


Meta-Analysis

Network Meta-Analysis on the Mechanisms Underlying Alcohol Augmentation of COVID-19 Pathologies

Wenfei Huang, Heping Zhou, Colin Hodgkinson, Angelo Montero, David Goldman, and
Sulie L. Chang 

Background: The coronavirus disease 2019 (COVID-19) pandemic is a worldwide crisis caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). Many COVID-19 patients present with fever in the early phase, with some progressing to a hyperinflammatory phase. Ethanol (EtOH) exposure may lead to systemic inflammation. Network meta-analysis was conducted to examine possible relationships between EtOH consumption and COVID-19 pathologies.

Methods: Molecules affected by EtOH exposure were identified by analysis with QIAGEN Knowledge Base. Molecules affected by COVID-19 were identified from studies in MEDLINE, bioRxiv, and medRxiv reporting gene expression profiles in COVID-19 patients, QIAGEN Coronavirus Network Explorer, and analysis of the RNA-sequencing data of autopsied lungs of COVID-19 patients retrieved from the GEO database. Network meta-analysis was then conducted on these molecules using QIAGEN Ingenuity Pathway Analysis (IPA).

Results: Twenty-eight studies reporting significant gene expression changes in COVID-19 patients were identified. One RNA-sequencing dataset on autopsied lungs of COVID-19 patients was retrieved from GEO. Our network meta-analysis suggests that EtOH exposure may augment the effects of SARS-CoV-2 infection on hepatic fibrosis signaling pathway, cellular metabolism and homeostasis, inflammation, and neuroinflammation. EtOH may also enhance the activity of key mediators including cytokines, such as IL-1 β , IL-6, and TNF, and transcription factors, such as JUN and STAT, while inhibiting the activity of anti-inflammatory mediators including glucocorticoid receptor. Furthermore, IL-1 β , IL-6, TNF, JUN, and STAT were mapped to 10 pathways predicted to associate with SARS-CoV-2 proteins, including HMGB1, IL-1, and IL-6 signaling pathways.

Conclusions: Our meta-analyses demonstrate that EtOH exposure may augment SARS-CoV-2-induced inflammation by altering the activity of key inflammatory mediators. Our findings suggest that it is important for clinicians to caution patients about the risk of alcohol consumption, which has increased during the COVID-19 pandemic. The findings also call for further investigation into how alcohol exposure affects viral infections.

Key Words: Ethanol, Ingenuity Pathway Analysis, Inflammation, Cytokine, COVID-19.

THE CORONAVIRUS DISEASE 2019 (COVID-19) pandemic is a serious global health threat caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2). Significant efforts have been undertaken from around the world to study SARS-CoV-2, characterize COVID-19, examine the mechanisms by which SARS-CoV-

2 causes COVID-19, and identify the factors that influence disease severity. COVID-19 is featured by fever and dry cough along with elevated levels of cytokines such as interleukin (IL)-1 β , IL-6, and tumor necrosis factor (TNF)- α (Huang et al., 2020). In severe COVID-19 cases, SARS-CoV-2 infection triggers intensive immune responses with high levels of inflammatory cytokines in blood plasma (Tay et al., 2020; Vardhana and Wolchok, 2020).

Alcohol is the most widely used addictive substance in the world. Chronic alcohol abuse that becomes severe is called "alcohol use disorder" (AUD) (NIAAA, 2020a). This chronic relapsing brain disease is characterized by compulsive alcohol use, loss of control over alcohol intake, and a negative emotional state when not drinking (NIAAA, 2020a). According to the 2019 National Survey on Drug Use and Health (NSDUH), 14.1 million adults aged 18 and older and 414,000 adolescents aged 12 to 17 in the United States have AUD (NIAAA, 2020b; SAMHSA, 2019). Alcohol consumption has been reported to increase the risk of acquired

From the Institute of Neuroimmune Pharmacology (WH, AM, SLC), Seton Hall University, South Orange, New Jersey; Department of Biological Sciences (WH, HZ, AM, SLC), Seton Hall University, South Orange, New Jersey; and Laboratory of Neurogenetics (CH, DG), NIAAA, NIH, Rockville, Maryland.

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Correspondence: Sulie L. Chang, PhD, Institute of Neuroimmune Pharmacology, Seton Hall University, 400 South Orange Avenue, South Orange, NJ; E-mail: Sulie.Chang@shu.edu

Huang and Zhou equally contributed to this study.

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community pneumonia (Simou et al., 2018). Alcohol use and abuse has been found to induce gut leakage, intestinal inflammation, and increased entry of bacteria and/or bacterial endotoxins into the circulation (Patel et al., 2015; Wang et al., 2005), promoting systemic inflammation with elevated levels of cytokines (Patel et al., 2015), which may further cause organ dysfunctions throughout the body, including chronic liver disease, neurological pathologies, and behavioral disorders (Bishehsari et al., 2017). We therefore hypothesized that alcohol may augment the inflammatory response to SARS-CoV-2 infection and that EtOH may worsen the severity of COVID-19 outcome through modulation of the inflammatory response in COVID-19 patients.

Sales of alcoholic beverages in the United States have increased by 55% compared with this time last year (BREMER, 2020), suggesting that the COVID-19 pandemic may have led to significantly increased alcohol consumption. This study conducted network meta-analysis using QIAGEN Ingenuity Pathway Analysis (IPA) to investigate the effects of alcohol in the context of COVID-19. IPA is a bioinformatics tool that uses computational algorithms to analyze the functional connectivity of genes through QIAGEN Knowledge Base (QKB), which is a comprehensive repository for manually curated information identified from over 7 million individually modeled relationships among diseases, drugs, biological entities including genes, proteins, and metabolites, biological processes including expression, molecular cleavage, and phosphorylation, and published results from omics experiments. Molecules affected by EtOH exposure were obtained from QKB. Molecules affected by COVID-19 were obtained from literature search of MEDLINE, bioRxiv, and medRxiv for studies reporting gene expression profiles in COVID-19 patients, genes differentially expressed in autopsied lungs of COVID-19 patients via RNA-sequencing analysis retrieved from Gene Expression Omnibus (GEO), and QIAGEN Coronavirus Network Explorer (QCNE) that consists of 69 biological networks generated to illustrate how SARS-CoV-2 might affect its host cells. Network meta-analysis using IPA was then performed to identify biological processes and key inflammatory mediators affected by both EtOH and COVID-19. IPA was also performed to identify how EtOH acted on these inflammatory mediators and how these inflammatory mediators impacted the pathways affected by SARS-CoV-2 proteins. Our network meta-analysis identified key inflammatory pathways common to EtOH and COVID-19 and provided potential mechanisms by which EtOH may impact the inflammatory response in COVID-19 patients.

MATERIALS AND METHODS

Ingenuity Pathway Analysis Software

The IPA Analysis Match CL license was purchased from QIAGEN (QIAGEN, Germantown, MD; QIAGEN Inc., <https://www.QIAGENbioinformatics.com/products>). IPA is a bioinformatics tool that uses computational algorithms to analyze the functional

connectivity of molecules building on QIAGEN Knowledge Base (QKB), a horizontally and vertically structured repository database composed of over 7 million individually modeled relationships between diseases, drugs, biological entities, such as genes, proteins, and metabolites, processes, such as expression, molecular cleavage, and phosphorylation, and the published results of omics experiments, such as increased or decreased expression. The information is manually curated and extracted primarily from scientific literature including journal articles, publicly available molecular content databases, and textbooks over the past 2 decades. The information used in this study was retrieved from QKB from June 15, 2020, to August 12, 2020.

Identification of Molecules Impacted by EtOH from QKB

Using IPA's "Build"- "Grow" tool, molecules, including genes, proteins, and complexes that exist in organisms in nature, affected by EtOH were obtained from QKB.

Collection of Molecules Impacted by COVID-19 Infection

Molecules affected by COVID-19 were identified from 3 different sources as described below. First, literature search of MEDLINE via PubMed and of bioRxiv and medRxiv via COVID-19 on COVID-19 patients was conducted before June 30, 2020. A total of 27,565 articles including possible duplicates among MEDLINE, bioRxiv, and medRxiv were identified. Among these articles, there were 218 articles reporting on gene expressions in COVID-19 patients with 28 studies profiling gene expression changes in COVID-19 patients and these 28 studies were included in our study (Fig. 1A). These 28 articles included 7 transcriptomic studies, one proteomic and metabolomic study, 6 studies on risk factors and treatment, 7 studies on clinical characterization, and 7 studies regarding molecular mechanisms, biomarker identification, and various perspectives on COVID-19. The list of these articles is provided in the Appendix S1. Molecules with reported significant expression level changes in COVID-19 ($p < 0.05$) were designated as molecules affected by COVID-19 via literature (Fig. 1A) and added to a pathway in IPA using its "Build"- "Add molecules/relationship" tools. Second, dataset search was performed in Gene Expression Omnibus (GEO) database for studies on COVID-19 before June 30, 2020, and 16 studies were identified. Among these studies, 3 studies were conducted in nonhuman species and therefore excluded, and 12 studies were *in vitro* studies and therefore excluded. One study was an *in vivo* study performed on the autopsied lungs from COVID-19 patients and therefore included in our study (Fig. 1A). Fastq RNA-sequencing data files of autopsied lungs from COVID-19 patients and COVID-19-negative controls were retrieved from GEO (Accession ID: GSE150316) and analyzed using QIAGEN CLC Workbench (QIAGEN, Germantown, MD). The standard RNA-seq analysis tools, "PCA for RNA-seq" and "Differential Expression in Two Groups," under "RNA-seq and small RNA analysis" group in CLC Workbench were used to identify genes differentially expressed in autopsied COVID-19 lungs as compared to COVID-19-negative lungs (Fig. 1B). These molecules were designated as molecules affected by COVID-19 via RNA-seq (Fig. 1A). Third, molecules affected by COVID-19 were identified by exploring the 69 networks (14 canonical pathways, 36 functions, and 19 diseases) on QIAGEN Coronavirus Network Explorer (QCNE) [QIAGEN, Germantown, MD]. QCNE was built on curated relationships among molecules in QKB, and the human host proteins were identified to interact with SARS-CoV-2 viral proteins through affinity purification-mass spectrometry screening by Gordon and colleagues (2020). Duplicate molecules were removed from the collected 50,290 molecules. The remaining 10,733 nonduplicate molecules were designated as molecules affected by COVID-19 via QCNE (Fig. 1A).

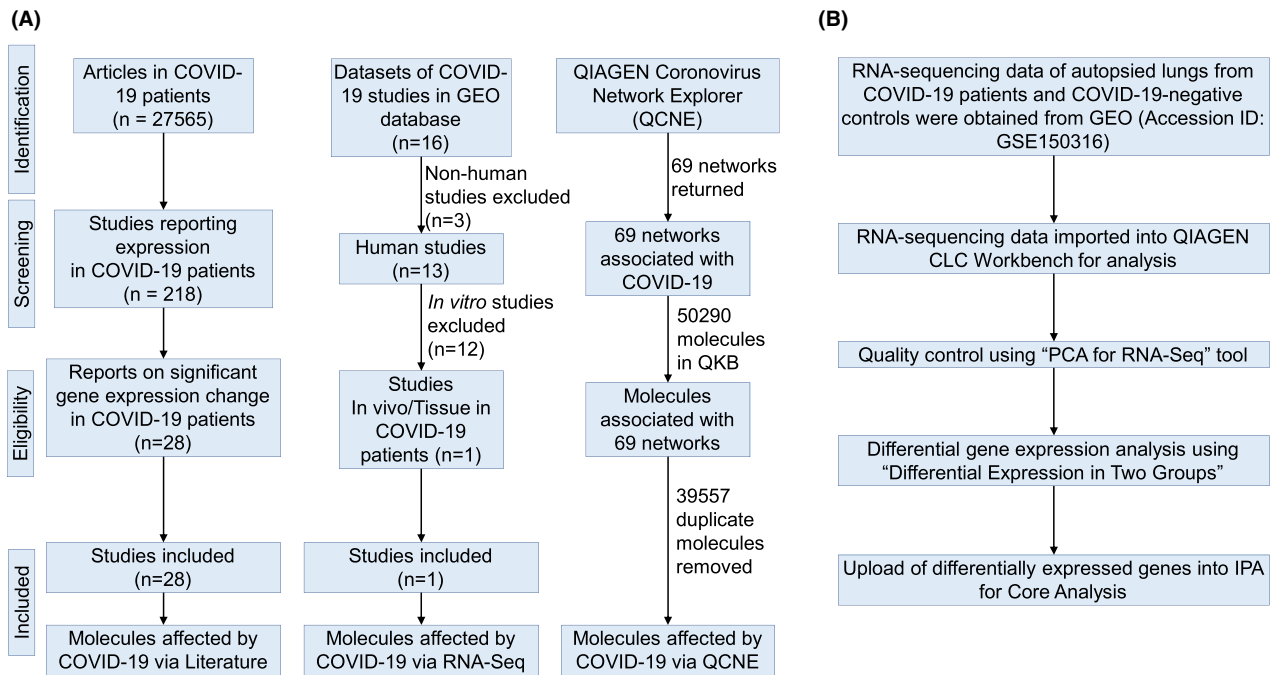


Fig. 1. Workflow to identify molecules affected by COVID-19 via literature, RNA-seq, and QCNE (A) and the workflow to identify genes differentially expressed in the autopsied lungs of COVID-19 patients as compared to COVID-19-negative controls via RNA-sequencing data analysis (B).

Overlapping of Molecules Affected by COVID-19 With Those Involved in Inflammatory Response

Molecules involved in inflammatory response were identified using "Grow"-"Functions and Diseases" tool in the following sets of molecules: (i) molecules affected by EtOH; (ii) molecules affected by EtOH and COVID-19 via literature; (iii) molecules affected by COVID-19 via literature but not affected by EtOH; (iv) molecules affected by EtOH and COVID-19 via RNA-seq; (v) molecules affected by COVID-19 via RNA-seq but not affected by EtOH; (vi) molecules affected by EtOH and COVID-19 via QCNE; and (vii) molecules affected by COVID-19 via QCNE but not affected by EtOH. These sets of molecules were mapped in pathway Windows, respectively. For the set of molecules affected by COVID-19 via QCNE, they were mapped in multiple pathways since no more than 1,000 molecules could be mapped in each pathway Window. The lists of molecules affected by inflammatory response from different pathways were then combined and compiled to obtain the final list.

IPA for Core Analysis

The molecules affected by EtOH alone or the molecules affected by both EtOH and each of the 3 sets of molecules affected by COVID-19 were uploaded into IPA for "core analysis," respectively. The analysis included Canonical Pathway Analysis, Diseases and Functions Analysis, and Upstream Regulator Analysis (Fig. 2). Canonical Pathway Analysis was used to identify pathways affected by EtOH alone or by both COVID-19 and EtOH based on the connections of molecules affected by EtOH alone or by both COVID-19 and EtOH to the 705 canonical pathways stored in QKB. The significance for the association with each of these 705 canonical pathways was calculated using a Benjamini-Hochberg-corrected Fisher's exact test. Its *p*-value indicated the likelihood of finding that number of overlapping molecules from the dataset in the canonical pathway. Disease and Function Analysis was used to identify diseases and functions affected by EtOH alone or by both COVID-19 and EtOH. The significance for the association between the

uploaded molecules and a given disease and function in QKB was calculated using right-tailed fisher's exact test. Upstream Regulator Analysis was used to identify possible upstream regulators that may account for observed changes in the expression levels of queried molecules by overlapping them with curated networks in QKB. Each upstream regulator was assigned an overlap *p*-value to measure the significance of genes in the dataset that were downstream of the upstream regulator using a suite of algorithms reported by Krämer and colleagues (2014).

Connectivity and Molecule Activity Predictor (MAP)

The common set of molecules that were identified to be affected by EtOH and COVID-19 via literature, RNA-seq, and QCNE were added into a pathway Window. EtOH was then added to this pathway Window. The connections among these molecule nodes were identified using "Connect" according to the curated findings in QKB. "Molecule Activity Predictor (MAP)" tool was then used to identify changes in the activity processes, including expression, transcription, activation, inhibition, or phosphorylation, in response to other molecules.

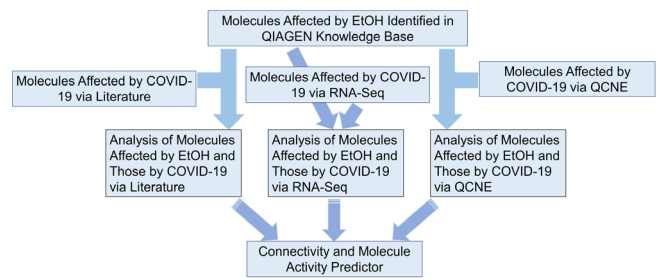


Fig. 2. Workflow of analysis of molecules affected by EtOH and those affected by COVID-19 via literature, RNA-seq, and QCNE.

RESULTS

Analysis of Molecules Impacted by EtOH

A total of 571 molecules were found to be affected by EtOH by searching QIAGEN Knowledge Base (QKB). These molecules were uploaded into IPA, and core analysis was performed on these molecules. The top 10 pathways identified by Canonical Pathway Analysis to be affected by EtOH with the lowest p -value and highest confidence are presented in Fig. 3A. Among these pathways, neuroinflammation signaling pathway was affected by EtOH with the lowest p -value ($p = 6.95E-76$), suggesting close relationship between EtOH and neuroinflammation. Canonical Pathway Analysis also identified hepatic fibrosis signaling pathway and hepatic cholestasis to be affected by EtOH with very high confidence, indicating the toxic effects of EtOH and its metabolites on liver (Fig. 3A). Furthermore, several immune response pathways, including role of macrophages, fibroblasts, and endothelial cells in rheumatoid arthritis, glucocorticoid receptor signaling, role of pattern recognition receptors in bacteria and viruses, systemic lupus erythematosus in B-cell signaling pathway, and role of double-stranded RNA-dependent protein kinase (PKR) in interferon induction and antiviral response, were found to be affected by EtOH with a p -value of less than $1.43E-39$ (Fig. 3A), suggesting a close relationship between EtOH and immune response.

Disease and Function Analysis was also performed on the 571 molecules affected by EtOH, and top 10 diseases and

functions affected by EtOH are presented in Fig. 3B. Necrosis, apoptosis, and quantity of cells were found to be very strongly affected by EtOH, suggesting toxic effects of EtOH and its metabolites on cell viability. Concentration and synthesis of lipid, glucose metabolism disorder, and cellular homeostasis were also found to be affected by EtOH, indicating that EtOH may have a significant impact on cellular metabolism and homeostasis. Furthermore, inflammation of organs, absolute anatomic regions, and body cavities was also found to be caused by EtOH, suggesting that EtOH exposure may lead to inflammation in the organ systems.

Upstream Regulator Analysis was then performed on the 571 molecules affected by EtOH, the top 10 molecules predicted as upstream regulators are shown in Fig. 3C. Among the top 10 upstream regulators predicted by the analysis, leptin was found to be an upstream regulator affected by EtOH, which is well documented in published studies (Otaka et al., 2007). Furthermore, 6 of the top 10 predicted upstream regulators were cytokines, including TNF, INF- γ , IL-1 β , IL-6, IGF-1, and TGF- β 1 (Fig. 3C). The schematic network regulated by TNF, the predicted upstream regulator with the lowest p -value ($p = 3.36E-145$), is shown in Fig. 3D. As the master upstream regulator, TNF was predicted to affect the expression or activity of other molecules involved in immune response, including TGF- β 1, inhibitor of nuclear factor kappa B kinase subunit beta ($\text{I}\kappa\text{B}\kappa\text{B}$), and prostaglandin E2, which, in turn, may affect the expression or activity of other molecules including signal transducer and activator of transcription (STAT), nuclear factor (NF)- κB , hypoxia-inducible

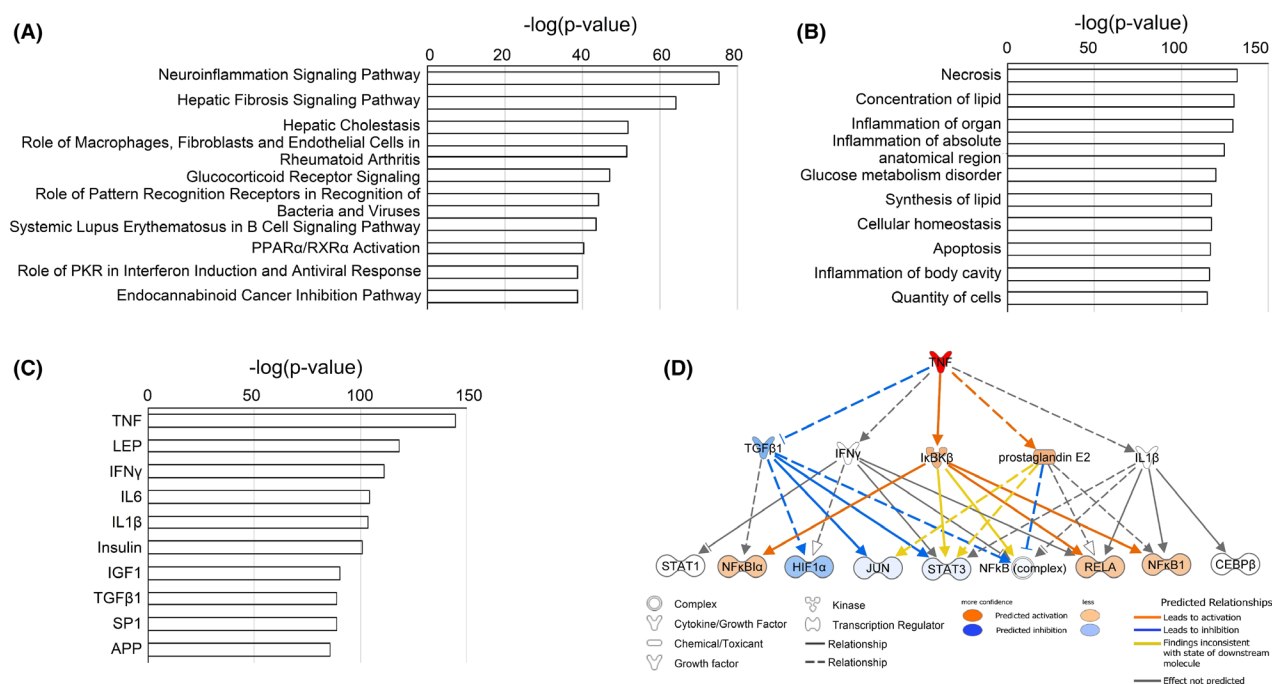


Fig. 3. IPA for core analysis of molecules affected by EtOH. The 571 molecules affected by EtOH were uploaded into IPA for core analysis. Top 10 enriched canonical pathways (A), related diseases and functions (B), and predicted upstream regulators (C) were plotted against their $-\log(p\text{-value})$, respectively. (D) Schematic network regulated by TNF, the top predicted upstream regulator.

factor (HIF)-1 α , and JUN. These key inflammatory mediators may represent the main contributors to EtOH-associated neuroinflammation and immune response.

Identification of Molecules Impacted by COVID-19

Several approaches were used to identify molecules impacted by COVID-19. The first set of molecules were obtained by literature search of MEDLINE via PubMed and of bioRxiv and medRxiv via COVID-19 (Fig. 1A). Twenty-eight studies were identified reporting gene expression profiles in COVID-19 patients as provided in the Appendix S1. A total of 363 molecules including genes, proteins, and complexes with reported changes in expression or activity were identified as described in Materials and Methods and added to “My Pathway” in IPA through its “Build” and “Add molecules/relationship” tool. These molecules were designated as molecules affected by COVID-19 via literature (Fig. 1A).

The second set of molecules impacted by COVID-19 were identified by searching the Gene Expression Omnibus (GEO) database. One study (accession number: GSE150316) was identified reporting on total RNA-sequencing data of autopsied lungs from 5 COVID-19 patients and 5 COVID-19-negative controls. RNA-sequencing data were retrieved from GEO and analyzed using QIAGEN CLC workbench (Fig. 1B). Expression levels of 797 genes were found to be significantly changed in COVID-19 lungs ($p < 0.05$) compared with negative controls. These molecules were designated as molecules affected by COVID-19 via RNA-seq (Fig. 1A).

QIAGEN Coronavirus Network Explorer (QCNE) has identified 14 pathways, 36 functions, and 19 diseases potentially affected by SARS-CoV-2 viral proteins, based on human host proteins identified to interact with SARS-CoV-2 viral proteins using affinity purification–mass spectrometry screening (Gordon et al., 2020) and their connections to biological functions or diseases in the QIAGEN Knowledge Graph derived from the biomedical literatures curated in QKB. A total of 10,733 molecules were identified in QCNE to be affected by COVID-19. These molecules were designated as molecules affected by COVID-19 via QCNE (Fig. 1A).

Analysis of Molecules Affected by EtOH and Those by COVID-19 via Literature Suggested Involvement of Inflammatory Response in EtOH and COVID-19

The 363 molecules affected by COVID-19 via literature were uploaded into IPA and analyzed using IPA’s “My Pathway” “Connect” tool together with the 571 molecules affected by EtOH. A total of 112 molecules were found to be affected by both EtOH and COVID-19 via literature. Among these 112 molecules, 53 molecules were found to be involved in inflammatory response with a p -value of $3.60E-43$ (Fig. 4). Among the remaining 251 molecules affected by COVID-19 via literature but not by EtOH, 79 molecules were found to be involved in inflammatory response with a p -value of $6.03E-39$ (Fig. 4).

The 112 molecules overlapped by EtOH and COVID-19 via literature were uploaded into IPA for core analysis. Canonical Pathway Analysis on these molecules was performed. Six of the top 10 canonical pathways were identified to be involved in immune response (Fig 5A). These pathways include neuroinflammation signaling pathway, glucocorticoid receptor signaling, role of macrophages, fibroblasts, and endothelial cells in rheumatoid arthritis, systemic lupus erythematosus in B-cell signaling pathway, acute-phase response signaling, and role of pattern recognition receptors in bacteria and viruses, suggesting significant involvement of inflammatory response in both EtOH exposure and COVID-19.

Disease and Function Analysis was also performed on the 112 molecules overlapped by EtOH and COVID-19 via literature. The top 10 diseases and functions predicted to be related to both EtOH and COVID-19 with the highest confidence include 3 main categories: cellular/organismal survival/death, such as necrosis, morbidity, or mortality, quantity of cells, organismal death, and cell survival; metabolism, such as concentration of lipid and glucose metabolism disorder; and immune functions, such as inflammation of organ, cell movement of leukocytes, and cellular infiltration (Fig. 5B).

Upstream Regulator Analysis was then performed on the 112 overlapped molecules to identify potential upstream regulators. Among the top 10 predicted upstream regulators, there were 4 cytokines, including TNF, INF- γ , IL-6, and IL-1 β , and 3 transcription factors that regulate the expression of key inflammatory mediators, including CCAAT/enhancer-binding protein (C/EBP) β , NF- κ B, and specificity protein (SP)1. Together with immunoglobulin, 8 of the top 10 predicted upstream regulators were involved in immune response (Fig. 5C). TNF, the upstream regulator predicted with the highest significance ($p = 8.61E-51$), was predicted to be the top regulator for insulin-like growth factor (IGF)1,

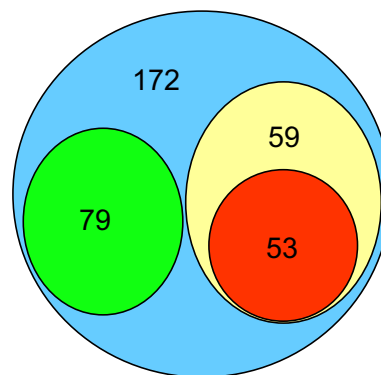


Fig. 4. Fifty-three molecules affected by both COVID-19 via literature and EtOH were involved in inflammatory response. A total of 363 molecules were identified to be affected by COVID-19 via literature (blue, green, yellow, and red), and 112 of the 363 molecules were affected by EtOH with 53 molecules (red) related to inflammatory response. Among the remaining 251 molecules affected by COVID-19 (blue and green) but not affected by EtOH, 79 molecules were related to inflammatory response (green).

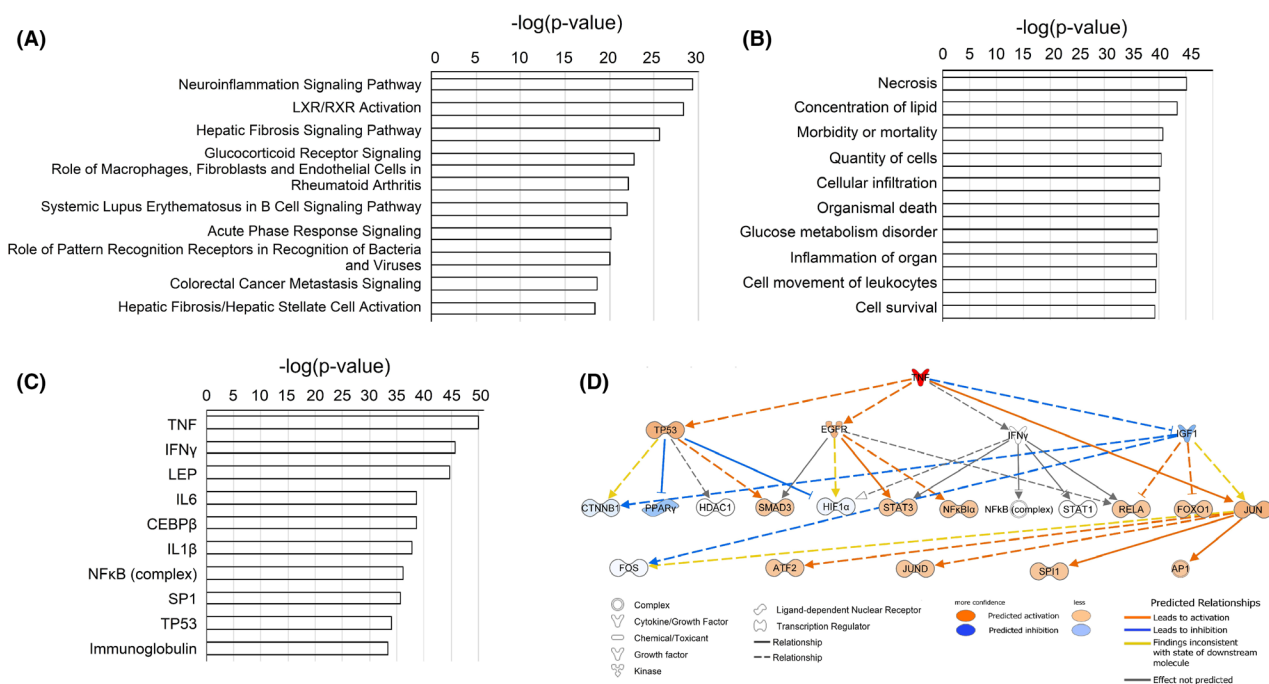


Fig. 5. Analysis of 112 molecules that were affected by EtOH and COVID-19 via literature. The 112 molecules affected by COVID-19 via literature and with EtOH were uploaded into IPA for core analysis. Top 10 enriched canonical pathways (A), related diseases and functions, (B) and predicted upstream regulators (C) were plotted against their $-\log(p\text{-value})$, respectively. (D) Schematic network regulated by TNF, the top predicted upstream regulator.

epidermal growth factor receptor (EGFR), and TP53, which, in turn, may regulate the expression or activity of other molecules, including STATs, NF- κ B, JUN, HIF-1 α , PPAR, and SMAD (Fig. 5D).

Analysis of Molecules Affected by EtOH and Those by COVID-19 via RNA-seq Suggested a Key Role of Inflammation in the Impact of EtOH Exposure on COVID-19

RNA-seq data of autopsied lungs from 5 COVID-19 patients and 5 COVID-19-negative controls were retrieved from GEO database (GSE150316) and analyzed using CLC Workbench. A total of 1,593 genes were found to be differentially expressed in the autopsied lungs of COVID-19 patients as compared to COVID-19-negative controls with 1,528 molecules affected by COVID-19 alone and 66 molecules affected by both COVID-19 via RNA-seq and EtOH. Among the 1,528 molecules affected by COVID-19 alone, 121 molecules were related to inflammatory response. Among the 66 molecules affected by both EtOH and COVID-19 via RNA-seq, 31 molecules were related to inflammatory response (Fig. 6).

The 66 molecules affected by both EtOH and COVID-19 via RNA-seq were uploaded into IPA for core analysis. Besides hepatic fibrosis / hepatic stellate cell activation and hepatic fibrosis signaling pathway, the top 10 pathways identified via Canonical Pathway Analysis included neuroinflammation signaling pathway, glucocorticoid receptor signaling, LXR/RXR activation, HIF-1 α signaling, role of

macrophages, fibroblasts, and endothelial cells in rheumatoid arthritis, and production of nitric oxide and reactive oxygen species in macrophages ($p < 3.87E-10$), suggesting oxidative stress and inflammation (Fig. 7A).

Disease and Function Analysis was performed on the 66 molecules affected by both EtOH and COVID-19 via RNA-seq. Top 10 diseases and functions predicted to be related to both EtOH and COVID-19 via RNA-seq included 3 functions involved in inflammation, namely inflammation of absolute anatomic region, inflammation of organ, and inflammation of body cavity, and 4 functions involved in movement of cells, namely migration of cells, movement of blood cells, cell movement, and leukocyte migration (Fig. 7B). These functions predicted to be affected by EtOH and COVID-19 via RNA-seq also suggested the involvement of inflammatory response.

Upstream Regulator Analysis was then performed on the 66 molecules affected by EtOH and COVID-19 via RNA-seq. Among the top 10 predicted upstream regulators, 7 of them were well known to be involved in inflammatory response, including TNF, IL-6, IFN- γ , IL-1 β , NF- κ B, NF κ B1 α , and AP1 (Fig. 7C). TNF was again predicted to be the upstream regulator with the highest significance ($p = 3.54E-40$). As the predicted top regulator, TNF was predicted to regulate the activity of PPAR γ , which, in turn, may regulate the expression or activity of other molecules, including AP1, PPAR α , JUN, nuclear factor erythroid 2-related factor 2 (NFE2L2), and STAT3 (Fig. 7D).

Analysis of Molecules Affected by EtOH and Those by COVID-19 via QCNE Indicated Involvement of Inflammatory Response in EtOH and COVID-19

The 571 molecules affected by EtOH were then analyzed together with molecules affected by COVID-19 via QCNE using “My pathway”-“BUILD”- “Grow” tool in IPA. A

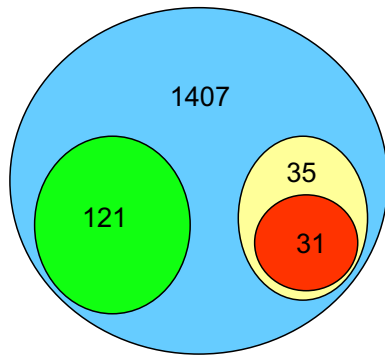


Fig. 6. Thirty-one molecules affected by both COVID-19 via RNA-seq and EtOH were involved in inflammatory response. RNA-seq data of autopsied lungs from 5 COVID-19 patients and 5 COVID-19-negative controls (GSE150316) were analyzed using CLC Workbench. 1,593 genes (blue, green, yellow, and red) were differentially expressed in COVID-19 lungs with 66 genes affected by EtOH (yellow and red). Among the 66 molecules affected by EtOH, 31 molecules were related to inflammatory response (red). Among the remaining 1,528 molecules affected by COVID-19 but not by EtOH, 121 molecules were related to inflammatory response (green).

total of 529 of 571 EtOH-associated molecules were overlapped with molecules affected by COVID-19 via QCNE (Fig. 8). Among these 529 molecules, 211 were found to be involved in inflammatory response. Among the 10,204 molecules affected by COVID-19 via QCNE only, a total of 1,057 molecules were found to be involved in inflammatory response.

The 529 molecules affected by EtOH and COVID-19 via QCNE were uploaded into IPA for core analysis. Canonical Pathway Analysis revealed involvement of key immune response pathways, including neuroinflammation signaling pathway, role of macrophages, fibroblasts, and endothelial cells in rheumatoid arthritis, glucocorticoid receptor signaling, role of pattern recognition receptors in recognition of bacteria and viruses, systemic lupus erythematosus in B-cell signaling pathway, and role of PKR in interferon induction and antiviral response ($p < 8.96E-42$). The top 10 predicted pathways also included hepatic fibrosis signaling pathway and hepatic cholestasis (Fig. 9A).

Disease and Function Analysis was performed on the 529 molecules affected by both EtOH and COVID-19 via QCNE. Top 10 diseases and functions predicted to be related to both EtOH and COVID-19 via QCNE were generally in 3 broad categories: inflammatory response, including inflammation of organ, inflammation of absolute anatomic region, inflammation of body cavity, and migration of cells; cell number, including necrosis, apoptosis, and quantity of cells; and homeostasis, including concentration of lipid and cellular homeostasis (Fig. 9B).

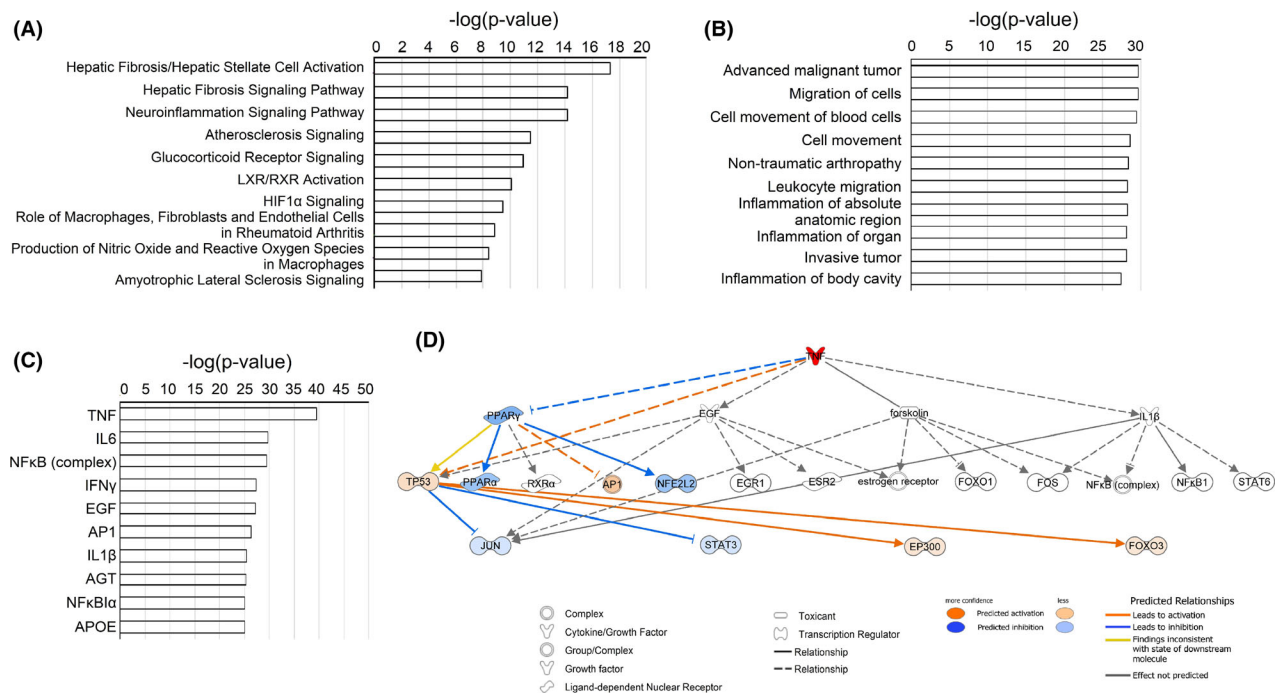


Fig. 7. Analysis of 66 molecules that were affected by EtOH and COVID-19 via RNA-seq. The 66 molecules affected by COVID-19 and EtOH were uploaded into IPA for core analysis. Top 10 enriched canonical pathways (A), related diseases and functions (B), and predicted upstream regulators (C) were plotted against their $-\log(p\text{-value})$, respectively. (D) Schematic network regulated by TNF, the top predicted upstream regulator.

Among the top 10 predicted regulators, 7 molecules were key inflammatory mediators, including TNF, IFN- γ , IL-1 β , IL-6, TGF- β 1, IGF1, and SP1 (Fig. 9C). TNF was predicted to be the upstream regulator with the highest significance ($p = 1.28E-153$), and the master regulator for TGF- β 1, IFN- γ , prostaglandin E2, IL-1 β , and I κ BK β , which, in turn, may regulate the expression or activity of other molecules, including NF- κ B, JUN, HIF-1 α , and SMAD3 (Fig. 9D).

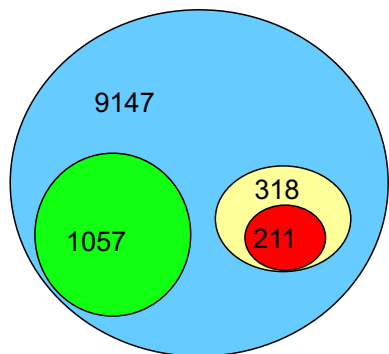


Fig. 8. Two hundred eleven molecules affected by both COVID-19 via QCNE and EtOH were involved in inflammatory response. A total of 10,733 molecules were affected by COVID-19 via QCNE (blue, green, yellow, and red), and 529 molecules were affected by EtOH with 211 molecules (red) related to inflammatory response. Among the remaining 10,204 molecules affected by COVID-19 (blue and green) but not by EtOH, 1,057 molecules were related to inflammatory response (green).

Actions of EtOH on Key Molecules Affected by Both EtOH Exposure and COVID-19

By comparing the IPA for core analyses of 3 sets of molecules affected by EtOH and COVID-19 via literature (Fig. 5), EtOH and COVID-19 via RNA-seq (Fig. 7), and EtOH and COVID-19 via QCNE (Fig. 9), we found 11 molecules were consistently identified among the top 10 canonical pathways, upstream regulators, or networks regulated by the predicted top regulator. These 11 molecules included proinflammatory cytokines, such as IL-1 β , IL-6, IFN- γ , and TNF- α , and transcription factors that regulate the expression of inflammatory mediators, including JUN, HIF-1 α , NF- κ B, STAT, PPAR/RXR, and glucocorticoid receptor (GR). Connectivity and Molecule Activity Predictor tool was then used to predict the actions of EtOH on these molecules. EtOH was predicted to activate proinflammatory cytokines including IL-1 β , IL-6, IFN- γ , and TNF- α , and activate such transcription factors as JUN, HIF-1 α , NF- κ B, and STAT while inhibiting PPAR/RXR and GR (Fig. 10).

Mapping of Key Molecules Affected by Both EtOH Exposure and COVID-19 Onto SARS-CoV-2 Pathways

The 11 key molecules consistently identified among the top 10 canonical pathways, upstream regulators, or networks regulated by predicted top upstream regulators in the IPA for core analysis, namely IL-1 β , IL-6, IFN- γ , TNF, JUN,

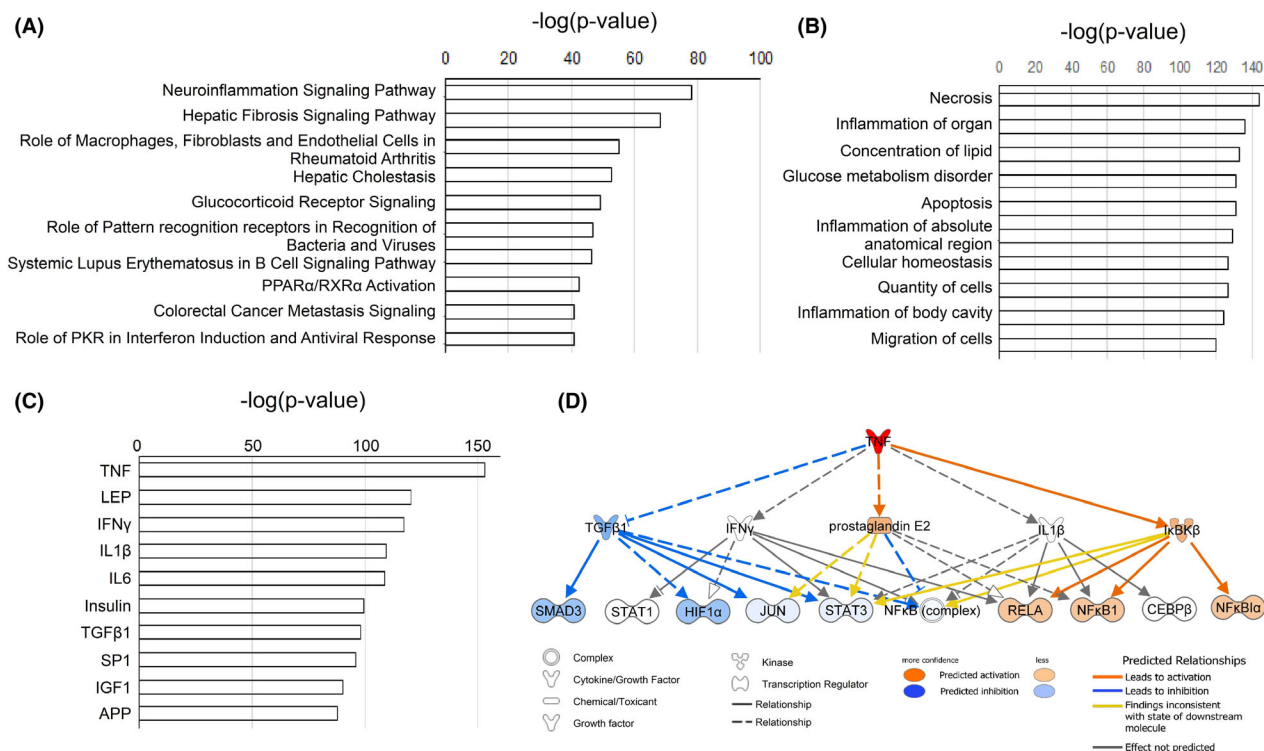


Fig. 9. Analysis of molecules affected by EtOH and COVID-19 via QCNE. The 529 molecules affected by COVID-19 and EtOH were uploaded into IPA for core analysis. Top 10 enriched canonical pathways (A), related diseases and functions (B), and predicted upstream regulators (C) were plotted against their $-\log(p\text{-value})$, respectively. (D) Schematic network regulated by TNF, the predicted top upstream regulator.

NF- κ B, HIF-1 α , STAT, PPAR/RXR, and GR, were then mapped to 14 pathways in QCNE predicted to be affected by SARS-CoV-2 proteins via protein–protein interactions. Five molecules, IL-1 β , IL-6, TNF, JUN, and STAT, were mapped to 10 of 14 pathways affected by SARS-CoV-2 proteins (Fig. 11). IL-6 was mapped to 9 of 14 pathways and IL-1 β to 7 of 14 pathways, with both mapped to the HMGB1 signaling, IL-1 signaling, and IL-6 signaling pathways. TNF was mapped to 3 of 14 pathways. JUN and STAT were mapped to 4 of 14 pathways, and they may regulate the expression of IL-1 β , IL-6, and TNF.

DISCUSSION

Chronic consumption of alcohol not only causes alcoholic liver disease with hepatic lesions such as steatosis, cholestasis, fibrosis, or cirrhosis (Louvet and Mathurin, 2015), but also leads to altered immune response to microbial infections (Barr et al., 2016; Szabo and Saha, 2015). Alcohol consumption has significantly increased since the outbreak of COVID-19 in December 2019 (Pollard et al., 2020; The Nielsen Company, 2020). While alcohol consumption has been suggested as a risk factor for COVID-19 (Chick, 2020; Ramalho, 2020; Testino, 2020), it is not clear how alcohol consumption may affect the progression and outcome following infection with SARS-CoV-2.

This current study was undertaken to examine how EtOH might interact with, and affect, responses in COVID-19 patients. EtOH, the key chemical present in alcoholic beverages and responsible for various effects of use and abuse of alcohol beverages, was the key word used for our literature search and *in silico* analyses. A total of 571 molecules were found to be affected by EtOH in QKB. Among these 571 molecules, 112 molecules were identified to be affected by COVID-19 via literature; 66 molecules were identified to be affected by COVID-19 via RNA-seq; and 529 molecules were identified to be affected by COVID-19 via QCNE, suggesting significant overlap of molecules affected by both EtOH and COVID-19.

Canonical Pathway Analysis of molecules affected by both EtOH and COVID-19 consistently revealed that EtOH and COVID-19 converge on their effects on hepatic fibrosis signaling pathway. Alcohol consumption is well recognized to cause liver cirrhosis, even in modest amounts (Iranpour and Nakhaee, 2019; Simpson et al., 2019). It should be pointed out that some molecules affected by COVID-19 via RNA-seq were found to be involved in hepatic fibrosis in our analysis even though RNA-seq data were obtained from autopsied lung samples. While fibrosis in histologically distinct organs may involve different pathways, there is evidence of evolutionarily conserved pathways involved in the fibrosis of both hepatic and pulmonary fibrosis (Makarev et al., 2016). While the cause of death is not reported for these patients whose lung samples were taken for RNA sequencing, it is possible that these patients may have suffered from pulmonary and/or liver fibrosis. Indeed, it has been reported that abnormal liver function tests were common in COVID-19 patients (Garrido et al., 2020), which may be due to direct actions by the virus or indirect actions by the drugs and/or systemic inflammation during the course of COVID-19 (Lee et al., 2020). Our studies suggest that chronic alcohol consumption–induced liver dysfunction may contribute to worsening the patients' response to SARS-CoV-2 infections.

Our Disease and Function Analysis of the 571 EtOH-associated molecules confirmed previous research findings that EtOH and its metabolites exert toxic effects on cells and affect cellular metabolism and homeostasis (Steiner et al., 2015; Wang et al., 2016; You and Arteel, 2019). Our IPA for core analysis of molecules affected by both EtOH and COVID-19 demonstrated that EtOH and COVID-19 converged on their effects on lipid and glucose metabolism and on the RXR and GR pathways (Figs 5, 7 and 9). As a nuclear receptor, RXR may form homodimer or heterodimer with PPAR or LXR. Activation of RXR and its partners plays key roles in the regulation of genes involved in lipid and glucose metabolism (Cha et al., 2001; Monsalve et al., 2013; Neuschwander-Tetri, 2015). RXR/PPAR are also known to inhibit the production of proinflammatory cytokines such as TNF- α and IL-6 (Monsalve et al., 2013). It has also been reported that RXR may attenuate the antiviral response by suppressing the production of type I interferon (Ma et al., 2014). Glucocorticoids are also well known to

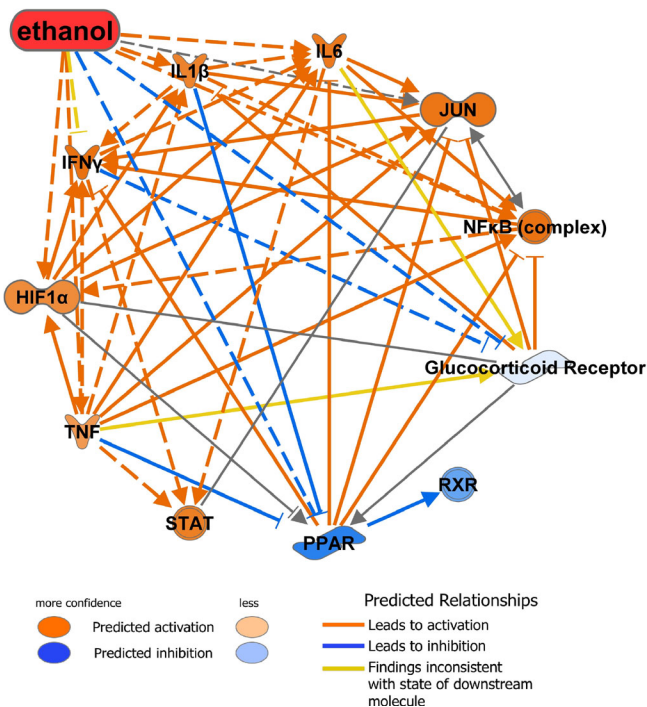


Fig. 10. Connectivity map and action of EtOH on the key molecules consistently identified in the top 10 canonical pathways, upstream regulators, or networks regulated by predicted top upstream regulators in the IPA for core analysis of molecules affected by both EtOH and COVID-19 via literature, RNA-seq, and QCNE.

Fig. 11. Ten of the 14 QCNE pathways that contain any of the key molecules consistently identified in the top 10 canonical pathways, upstream regulators, or networks regulated by predicted top upstream regulators in the IPA for core analysis of molecules affected by both EtOH and COVID-19 via literature, RNA-seq, and QCNE: HMGB1 signaling, IL-1 signaling, IL-6 signaling, IL-2 signaling, SAPK/JNK signaling, PGDF signaling, PTEN signaling, CDK5 signaling, IGF-1 signaling, and inhibition of ARE-mediated mRNA degradation pathway.

regulate glucose and lipid homeostasis besides their immunosuppressive properties (John et al., 2016; Kuo et al., 2015). Our EtOH Activation Analysis showed that EtOH may inhibit PPAR/RXR either directly or indirectly via TNF and that EtOH may inhibit GR pathway directly or indirectly via IFN- γ (Fig. 10). Our study suggests that EtOH-induced inhibition of GR and RXR pathways may worsen COVID-19 patients' defects in cellular metabolism and homeostasis, and enhance inflammation caused by SARS-CoV-2 infection.

Our overlapping analysis found that many molecules affected by both EtOH and COVID-19 are involved in inflammation (Figs 4, 6 and 8). Canonical Pathway Analysis of molecules affected by both EtOH and COVID-19 demonstrated that both EtOH and COVID-19 affect the pathway on the role of macrophages, fibroblasts, and endothelial cells in rheumatoid arthritis (Figs 5, 7 and 9). It is important to note that our discovery on the involvement of the pathway on the role of macrophages, fibroblasts, and endothelial cells in rheumatoid arthritis does not necessarily mean that EtOH and SARS-CoV-2 may cause rheumatoid arthritis, but rather suggest that the inflammatory response in rheumatoid arthritis, EtOH, and COVID-19 involve a common set of key mediating cells and molecules. Indeed, no causal association has been established between alcohol intake and occurrence of rheumatoid arthritis (Bae and Lee, 2019). Studies on the potential link between respiratory viral infections and the development of rheumatoid arthritis have also been very limited (Arleevskaya et al., 2017) even though ambient respiratory viral infections have been suggested as a risk factor for the development of rheumatoid arthritis (Joo et al., 2019). Angiotensin-converting enzyme 2 (ACE2), a gene widely expressed in many human tissues including kidney, heart, gastrointestinal tract, blood vessels, lung, and brain (Hamming et al., 2004) and on the surface of various cells including type II alveolar cells (AT2), type I alveolar cells (AT1), airway epithelial cells, fibroblasts, endothelial cells, and various types of immune cells including macrophages (Zhao et al., 2020), has recently been identified as the coreceptor for SARS-CoV-2 envelope spike glycoprotein (Hoffmann et al., 2020). Therefore, it is no surprise that fibroblasts, endothelial cells, and macrophages that were involved in rheumatoid arthritis may also be involved in the inflammatory response during COVID-19. Indeed, the increasing knowledge about the pathophysiology of SARS-CoV-2 infection has led to the consideration of some antirheumatic drugs as potential treatment options for COVID-19 (Favalli et al., 2020).

One of the key causes of the alcohol-related diseases is alcohol consumption-induced inflammation. It is known to induce gut leakage and intestinal inflammation, which, in turn, increases the entrance of bacteria or bacterial

endotoxins into the circulation, leading to the release of proinflammatory cytokine (Patel et al., 2015; Wang et al., 2005) and systematic inflammation (Patel et al., 2015). EtOH is reported to increase IL-1 α , IL-1 β , TNF, IL-6 (Achur et al., 2010), and RELA (Alfonso-Loeches et al., 2010; Shinohara et al., 2010). Indeed, chronic alcohol consumption has been reported to accelerate inflammatory response, which affects multiple organs and tissues including alcoholic liver disease and pancreatitis (Szabo and Saha, 2015, Alcohol Res). Our IPA for core analysis consistently identified that both EtOH and COVID-19 converged on their effects on proinflammatory mediators, including cytokines such as IFN- γ , IL-6, IL-1 β , and TNF, and transcription factors such as NF- κ B, JUN, HIF-1 α , and STAT, as well as on anti-inflammatory mediators, including GR and PPAR/RXR (Figs 5, 7 and 9). Our EtOH activation analysis on these molecules showed that EtOH activated proinflammatory cytokines including IL-6, IL-1 β , IFN- γ , and TNF, and transcription factors including HIF-1 α , JUN, NF- κ B, and STAT, which may in turn enhance the production of more inflammatory mediators while inhibiting PPAR/RXR and GR signaling (Fig. 10). Furthermore, 5 of these 10 molecules, IL-1 β , IL-6, TNF, JUN, and STAT, were then mapped to 10 of the 14 COVID-19 pathways in QCNE predicted through interactions with SARS-CoV-2 proteins, including the high-mobility group box 1 (HMGB1) pathway, IL-1 pathway, and IL-6 pathway. Potential benefits of inhibiting IL-1, IL-6, and HMGB1 have been explored. For example, blockade of IL-6 with sarilumab has been reported in severe COVID-19 patients with systemic hyperinflammation (Della-Torre et al., 2020). HMGB1, a damage-associated molecular patterns (DAMPs), may be released by damaged cells, and initiate and sustain the inflammation during SARS-CoV-2 infection by inducing the release of inflammatory mediators (Cicco et al., 2020; Street, 2020). Our studies suggest that EtOH may worsen inflammation in COVID-19 patients via multiple signaling pathways including IL-6, IL-1, and HMGB1 signaling.

Our Canonical Pathway Analysis of molecules affected by both EtOH and COVID-19 consistently demonstrated their effects on the neuroinflammation signaling pathway. This is of importance since normal immune function not only involves interactions among immune cells and communications between local immune cells and nonimmune cells, but also involves crosstalk between the brain and the periphery (Sarkar et al., 2015). Neuroimmune activation is one of the key pathologies of numerous psychiatric disorders including alcoholism, depression, and anxiety (Coleman et al., 2018). The expression of HMGB1 and TLRs has been reported to be increased in alcoholic brain and in mice treated with

EtOH, and EtOH-induced HMGB1/TLR signaling has been suggested to contribute to the induction of proinflammatory cytokines, such as IL-1 β , thereby inducing neuroimmune activation in alcoholic brains (Crews et al., 2013). While there have been limited studies on neuroinflammation in COVID-19 patients, these patients have been reported to exhibit neurological consequences including nausea, damage to respiratory centers, and cerebral infarction (Jarrahi et al., 2020). SARS-CoV-2 infection may cause neurological diseases (Mao et al., 2020; Wu et al., 2020) and potential neuropsychiatric sequelae (Troyer et al., 2020). Our studies suggest that EtOH may worsen the neuroinflammation taking place in the brain of COVID-19 patients and that neuroinflammation may contribute to both non-neuronal and neurological consequences in COVID-19.

Upon infection with SARS-CoV-2, a proper coordinated antiviral immune response is needed to successfully combat the infection. However, in some patients, their immune activation progresses to an uncontrolled hyperinflammatory stage with abnormally high levels of cytokines and chemokines in their system, thereby magnifying the severity of COVID-19 and promoting the progression to acute respiratory distress syndrome (Girija et al., 2020). Our studies suggest that chronic alcohol consumption may also contribute to systemic inflammation, which may worsen the patients' response to SARS-CoV-2 infection, which may escalate or expedite the onset of respiratory failure and multiorgan failure in COVID-19 patients (Testino, 2020). Therefore, dampening the immune response has been used as a treatment strategy for COVID-19. There is a recent report suggesting the effectiveness of synthetic glucocorticoid dexamethasone as a potential treatment for COVID-19 (Horby et al., 2020), while there are also reports that glucocorticoid has no effects on the outcome of COVID-19 patients (Lu et al., 2020). The timing and dose of dexamethasone administered may impact the outcome since appropriate inflammatory response is critical for the antiviral response.

LIMITATIONS

Several limitations of our meta-analysis could potentially affect our analysis results. First, for the list of molecules affected by COVID-19 via literature, the list may not be all-exhaustive since there may be studies that have met the inclusion criteria but not included in our search considering that our comprehensive literature search of MEDLINE, bioRxiv, and medRxiv was conducted before June 30. Four of the 28 identified studies were preprint studies from bioRxiv and medRxiv. For the list of molecules affected by COVID-19 via RNA-seq, only one study was available from GEO reporting significant gene expression changes in the autopsied lung of COVID-19 patients as compared to non-COVID-19 patients before June 30, 2020. No demographic information was available on the GEO GSE150316 RNA-sequencing study. The underlying cause of death for non-COVID-19 patients was not available. It is not known

whether COVID-19 patients had other maladies, which may have affected their gene expression profile. In addition, the list of genes affected by COVID-19 via RNA-seq would be expected to vary depending on the cutoff threshold selected. For our study, a cutoff threshold of $p < 0.05$ was used. Despite the limitations associated with molecule lists, our studies used 3 different approaches, that is, literature, RNA-seq, and QCNE, to identify the list of molecules affected by COVID-19 and discovered a common set of molecules affected by both EtOH and COVID-19 via literature, RNA-seq, and QCNE. Second, IPA uses the overrepresentation analysis (ORA) in which the statistical relevance of a given pathway is based on evaluating the proportion of differentially expressed components within the pathway above the proportion of molecules that could be randomly expected. It gives every component in the pathway equal weight regardless of whether the component is inherent to the interactions. Third, considering that the information on the drinking history of COVID-19 patients is sparse, it is difficult to examine the action of alcohol exposure in COVID-19 patients. Despite this limitation, our meta-analyses with molecules affected by both EtOH and COVID-19 from 3 different sources, that is, literature, RNA-seq, and QCNE, were able to examine how EtOH augmented SARS-CoV-2-induced inflammation by altering the activity of key inflammatory mediators.

CONCLUSION

In silico methodologies have been widely used to study COVID-19, such as searching for therapeutic agents (Hall and Ji, 2020; Shah et al., 2020) and identifying potential pathologies based on prior knowledge gained from diseases caused by viral infection of the same betacoronavirus family (especially SARS-CoV and MERS-CoV) (Mousavizadeh and Ghasemi, 2020). With the recent rapid spread of COVID-19 pandemic around the world, significant global efforts are being undertaken to characterize COVID-19. Although it has not been possible to directly determine the interaction of alcohol consumption on the severity of COVID-19 symptoms in patients, the overlap of pathways activated by SARS-CoV-2 infection and alcohol consumption suggests that alcohol consumption might augment the disease progression and potentially lead to poorer clinical outcomes. This is particularly concerning given the appearance of a second wave of infections around the globe, and the rise of binge drinking in the group aged 50 and older, a group at elevated risk of serious COVID-19 symptoms. Our findings suggest the importance to caution against alcohol consumption during the COVID-19 pandemic and facilitate further investigation into how alcohol exposure may affect viral infections.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Appendix S1. References from which 363 genes were found to be affected by COVID-19.