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

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Abstract

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

Author Summary

SARS-CoV-2 infection caused Covid-19 which has reached the category of a worldwide pandemic. However, no treatments or vaccines are still available. For this reason, it is still necessary the molecular study of this disease. In this study, we reanalyzed data from peripheral blood mononuclear cells from Covid-19 patients and healthy controls using computational techniques that allow the study of differential biological processes and metabolic pathways. The results suggested a complex inflammatory response, involving genes related to response to bacterial infection and allergic processes, and alterations in metabolic pathways such as glutamate metabolism, cysteine and methionine metabolism or

57 tetrahydrobiopterin metabolism. These processes could be used in the future as therapeutic
58 targets in Covi-19 infection.

Introduction

The emerging coronavirus SARS-CoV-2 has rapidly expanded from its origin in Wuhan, China, to become a worldwide pandemic only after four months since its first identification. At June 2nd of 2020, 6,194,533 cases and 376,320 deaths have been reported worldwide, according to the World Health Organization [1].

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

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

Probabilistic graphical models (PGMs) have demonstrated their utility in analyzing gene expression data by identifying relevant biological processes [5, 6]. These models allow making associations between genes according to their expression patterns across a series. Interestingly, the PGM networks have functional structure, allowing study expression data from a functional point of view. We thus set out to re-analyze Xiong. et. al. data using PGMs, aiming for a deeper understanding of biological processes involved in SARS-CoV-2 pathogenesis.

Results

Processing of RNA sequencing data

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

Analysis of differential genes between healthy controls and patients

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

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

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

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

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

115 **Metabolism functional node**

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

121 **Inflammatory response A functional node**

122 This node included 236 genes, of which 18 were implicated in an inflammatory response. This
123 node mostly comprised chemokines.

124 **Inflammatory response B functional node**

125 This node included 129 genes, of which 17 were involved in inflammatory response. This
126 functional node included three toll-like receptors, TLR2, TLR5 and TLR4. Among other
127 functions, TLR2 promotes apoptosis in response to bacterial lipoproteins. TLR5 protein
128 recognizes bacterial flagellin, the principal component of bacterial flagella. Additionally, TLR4
129 has been implicated in signal transduction events induced by lipopolysaccharide found in most
130 gran negative bacteria. This node also contained AOAH and CD14, both genes implicated in
131 response to bacterial lipopolysaccharides as well. Finally, this functional node included VNN1
132 which plays a suppressive role in influenza virus replication in human alveolar epithelial cells
133 [7]. Therefore, this node is mostly related to the response to bacterial infections.

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

135 We obtained an alternative PGM network, now based on the 2,000 most variable genes
136 according to their SD and functionally characterized. The resulting network was divided into
137 nine functional nodes: apoptosis, oxygen binding, blood coagulation, response to the virus, T
138 cell, cell division, and three nodes related to an inflammatory response (Fig 3, S2 File).

139 **Fig 3: Probabilistic graphical model based on the expression of the 2,000 most variable**
140 **genes.**

141 Cell division and inflammatory response B functional node activities were differential between
142 controls and patients. On the one hand, patients had a higher activity of response to virus, cell
143 division, and one of the inflammatory functional nodes. On the other hand, healthy individuals
144 had a higher activity of T cell and two out of the three functional nodes related to
145 inflammatory processes (Fig 4).

146 **Fig 4: Functional node activities from the network based on the expression of the 2,000 most**
147 **variable genes.** In the Y axis the activity of the functional node in arbitrary units,
148 understanding as the mean expression of those genes in each node that were related to the
149 overrepresented function. In the X axis, healthy controls and Covid-19 patients. ***, ≤ 0.001;
150 ** , ≤ 0.01 ; * ≤ 0.05.

151 **Inflammatory response A functional node**

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

156 **Inflammatory response B functional node**

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

160 **Inflammatory response C functional node**

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

163 **T cell functional node**

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

167 **Cell division functional node**

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

171 **Response to virus functional node**

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

174 **Metabolic modeling**

175 FBA was performed to characterize metabolic alterations caused by SARS-CoV-2 infection (S3
176 File). Glutamate metabolism, methionine and cysteine metabolism, and tetrahydrobiopterin
177 metabolism flux activities were differential between healthy controls and Covid-19 patients
178 (Fig 5). In addition, several tendencies in other metabolic pathways such as TCA cycle and
179 steroid metabolism that need to be confirmed were shown (S1 Figure).

180 **Fig 5: Differential flux activities between healthy controls and patients.** a.u. = arbitrary units.
181 * $p < 0.05$.

182

183 ## Discussion

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

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

194 Additionally, our analysis offered complementary information. For instance, in the network
195 that characterizes differences between healthy individuals and SARS-CoV-2 patients, there
196 were two functional nodes related to inflammatory response. Strikingly, inflammatory
197 response A functional node activity was higher in patients than in healthy controls. This node
198 was composed by cytokines and chemokines. However, inflammatory response B node activity
199 was higher in healthy controls than in patients and it was related to response to bacterial
200 infections. SARS-CoV-2 can carry with a bacterial co-infection of *Mycoplasma pneumoniae* so
201 the study of those genes in relation to the presence of a bacterial co-infection may be relevant
202 [9]. Metabolism node had also a higher functional node activity in Covid-19 patients than in
203 healthy controls. The increase in glycolysis reactions implies an increase in Krebs cycle
204 reactions as well and therefore in ATP production, essential for the virus replication [10, 11].
205 The differences on metabolism functional node suggested that a deeper analysis of
206 metabolism could supply more detail information. Glutamate metabolism showed differences
207 between controls and Covid-19 patients. Interestingly, an alteration caused by another RNA
208 virus, the HIV-1, has been previously described [11]. Moreover, it has been previously
209 suggested that methionine plays a relevant role in viral replication of other coronaviruses [12].
210 No alterations in tetrahydrobiopterin metabolism have been previously described.

211 On the other hand, in the network obtained for the 2,000 most variable genes, two functional
212 nodes related to inflammatory response were also identified. Inflammatory response A
213 functional node was again mostly composed by cytokines and chemokines. Inflammatory
214 response B functional node had a lower activity in SARS-CoV-2 patients. Interestingly, the
215 inflammatory response B node was composed of genes related to allergic response and

216 regulation of immunological self-tolerance. This fact may be related to the severe acute
217 respiratory syndrome, associated with a dysregulation of the immune response [2].

218 In this inflammatory response B node is included CCR3, a chemokine highly expressed in
219 eosinophils and basophils, and is also detected in TH1 and TH2 cells, as well in airway epithelial
220 cells [13, 14]. This receptor may contribute to the accumulation and activation of inflammatory
221 cells in allergic airway and it is also known to be an entry co-receptor for HIV-1. MS4A2 is also
222 implicated in allergic processes [15]. Therefore, this node seems to be more related to the self-
223 control of the inflammatory response instead of the other inflammatory functional nodes,
224 more related to chemokines and cytokines.

225 Cell division functional node had a significantly higher activity in Covid-19 patients than in
226 controls. This node is mainly composed by genes related to M-phase and mitosis process,
227 which may be related to viral infection. An accumulation of G2/M phase cells in other
228 coronaviruses has been previously described in order to promote favorable conditions for viral
229 replication [16].

230 As expected, functional nodes related to response to the virus were relevant in both networks.
231 In the case of the network built based on the 2,000 most variable genes, this functional node
232 was mainly related to interferon response. Remarkably, this node included IFITM3 gene,
233 whose genetics is associated with immunity to other well-known viruses such as influenza A or
234 dengue virus [17, 18]. IFITM3 protein has been described as related to the entry of MERS- CoV
235 and SARS-CoV[19]. The first response to a viral infection of the immune system is mediated by
236 interferons so it seems logical that these genes were overexpressed in patients infected by
237 SARS-CoV-2.

238 In addition, in the T cell functional node appeared GATA3 gene which has been previously
239 related to nasopharyngeal virus infections [20]. Since SARS-CoV-2 presents mainly respiratory
240 tropism, GATA3 may play an essential role.

241 Our study had some limitations. Most importantly, the reduced number of samples severely
242 limited the statistical power and the information that could be obtained by functional
243 analyses. A larger number of samples will be useful to deepen in the molecular
244 characterization of this disease. Also, studying a larger cohort stratified according the severity
245 of the disease could be of much interest as it may help define how functional modules vary in
246 relation to the virulence of the infection.

247 In this study, some previously not described relevant processes in SARS-CoV-2 pathogenesis
248 such as bacterial inflammatory response processes, metabolism or allergic processes, were
249 proposed. In the absence of treatments for these patients, molecular characterization of the
250 disease could be helpful to define targetable processes. Therefore, these results may be
251 relevant to propose new therapeutic treatments.

252 ## Materials and Methods

253 ### Patient cohort

254 Three samples from PBMCs from three patients infected with SARS-CoV-2 and three samples
255 from healthy controls were analyzed. These samples are all from the work of Xiong et al. [4].

256 ### Processing of RNA sequencing data

257 Before processing fragments per kilobase of exon model per million of reads (FPKM) data, we
258 checked their quality using FastQC (v0.11.9, Brabaham, UK). Reads longer than 100 nt showed
259 the presence of Illumina adapter sequences which were removed by trimming using Prinseq
260 [21]so all samples were matched to 2x100 format. Then, reads were mapped against the
261 human genome (GRCh38.96) using TopHat, using an estimated paired-end inner size of 25 and
262 finally FPKM data were obtained using CuffDiff. All these programs were accessed using the
263 integrated GPRO suite (Biotechvana, Valencia, Spain) [22].

264 After FPKM processing, Perseus v1.6.5 software was used to filter RNAseq data [23]. Log2 was
265 calculated and only those genes with at least 50% of the detectable readings were used for the
266 subsequent analyses.

267 ### Probabilistic graphical models

268 2,000 most variable genes were selected according to their standard deviation (SD) of
269 expression across the series and used to build a PGM network. RNAseq expression data was
270 used without other a priori information.

271 The resulting network was divided into functional nodes by gene ontology analyses. These
272 gene ontology analyses were performed in DAVID webtool v8 using "Homo sapiens" as
273 background and KEGG, Biocarta and GOTERM-FAT as categories [24].

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

277 These analyses was done using *grapHD* package [25] and R v3.2.5. Network visualization was
278 done in Cytoscape [26]. PGMs were built in two steps, first, the spanning tree with the
279 maximum likelihood was found, and then, the edges were refined based on the minimization
280 of the Bayesian Information Criterion (BIC) [27].

281 ### Statistical analysis

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

286 **Flux Balance Analysis and metabolic models**

287 Flux Balance Analysis (FBA) allows metabolic modeling from gene expression data. It is widely
288 used in microbiology and cancer [28]. The complete human metabolic reconstruction Recon 3D
289 was used to perform these analyses. It contains 10,600 reactions, 5,835 metabolites and 5,939
290 Gene-Protein-Reaction rules (GPRs), which contain information in the form of Boolean
291 expressions about what genes are involved in each metabolic reaction. GPRs were solved using
292 a modification of Barker et al. algorithm [29, 30], solving "AND" expressions as the minimum
293 and "OR" expressions as the sum. Then, the obtained values were introduced as the reaction
294 bounds by a modified E-flux algorithm based on the Max-min function [30, 31]. Finally, FBA
295 was solved using COBRA Toolbox library v2.0 [32] and MATLAB.

296 The 10,600 metabolic reactions are grouped into 103 metabolic pathways or subsystems. In
297 order to compare metabolic activity between controls and Covid-19 patients, flux activities
298 were calculated as previously described as the sum of fluxes of the reactions contained in a
299 concrete metabolic pathway [5, 33]. To compare flux activities between control and patients a
300 T-test was used.

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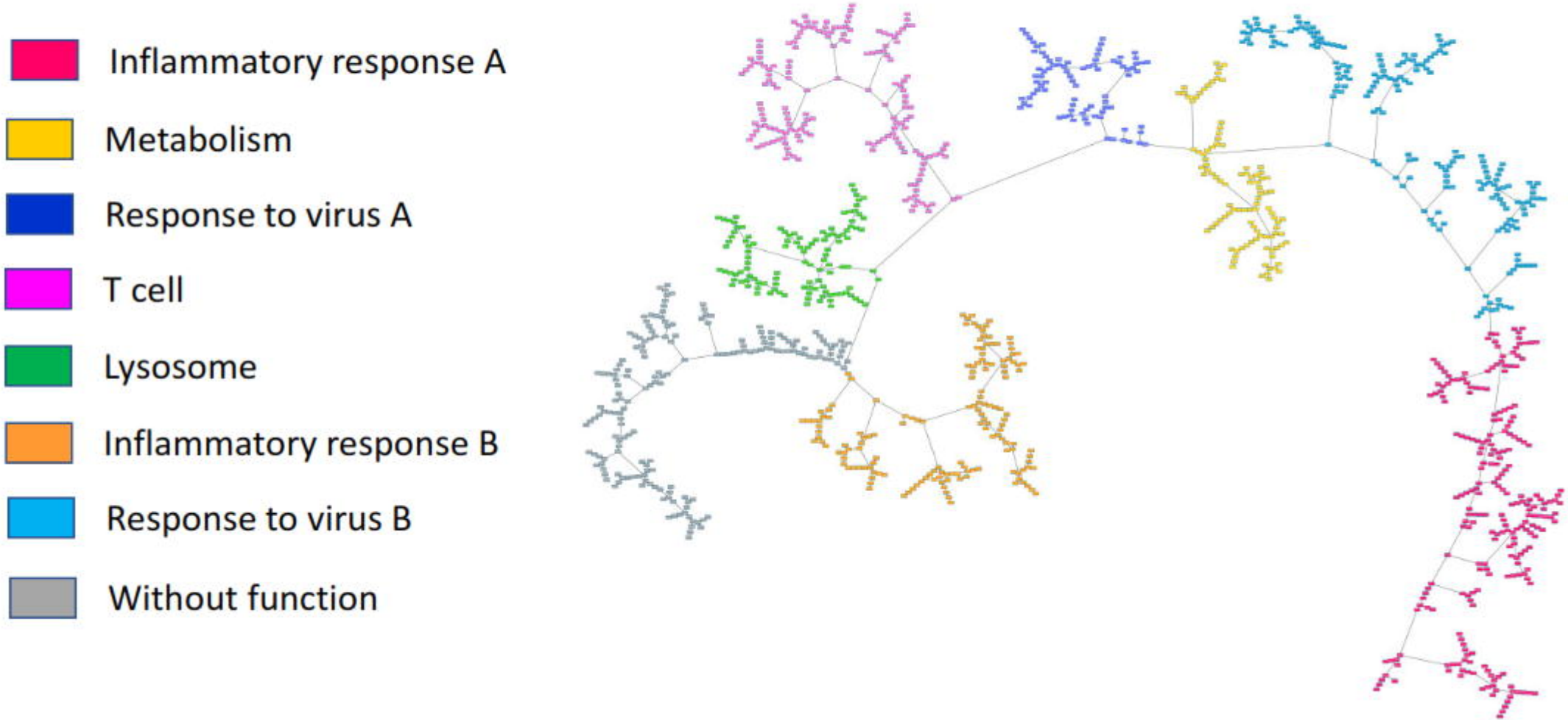
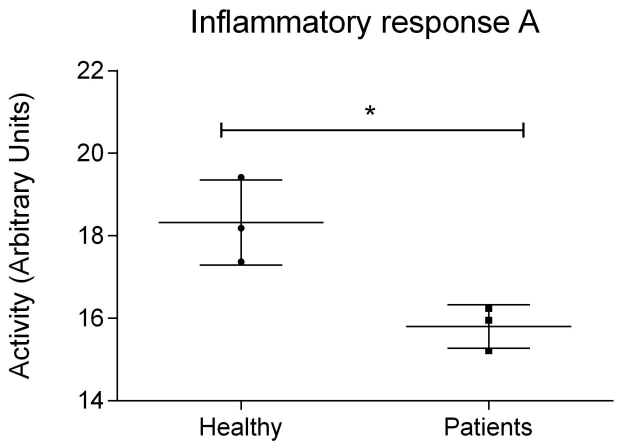
Supporting information

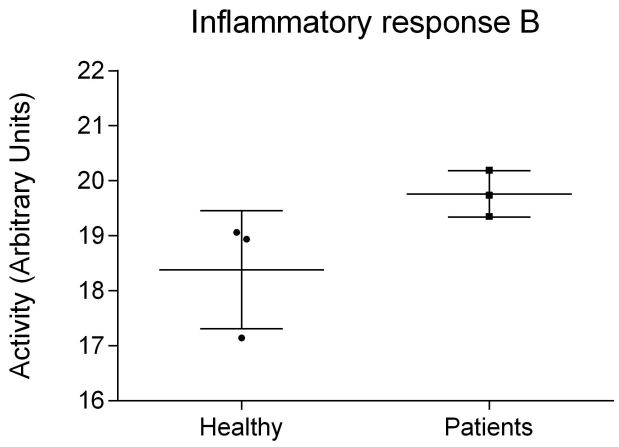
S1 File: Genes included in the probabilistic graphical model based on the expression of 1,234 differential genes between healthy individuals and patients.

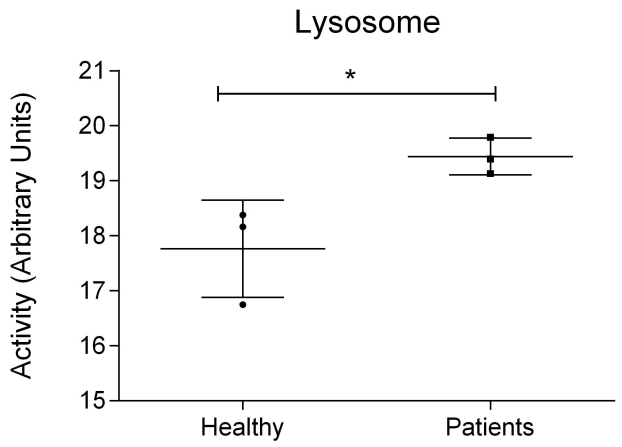
S2 File: Genes included the probabilistic graphical model based on the expression of the 2,000 most variable genes.

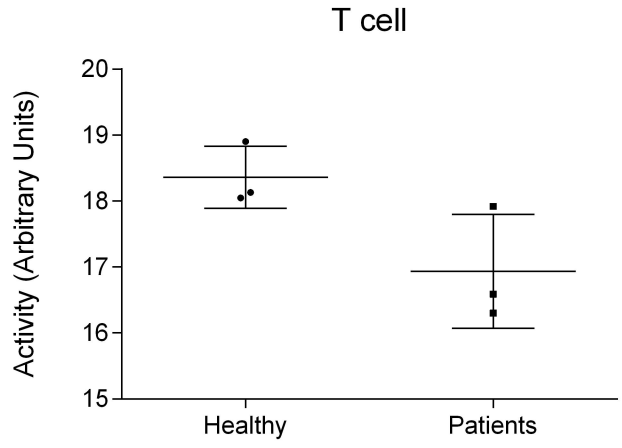
S3 File: Flux Balance Analysis results.

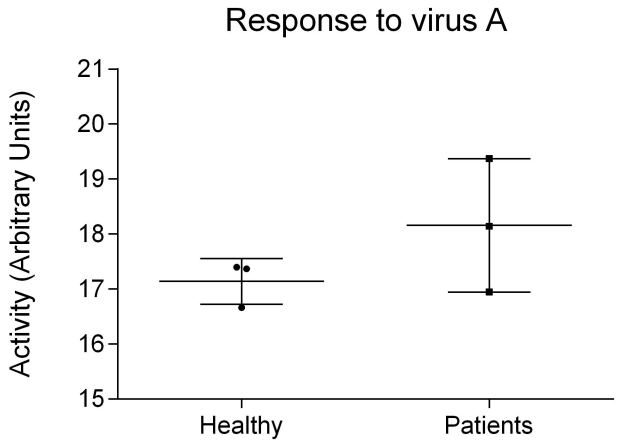

Inflammatory response A
Metabolism
Response to virus A
T cell
Lysosome
Inflammatory response B
Response to virus B
Without function

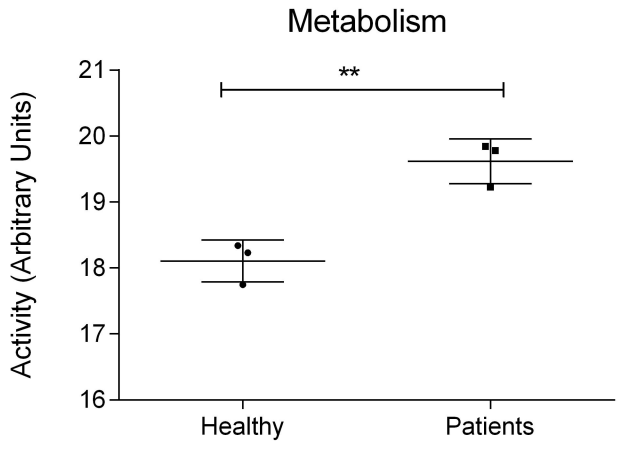

Inflammatory response A
Activity (Arbitrary Units)
22
20
18
16
14
*
Healthy
Patients

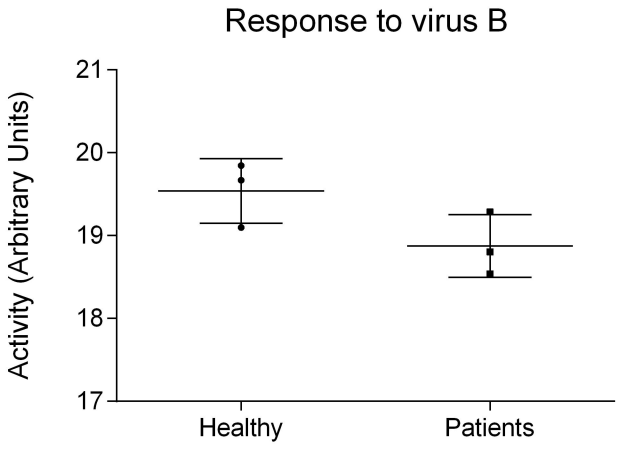

Inflammatory response B
Activity (Arbitrary Units)
22
21
20
19
18
17
16
Healthy
Patients

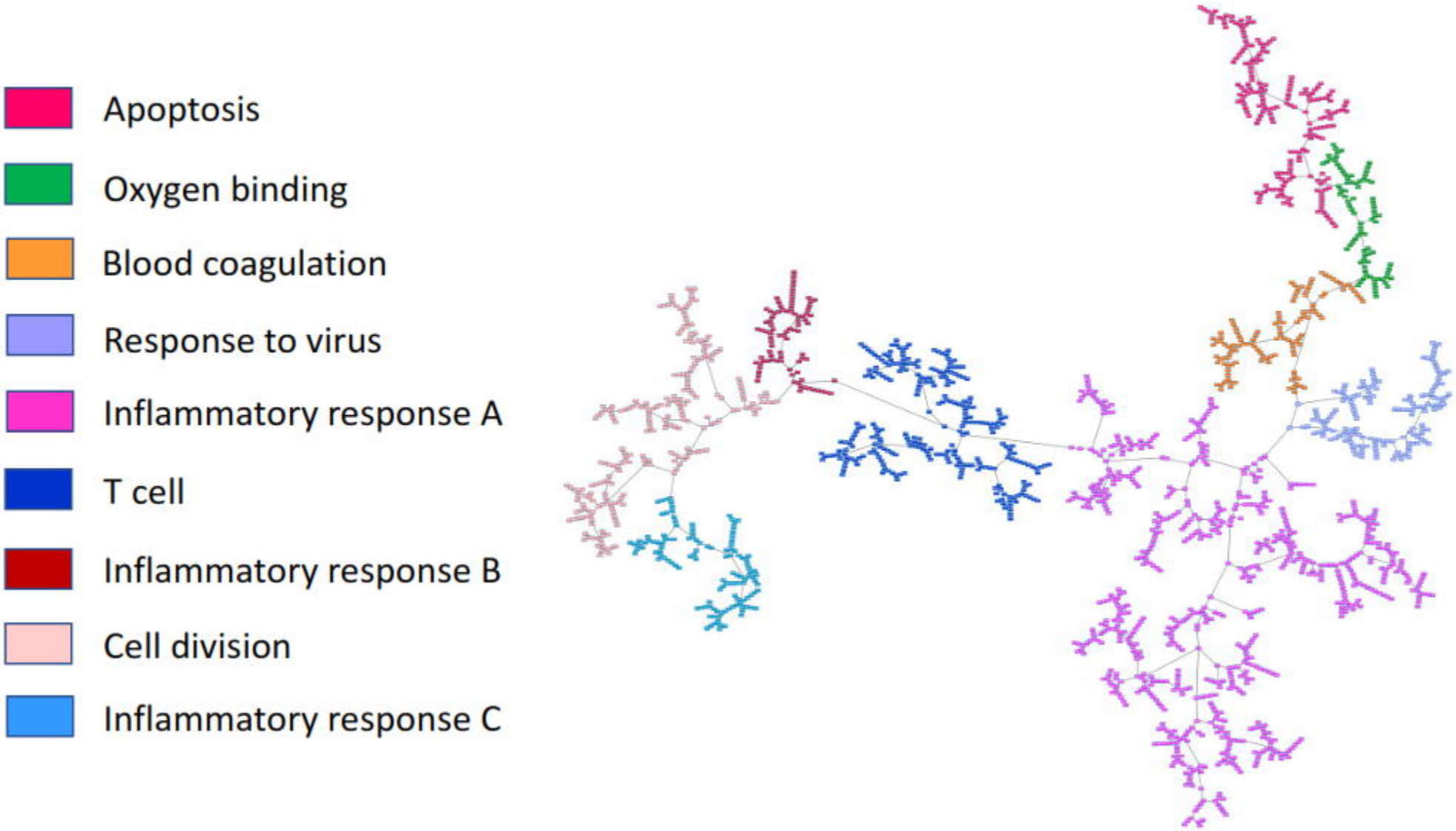

Lysosome
Activity (Arbitrary Units)
21
20
19
18
17
16
15
*
Healthy
Patients

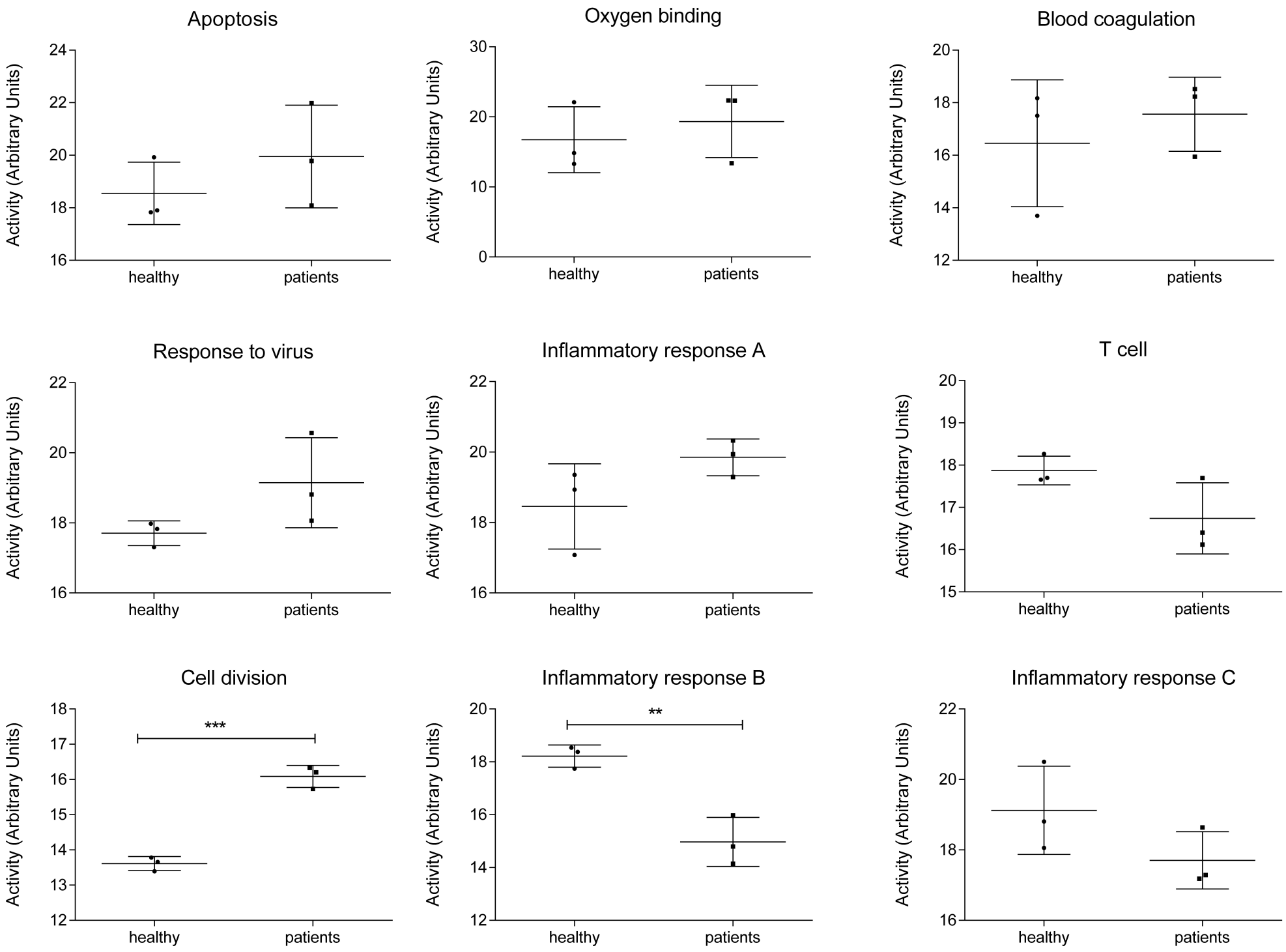

T cell
Activity (Arbitrary Units)
20
19
18
17
16
15
Healthy
Patients

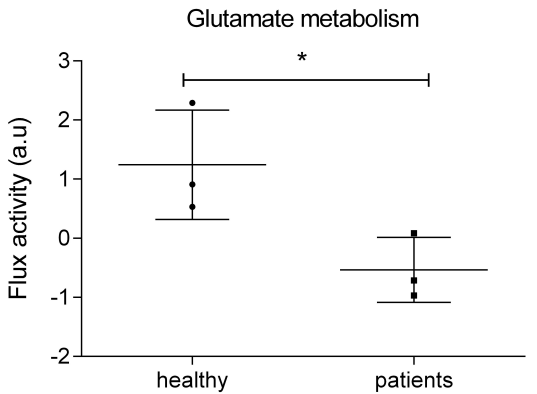

Response to virus A
Activity (Arbitrary Units)
21
20
19
18
17
16
15
Healthy
Patients

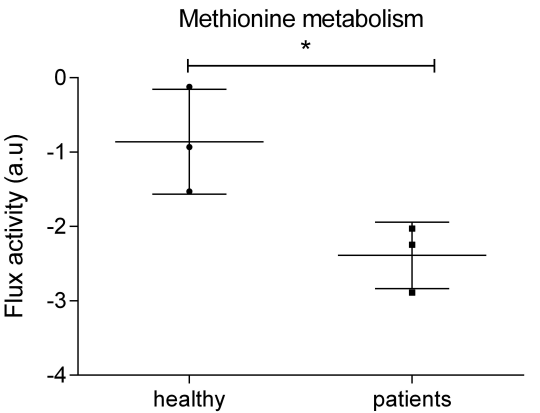

Metabolism
Activity (Arbitrary Units)
21
20
19
18
17
16
**
Healthy
Patients

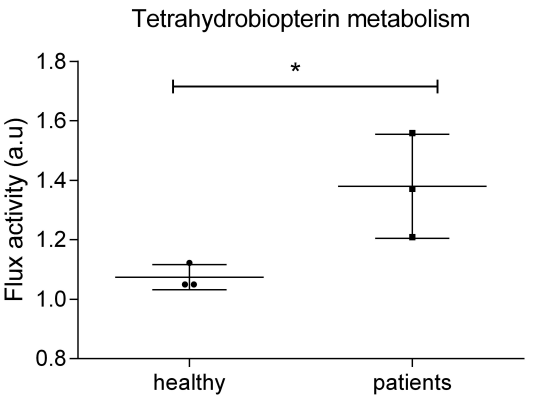

Response to virus B
Activity (Arbitrary Units)
21
20
19
18
17
Healthy
Patients

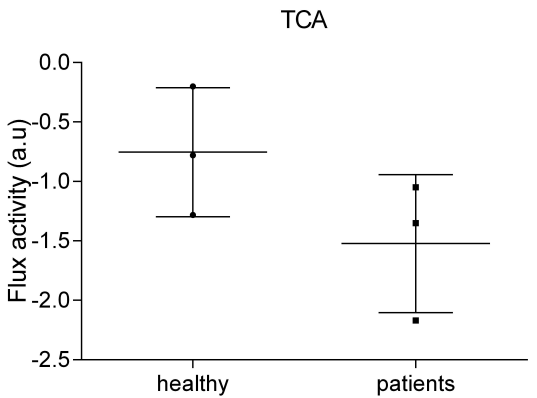

Apoptosis
Oxygen binding
Blood coagulation
Response to virus
Inflammatory response A
T cell
Inflammatory response B
Cell division
Inflammatory response C

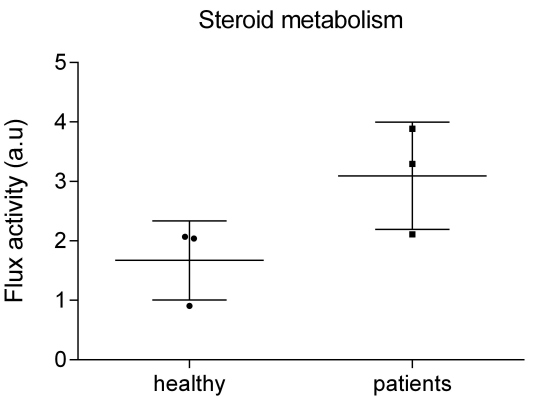

Apoptosis
Activity (Arbitrary Units)
24
22
20
18
16
healthy
patients
Oxygen binding
Activity (Arbitrary Units)
30
20
10
0
healthy
patients
Blood coagulation
Activity (Arbitrary Units)
20
18
16
14
12
healthy
patients
Response to virus
Activity (Arbitrary Units)
22
20
18
16
healthy
patients
Inflammatory response A
Activity (Arbitrary Units)
22
20
18
16
healthy
patients
T cell
Activity (Arbitrary Units)
20
19
18
17
16
15
healthy
patients
Cell division
Activity (Arbitrary Units)
18
17
16
15
14
13
12
***
healthy
patients
Inflammatory response B
Activity (Arbitrary Units)
20
18
16
14
12
**
healthy
patients
Inflammatory response C
Activity (Arbitrary Units)
22
20
18
16
healthy
patients


Glutamate metabolism
*
Flux activity (a.u)
3
2
1
0
-1
-2
healthy
patients


Methionine metabolism
*
Flux activity (a.u)
0
-1
-2
-3
-4
healthy
patients


Tetrahydrobiopterin metabolism
*
Flux activity (a.u)
1.8
1.6
1.4
1.2
1.0
0.8
healthy
patients


TCA
Flux activity (a.u)
0.0
-0.5
-1.0
-1.5
-2.0
-2.5
healthy
patients


Steroid metabolism
Flux activity (a.u)
5
4
3
2
1
0
healthy
patients