Gene Co-Expression Networks Offer New Perspectives on Sepsis Pathophysiology

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***Abstract*—Sepsis is among the most common causes of death in intensive care units. Septic shock is a type of circulatory shock that shows signs and symptoms that are similar to non-septic shock. Despite the impact of shock on patients and the economic burden, knowledge of the pathophysiology of septic shock is scarce. In this context, weighted gene co-expression network analysis can help to elucidate the molecular mechanisms of this condition. The gene expression dataset used in this study was downloaded from the Gene Expression Omnibus, which contains 80 patients with septic shock, 33 patients with non-septic shock, and 15 healthy controls. Our novel analysis revealed five gene modules specific for patients with septic shock and three specific gene modules for patients with non-septic shock. Interestingly, genes related to septic shock were mainly involved in the immune system and endothelial cells, while genes related to non-septic shock were primarily associated with endothelial cells. Together, the results revealed the specificity of the genes related to the immune system in septic shock. The novel approach developed here showed its potential to identify critical pathways for the occurrence and progression of these conditions while offering new treatment strategies and effective therapies.**

***Index Terms*—Biology and genetics, gene co-expression network analysis, sepsis.**

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I. Introduction

THE last definition of sepsis states that is an organ dysfunction caused by a dysregulated host response to infection [1]. This condition is one of the main healthcare problems in intensive care units (ICUs) [2] and represents a challenge for physicians due to its high mortality rate. Despite the advances in the care of patients, the incidence of sepsis has increased while, fortunately, the mortality rate has decreased [3]. In fact, a recent study has estimated around 31.5 million cases of sepsis worldwide, with 19.4 million cases being considered severe sepsis, and 5.3 million deaths annually [4]. Moreover, sepsis represents the first cause of mortality in non-coronary ICUs [5], [6], with a mortality rate of 38% in the case of septic shock in Europe and North America [7]. In addition to the negative impact of sepsis on patients, the economic burden of sepsis has been increasing over the last several years and represents a challenge for healthcare systems, with an increase in cost due to longer hospital stays. Supporting this, the average hospital cost per stay was estimated at $37424, $32421, $13292, and $24384 for Europe, the United States, Asia, and South America, respectively [8]. For these reasons, the World Health Organization recognizes sepsis as a global health priority [9].

Despite the significant health problem that sepsis represents and the advances made in understanding its pathophysiology in the last several years, knowledge about the dysregulation of the complex molecular signaling network in patients with sepsis and septic shock is scarce. Currently, one of the methods used to know the specific pathological state of sepsis is the analysis of gene expression patterns [10], [11], [12], [13], allowing the identification of diagnostic and prognostic gene

signatures, as well as novel therapeutic targets. However, this has not been enough to elucidate the molecular mechanisms of this condition. In this sense, weighted gene co-expression network analysis (WGCNA) applied to the gene expression values of patients with septic shock can help to uncover the underlying biological functions of genes and describe the huge and complex relationships in this condition. Thereby, the use of WGCNA could reveal relevant routes in the context of septic shock that, until now, have remained hidden. In recent years, an increasing number of works have successfully applied this method to discover the genes associated with various diseases, such as chronic kidney disease [14], cancer [15], [16], [17], asthma [18], and diabetes [19]. These precedents indicate that this method could be also used to identify key genes as novel candidate biomarkers or therapeutic targets in septic shock. Moreover, sepsis lacks a quick and accurate gold standard for its diagnosis, making it difficult to differentiate between septic and non-septic shock following surgery, conditions that show similar signs and symptoms [20]. Hence, this technique could allow us to identify specific and unique genetic patterns of septic shock, opening new doors for personalized treatment in this pathology.

Previous reports have revealed the gene co-expression patterns in sepsis [21], [22], [23], but these studies were focused on medical sepsis. Thus, the goal of the present study is to increase the knowledge about the correlation network in patients with septic shock and non-septic shock by the identification of specific gene clusters to understand the pathophysiology of septic shock (i.e., the correlation between genes of unknown function with biological processes or distinguish transcriptional regulatory programs). Therefore, the potential findings of this study can help advance the understanding of septic shock and non-septic shock transcriptomes and provide novel therapeutic targets.

II. Methods

A. The Gene Expression Omnibus Dataset

A microarray dataset with accession number GSE131761 was obtained from the Gene Expression Omnibus (GEO) public database. This dataset includes 129 samples comprising 15 healthy controls, 80 patients with septic shock, and 33 patients with non-septic shock. Pre-processing was performed as previously described by Martínez-Paz et al. [10]. Briefly, dataset files were imported into the Bioconductor R package ecosystem and were normal-exponential background corrected. Normalization was performed by the quantile method, and gene expression values were calculated using the *lmFit* function.

B. Network Analysis

After pre-processing, a correlation network was generated independently for each group by WGCNA. This method is a data mining method based on representing each gene as nodes and pairwise correlations between them as network links [24]. Pearson's rank correlations were used to recurrently assess the relationship between all pairs of nodes using the Matlab 'Statistics and Machine Learning Toolbox'. Due to the large size of the resulting networks (more than 500 million connections), Cohen's threshold for large correlations [25] or very strong correlations [26] was applied. Thus, two weighted networks per group were generated, one representing high positive correlations ($R>0.8$) and the other with high negative correlations ($R<-0.8$). Before thresholding, the generated correlation networks had the same number of nodes, only differing in the value of each correlation. However, after thresholding, each network can show a different number of connections.

To assess the specific genes involved in septic shock, we were interested in analyzing the group-specific strong relationships, that is, those correlations between specific genes above the threshold (0.8) in the septic shock group but not in the non-septic shock group and vice versa. These networks are called differential networks. For this purpose, we developed a novel approach consisting of obtaining the characteristic and specific gene expression patterns of each group. Thus, after applying the threshold $|R|>0.8$, the networks were binarized, that is, a value of 1 was assigned to those connections higher than the threshold and a value of 0 to the rest. Finally, the shared links between the groups were removed. In this way, new networks consisting of non-shared links were obtained. These networks only show specific strong connections (above 0.8) from each group, allowing analysis of the particular patterns of each group.

To increase the robustness of the results while statistically comparing the properties of the networks, a previously validated bootstrap procedure [27], [28] was applied for the first time in genetic data. Thereby, for each group, 100 random selections of 33 subjects with possible repetition (the number of subjects in the group of patients with non-septic shock, i.e., the more restrictive of the two groups) were used to generate the networks. Seven complementary graph parameters derived from Complex Network Theory were then calculated on each resulting network, including the number of links, node degree, characteristic path length, diameter, average clustering coefficient, modularity, and eigenvector centrality [29], [30], [31], [32].

Finally, Gephi software (version 0.9.2) was used for network visualization [33]. Depending on the nature of the networks, two different force-based algorithms were used. The ForceAtlas2 algorithm [34] was applied to the weighted networks, which considers both the distance and the node degree of the connected nodes. On the other hand, the Fruchterman-Rheingold algorithm [35] was used to represent the binary networks, which uses custom forces of attraction and repulsion, depending only on the distance between the connected nodes. Despite the non-deterministic nature of these methods, they usually reach stable stages (as in the case of our networks) and have the advantage of turning structural proximities into visual proximities. Thereby, genetic communities or clusters emerge spatially separated, providing information about hidden genetic structures [36].

C. Pathway Enrichment Analysis

Pathway enrichment analysis identifies biological pathways that are enriched in a gene list more than would be expected by chance. Analysis was developed using g:Profiler [37], a

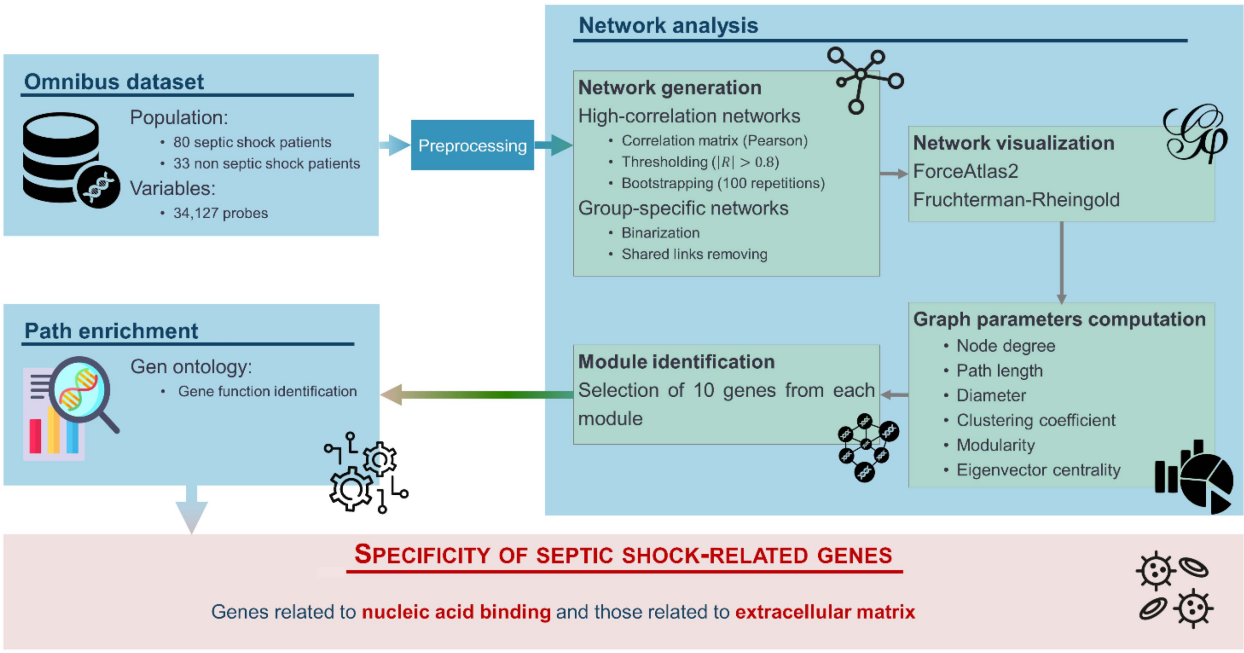

Fig. 1. Study workflow. Path enrichment after correlation network analysis identified genes particularly relevant in septic shock.

TABLE I
GRAPH-THEORY PARAMETERS ASSOCIATED WITH THE HIGH-CORRELATION NETWORKS (POSITIVE AND NEGATIVE) FOR EACH GROUP

Feature	Graph parameter	High positive correlation networks			High negative correlation networks		
		Septic shock	Non-septic shock	*p*-value	Septic shock	Non-septic shock	*p*-value
Basic	Number of links	502,031	616,045	<0.05	531	28,783	<0.05
	Average node degree	29.421	36.103	<0.05	2.855	8.489	<0.05
Integration	Characteristic path length	8.060	7.116	<0.05	3.131	5.651	<0.05
	Diameter	24	24	N.S.	8	20	<0.05
Segregation	Average clustering coefficient	0.570	0.422	N.S.	0.000	0.000	N.S.
	Modularity	0.257	0.349	<0.05	0.527	0.563	N.S.
Centrality	Eigenvector centrality	0.030	0.051	<0.05	0.007	0.074	<0.05

N.S.: Non-significant

database for annotation, visualization, and integrated discovery (DAVID) [38], and protein annotation through evolutionary relationship (PANTHER) [39]. These techniques search a collection of gene sets representing Gene Ontology (GO) terms, pathways, networks, regulatory motifs, and disease phenotypes. Pathway enrichment methods use Fisher's exact test or binomial test, with Bonferroni correction for multiple testing, by considering all annotated protein-coding genes as background genes for comparison purposes. The general study design is summarized in Fig. 1.

III. RESULTS

A total of 113 patients from the Gene Expression Omnibus database (GSE131761) were included in the current study, of which 80 patients had septic shock and 33 patients had non-septic shock. The clinical characteristics of the postsurgical patients that were enrolled have been described previously [10].

A. Consensus Network Construction and Module Detection

In this work, we applied WGCNA using 34127 probes from the microarrays of 133 patients to construct the gene modules from the matrix of gene expression values. The first step of the present study was to analyze the correlation structure in postsurgical patients with septic shock and non-septic shock, to evaluate the behavior of gene clusters and identify changes in gene-to-gene interactions that can be associated with these conditions. Table I shows the graph-theory-related parameters of the high positive and high negative weighted correlation networks considering all the possible pairs of nodes. The focus was on different complementary characteristics, including basic features, integration, segregation, and centrality, to compressively characterize the networks. Two basic features of the network were provided. First, the number of links in the network indicates the number of correlations higher than 0.8 for high positive networks or lower than −0.8 for high negative networks. The

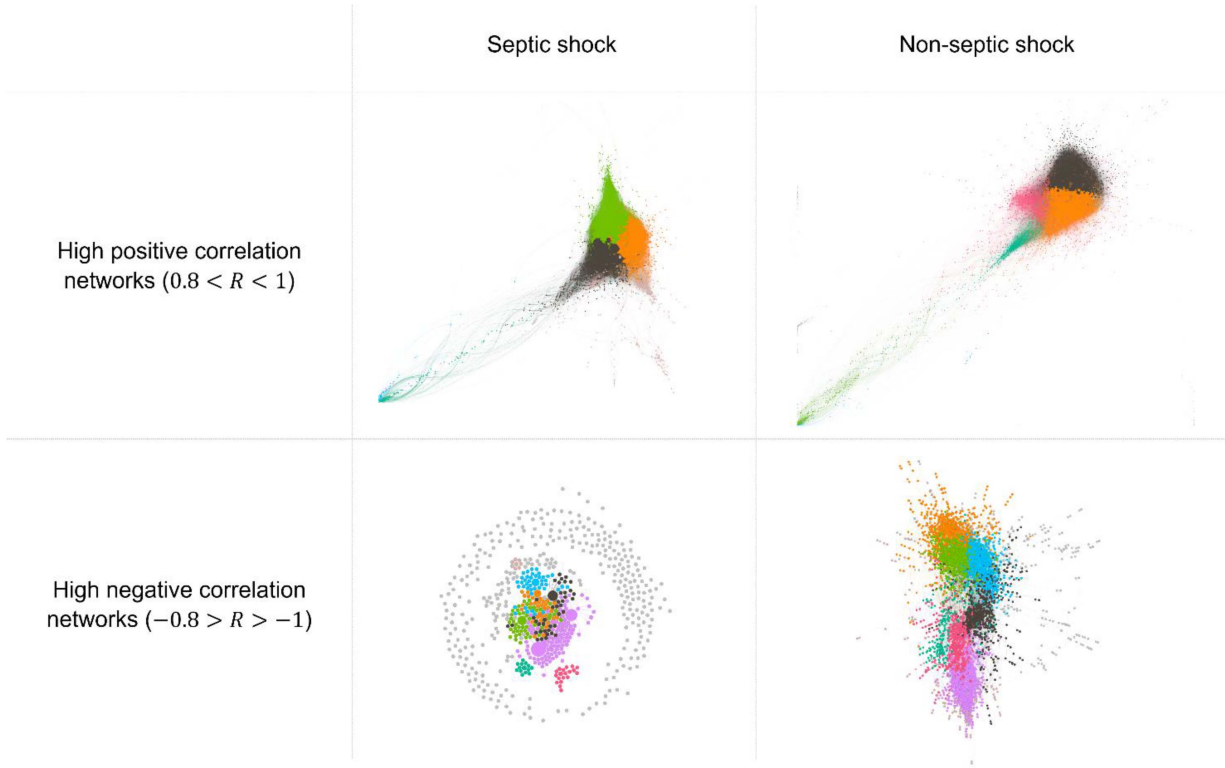

Fig. 2. High positive and high negative correlation networks for patients with septic shock and non-septic shock. The color of each node represents the membership of a specific module obtained by applying Blondel's modularity [32]. The arrangement of the nodes follows the ForceAtlas2 algorithm [34], which is based on attractive and repulsive forces between nodes, appropriate for weighted networks. In this way, the nodes (and modules) with the greatest relationship between them tend to appear spatially close.

node degree also was calculated, which provides information on the connectedness of the considered gene by adding all the correlations that start from that node in a single index [29]. Thus, the average node degree summarizes the density of the network. The integration measures give an estimate of the degree of compactness of the network. Here, the characteristic path length and the diameter were reported. While the characteristic path length is the average shortest path length between all pairs of nodes [30], the diameter is the shortest distance between the two most distant nodes in the network [24]. On the other hand, network segregation is the capability of the network to be divided into different units with high intra-unit connectivity. The average clustering coefficient [31] and Blondel's modularity [32] were used for this work. The average clustering coefficient measures the presence of clusters inside the network by computing the ratio between the existing triangles and the total number of triangles that could exist. The modularity index provides information on how different modules inside the network are separated from each other in terms of correlations. Finally, the centrality of a node provides an estimate of the degree of relevance of that node within that network. In this context, if a node is very relevant (usually named 'hub'), it means that it is well connected and, therefore, many paths pass through it. The degree of centrality of the network gives an idea of its global topology. In particular, the eigenvector centrality [40] measures the average influence of all the nodes, that is, its connectedness to other important/highly connected genes.

The results obtained from this analysis show that patients with non-septic shock presented with larger high-correlation networks as indexed by the number of links, both positive and negative (Table I). In addition, the overall connectivity of the network is diminished in septic shock, meaning a lower global relationship between gene expressions. This is particularly noticeable in the negative correlation network. Differences between networks are also evident regarding network integration and segregation. Finally, higher degrees of centrality are shown by non-septic shock, supporting the lower number of hubs (highly connected nodes) in them. In summary, the high positive correlation network presented a higher size and connectedness in patients with non-septic shock. Similarly, when the high negative correlation network was analyzed, these parameters were higher in patients with non-septic shock. These results are depicted in Fig. 2, where the structure of these correlation networks and the presence of different clusters are shown, allowing the possibility to characterize and inspect the differences between these kinds of postsurgical patients.

B. Septic Shock and Non-Septic Shock Network Analyses Without Shared Links

To analyze the specific relations between genes particularized for each group, new correlation networks, called differential networks, were performed by removing the non-shared links between groups and binarizing the resulting weighted networks.

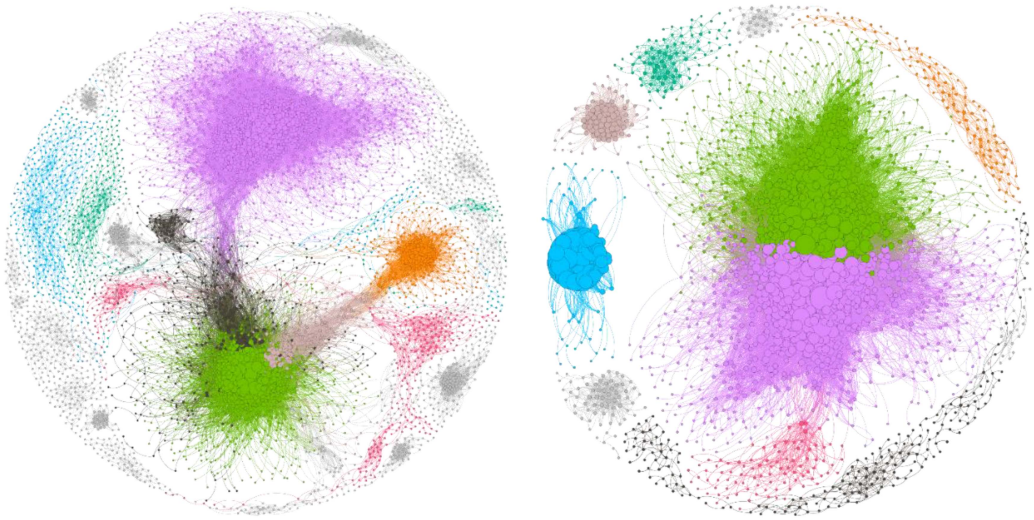

Fig. 3. Correlation networks (|R|>0.8) after removing shared links in patients with septic shock (left) and non-septic shock (right). The color of each node represents the membership of a specific module obtained by applying Blondel's modularity [32]. The arrangement of the nodes follows the Fruchterman-Reingold [35] algorithm, which is based on attractive and repulsive forces between nodes, appropriate for binary networks. The first goal of this study was to evaluate the behavior of gene clusters and identify changes in gene-to-gene interactions that can be associated with postsurgical patients with septic shock and non-septic shock through gene correlation networks. Non-septic patients presented a higher number of positive and negative links, connectivity, and centrality than septic patients. These results show marked genomic differences between both patient groups. However, to reinforce the hypothesis of a differentiated genetic signature in postsurgical patients with shock, a new network analysis was performed without shared links. In this case, the septic shock network presents a higher degree of specificity for its correlations. Therefore, the comparison between the networks with shared and non-shared links shows that the correlation network of patients with non-septic shock was significantly changed after the exclusion of shared links.

TABLE II
GRAPH-THEORY PARAMETERS ASSOCIATED WITH THE DIFFERENTIAL CORE NETWORK

Feature	Graph parameter	Septic shock	Non-septic shock
Basic	Number of links	259,222	106,437
	Average node degree	15.192	6.238
Integration	Characteristic path length	5.772	3.180
	Diameter	31	24
Segregation	Average clustering coefficient	0.464	0.473
	Modularity	0.423	0.570
Centrality	Eigenvector centrality	0.078	0.396

N.S.: Non-significant

Thus, these networks consisted of a variety of links representing high correlations (|R|>0.8) that only appear in one group of patients but not in the other. The visual representation of the new correlation networks of patients with shock after removing the shared links confirms the existence of a reduced number of well-defined and separated clusters in each condition (Fig. 3). Concerning these correlations, the septic shock network has a higher number of links, average node degree, path length, and diameter when compared with the non-septic shock network, showing a high degree of specificity with the septic shock network (Table II).

C. Cluster Analysis

Cluster analysis of septic shock and non-septic shock-specific modules allows us to study their characteristics and identify the differences between both groups.

A GO enrichment analysis on the genes in modules was first performed. The main processes obtained in this analysis were related to nucleic acid binding and the extracellular matrix (see Table S1 in the Supplementary Material for details, available online).

In addition to the previous analyses, genes with the highest node degree for each cluster from these non-shared networks were assessed (Fig. 3). The shortlist of the top 10 genes in each cluster is shown in Table III. From the patients with septic shock, the genes with higher node degree for the purple, green, blue, gray, and orange modules were IGLV5-48, COLEC10, MICU2, NDNF, and ST7-OT4, respectively; for non-septic shock, the genes with highest node degree for the purple and green modules were FLJ36000 and AGGF1, respectively.

IV. DISCUSSION

Most previous studies have focused on the transcriptional profiling of sepsis and septic shock using microarrays to identify biomarker candidate genes [10], [11], [12], [13]. However, while there are previous reports that have analyzed gene co-expression

TABLE III
GENES WITH THE HIGHEST NODE DEGREE IN THE DIFFERENTIAL NETWORK

Septic shock					Non-septic shock		
Purple	Green	Blue	Gray	Orange	Purple	Green	Blue
IGLV5-48	*COLEC10*	*MICU2*	*NDNF*	*ST7-OT4*	*FLJ36000*	*AGGF1*	A_33_P3875570
HSPG2	*KATNBL1P6*	*CCAR1*	*APOL5*	*ETNK2*	*TNXB*	*PRRT1*	*C7orf65*
SAMD11	*WBP2NL*	*USP1*	*IGF2BP1*	*SCN10A*	*ZBTB3*	A_33_P3289456	A_33_P3278211
A_33_P3209321	*FAM154B*	*RDH14*	A_33_P3380783	A_33_P3312034	*FBXL17*	A_33_P3376026	*SLC18A1*
GPR25	*TMEM207*	*GOPC*	A_33_P3375299	*CCT7P2*	A_33_P3222664	A_33_P3300877	*SP5*
CDKN2A	A_33_P3555368	*TDG*	*IL26*	*GPX8*	*ATP6V1G2*	*RIMBP2*	A_33_P3420347
SLC22A11	*ADH1B*	*ATF1*	*TMCO5B*	*LGALS14*	A_24_P170726	*TEAD1*	A_33_P3383292
CELA2B	A_33_P3223059	*ZNF721*	A_33_P3266609	*RGS13*	A_33_P3306802	*AAK1*	*CCDC40*
MUC3A	*TTPA*	*FNTA*	*ACTRT2*	*NUTM1*	*BCL2L15*	A_33_P3713035	*AK127999 / KCNIP4*
TNFRSF14	*BSN*	A_32_P45493	A_33_P3392213	*PM20D1*	*CEP104*	*CHD1L*	*DEFB136*

patterns in sepsis [21], [22], [23], there are no works about the scenario involving septic shock vs. non-septic shock to assess their pattern specificity. Moreover, compared with the classical analysis of transcriptional profiles, the study of gene networks-based methods allows one to gain insight into the pathophysiology of both septic shock and non-septic shock, as well as global biology activity, considering that both conditions show similar signs and symptoms [20].

The differences between specific modules with regard to patients with septic shock and non-septic shock open the possibility to study the particular characteristics of those clusters and to identify the arising differences between them. Related to both conditions, the GO results show that the most prominent processes in the detected modules are related to nucleic acid binding and the extracellular matrix. However, previous reports show that the pathways involved in septic shock and non-septic shock were mainly related to the immune system, inflammatory processes, or endothelial barriers [41], [42], [43], [44], [45]. Despite inconsistency in the module's annotation, these differences could be due to the way that genes were obtained for the analysis. While the previous works explored the GO using the differentially expressed genes, in the present study, the analyzed genes were obtained from correlation networks. Particularly, genes were selected as those with a higher node degree in each module (i.e., genes highly correlated with genes of the same module). These results could indicate the existence of crossover effects that could have been hidden by the classical analysis and revealed for the first time. In this sense, it has been reported the modulation of the extracellular matrix into the immune cell function [46] and how the composition of the extracellular matrix undergoes changes during infections [47]. Regarding the nucleic acid binding pathways obtained, previous studies have shown that nucleic acid binding proteins are associated with poor prognosis in septic patients [48] and are required for interferon production in response to viral infection [49]. Moreover, bacterial and viral nucleic acids can act as inductors of inflammation [50]. Thus, the present routes reported in this work have received less attention than other pathways in the infection processes and can offer a new research line in the pathophysiology of sepsis.

In this study, WGCNA was used with a bootstrapping procedure to analyze, in a robust way, those genes with higher node degrees for each cluster from differential networks. Genes IGLV5-48, COLEC10, MICU2, NDNF, and ST7-OT4 presented the most node degree for septic shock. IGLV5-48 encodes for immunoglobulin lambda variable 5-48, involved in the immune response; however, its function and molecular mechanism are not clear [51]. In addition, the gene expression of members of the IGLV family was upregulated in patients with cardiogenic shock and septic shock [45]. COLEC10 encodes for a protein C-lectin family member, a collectin subfamily member, with one of its functions being binding to antigens on microorganisms facilitating their recognition and removal. It has been reported that vascular endothelial cells have receptors for collectins [52]. As a result, these cells play a major role in the systemic response to bacterial infections [53]. In addition, the protein encoded by this gene activates the complement system [54]. MICU2 encodes for a transporter protein called mitochondrial calcium uptake 2. It has been reported its upregulated expression in cells in vitro after infection with Salmonella enterica Serovar Typhimurium [55].

Further, mitochondrial calcium uptake 2 plays an important role in the regulation of the Pseudomonas aeruginosa-dependent inflammatory response [56]. This protein has been associated

with the induction of autophagy and apoptotic cell death in endothelial cells in response to oxygen-glucose deprivation [57]. NDNF encodes for neuron-derived neurotrophic factor, which is secreted in cultured endothelial cells stimulated by hypoxia, promotes endothelial cell survival and vessel formation, and plays an important role in the process of revascularization [58]. ST7-OT4 encodes for a long non-coding RNA whose expression is upregulated in cardiac CCR2- macrophages [59]. Altogether, the node genes fit with the pathophysiology of sepsis, where this condition is defined as organ dysfunction caused by a host response to infection [1]. In this sense, the endothelial cells play a central role in the systemic response to bacterial infection, leading to multiorgan failure syndrome [53], [60]. Moreover, these node genes are involved in the immune system, which is consistent with the key role of this system in sepsis and with previous studies that suggest that this condition is accompanied by overall immune dysregulation [10], [61], [62], [63]. Regarding patients with non-septic shock, the most node degree genes for the purple and green modules were FLJ36000 and AGGF1. In the case of the blue module, the most degree gene corresponded with an uncharacterized DNA sequence. FLJ36000 is a lncRNA, while AGGF1 encodes an angiogenic factor that acts as an anti-inflammatory factor by suppressing endothelial activation responses to TNF-α [64]. On the other hand, a brief description of the role of the top 10 genes of each cluster is described in Table S1 (Supplementary Material, available online). Overall, these results can help to identify new gene signatures that help to understand the pathophysiology of septic shock and non-septic shock. However, the 10 genes with the highest node degree for each cluster from these non-shared networks were analyzed, aiming to find their relation to septic shock and non-septic shock. As shown in Table S1, available online, these genes maintained a relationship with each condition. While septic shock genes are mostly involved in inflammatory processes, the immune system, and endothelial cells, non-septic shock genes are mainly related to endothelial cells.

This work presents limitations that we must acknowledge. First, no distinction of different subgroups within non-septic shock was made. This would allow an even more specific study of the patterns within this heterogeneous group. However, due to the sample size of the database, this would also reduce the statistical power of studying each group separately. Second, it is a single-center study; therefore, a multi-center study would provide possible inter-hospital variation, providing more robustness to the results. Third, a compatible database with characteristics similar to the one reported here was not found. This would have made it possible to validate the results, which would help to reinforce their robustness. However, for that reason, a bootstrap-based approach with 100 iterations was used. Since each iteration produces different networks, the results found here are more robust and generalizable than with a classical approach. The last limitation is about the nature of the samples, where the peripheral blood provides the gene expression patterns of white blood cells and offers mainly insight into immune pathways. Thus, future works should keep in mind the kind of shock to analyze the relationship with its specific gene expression patterns and analyze its mRNA level in the endothelial cells, which appear to be the target tissue for these conditions.

V. Conclusion

The present study identified novel genetic modules from correlation networks associated with septic shock and non-septic shock in post-surgical patients using gene co-expression network analysis. This was achieved by using a novel procedure that combines correlation networks, differential networks, and a bootstrap procedure to increase the robustness of the results. Of each module, the most representative genes in septic shock were mainly related to the immune system and endothelial cells, while genes encoding aspects related to endothelial cells were the most representative for non-septic shock. This novel way of selecting the most relevant genes could provide new pathways that might have remained hidden until now. Therefore, these results offer new insight into patients with shock to promote the identification of critical pathways and provide new treatment strategies in future clinical studies.

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