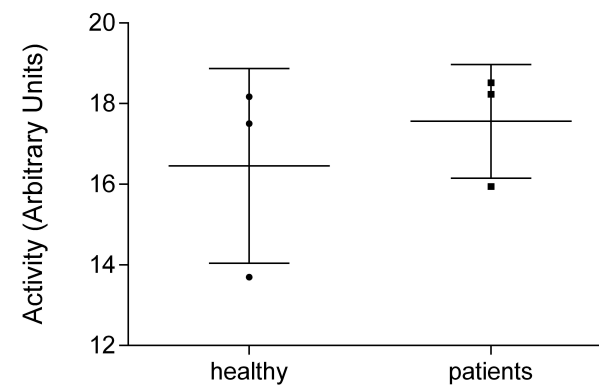
Apoptosis



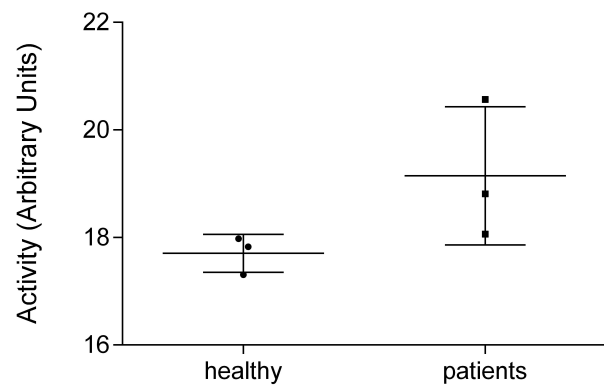
Oxygen binding



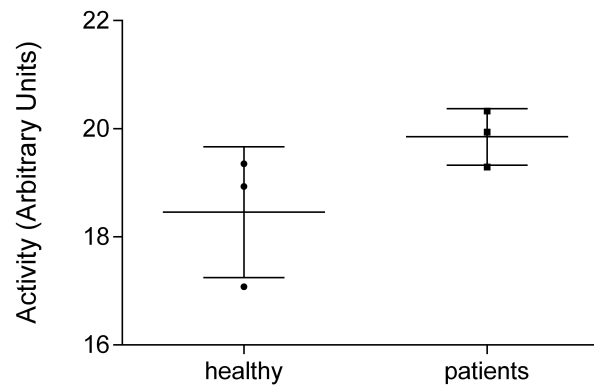
Blood coagulation



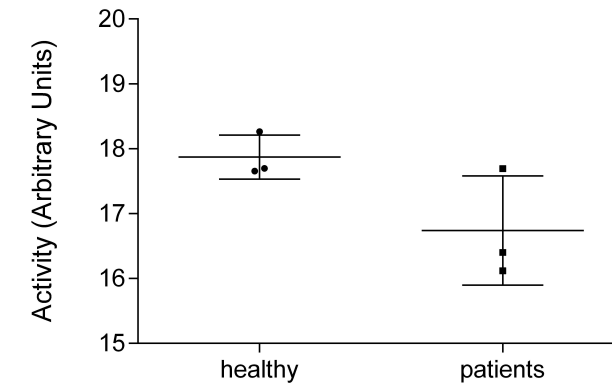
Response to virus



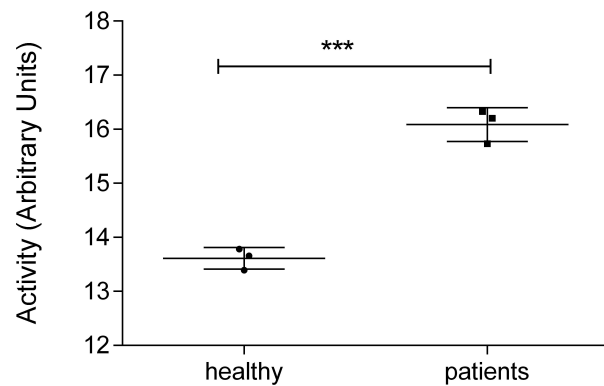
Inflammatory response A



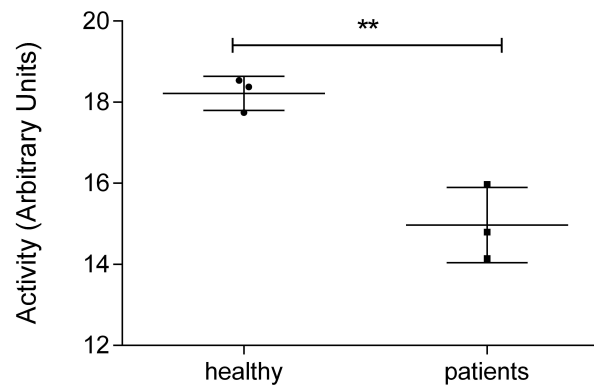
T cell



Cell division



Inflammatory response B



Inflammatory response C

