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A CASE STUDY CORRELATING CARDIOMETABOLIC MARKERS AND T HELPER INFLAMMATORY PATHWAYS IN COVID-19 PATIENTS

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A CASE STUDY CORRELATING CARDIOMETABOLIC MARKERS AND T HELPER INFLAMMATORY PATHWAYS IN COVID-19 PATIENTS.

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Abstract

COVID-19 is one of the deadliest pandemics in the history of mankind and the infection and mortality rate to this date has been immense. There is several information and data available to analyze patient data regarding both deceased and non-severe patients. A vast amount of research and study is ongoing to understand the implications and cure for this disease. The main purpose of this paper is to find the correlation between inflammatory pathway markers and cardiometabolic markers of COVID-positive patients and to see which T-helper pathway markers are enriched and which markers have reduced count amongst three categories of patients.

This paper includes several analyses, initially finding the difference in blood markers between covid positive and covid negative patients to look at an overall picture of how the protein markers between these two categories differ. The next steps include analyzing only the positive patients using a linear model to see how the T-helper pathway markers differ between the 3 categories of patients. Several interesting outcomes were found where the deceased patient had reduced values for many infection-fighting markers compared to the non-severe patients.

Lastly, a generalized linear model was run to generate a network to observe the cardiometabolic markers are activated and the ones that are suppressed by the

T-helper pathway markers, and the results showed activation of markers like LTBP2 that are directly related to heart failure, other markers are explained in the paper.

Overall, it can be concluded that T-helper cell enrichment analysis can help understand the implications of covid on patients with underlying conditions and help detect activated markers that are directly correlated to fatal outcomes. With the help of this research, targeted drugs can be used to reduce mortality in several patients

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I. INTRODUCTION:

COVID-19, also known as the noble coronavirus CoV has been declared as a global pandemic, and rightfully so since to date, 219 million people have been infected by this disease. The death toll has reached 4.55 million worldwide, making it one of the worst pandemics in the history of mankind. Everything from businesses to schools as well as religious gatherings came to a complete halt. The economy has tumbled and traveling even to local areas has been restricted as the world has been forced to go at times to a complete lockdown. People have been forced to wear facial masks and many public gatherings have been canceled completely.

Covid-19 is the third coronavirus after SARS-Cov and MERS-Cov and has been suspected to have originated from bats and then passed on to humans in Wuhan, China in 2019 [1]. According to the World Health Organization - Western Pacific[website], Covid-19 is more severe amongst patients who are 60 years or older with underlying health conditions such as diabetes, obesity, heart and lung disease. A compromised immune system and complications like ARDS, complete respiratory failure, and renal insufficiency is common in the most extreme cases [2]. Although there have been several vaccines that have reduced the symptoms and serious illness, their ability to prevent the virus is

still unknown. Hence further studies are needed in order to understand the cause and effect of this virus as the threat still looms large.

One of the biggest risk factors in COVID-positive patients is their elevated expression levels of inflammatory helper T-cell-associated cytokines as most of the illness correlated with infection is found to be cytokine storm which results in lung tissue damage and other severe complications.

There have been many research projects on T-helper cell response and Covid-19 progression where studies have found that Th2 cells had a higher activation compared to Th1 cells and Th17 cells had drastic reductions [3]. And a higher count of Th2 cells was found on deceased patients [3]. Studies have shown that severely ill patients tended to have an elevated concentration of pro-inflammatory cytokines with the major one being interleukin (IL-6) compared to non-severe patients. Researchers have also found an association between increased mortality and a shift in the balance of Th1-Th2[Ana. B Pavel]. Other studies have also shown that cytokines are capable of myocardial infarction. E.g. myocardial injuries have been associated with ACE2 mainly due to the elevated expression of ACE2 in the cardiovascular system in Covid-positive patients [4].

The main goal of this paper is to perform gene enrichment analysis which is a computational study to determine whether a priori defined set of genes shows statistical differences between two biological states which, in this case, is between COVID-positive and COVID-negative patients. There are also multiple other analyses performed on only COVID-positive patients which includes making a generalized linear model to predict the cardiovascular changes by the synergistic effect of immune biomarkers from different immune pathways (Th1, Th2, Th17) and to find the correlation between cardiometabolic markers and the T-helper cell pathway markers in order to have better insight about the interaction of cytokines with the cardiovascular system. Moving forward with this study a potential targeted treatment through experimental medicines such as immunomodulatory therapies is achieved.

II. WHAT IS A CYTOKINE STORM?

Cytokine storm, also known as cytokine release syndrome, is very similar to another disorder known as macrophage activation syndrome. Before moving forward here is a brief summary of what cytokines are. Cytokines are small soluble molecules that act as a messenger for the immune system and consist of several proteins and glycoproteins. They are produced by a variety of immune cell types such as Basophils, eosinophils, macrophages, B-cells, and T helper cells. Cytokines bind to specific receptors called cytokine receptors on the surface of cells and change the activity of cells by altering the functions of proteins by changing the expression of certain genes. Ultimately, the cytokines have an impact on the maturation, growth, development, activation, and suppression of immune cells. The four structural groups of cytokines are Interleukins (IL), Interferons (IFN), Tumor Necrosis Factor (TNF), and Colony Stimulating Factors (CSF).

Simply put, when our immune response overreacts, in normal cases, we see that the immune system ramps up and slowly normalizes but in COVID-positive patients it stays up and becomes uncontrollable and deadly. Cytokine storm leads to a powerful attack by the immune system on the body which can begin in the lungs and spread to the rest of the body. There is overproduction of pro-inflammatory immune cells in the

lungs that cause inflammation and edema which can cause respiratory distress, acute respiratory distress syndrome (ARDS), and might also cause secondary bacterial infection. Our T cells and natural killer cells are the main contributors of cytokines to this inflammatory response.

III. WHAT IS T-HELPER CELL?

There are three main types of T lymphocytes or T cells.

1. Cytotoxic t cells that directly kill infected host cells that are infected by microbes
2. T regulatory cells, which suppress the immune system by inhibiting Helper T cells, Dendritic cells, and Cytotoxic T cells.
3. Helper T cells help activate other immune cells like macrophages, Dendritic cells, and B-cells.

The focus of this paper is the T helper cells. The type of cytokine released from the antigen-presenting cell is determined by the type of antigen that activated the naive-helper T-cell. Once activated, these helper T-cells activate other cells to generate certain immune responses.

TH1: The naive helper cell matures into a T1 helper cell IF IL-12 cytokine is released by the antigen-presenting cell. That T helper cell then releases IFNγ which results in the activation of macrophages and causes B-cells to produce IGG antibodies that play a vital role in activating the classical component pathway. Hepler T1 cells also activate cytotoxic T-cells. T1 cells play an important role in defense against intracellular microbes. Overactivation of T1 cells results in Type 4 hypersensitivity

TH2: T2 cells mature from the release of Interleukin (IL-4). The matured T2 cells then activate IL-4 which stimulates B-cells to produce IGG and IL-5 which activates eosinophils and IL-13 which produces mucosal epithelial cells. TH2 helps against multicellular pathogens like parasites and is predominant in allergies. Subjects prone to hyperallergic reactions have a greater T2 to T1 ratio and over-activation results in Type 1 hypersensitivity.

TH17: TH17 cells mature from the secretion of Interleukin (IL-6) and Transforming Growth Factor beta (TGF) which then produces Interleukin (IL-17) and recruits neutrophils and macrophages to fight against extracellular bacteria and fungi. Overactivation of TH17 results in autoimmune disorders and other inflammatory disorders.

IV. PROCEDURE:

Dataset:

The dataset used was acquired from the Massachusetts General Hospital which consists of a sample of 384 patients, where 306 were covid positive and 78 were covid negative. 1352 plasma protein data were collected from these patients. These patients were further categorized with different categorization techniques. For this research, the who-max score was used to categorize these patients and only used samples of covid positive patients in the later studies. There were five levels of WHO scores and to simplify the division, number 1 was assigned to patients that were deceased, 2 for patients that were intubated, and 3, 4, 5 were grouped together as 3 for patients that were non-severe.

Data cleaning:

There was a vast array of information acquired from the dataset, but the dataset had to be prepared for research based on this paper's needs. The first step of data preparation was to separate the normalized Protein expression values of the patients based on days and only day 0 was used. The protein data was further categorized into 4 categories: CARDIOMETABOLIC, NEUROLOGY, ONCOLOGY, and INFLAMMATION. T-test was performed for all the normalized Protein expression values of the positive against negative patients and the markers were selected that correspond to less than or equal to a p-value of 0.05 to prove that the null hypothesis theory does not hold. Exploring

the data further, subject 365 was found to be missing the Interferon-gamma value and that patient was completely removed as Interferon-gamma is a vital part of this research.

The 2 datasets are read and joined based on the sample_id(assigned to each patient). The next step was to join the markers based on the category, there are 4 categories, Inflammatory, Neurology, Oncology, and Cardiometabolic. Since this study is divided into 2 parts based, 2 separate data frames were created. One contains the makers of both the positive and negative patients and the other only contains the sample of the positive patients. The last process includes subsetting the data to only include samples from day 0, hence the time point of D0 is chosen.

```
df <- read.csv("C:/Users/17742/Documents/thesis/Example_heatmap/Thesis_Work/Thesis_Work/COVID_Blood_Markers.txt", sep=";",skip = 3)
ann <- read.csv("C:/Users/17742/Documents/thesis/Example_heatmap/Thesis_Work/Thesis_Work/COVID_Clinical_Info.csv", sep=";",skip = 3)

df$Timepoint
df$Timepoint <- as.character(df$Timepoint)

df$SampleID

df$SampleID <- as.character(df$SampleID)
df$SampleID

df$SampleID2 <- sapply(strsplit(df$SampleID,"_"), `[`, 1)
df$SampleID2

df$subject_id
df$subject_id <- as.character(df$subject_id)

nrow(df[df$subject_id==df$SampleID2,])==nrow(df)

ann$subject_id <- as.character(ann$subject_id)

dfm <- merge(ann,df, by.x="subject_id", by.y="subject_id")  #merge 2 df by subject ID


dfm$marker <- paste0(dfm$Assay,"_", dfm$Panel) #merge assay and panel into another column
dfm$covstat <-paste0(dfm$subject_id,"_", dfm$COVID)

dfm_D0 <- dfm[dfm$Timepoint == "D0",]  #select time point D0
unique(dfm_D0$Panel)

nrow(dfm_D0)
length(unique(dfm_D0$marker))
length(unique(dfm_D0$subject_id))

dfm_D0_pos <- dfm[dfm$Timepoint == "D0" & dfm$COVID == "1",]
dfm_D0_neg <- dfm[dfm$Timepoint == "D0" & dfm$COVID == "0",]
```

Fig: R code used to clean and prepare the initial dataset

The 2 datasets are joined together by subject_id. To panel data was added to the markers to differentiate the category better and cov_stat was used to differentiate the subject id, where the covid positive patients were assigned a value of 1 and negative patients were given a 0. The last step was to subset the dataset only to include day 0 normalized Protein expression values.

V. DIFFERENCE BETWEEN COVID POSITIVE AND COVID NEGATIVE PATIENTS

The first analysis was done to observe how the covid positive patients differ from covid negative patients and our focus was on the inflammatory marker. We had 2 files to work with, first one was the normalized Protein expression value file for all the markers which had samples of 386 patients and the second file included the other category values including the WHO values and many others, but our main analysis revolved around the WHO values. The normalized Protein expression values were taken on a weekly interval for 4 weeks, but this research only includes data from the first week. The next step was to join the sample ID of the patients with the sample day for ease of splitting the data and the marker and assay were joined together into one column so that I can split the inflammatory markers from the rest. The summary function was used to check for the mean and the median of all the data and to find outliers. We then ran a t-test on the positive versus the negative patient's data frames using the t-test function in R. We found the mean value for the covid positive against the covid negative patients along with the t-value, the p-value, and the false discovery rate to assert that null hypothesis is not of concern. We ordered our results data frame based on the p-value and only picked the markers that had a p-value of less than 0.05 and FDR value of less than 0.05

Since our focus is on cardiometabolic and inflammatory markers, the 2 heat maps are provided down below.

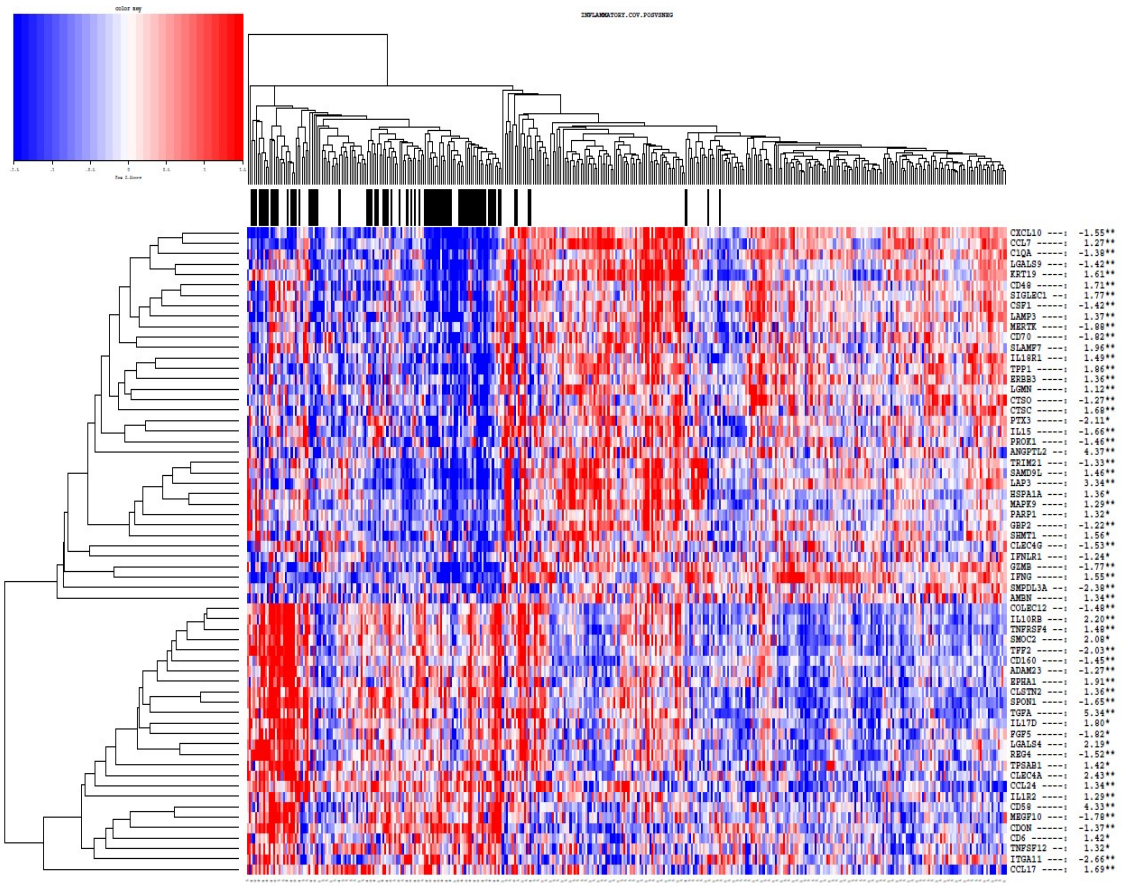

Fig 1: Top Inflammatory markers with fold change values for covid positive vs negative patients.

The solid black shades on the top column represents the covid positive patients and the markers along with their fold change values are represented on the right side of the diagram.

The heatmap above only contains the inflammatory markers that had the biggest fold changes between COVID positive and Covid negative patients. The darker shades on the column are the samples of the covid positive patients and the lighter ones

represent the negative patients. Markers that are matched with the jklist are explained further.

VI. PATHWAY ANALYSIS BETWEEN POSITIVE PATIENTS:

There is a vast array of markers present in this dataset, since the focus of this paper is the inflammatory markers, only the markers that correspond to the T-helper pathways were chosen. The initial data preparation step was to have all the marker names on rows and the subject id_numbers on the columns and the matrix was filled with the normalized Protein expression values. Patient differentiation for this analysis and all the others moving forward was based on Covid-positive patients and the WHO scores. As mentioned before, the patients were differentiated into 3 categories and labeled as deceased, intubated, and non-severe.

After preparing the dataset, the gsva library was used and we used the gsva function to estimate the enrichment scores.

R code for GSVA function

```
resetGSVA.zscore <- gsva(who_df, JKList,verbose = FALSE, method='zscore', parallel.sz=4)
```

The first input is the data frame with all the normalized Protein expression values that are differentiated by the WHO scores. The second input is the JKList that consists of a matrix of gene collection that contains annotations for the genes that are the row names from the WHO data frame. The ‘z-score’ method [6] was used which standardizes the

initial expressions into z-score over the sample and combines them together as their sum divided by the square root of the size of the gene set.

Separating the pathways:

Once the gene enrichment was calculated using the gsva function and the scores were compared against the JKList database, the pathways were separated into 4 categories. Th1, Th2, Th17, and Atheros. Before these pathways were further explored by each individual assay, a heatmap was generated to have a general analysis to see the difference between the three categories of patients. In order to generate the heatmap the data needed further preparation.

The first step for generating the heatmap was to create a data frame that included all the selected pathway z-scores for all the patients that were categorized using the WHO score category. A linear model was generated from the data frame with the 3 categories of patients being the independent variables and the mean values of the pathways as the dependent variables to find a relationship between the different categories of the COVID-positive patients and the inflammatory pathways.

```
w.pathway.results <- c()

for (i in 1:(ncol(who.pathway.df.cp)-1)){

  indp1 <- who.pathway.df.cp$`7`
  dept1 <- who.pathway.df.cp[,i]

  lm1 <- lm(dept1 ~ indp1)
  m1<- lsmeans(lm1, specs = ~ indp1)

  D1<- contrast(m1,'pairwise', name='Contrast', adjust='none')

  m1 <- as.data.frame(m1)
  D1 <- as.data.frame(D1)

  w.pathway.results <- rbind(w.pathway.results, c( m1[1,"lsmean"], m1[2,"lsmean"], m1[3,"lsmean"],
                                                   D1[1,"estimate"], fch_compute(D1[1,"estimate"]), D1[1,"p.value"],
                                                   D1[2,"estimate"], fch_compute(D1[2,"estimate"]), D1[2,"p.value"],
                                                   D1[3,"estimate"], fch_compute(D1[3,"estimate"]), D1[3,"p.value"]

  )
  )

}
```

Fig: R code for generating the linear model

VII. GENERATING THE HEATMAPS:

The results from the linear model were organized in a data frame with the means, the estimates, and the p-values. A function called ‘doClusterTableFCH_fromMatrix’ was used to create the heatmap. The parameters used for this function for the analysis are.

- A matrix consisting of the names of the pathways as rows and the mean values of the three categories, p-value, and false discovery rate values of the 3 categories.
- Ps parameter takes names of all the pathways in the data frame
- Colfac (column factor) are the three factors which in this case are the 3 WHO categories of patients.
- Dmeth is the agglomeration distance to cluster.
- Hmeth; The method to measure the distance between the 2 matrix rows, and in our case I used the Euclidean distance between 2 points and used the absolute value of the delta.
- We used the ‘average’ option for the hierarchical clustering method.
- Coefs; The fold change values for the individual pathways were used for the coefficient parameter and the false discovery rate values were used for the fdrs parameter. The fold change was calculated by keeping the sign of subtracting the positive mean from the negative mean and then multiplying

the sign with 2 to the power of the absolute value of the difference between the positive and the negative mean.

- Fdrs contains the false discovery rate values of all the pathways.

```
pdf(paste0('./pathway_heatmap.',Sys.Date(),'.pdf'),height=15,width=30)
doClusterTableFCH_fromMatrix(w.pathway.results.f.means, ps=wDEG,
                             ss=ss,
                             colfac=as.factor(c('Deceased','Intubated','Non-severe')),
                             dmeth=my.cordist, hmeth='average', cexg=2.5, ColD=TRUE,
                             main='who Path',
                             margins=c(30,70),
                             scl=NULL, breaks=seq(-1.5,1.5,.1), coefs = (w.p.COEF),
                             fdrs=w.p.FDRS)
dev.off()
```

Fig: R code to generate the heatmap

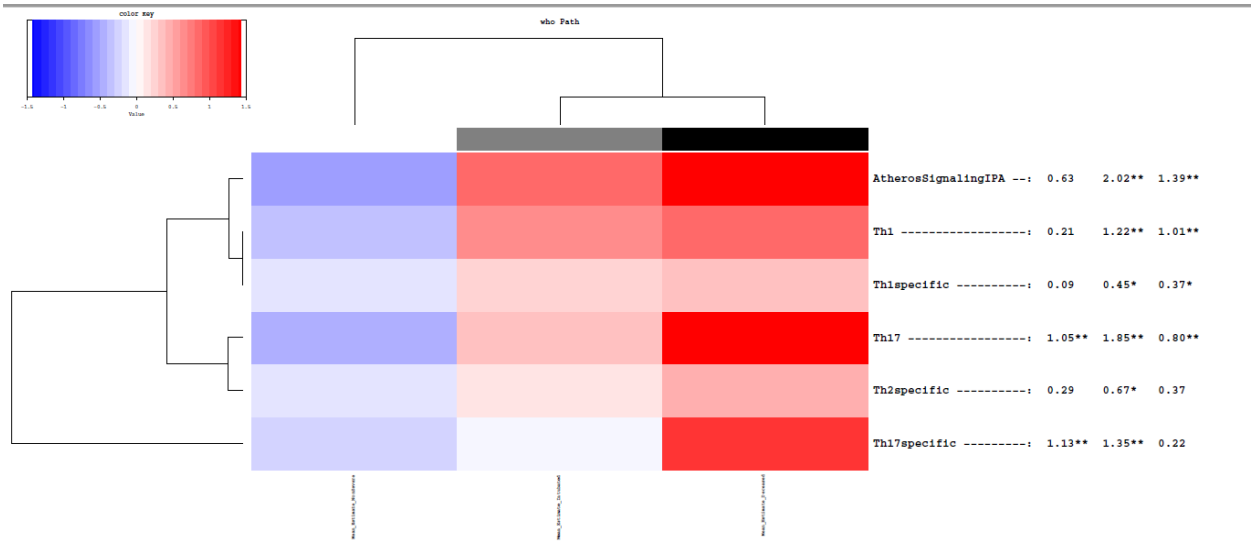

Fig: Heatmap based on several pathways for COVID positive patients

The first blue column from the right represents the non-severe patients, followed by the intubated and the deceased patients in red and bright red. The column of numbers represents the fold changes between the deceased versus intubated patients followed but the deceased versus non-severe and the last column is the intubated versus non-severe.

The white bar represents the non-severe patients followed by the intubated patients represented in gray and then the deceased patients in black. For additional analysis, the Atheros pathway was also included. The scores to the left are the fold change values obtained by dividing the mean values of each pathway and we can clearly see that there are significant fold changes between all the T-helper cell pathway markers between the deceased and the non-severe patients. A deep analysis and explanation of these individual pathways are done in the next part of the paper.

Atheros pathway:

Atherosclerosis is the hardening of the arteries of the heart by plaque formation. A plack is a waxy substance usually made of lipids. Atherosclerosis is one of the most common causes of heart attacks and other heart-related problems. Ischemia of the cardiac muscle cells occurs when blood supply is cut off due to the plaque formation.

Only the assays corresponding to the Atheros pathway were selected for this analysis. A separate data frame had to be created with the 26 selected assays (from the jklist) as the columns as the normalized Protein expression values as the rows of the WHO categorized patients. A linear regression model was made with the categories as the independent variables and the assays as the dependent variables. The output from the linear regression consists of the means of all the categories against each other, including the false discovery rate and the log fold change. The heatmap functions were provided with the same parameters as before and the heatmap is down below;

	Deceased	Intubated	Non-Severe
CCL2	↑ 6.62811219512195	6.3834909	5.93855479
CD40	↑ 7.16017804878049	6.8489288	6.62610798
CXCL12	↑ 1.78835609756098	1.4657303	1.32890585
F3	↑ 5.68086341463415	5.4528621	4.89768404
ICAM1	↑ 6.27356829268293	6.3072576	6.16576862
IFNG	↓ 2.91263414634146	3.1910818	3.58214628
IL1A	↑ 0.295560975609756	0.3088606	0.24004521
IL1B	↑ 0.973985365853659	0.7135530	0.55560957
IL1RN	↑ 3.50419024390244	3.2272742	2.22757181
IL33	↑ 0.145146341463415	0.1662727	0.05597713
IL6	↑ 6.81597560975611	6.3769379	4.84699362
ITGB2	↑ 4.93110243902439	4.8064152	4.75257713
LPL	↑ 5.12305609756098	4.4329500	4.59376064
MMP1	↑ 10.3256268292683	10.0618197	9.66102181
MMP9	↑ 3.38876097560976	3.2663955	2.75148457
PDGFA	↓ 1.56549024390244	2.0466591	1.92735266
PDGFB	↓ 4.73201707317073	5.4379500	5.25149255
PDGFC	↓ 0.329587804878049	0.4272121	0.36097926
PLA2G2A	↑ 4.62591219512196	4.4518000	3.21855798
PRDX6	↑ 2.58237073170732	2.6352455	2.31945053
SELE	↑ 5.66111951219512	5.8582939	5.66029149
SELP	↑ 2.90970731707317	2.8541364	2.76402340
SELPLG	↑ 3.61214146341464	3.5118212	3.58610053
TNF	↑ 2.07664146341464	2.0237758	1.77815638
TNFRSF12A	↑ 3.15669756097561	2.4495773	2.01696968
TNFSF12	↓ 5.20636341463415	5.1085439	5.31239255

Table: Atheros means values

	FCH_Deceased_vs_Intubated	FCH_Deceased_vs_NonSevere	FCH_Intubated_vs_NonSevere
CCL2	1.18478171956078	1.61278866885669	1.36125384299023
CD40	1.24078165461627	1.4480084987974	1.16701314321513
CXCL12	1.25060465687263	1.37501775390635	1.09948235547503
F3	1.17121123124649	1.72091921196276	1.4693499908904
ICAM1	-1.02362641564371	1.0775835076049	1.10304294344638
IFNG	-1.21288912317022	-1.59053501242669	-1.31136060340733
IL1A	-1.00926122352653	1.03923056136514	1.04885510788954
IL1B	1.19783760943265	1.33642214261147	1.11569559353246
IL1RN	1.21160211485643	2.42270447819114	1.99958752835144
IL33	-1.01475143893913	1.06375743376142	1.07944938659159
IL6	1.35569978210764	3.91491773651446	2.88774682137082
ITGB2	1.0902713844255	1.13172646991399	1.03802272175595
LPL	1.61340216565356	1.44322422423618	-1.11791510879568
MMP1	1.20064290796412	1.58513423774108	1.32023787191558
MMP9	1.08851819359249	1.55539003522189	1.4289058688937
PDGFA	-1.39587412184432	-1.28508377808297	1.08621254555607
PDGFB	-1.63119914789075	-1.43343400031974	1.13796599461635
PDGFC	-1.07001002717453	-1.02199734646775	1.0469792616123
PLA2G2A	1.12826987703475	2.65250269398595	2.3509470100869
PRDX6	-1.03732985558591	1.19990500984514	1.24469729057948
SELE	-1.14645078751571	1.00057410642847	1.14710897228275
SELP	1.03927031810265	1.10625495167749	1.06445352321534
SELPLG	1.0720114025907	1.01821408767043	-1.05283497407049
TNF	1.03732337218295	1.22985231361469	1.1856016615403
TNFRSF12A	1.63254220385001	2.20339459019707	1.34967082933649
TNFSF12	1.07015478133619	-1.07626186215753	-1.15176677775767

Fig: Atheros pathway-specific marker fold changes.

The first column represents the selected markers from the jk list followed by the fold change values. The highest fold changes values are highlighted in red followed by the second highest values.

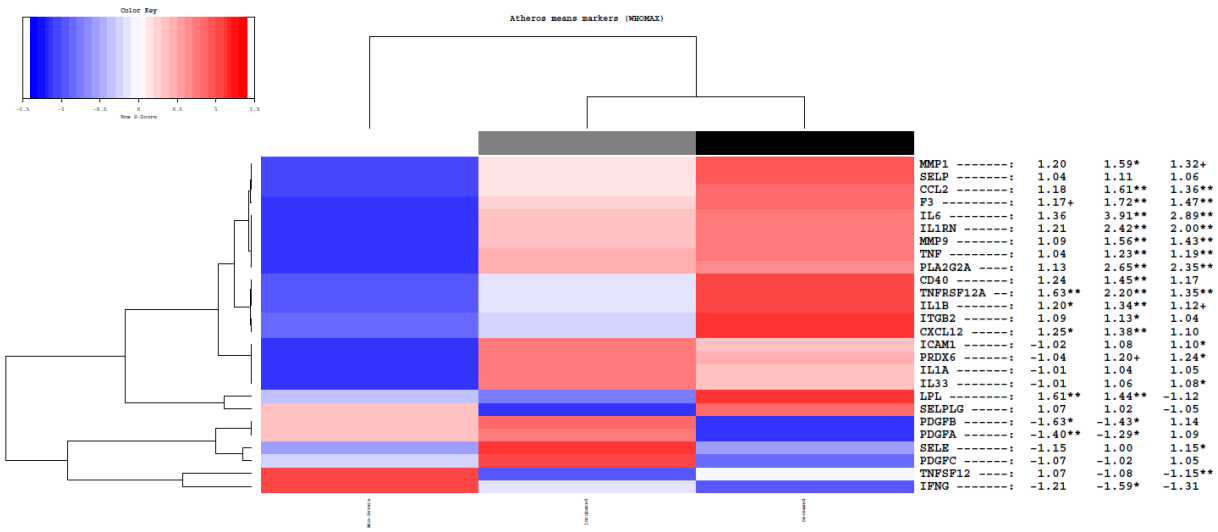

Fig: Heatmap generated from Atheros pathway assays

The results are consistent with what was seen before in the pathway analysis. The deceased against the non-severe markers have the highest fold changes. Severe covid patients have shown higher levels of CCL2(chemokine ligand 2) in their lung macrophages [5] and this study confirms where the fold change between the deceased and non-severe is one of the highest.

Changes in F3(Coagulation Factor III) have been directly correlated with poor outcomes in covid positive patients [6].

IL-1 receptor antagonist is elevated in deceased patients, and according to [6] it is evident that suppressing/ blocking IL-1 receptor alleviates acute hyperinflammatory respiratory failure in COVID-19 patients.

Th1 pathway:

T cells play a vital role in the immune system, specifically in the adaptive immune system and each of them is activated by a specific type of cytokines and transcription factors. Since the main pathway for covid to enter our body is through the nasal passage, Th1 cells are important as the defense mechanism as they promote cell-mediated immune responses and are required for host defense against intracellular, viral and bacterial pathogens. The data set preparation follows the same procedure where the z-score values were compared with the jk list and these are the selected TH1 markers; “CCL3" "CCL4" "CCL5" "CXCL10" "CXCL11" "CXCL9" "IFNγ" "IFNγR1" "IFNγR2" "IL12B" "IL1B" and "IL2RA".

The next procedure is to run a linear model where the dependent variables are the assays and the independent variables are the patients that are categorized in 3 categories. After running the linear model, a data frame was generated with mean values of each category against the other, the fold change, and the p values.

	Deceased	Intubated	Non-Severe
CCL3	↑ 3.82255853658537	3.759805	3.3875085
CCL4	↑ 4.5132512195122	4.449464	4.1992367
CCL5	↓ 3.02582682926829	3.473033	3.5043255
CXCL10	↑ 8.08715853658537	8.017332	7.0742090
CXCL11	↑ 5.43631951219512	5.434698	5.0764830
CXCL9	↑ 4.70074634146342	4.624453	3.9695250
IFNG	↓ 2.91263414634146	3.191082	3.5821463
IFNGR1	↑ 1.70759512195122	1.369600	1.1835936
IFNGR2	↑ 2.29893170731707	2.539286	2.2252293
IL12B	↓ 5.54744634146341	5.581524	5.6750787
IL1B	↑ 0.973985365853659	0.713553	0.5556096
IL2RA	↑ 5.34139268292683	5.163850	4.9182899

Table: Mean values of TH1 pathways for different categories, red shows elevated level compared to non-severe subjects

From the table above it can be clearly seen that pro-inflammatory cytokine CXCL10 has the highest mean amongst the deceased patients followed by the intubated patients. CCL3, CCL4 are also elevated in deceased patients compared to the rest. The opposite can be seen for IFNγ as deceased patients and intubated patients have low means compared to the non-severe patients. Before proceeding further here is a brief summary of what Interferons are.

Interferons (IFNs) are proteins that are created and released by host cells that result from the presence of pathogens such as viruses, tumor cells, parasites, and

bacteria. They enable cell communications for triggering the protection of the immune system. There are 2 types of interferons, Type 1 IFN and Type 2 IFN.

The functions of interferons are as below.

1. They activate natural killer cells.
2. They upregulate antigen presentation to T lymphocytes which results in increased recognition of infection or tumor cells.
3. They enhance the ability to resist new infections of uninfected host cells.

Hence it can be seen that lower means of IFNγ plays a crucial role in the survival of patients.

	FCH_Deceased_vs_Intubated	FCH_Deceased_vs_NonSevere	FCH_Intubated_vs_NonSevere
CCL3	1.04445764639075	1.35195771181431	1.29441123485108
CCL4	1.0452061967477	1.24316217838936	1.18939419059859
CCL5	-1.36339774694819	-1.39329302307284	-1.02192703940693
CXCL10	1.04959061009105	2.01803261902716	1.92268547338862
CXCL11	1.00112424202174	1.28328048548023	1.28183938777537
CXCL9	1.05430575008948	1.66004383924028	1.5745374044478
IFNG	-1.21288912317022	-1.59053501242669	-1.31136060340733
IFNGR1	1.26399882460548	1.4379380328139	1.13761026104017
IFNGR2	-1.18128301939506	1.05241407764769	1.24319887929753
IL12B	-1.02390218640948	-1.09249932014056	-1.06699578791957
IL1B	1.19783760943265	1.33642214261147	1.11569559353246
IL2RA	1.13095590652386	1.34080811823534	1.18555295613291

Table. Fold changes for TH1 markers, highest fold changes between deceased and non-severe shown in red, followed by yellow.

The same procedure was followed as above to generate a heatmap with the Th1 assays as the rows and the three categories of patients as the columns and the values consisting of the mean values from our linear model. The heatmap generated is as below:

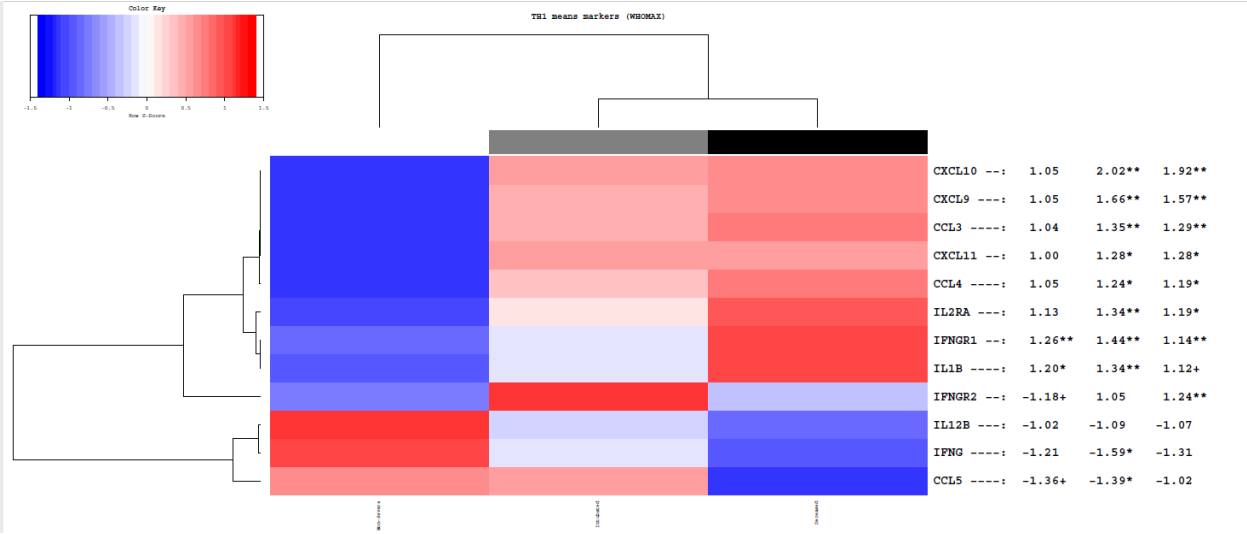

Fig: Th1 heatmap

The heatmap clearly shows that pro-inflammatory cytokines like CXCL10 have one of the highest [fold change value of 2.02(deceased vs non-severe)] indicating positive association of T-helper-1 cell function. The driving factors of these cytokines can be related to the much-discussed “cytokine storm”[7]. There is also evidence of overexpression of CCL3[Fold change of 1.35(deceased vs non-severe)], CXCL11[fold change of 1.28(deceased vs non-severe)], IFNγ1[fold change of 1.43(deceased vs non-severe)], and IFNγ2[fold change of 1.24(intubated vs non-severe)] in intubated patients further solidifying evidence of higher T-helper-1 cell function activity.

Th2 pathway:

Th2 cells mediate the activation and maintenance of the antibody-mediated, immune response against extracellular parasites, bacteria, allergens, and toxins. Th2 cells like Th1 cells also produce various cytokines such as IL-4, IL-5, IL-6, IL-9, and IL-17E(IL-25). Th2 cells stimulate and recruit specialized subsets of immune cells such as eosinophils and basophils. Basophils and eosinophils are vital effector cells in human allergic diseases, they play an important role in promoting allergic inflammation through the release of proinflammatory mediators. The th2 markers are selected the same way as the th1 markers and the list consists of "CCL11" "CCL13" "CCL17" "CCL18" "CCL22" "CCL24" "CCL26" "CCL7" "IL10" "IL13" "IL33" "IL4R" "IL5" "IL7R" and "TSLP".

	Deceased	Intubated	Non-Severe
CCL11	↑ 6.84570731707317	6.33067727	6.35384362
CCL13	↑ 6.37981951219512	6.22762121	6.17464309
CCL17	↓ 4.15869512195122	4.22982273	4.61510798
CCL18	↑ 5.79383902439025	5.78655758	5.63154574
CCL22	↓ 5.17206829268293	5.27278788	5.44260213
CCL24	↑ 5.11542682926829	4.69108939	4.64971649
CCL26	↑ 1.94672195121951	1.84481061	1.74249894
CCL7	↑ 5.42666585365854	5.68446212	4.34779947
IL10	↑ 0.395817073170732	0.37814242	0.09606170
IL13	↓ -0.199251219512195	-0.08607879	-0.09671277
IL33	↑ 0.145146341463415	0.16627273	0.05597713
IL4R	↑ 3.94079756097561	3.61803333	3.09242074
IL5	↓ 0.0981731707317064	0.39597727	0.18509947
IL7R	↑ 4.03603414634147	3.71333485	3.96837553
TSLP	↓ 0.123090243902439	0.15944091	0.17098511

Fig: Mean values of TH2 pathways for different categories, red shows elevated level compared to non-severe subjects

CCL7, CCL11, IL4R, and IL7R have the highest means amongst the deceased patients. C-C motif chemokine is a protein that in humans is encoded by the CCL11 gene. Increased levels of CCL7 are directly related to loss of lung function and fatal outcomes [8] and this study confirms this as the deceased patients have a CCL7 mean value of 5.43 compared to 4.35 in non-severe patients.

IL(Interleukin) receptors are a type of cytokine receptors that belong to the immunoglobulin superfamily. The binding of IL-4 on the surface of macrophages causes alternative activation of macrophages which downregulates inflammatory mediators like IFNγ during an immune response and this was evident in the Th1 heatmap where the deceased patients had a lower mean value of IFNγ compared to that of the non-severe patients. The difference in means in IL4R between deceased and non-severe patients is also quite significant and is visualized in the heatmaps.

	FCH_Deceased_vs_Intubated	FCH_Deceased_vs_NonSevere	FCH_Intubated_vs_NonSevere
CCL11	1.42902389907349	1.40626033589134	-1.01618730373116
CCL13	1.11126146230979	1.15282731736152	1.03740420815577
CCL17	-1.05053745917169	-1.37212589437552	-1.30611800883083
CCL18	1.00505987373293	1.1190645680784	1.11343074907771
CCL22	-1.07230817405808	-1.20625409074938	-1.12491363950383
CCL24	1.34195606143142	1.38099714773624	1.02909267108431
CCL26	1.07319433565959	1.15206571749918	1.07349217119294
CCL7	-1.19565093912813	2.1123756051116	2.52566387604305
IL10	1.01232648560638	1.23093567342935	1.21594731633641
IL13	-1.08160402961349	-1.07366093147621	1.00739814396185
IL33	-1.01475143893913	1.06375743376142	1.07944938659159
IL4R	1.25072466380095	1.80047406247252	1.43954470123015
IL5	-1.22927193781842	-1.06210492558691	1.15739218245234
IL7R	1.25066837508115	1.04801445404391	-1.19336939510259
TSLP	-1.02551647228405	-1.03375539777686	-1.00803392799187

Fig. Fold changes for TH2 markers

The data set preparation also follows the same procedure as that of th1 and the heatmap produced is as below

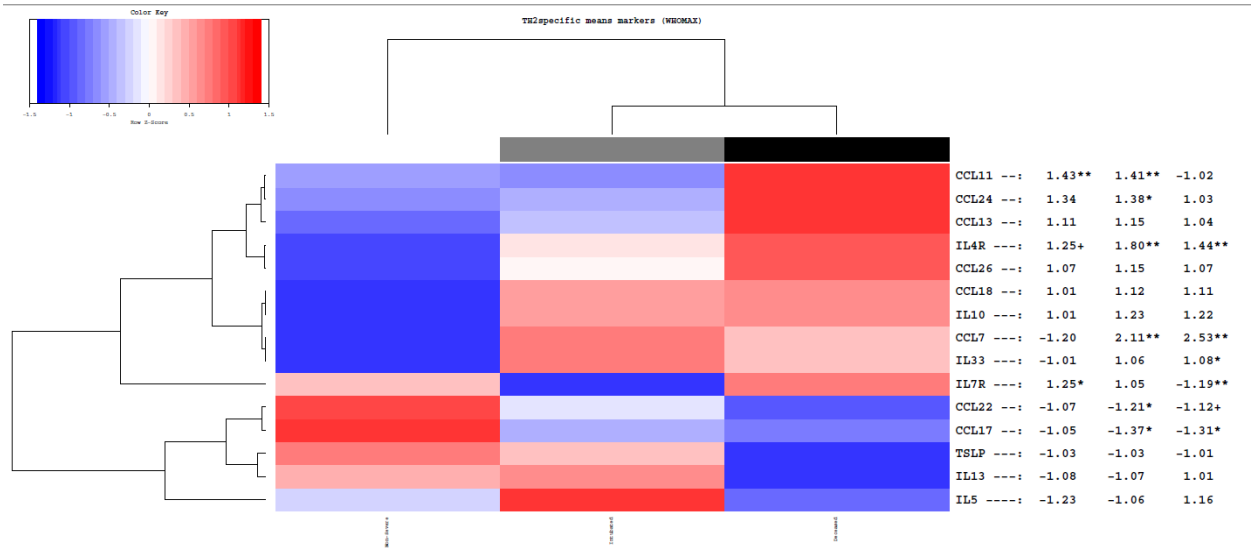

Fig: Th2 heatmap

Th17 pathway:

The process for studying the TH17 cells arises like that of Th1 and Th2. The mean values are in the table below.

	Deceased	Intubated	Non-Severe
CCL20	↑ 6.2170756097561	5.6334561	4.9017176
CXCL1	↑ 3.89020487804878	3.5670000	3.5413926
CXCL3	↓ 4.74091219512195	4.7368864	5.0763207
IL12B	↓ 5.54744634146341	5.5815242	5.6750787
IL17A	↑ 0.803480487804879	0.4156970	0.4512489
IL17F	↑ 0.50649756097561	0.3746712	0.4249404
IL6	↑ 6.81597560975611	6.3769379	4.8469936
IL6R	↑ 4.35270487804877	4.2790773	4.3140809
LCN2	↑ 3.03954878048781	2.5244576	2.2108170
PI3	↑ 2.79628536585366	1.8013045	1.6854941
S100A12	↑ 6.27498536585366	6.3617197	5.7134809
S100P	↑ 2.39181951219513	2.0339333	1.4431271

Table: Mean values of TH17 pathways for different categories, red shows elevated level compared to non-severe subjects

The markers with the highest means and significant fold changes amongst the deceased and the rest are CCL20, S100P, IL6, LCN2, and AS100A12.

	FCH_Deceased_vs_Intubated	FCH_Deceased_vs_NonSevere	FCH_Intubated_vs_NonSevere
CCL20	1.49860435503525	2.48864087552891	1.66063902534861
CXCL1	1.25110673796922	1.2735117978631	1.01790819217411
CXCL3	1.00279439079537	-1.26173466254313	-1.26526044227033
IL12B	-1.02390218640948	-1.09249932014056	-1.06699578791957
IL17A	1.30838172905692	1.276533635223	-1.0249488873267
IL17F	1.09567987612331	1.05815952144903	-1.03545812697777
IL6	1.35569978210764	3.91491773651446	2.88774682137082
IL6R	1.05235948007131	1.02713372868634	-1.02455936425848
LCN2	1.42908448117921	1.77612332575722	1.24283997842567
PI3	1.99305402924877	2.15964055947238	1.08358354955706
S100A12	-1.06196361039812	1.47580746115616	1.56725381970188
S100P	1.28154681279085	1.93012245976535	1.50608814325096

Table: Th17 fold change values

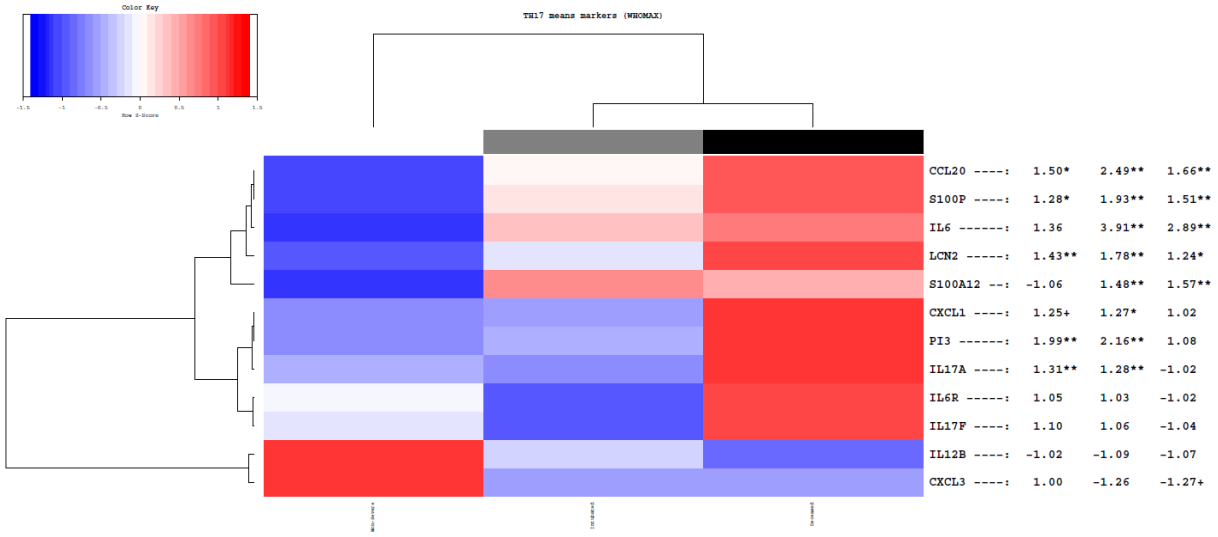

Fig: Th17 heatmap

The markers that had significant fold changes here are CCL20, S100P, IL6, LCN2, PI3 and AS100A12. Chemokine ligand 20 (CCL20) is a liver activation regulated chemokine. Gene expression of this assay can be induced by inflammatory cytokines such as IFNγ.

It can be seen that patients that are intubated have the highest enrichment of CCL20, similar results are seen from Anno et.al research [9]. Moving to S100P, other studies have shown higher levels of this marker to be strongly related to lung inflammation in covid positive patients [10].

V. GENERAL LINEAR MODEL AND LASSO REGRESSION:

The next analysis step is to create a linear model to find an association between cardiometabolic markers and the T helper pathways. The data preparation was similar as the rows contained all the cardiometabolic markers and the columns consisted of all the positive patients and the values were the normalized Protein expression values. The T-helper cell marker pathway values were selected from the JK list as mentioned above and 8 Th1 markers were selected along with 15 Th2 markers and 11 Th17 markers. Th1 markers are CCL3, CCL4, CXCL10, CXCL9, IFNγ, IFNγR1, IL12B, and IL1B. The 15 Th2 markers are CCL11, CCL13, CCL17, CCL22, CCL24, CCL26, CCL7, IL10, IL10RA, IL10RB, IL13, IL33, IL4R, IL5, and IL5RA. The 11 Th17 markers are CCL20, CXCL1, CXCL10, CXCL12, CXCL14, CXCL17, CXCL3, IL12B, IL17A, IL17F, and IL6.

The first step was to create a regression model against the Th1 helper cells and lasso regression was chosen to determine the coefficients. The dependent variables were the cardiometabolic markers and the independent variables were the T-helper cell pathway markers. The decision to use lasso regression instead of ridge regression was mainly due to setting a specific cut-off point for the T -helper cell pathway markers and ridge regression tend to asymptotically make the slope close to 0 as the penalty is increased, and markers with weaker association are not needed. Lasso regression on the

other hand takes the absolute value of the slope multiplied by the penalty and hence can completely shrink the slope to 0 which helps in having markers with the better association

and since lasso regression excludes markers with the minimal association, and it is better at reducing the variance of our model.

Glmnet package in R was used to perform lasso regression and the arguments chosen are as follows. The first input matrix was the T helper cell pathway marker matrix, and the second input was the cardiometabolic matrix and the distribution family is chosen as binomial. The maximum coefficients for each independent marker were chosen and then the matrix was multiplied with each individual pathway matrix. Similar procedures were followed to generate the rest of the T helper pathway markers.

The prediction results were the matrix multiplications performed in the previous step and the Pearson Correlation test was performed in order to find the linear association between the cardiometabolic markers and the T helper cell markers. The reasoning behind choosing Pearson Correlation is as below:

.

1. The blood marker values that are used to perform our analysis were all acquired from random patients.
2. Both variables are continuous data, i.e., the normalized Protein expression values of the cardiometabolic markers and the pathway markers are both continuous.
3. All the data contains paired samples, i.e., every individual patient sample contains both cardiometabolic values and T helper cell marker values. Patient number 365 was discarded as the subject was missing the Th1 Interferon Gamma marker value.
4. There is no relationship between the values of the variables of the subjects, i.e, none of our patients are related to each other.
5. All the variables are (approximately) normally distributed. (Pathway transformation needed)
6. A linear correlation exists between the cardiometabolic markers and pathway markers.
7. No outliers exist in the data as they were already removed.

Once it was ensured that the variables fulfill all these criteria for a Pearson correlation test, the test was carried out and three separate data matrices for the individual pathways were generated that consisted of the estimates, the adjusted p-values, and the false discovery rates, and all the three pathways were combined into one data matrix.

Results of Pearson correlation

Using the Pearson correlation, the cardiometabolic markers with a correlation value of 0.8 or greater for each pathway were selected. There were 41 markers with a higher than 0.8 correlation for TH1 and the top 5 cardiometabolic markers were COL6A3, THBD, NECTIN2, HSPG2, and EPHB4. There were 37 markers for TH2 with the top 5 markers being the same as TH1 and 10 markers for TH17 with the top 5 markers being CNST, SNAP23, GRAP2, CD69, IL6.

Alternative correlation testing:

After performing the Pearson, a Spearman correlation test was performed to see if the results vary, and hence the necessary steps were taken as below to see first if the data qualifies for Spearman testing. There are two major differences between Pearson and Spearman's testing, the first being that there must be a monotonic relationship between our two variables, and the figure below shows that the test holds.

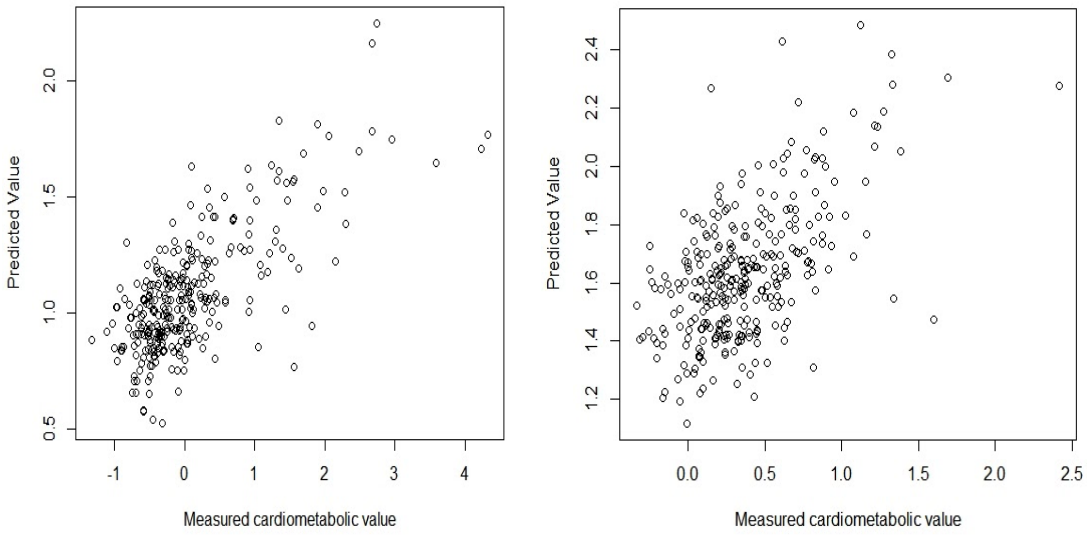

Fig: Spearman verification TH1 and TH2

The data points show a positive linear trend and as the predicted values increase, the cardiometabolic values also increase. And the same can be seen for the TH17 markers in the figure below.

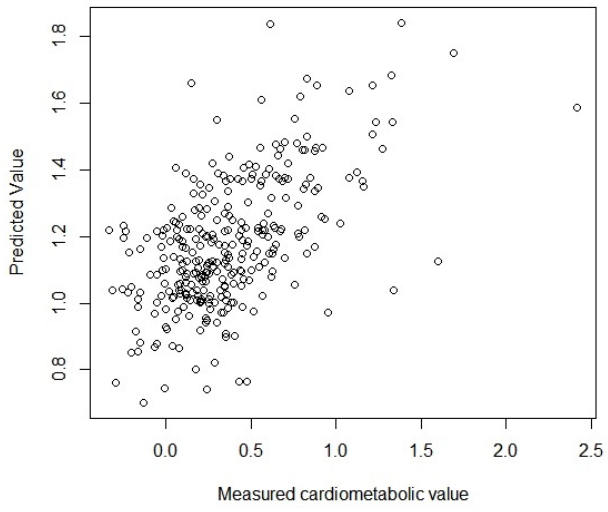

Fig: Spearman verification TH17

The next step includes combining all the estimates, p-value, and adjusted p-value (False Discovery Rate) like the Pearson correlation and detecting the markers with a correlation greater or equal to 0.8.

Spearman correlation results:

The same steps were performed and a threshold of 0.8 was used to select the cardiometabolic markers. 41 markers with TH1 markers were selected and the top 5 markers were COL6A3, HSPG2, EPHB4, IL18BP, NECTIN2, and 2 differences were found compared to Pearson correlation with the new inclusions being EPHB4, IL18BP.

Th2 yielded 32 markers with the threshold limit and the top 5 were similar to TH1 as seen on Pearson correlation. Finally, 10 markers were selected for TH17 with the top 5 being CA13, CD69, CCL5, IL6, LTBP2, and 3 different markers were found on the top 5 compared to Pearson correlation with the changes being CNST, SNAP23, GRAP2. Most of the differences fall within the threshold of error and hence the results of Pearson Correlation were chosen for the next step of the analysis.

VI. MAPPING AND NETWORK PLOTTING:

All the previous steps were carried out to see how the Th pathways interacted with the cardiometabolic markers and after performing regression and multiple correlation tests in the previous section, several selected markers were chosen. There are multiple ways to visualize these interactions, and mapping was chosen with each white vertex representing a cardiometabolic marker and each light green arrow representing a T helper positive association pathway, and red for negative association.

Data preparation for mapping:

For this procedure, the correlation results from the previous test were chosen. A symmetric threshold was used for detecting positive and negative association where any correlation value higher than 0.05 represents positive association and values less than -0.05 represents negative association. Only 10 cardiometabolic markers with the highest correlation of each pathway were chosen and 1 from each pathway is visualized below.

```r
x <- subset(th1df, rownames(th1df) %in% TOP10TH1CM)

mapping <-c()

for (j in(1:nrow(x))){

  for (i in (1:ncol(x))){
    if ((x[j,i])> 0.05){
      print(colnames(x[i]))
      print('activation')
      mapping <- rbind(mapping, cbind(rownames(x[j,]),colnames(x[i]),'activation'))

    }
    else if (x[j,i]< -0.05) {
      print(colnames(x[i]))
      print('supression')
      mapping <- rbind(mapping, cbind(rownames(x[j,]),colnames(x[i]),'supression'))
    }
  }
}
```

R code to determine positive and negative association

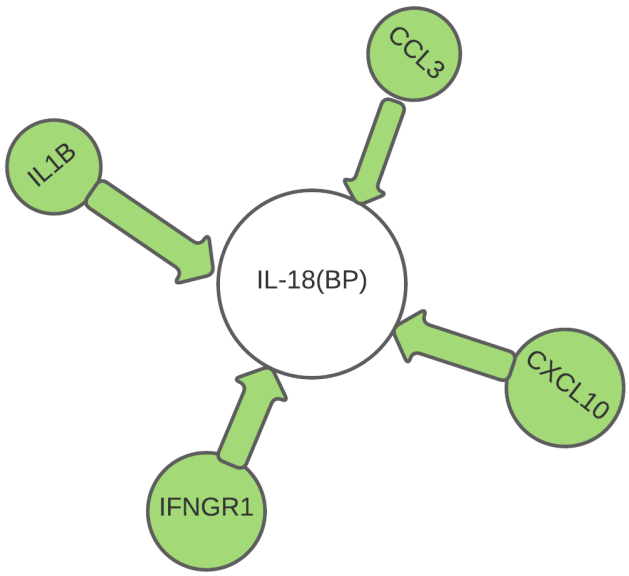

Fig: IL-18(BP)(cardiometabolic marker) being invigorated(green) by TH1 markers

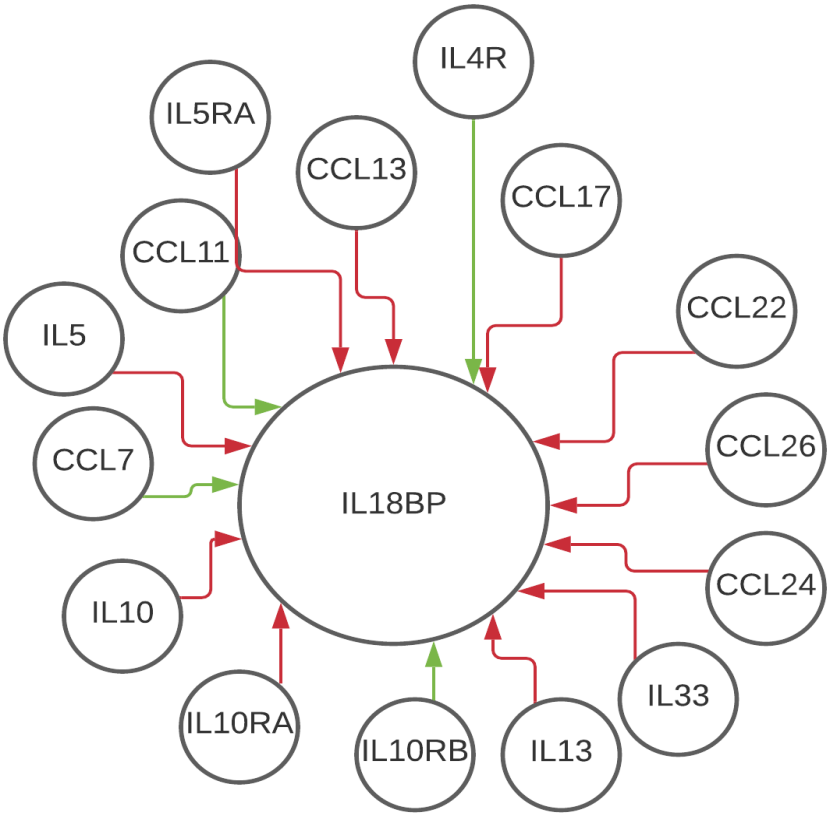

Fig: IL18BP (cardiometabolic marker) being invigorated(green) and subdued(red) by TH2 marker and

The effects of IL18-(BP) and LTBP2 are explained in the conclusion section.

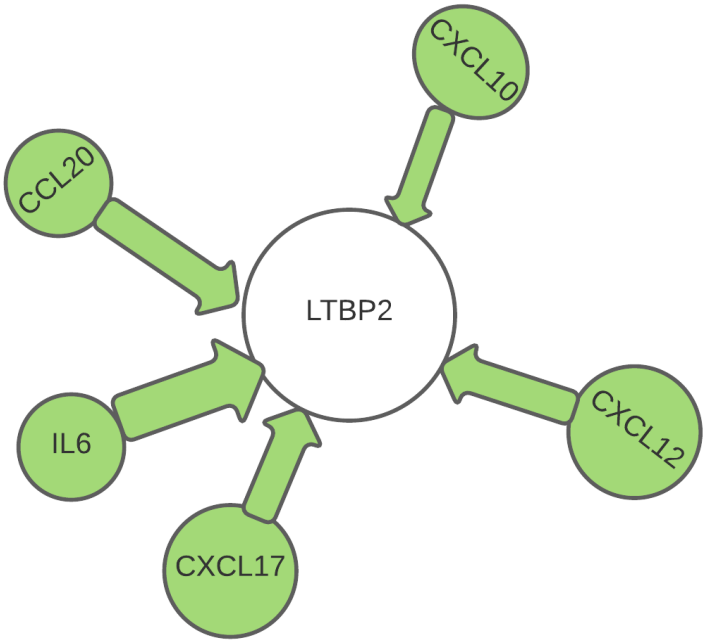

FIG: LTBP2 being invigorated(green) by TH17 markers.

VII. CONCLUSION:

Covid-19 has changed human life and the way people live, behave and interact forever and has been labeled as one of the worst pandemics in the history of mankind. The mortality rates are one of the highest ever recorded for any disease and have affected people of all ages, races, and gender but especially ones with old age and underlying health conditions. This was a never-before-seen disease that still needs a lot of study and research to understand the implications on the human body in order to find better vaccines and better cures. Many institutions have been very helpful and released data that can be analyzed to find correlations and driving factors of COVID-19.

With the help of the blood protein marker data collected from Massachusetts General Hospital, multiple analyses have been performed that include finding differences between covid positive against covid negative patients.

The second analysis focused mainly on 3 inflammatory pathways, TH1, TH2, and TH17. The main purpose for choosing this pathway was to analyze and find how the inflammatory markers react on 3 categories of patients that are all covid positive as one of the main sources of covid entering the human system is through the nasal passage and mouth, and the body's first line of defense is the inflammatory markers. Using a general linear model, it was observed that all the inflammatory markers were enriched in the severe patients compared to the intubated and non-severe patients. Upon inspecting

each pathway separately, Th1 had several enriched markers that are directly related to cytokine storm, and one of the most important markers, Interferon-gamma that helps in recognition of infection and help in resisting infection in uninfected cells were severely lower in deceased patients. Several immune markers that help fight viruses and infection have been also seen to have a lower count amongst deceased patients compared to non-severe subjects. Th2 and Th17 results also followed the same path where it was observed that deceased patients had an elevated level of CCL7, which in other studies have also been shown to have a direct relation to loss of liver function and fatal outcome.

The last analysis step was to run a generalized linear model to correlate all the cardiometabolic markers to the helper cell pathway markers to observe pathway markers positively and negatively associated with cardiometabolic markers. Several interesting results were observed from the network map generated and the most noteworthy were as follows.

It is seen that IL-18(BP) is invigorated by IFNGR1 and other TH1 markers, and according to [15] it can be concluded that high serum level of IL-18 can be correlated with severe diseases and death in viral infections led by a cytokine storm.

LTBP2, a marker that has been seen in previous studies to be associated with heart failure, was invigorated by the TH17 markers like CXCL17 and others is directly related to heart failure [16].

VIII. FUTURE WORK

As more and more COVID-19 data are being available, some of the future goals include but are not limited to, analyzing time-series data of COVID-19 positive patients against COVID-19 negative patients throughout other pathway markers. There were three other protein markers in the data set used including oncology, neurology, and inflammatory, and there are plans to see how they fare when put under the same treatment as the cardiometabolic markers. Further research plans also include taking previously known underlying conditions between covid positive and covid negative patients to see which of them contribute to the severity of COVID.

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