AN EXHIBITION OF BLOOD MOLECULAR PROFILING FOR DIAGNOSTICS AND DISCOVERY

MOLECULAR PROFILING OF BLOOD FOR DIAGNOSTICS AND DISCOVERY

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Lay Abstract:

Every cell of the body has the opportunity to secrete molecules into the blood. These molecules: proteins, ribonucleic acids (RNAs), and deoxyribonucleic acids (DNAs), can be secreted freely, or within small membrane compartments called extracellular vesicles (EV). Specific molecules are secreted more or less by cells depending on changes to their immediate environment, such as disease in a particular organ. We leveraged this to the benefit of medical science in three separate scenarios: 1) using the molecular contents of EVs to determine when someone has prostate cancer, and at what stage; 2) examining RNAs of the blood to determine why so many with asthma also have depression or PTSD; 3) measuring RNAs in the blood and hippocampus of mice to better understand how certain bacteria in the gut can alleviate depression. This work illustrates the utility of blood in tackling many challenging problems within medical science.

Abstract:

Every cell of the body has the opportunity to secrete molecules into the blood. These molecules: proteins, RNAs, and DNAs, can be secreted freely, or within extracellular vesicles (EV). The complement of specific molecules secreted by cells can vary in accordance with changes to their immediate environment, such as disease in a particular organ. Cells of the immune system which circulate in the blood may also change the rates at which they produce these molecules in response to a disease or unusual event occurring somewhere within the body. The full complement of proteins, RNAs, or DNAs from all sources within the blood can therefore be measured to garner information about disease states and communication between every tissue of the body. In this body of work, we leveraged this to address three separate challenges within medical science. First, we utilized blood as a source of biomarkers for disease and disease severity; isolating EVs from the blood of prostate cancer patients and healthy subjects and characterized their proteins with mass spectrometry to identify potential biomarkers for prostate cancer and its stages. Next, we explored the ability of blood to identify commonalities between distinct but often comorbid diseases; here we utilized publicly available datasets to identify transcripts or gene sets potentially facilitating the relationship between PTSD, MDD, and asthma. Finally, we utilized differential gene and gene sets expression to gain mechanistic insight into microbiota-gut-brain axis; investigating the hippocampus and blood of mice fed one of two psychobiotic bacteria: *Lactobacillus rhamnosus* JB1, *Lactobacillus reuteri* 6475. The analysis identified several mRNA expression differences potentially responsible for the mood-altering

characteristics of these psychobiotic bacteria. This body of work illustrates the utility of blood omics data for addressing many problems within medical science, and highlights the large scale of information stored within the blood.

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List of Abbreviations and Symbols:

EV	Extracellular vesicles
JB1 & JB-1	Lacticaseibacillus rhamnosus JB1
LR6475	Limosilactobacillus reuteri 6475
MDD	Major depressive disorder
PTSD	Post-traumatic stress disorder
PBS	Phosphate buffered saline
DNA	Deoxyribonucleic acid
RNA	Ribonucleic acid
mRNA	Messenger ribonucleic acid
Ig	Immunoglobulin
INF	Interferon
GABA	γ-aminobutyric acid
CNS	Central nervous system
IL	Interleukin
RNAseq	Ribonucleic acid sequencing
qPCR	Quantitative PCR
CD4 & CD8	Cluster of differentiation 4/8
TH	T helper
Treg	Regulatory T Cell
TNF	Tumor necrosis factor
TLRs	Toll-like receptors
MHC	Major histocompatibility complex

ANOVA	Analysis of variance
BH	Benjamini-Hochberg
FDR	False discovery rate
CEA	Carcinoembryonic antigen
CA	Cancer antigen
TPS	Tissue polypeptide-specific antigen
HER2	Human epidermal growth factor receptor 2
ctDNA	Circulating tumor deoxyribonucleic acid
BBB	Blood brain barrier
MV	Membrane vesicles
BPD	Borderline personality disorder
CRP	C-reactive protein
PCa	Prostate cancer
PSA	Prostate specific antigen
MS	Mass spectrometry
ID	Identification
CFB	Complement factor B
OC	Organ confined
EC	Extracapsular extending
SI	Seminal vesicle invading
RBP4	Retinol binding protein 4
PBIP1	PBX Homeobox Interacting Protein 1
APOC2	Apolipoprotein C2
PEDF	Pigment epithelium-derived factor

IGJ	Immunoglobulin joining chain
AMBP	Alpha-1-microglobulin/bikunin precursor
ITIH2	Inter-alpha-trypsin inhibitor heavy chain H2
MBB1A	Myb binding protein 1A
PCNT	Pericentrin
RET4	Retinol binding protein 4
ITI	Inter-alpha-trypsin inhibitor
AR	Androgen receptor
EGFR	Epithelial growth factor receptor
ADT	Androgen deprivation therapy
RIPA	Radioimmunoprecipitation
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
ACN	Acetonitrile
TRIS	Trisaminomethane
PPM	Parts per million
DE	Differential expression
GSEA	Gene set enrichment analysis
ORMDL3	ORMDL sphingolipid biosynthesis regulator 3
STX8	Syntaxin 8
TRKA	Tropomyocin receptor kinase A
DALY	Disability-adjusted life years
GWAS	Genome-wide association studies
GEO	Gene expression omnibus
C2	Curated gene sets

C7	Immune signature gene sets
PC	Principal component
Adj. P-Val	Adjusted p-value
FC	Fold change
KEGG	Kyoto encyclopedia of genes and genomes
ARHGAP24	Rho GTPase activating protein 24
SHISA4	Shisa family member 4
PTP4A3	Protein tyrosine phosphatase 4A3
TPPP3	Tubulin polymerization promoting protein 3
SPT	Serine palmitoyl transferase
ER	Endoplasmic reticulum
t-SNARE	Target soluble *N*-ethylmaleimide-sensitive factor attachment protein receptor
NGF	Nerve growth factor
CFTR	Cystic fibrosis transmembrane conductance regulator
GTP	Guanosine triphosphate
GDP	Guanosine diphosphate
RHO	Rhodopsin
PBMC	Peripheral blood mononuclear cells
RSV	Respritory syncytial virus
DGE	Differential gene expression
PCA	Principal component analysis
LPS	Lipopolysaccharide
U-BIOPRED	Unbiased Biomarkers for the Prediction of Respiratory Disease Outcomes

TMM	Trimmed mean of M-values
RMA	Robust multichip average
mTORC1	Mammalian target of rapamycin complex 1
COVID-19	Coronavirus disease 2019
UK	United Kingdom
US	United States
FKBP1A	FK506 binding protein prolyl isomerase 1A
SGK1	Serum/glucocorticoid regulated kinase 1
H2-Aa	Histocompatibility 2, class II antigen A, alpha
IFI30	Gamma-interferon-inducible lysosomal thiol reductase
CD79a & CD79b	B-cell antigen receptor complex-associated protein alpha chain and beta chain
SSRI	Selective serotonin reuptake inhibitors
HPA	Hypothalamic-pituitary-adrenal
CD25	Interleukin-2 receptor alpha chain
SNS	Sympathetic nervous system
ASD	Autism spectrum disorder
PTEN	Phosphatase and tensin homolog
BDNF	Brain-derived neurotrophic factor
VEGF	Vascular endothelial growth factor
NF-KB	Nuclear factor kappa B
RAN	NAs-related nuclear protein
FOXO3A	Forkhead box protein O3
CORT	Corticosterone

MAPK	Mitogen-activated protein kinase
PGK	Phosphoglycerate kinase
JAK-STAT	Janus kinase-signal transducer and activator of transcription
VCaP	Vertebral cancer of the prostate
STAR	Spliced transcripts alignment to a reference
GAGE	Gene-set enrichment for pathway analysis
PC346	Prostate carcinoma cell line 346
TMPRSS2:ERG	Transmembrane protease serine 2:v-ets erythroblastosis virus E26 oncogene homolog
PCA3	Prostate cancer antigen 3
PSMA	Prostate specific membrane antigen
SNRPN	Small nuclear ribonucleoprotein polypeptide N
CELF4	CUGBP elav-like family member 4
GPM6B	Glycoprotein M6B
RUNDC3A	RUN domain containing 3A
CPE	Carboxypeptidase E
CLSTN1	Calsyntenin 1
APBB1	Amyloid beta precursor protein binding family B member 1
NCDN	Neurochondrin
rsiR	Regularized sliced inversed regression

Declaration of Academic Achievement:

The author thankfully acknowledges the following individuals for their contributions toward this project: Dr. Jolene Read for her preparation of EVs for mass spectrometry in Chapter 2; Dr. Khalid Al-Nedawi for his preliminary draft of the methods and introduction sections, created figures 2, 3, and 4, as well as his analysis of the mass spectrometry data in Chapter 2; Dr. Anna Dvorkin-Gheva for her bioinformatics consultation for Chapters 3 and 4; Eiko Iwashita for help with maintaining and performing feeding experiments on mice, collecting brain and blood tissue, as well as instructing on hippocampus isolation in chapter 4; Dr. Paul Forsythe for his editing and writing contributions in Chapter 3 and 4.

The author's contributions were as follows: in Chapter 2, performing nanosight analysis, creating the tables and first figure, significantly adding to the preliminary manuscript draft for introduction and results, writing the discussion; in Chapter 3, searching for, selecting, downloading GEO datasets, preprocessing RNAseq data, performing principal component analysis, performing differential expression analysis, performing gene set enrichment analysis, creating figures, writing the first draft of the manuscript; and in Chapter 4, growing, isolating, and resuspending JB1 and LR6475 for mouse consumption, isolating hippocampus from mouse brain tissue, isolating total RNA from both whole blood and hippocampus, aligning and preprocessing RNAseq data, performing principal component analysis, performing differential expression analysis, performing gene set enrichment analysis, writing the initial draft of the manuscript and creating the figures and tables.

Chapter 1: Introduction:

1.1 The Blood

The survival of every cell and tissue in the body depends on the blood and circulatory system, which deliver oxygen and nutrients to their immediate environments, or, interstitial fluid (Pittman, 2011). Freshly oxygenated arterial blood satisfies this end, flowing from the heart through arteries to all tissues of the body to maintain homeostasis of the interstitial fluid (Pittman, 2011). Venous blood, on the other hand, flows from all tissues and cells of the body back through capillaries and veins to the heart, often with manifold molecular cargo secreted into the blood by those tissues and destined for other organs of the body (Pittman, 2011; Ross and Pawlina, 2003; Al Saleh *et al*., 2018). Venous blood enters the right atrium of the heart and via the right ventricle is pumped to the lungs to be re-oxygenated; freshly-oxygenated blood enters the left atrium and via the left ventricle is pumped to the rest of the body, carrying in it molecular cargo that traveled there in the venous blood (Betts, 2013). In this way, organs and tissues can transfer metabolites and other molecules to each other across the entire length of the body through circulation. Perhaps the most important example of this is seen in the digestive system, where food-derived molecular nutrients absorbed by enterocytes are secreted into submucosal blood vessels which feed into the superior and inferior mesenteric veins, these nutrients are pumped to the heart where they are then distributed throughout the body (Miron and Cristea, 2012; Ross and Pawlina, 2003; Kvietys, 2010).

A biomarker is a molecule or any measurable substance present in a particular circumstance, that serves as an indicator that a biological event is occurring (Strimbu and

Tavel, 2010). They are used currently, and are being developed to predict clinical outcomes such as the presence of a disease in the body, the stage or severity of a disease (ie. cancer), or outcomes following clinical procedure (Hampel *et al*., 2010; Devarakonda and Govindan, 2019; Al-Nedawi *et al*., 2019). Blood-based biomarkers are a popular choice for identifying diseases because collection of blood is relatively non-invasive compared with biopsy (Pittman, 2011; Pang *et al*., 2020). The blood is also an ideal place to look for biomarkers as previously mentioned, because it is constantly updated with secreted molecules of all types from tissues all over the body (Uhlen *et al*., 2015). Hypothetically, the information contained within the blood on the health status of every tissue and organ is comprehensive, and it is freely accessible provided one knows the type of molecule they are measuring, and have an instrument sensitive enough to find it.

1.1.1 Extracellular Vesicles

One of the ways in which cells secrete molecules into the blood is within extracellular vesicles (EV), an encompassing term that includes both microvesicles and exosomes. Both microvesicles and exosomes are small lipid capsules; but while microvesicles (200-1000 nm in diameter) are shed by direct budding from the cell membrane, exosomes (40-200 nm in diameter) are produced through invaginations in multivesicular bodies before the organelle fuses with the plasma membrane to release them (van Doormal *et al*., 2009). EVs are known to contain many types of molecular cargo including protein, miRNA, mRNA, and DNA (van Doormal *et al*., 2009). EV have been shown to contain many of the hallmark proteins and nucleic acids that their parent cells are known for, and they can carry out many of the same activities as their parent

cells, or to act biologically in their interest. For example, human prostate cancer cells will load their EV with many pro-inflammatory molecules to extend the influence of the tumour micro-environment throughout the body (Al Saleh *et al*., 2018). It was originally believed that EV were shed solely for the purpose of disposing of cellular waste, but it is now understood that they are also involved in capture and degradation of materials in multivesicular bodies, intercellular signaling, and even transfer of nuclear receptors and other proteins to different parts of the body (Karla *et al*., 2016; Al-Nedawi et al., 2008; Mir and Goettsch, 2020). This latter activity can be enhanced with tissue-specific integrins and tetraspanins embedded on the surface of EVs, which can determine the destination organ or tissue of the molecular cargo before even leaving its parent cell (Murphy et al, 2019; Lotvall *et al*., 2014 Hoshino *et al*., 2015). The brain is not off-limits for blood-based cargo delivery of EV, as they are also capable of crossing the blood-brain-barrier (Matsumoto *et al*., 2017; Yang *et al*., 2015).

Although EV can be isolated from blood, their contents are de facto part of any whole blood analysis as well. Different molecular species fare differently free and in EV however. Proteins have been shown to be quite stable when free in the blood, and even in storage only modest and uniform degradation of proteins across the proteome is observed (Zimmerman, 2010). One concern of blood proteomics analysis is that proteins that are already high in abundance in the blood, such as albumin and immunoglobulins, obfuscate the measurement of a mass spectrometer and make it harder to detect lower abundance proteins (Adkins *et al*., 2002; Govorukhina et al., 2003). Many of these high abundance proteins are also more stable than other, lower abundance proteins, further adding to the

difficulty detecting the full complement of proteins (Zimmerman, 2010). Isolating circulating EV instead offers a work around for this, as when isolated correctly they contain a more even distribution of proteins with less albumin (Baranyai *et al*., 2015). Because EV also have a lipid bilayer membrane, they allow for transport and detection of transmembrane proteins in the blood as well (Cvjetkovic *et al*., 2016). DNA and miRNA are quite stable free in the blood as well (Huang *et al*., 2017; Enelund *et al*., 2017), and even blood mRNA, though the least stable of the bunch, has been shown to last up to 24 hours *ex vivo* (Holford *et al.,* 2008). In addition to still increased stability, analyzing EV encapsulated cargo can offer a unique look at which specific cell or tissue types the cargo came from as well as the tissue or cell types for which it is destined based on markers on the surface of the EV (van Doormal *et al*., 2009; Murphy et al, 2019; Lotvall *et al*., 2014 Hoshino *et al*., 2015).

1.1.2 Immune Cells

The last source of protein and nucleic acids in whole blood are blood and immune cells, including monocytes, macrophages, erythrocytes, lymphocytes, platelets, and granulocytes, which include neutrophils, eosinophils, and basophils (Dean, 2005). First looking at cells with innate immune function; neutrophils are the most abundant circulating immune cell, they patrol the bloodstream for dysfunction and threats (Rosales, 2018). Neutrophils are capable of phagocytosing bacteria, removing them from the environment and degrading them within vesicles (Rosales, 2018). Neutrophils also play a significant role in wound healing, signaling to one another and forming cellular swarms around the site of the injury (Wilgus *et al*., 2013). The other granulocytes eosinophils and

basophils, along with mast cells (a type of leukocyte), help defend against parasites, and mediate the allergic response (Stone *et al*., 2011). Macrophages, a type of monocyte, are best known for their ingestion and degradation of bacteria, but in the absence of an immune response, they also play 'housekeeping' roles in that they recycle dead cells, and clear debris (Fond and Ravichandran, 2016). Also developing from monocytes, dendritic cells, which process large molecules from pathogens, allergens, and host cells to produce antigens, which dendritic cells then present to adaptive immune cells (Mellman and Steinman, 2001).

Natural killer cells, a type of lymphocyte, identify and destroy both tumor cells and virus-infected cells (Anfossi *et al*., 2006; Vivier *et al*., 2008). They release granules that contain proteins that bring about apoptosis, and puncture target cells (Cullen and Martin, 2007). Other lymphocytes, B and T cells, comprise the adaptive immune system (Alberts *et al*., 2002). The dual functions of B cells are antigen presentation (similar to dendritic cells) to T cells, and antibody production (Rivera *et al*., 2001; Popi *et al*., 2016). Antibodies themselves are secreted into the blood in order to neutralize pathogens, or opsonize them, binding to them as part of a signaling pathway that allows macrophages to identify and destroy them (Roos *et al*., 2004). Although the binding arms of antibodies are unique to particular pathogens, they generally fall into several categories of immunoglobulins (Ig), IgA, IgM, IgE, IgG, and IgD, which have some different and some overlapping roles (Schroeder and Cavacini, 2010). IgA is necessary in the gastrointestinal tract for the neutralization process (Johansen *et al*., 1999), complement activation relies on IgM (Sharp *et al*., 2019), mast cells in the allergic and parasitic

responses are activated by IgE (Mukai *et al*., 2016), IgG plays a role in opsonization, complement activation, and neutralization (Mak and Saunders, 2006), and basophils are activated by IgD (Chen *et al*., 2009). Both these broad classes of antibodies, and the transcripts that code them are abundant in the blood, and easily detected.

T cells have multiple functions and can be broadly classified as either CD8+ or CD4+, in accordance with the protein appearing on their surface (Olsen Saraiva Camara *et al*., 2012). T cells hosting CD8 are termed cytotoxic T cells and are essential for recognition and destruction of cancer cells and cells infected by virus (Janeway *et al*., 2001). Like natural killer cells, CD8+ T cells also shed granules that bring about apoptosis in the target cells (Janeway *et al*., 2001). CD4+ T cells can be further classified as either a T helper cell: TH1, TH2, and TH17; or as a regulatory T cell (Treg)(Luckheeram *et al*., 2012). Immune responses against intracellular pathogens, and in particular, bacteria are coordinated by TH1 cells (Del Prete, 1992); TH2 cells alert granulocytes, B cells, and mast cells which helps mount an immune response against extracellular pathogens (Del Prete, 1992; Sokol *et al*., 2009), and TH17 cells not only recruit neutrophils, but activate non-immune and immune cells alike, with their secretion of interleukin-17 (IL-17)(Gaffen, 2009). Tregs mediate the activity of other T cells, both preventing immune responses against the cells and antigens of the host, and adverse immune activation in general (Luckheeram *et al*., 2012).

Transcripts and molecules related to immune communication and signaling can also be found in the blood, including cytokines (Karsten *et al*., 2018). Small proteins with many functions, cytokines can be broken up into several categories, including:

interferons, which are required for the activation of immune cells (Zhang and An, 2007). Interferons can be further classified as either type I, which oversee antiviral immune responses; or type II, which are required for mounting a response to bacteria (Meurs *et al*., 1990; Lee and Ashkar, 2018). Interleukins, another class of cytokines, aid in activating inhibitory responses (Justiz Vaillant and Qurie, 2021), while chemokines recruit specific immune cells to a particular site for specific purposes (Oo and Adams, 2010). In immune cell differentiation and development, cytokines called colony-stimulating factors are necessary (Bezbradica *et al*., 2006); and in immune cell activation and proliferation, the tumor necrosis factor (TNF) cytokine family is essential (Mehta *et al*., 2018). TNF cytokines, as well as interferons, also play a big role in the activation of the inflammatory response seen frequently in the blood (Cantaert *et al*., 2010). Measuring the molecules of immune communication in the blood can give insight into the general immune response in a changing circumstance.

Several immune cell surface receptors also play critical roles in immune cell communication; present on innate immune cells such as dendritic cells and macrophages, Toll-like receptors (TLRs) are essential for the inflammatory and innate immune response, as they recognize microbial antigens (Kawasaki and Kawai, 2014). T and B cell receptors are found on cells of the adaptive immune system and essential to that system, in that these receptors are all unique so as to be able to bind to nearly anything (Janeway *et al*., 2001). The genes that code T and B cell receptors are constantly mutating with less DNA repair than the rest of the genome, this allows them to be versatile and bind to any foreign antigen that may exist (Janeway *et al*., 2001). The major histocompatibility

complex (MHC) are the proteins that do the antigen presentation on cell surfaces, and are used to indicate whether a cell is foreign or host (Alberts *et al*., 2002). Viral antigens are presented by MHC class I proteins in nearly all cell types, and are then recognized by CD8+ T cells which kill infected cells in response if necessary (Alberts *et al*., 2002). Antigen presenting cells such as macrophages and dendritic cells are what primarily express MHC class II proteins, which present antigens to CD4+ T cells (Alberts *et al*., 2002).

Altered gene and protein expression in circulating immune cells is often indicative of disease state because these immune cells are involved in the response to disease (Hagai *et al*., 2018; Saleh *et al*., 2020; Danne *et al*., 2017). Gene expression within circulating immune cells has been posited as a robust biomarker system for certain diseases and treatment states, independent of the rest of blood gene expression (Wang *et al*., 2020; Lyons *et al*., 2017). Furthermore many immune cells have been shown to cross-talk between different cells and tissues of the body, including facilitating much of the communication between the gut and brain referred to as the gut-brain-axis (Deng *et al*., 2021; Elmentaite *et al*., 2019; Jacobson *et al*., 2021; Liu *et al*., 2020). There is also cross-talk between cancer cells and immune cells in the area immediately surrounding the tumour, known as the tumour microenvironment (Wang *et al*., 2019). With all these things considered, it is apparent that gene and protein expression secreted by, and within circulating immune cells offers a unique look into the disease and treatment states of the body.

1.2 Analyzing Omics Data

At the cellular level, any of the manifold reasons mentioned above are sufficient for a protein or RNA to be either differentially expressed in immune cells, secreted into the blood either directly or within EV. However, the biological purpose of this secretion does not need to be understood for a biomarker to be predictive of a disease state. Rather, the robust and consistent presence of a particular molecule in the blood during a particular disease or treatment condition can serve as an excellent jump off point for understanding the condition. This approach, often referred to as hypothesis-generating research (Hartwick and Barki, 1994), was made possible by the advent of high-throughput techniques for assessing protein (ex. mass spectrometry) and nucleic acids (ex. next-generation sequencing, RNAseq, (whole genome) arrays).

A common approach to analyzing omics data is to compare the proteome or transcriptome of biological models treated to an experimental condition, with the proteome or transcriptome of wild type biological models as a control. This approach generates a total snapshot of all the changes to the proteome or transcriptome that occur between groups and is called differential representation or differential expression analysis. High-throughput techniques generate data containing far more variables, nucleic acid or protein species, than more traditional analyses like quantitative polymerase chain reaction (qPCR) however, and therefore require different statistical analysis to prevent false positives from slipping through during analysis (Benjamini and Hochberg, 1995). For example, when measuring a small number if genes in qPCR, a t-test or ANOVA are sufficient to determine if a change in expression is significant (meaning that there is a 5%

or less chance that the groups could have been randomly sampled from a single normal distribution). In a high-throughput analysis, the number of variables in the analysis is so great that ANOVA and t-test are guaranteed to generate false positives. Instead adjusted p-values that generate a measure of a true false discovery rate are used. This method of generating Benjamini-Hochberg (BH) adjusted p-values can be expressed by:

$$p_{(i)}^{\mathrm{BH}} = \min\left\{ \min_{j \geq i} \left\{ \frac{m p_{(j)}}{j} \right\}, 1 \right\}.$$

Where p-values, ordered from small to large *p(j)*, are multiplied by *m* = the total number of tests, and then divided by rank order. These values are then coerced into a non-decreasing sequence, and any p-values larger than 1 are made equal to 1 (Benjamini and Hochberg, 1995). Doing this corrects for the p-values <0.05 that one would expect to occur by chance in a single normal distribution, and with these adjusted p-values once again reflect the true false discovery rate (FDR), even with tens of thousands of variables. To illustrate the adjusted p-values, consider that all p-values were organized into 20 bins (0.00-0.05, 0.05-0.10… 0.95-1.00) that when taken from a single normal distribution, form a near-uniform distribution of p-values. Where there are two truly different experimental groups however, these bins of p-values form a skewed distribution, where there are disproportionately more significant p-values than non-significant. The p-value adjustment keeps only those p-values <0.05 that are ‘overflowing’ in their bin - that is to say, the most significant of the significant p-values.

Differential expression analysis on omics data with a high number of variables can be performed with R code or through automated online tools that offer increased ease

with less control. In this thesis, both methods were used in three separate differential expression analysis of proteins and nucleic acids in whole blood. First, a comparison between prostate cancer patients at different stages of the disease, and healthy individuals, to identify potential cancer biomarkers; second, a comparison of mRNA in the blood of three comorbid diseases, asthma, major depressive disorder, and post-traumatic stress disorder, to identify drivers of comorbidity. Finally, a comparison of mRNA in both the whole blood and hippocampus of mice fed one of two mood-altering bacteria, or a phosphate buffered saline (PBS) control.

1.3 Blood-Based Biomarkers in Cancer

DNA was first detected in the blood of cancer patients in 1977 (Leon *et al*., 1977), and in 1994 this DNA was shown to harbor cancer-hallmark mutations (Sorenson *et al*., 1994). The blood was at first thought to be a valuable alternative to detecting cancer through biopsy because the genetics in one section of a tumour are not indicative of the genetics of the tumour as a whole (Gerlinger *et al*., 2012; Yong, 2014). In fact, Gerlinger *et al*. (2012) found that cells found on one end of an individual kidney tumour only had one third of their mutations in common to cells at the other end, and that metastasized tumours differed still. When, over time, cancer cells from all parts of a tumour rupture and die, their molecular signature appears in the blood, leaving a more comprehensive signature of their mutant genome than could be seen looking at biopsy (Yong, 2014). A relatively non-invasive cancer biomarker detection system is also welcome, as many cancers are often not detected until it is too late (Incisive Health, 2014), while simultaneously, cancer patients often benefit more from an earlier treatment (Hamilton *et*

al., 2015). Cancer biomarkers are developed for many purposes beyond detection of cancer however, including determining the stage of the cancer and estimating its prognosis, and to detect response to and efficacy of a cancer treatment (Duffy, 2001; Duffy, 2013; Lennon *et al*., 2020; Bettegowda *et al*., 2014).

Free circulating proteins are one of the most widely used biomarkers across many cancers, although naturally the specific proteins differ between cancers (Henry and Hayes, 2012). For example, in breast cancer, carcinoembryonic antigen (CEA), cancer antigen 15-3 (CA 15-3), the soluble form of the human epidermal growth factor receptor 2 (HER2), and tissue polypeptide-specific antigen (TPS) (Di Gioia *et al*., 2016). Proteins, miRNAs and mRNAs from circulating EV have become more popular as cancer biomarkers in recent years, however, as they can be isolated from the blood prior to analysis, effectively lowering the sensitivity requirement for the measuring instrument, they can be used to detect cancer in earlier stages, and distinguish between different stages of tumour development (Lane *et al*., 2018; Melo *et al*., 2015; Hornick *et al*., 2015; Fu *et al*., 2018).

Research articles and clinical trials suggesting the use of vesicular biomarkers for many types of cancer (lung, breast, prostate, pancreas, leukemia, general cancer, colon cancer, gastric cancer, glioma, oropharyngeal, thyroid, gallbladder, and melanoma) have been published or are ongoing, but at this point only one EV biomarker test kit has been approved by the U.S. food and drug administration for clinical use in detecting or characterizing cancer, specifically urine EV biomarkers for distinguishing high- and low-grade prostate cancer (Zhao *et al*., 2019; Han *et al*., 2022). The main reason for this is

that, although the potential of EV as predictors of disease is apparent, there are many standardized procedures still to be developed (Zhao *et al.*, 2019; Lane *et al*., 2018). While great effort has been made to standardize blood collection methods, sample storage, processing, and type, there is no consensus on quantification and isolation methods (Lotvall *et al*., 2014; Coumans *et al*., 2017; Witwer *et al*., 2013). The debate around quantification asks, should vesicle dose be characterized as total protein content of vesicles, number of vesicles, or vesicle number to protein ratio; and for isolation, ultracentrifugation or chromatography (Xu et al., 2016).

Another school of thought seeks to replace the antiquated free circulating protein biomarkers with free circulating tumour DNA (ctDNA) (Duffy *et al*., 2018). The main advantage of ctDNA is that because it comes from burst tumour cells, it can be sequenced to give a very direct picture of the exact mutations from all parts of the tumour (Yong, 2014; Pessoa *et al*., 2020); and because it is less invasive than biopsy, it can be repeated more easily to monitor the progression of tumour mutations in closer-to-real-time (Pessoa *et al*., 2020; Duffy *et al*., 2018). Like free circulating protein biomarkers, however, the total amount in the blood increases as tumour stage progresses, varying from 0.01% to >90% of all DNA in circulation (Wan *et al*., 2017). This makes ctDNA a good option for identifying the full complement of mutations in later stage cancers when it is easy to detect, but a poor option for diagnosing cancers at an early stage when it is difficult to detect (Pessoa *et al*., 2020). Although these procedures are still in development, in the near future, high-throughput measurements and analysis of biomarkers in the blood will

have life-changing impacts in the detection and stage assessment of cancer patients in a clinical setting.

1.4 The Brain and Gut Microbiota Communicate Through Blood and Nerves

Protozoa, fungi, viruses, and bacteria are among the 100 trillion microorganisms that live in the human gastrointestinal tract (Bull and Plummer, 2014; Rath and Dorrestein; 2012). The microbiome, sometimes thought of as the population of genomes in an environment, in the human gut is represented by 3 million genes (Valdez *et al*., 2018). In that sense, the microbiome can therefore be considered a virtual organ, and the need for several host functions is replaced by the functional metabolites its genes encode (Bull and Plummer, 2014; Vyas and Ranganathan, 2012). Diet, drug intake, and other environmental factors, and to a lesser extent genetic factors dictate the biodiversity of the human gut microbiome (Goodrich *et al*., 2014; Rothschild *et al*., 2018). These factors result in different individuals possessing different species in their gut than others.

Native gut microbiota participate with their host in many biological processes, including: metabolism, drug efficacy, gene expression, the development of disease, and immunity (Wu and Wang, 2018). Intestinal bacteria help mediate proper brain and immune development via the microbiota-gut-brain axis (Yong *et al*., 2020). Lifestyle changes often proceed adverse changes in gut biodiversity that are thought to lead to conditions like obesity, schizophrenia, inflammatory bowel syndrome, major depressive disorder, and Parkinson's (Sherwin *et al*., 2016; Thursby and Juge, 2017; van de Guchte *et al*., 2018). The mediation of brain diseases by gut bacteria appear to occur through both the vagus nerve, and the circulatory system, in which immune cells and EV traveling

between gut and brain facilitate changes (Haas-Neill and Forsythe, 2020; Bharwani *et al*., 2017; Bravo *et al*., 2011; Liu *et al*., 2020).

Following host ingestion, some commensal bacterial species have been shown to alter brain, behaviour, and immune systems positively (Kandasamy *et al*., 2016; Alvarez *et al*., 2019; Mohsin *et al*., 2015; Bercik *et al*., 2011), and many of these same species are even known to produce neurotransmitters including GABA, serotonin, dopamine, and nor-epinephrine, although it remains unclear whether this latter activity influences the brain (Roshina, 2010; Lyte, 2011; Haas-Neill and Forsythe., 2020). Alone, *Lactobacillus*, a genus of anaerobe, produces a whole range of neurotransmitters; with single species such as *Lactobacillus plantarum* producing multiple neurotransmitters (Yong *et al*., 2020).

EV have been shown to have major involvement in communication between bacteria of the gut, and the brain (Summarized in Figure 1)(Haas-Neill and Forsythe, 2020). Like their parent cells, EV derived from commensal bacteria stimulate the nervous system, module immunity, can enter into the blood and pass the blood brain barrier (BBB), carry psychoactive cargo, and have been shown to alter gene expression and behaviour without their parent cell (Kaparakis *et al*., 2010; Meganathan *et al*., 2020; Al-Nedawi *et al*., 2015; Zakharzhevskaya *et al*., 2017; Choi et al., 2019).

It is hypothesized that membrane vesicles (MV) released by probiotics cross into the circulation from the gut and travel with their psychoactive or behaviour altering cargo to the brain, as an additional mechanism of probiotic control over behaviour (Haas-Neill and Forsythe, 2020). It follows then, that some component of the immune cells and EV in

the blood are derived from gut or brain, and are involved, or representative of the crosstalk between the two.

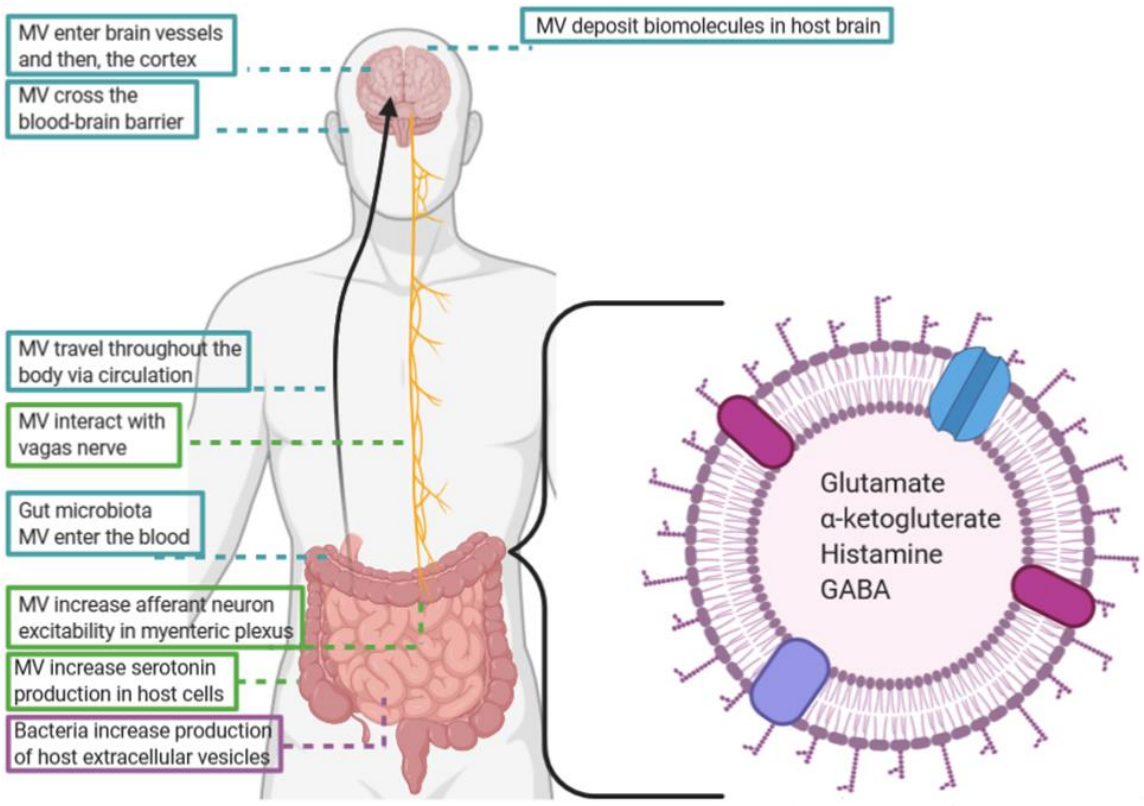

Figure 1: The cognition and behaviour influencing pathways of commensal derived bacterial membrane vesicles. Image adapted from: Haas-Neill, S., and Forsythe, P. (2020). A budding relationship: Bacterial Extracellular Vesicles in the Microbiota-Gut-Brain Axis. *International Journal of Molecular Sciences*. **21**(23):8899.

1.5 Blood Signatures of Mood Disorders

In recent years, evidence continues to suggest that a molecular signature of almost everything that happens in the body appears in the blood, and it appears that there could be blood biomarkers even for mood disorders, previously thought to be pathology exclusive to the brain (Le-Niculescu *et al*., 2009; Brand *et al*., 2015). Like cancer, mood

disorders are difficult to diagnose, and are frequently misdiagnosed. This is true of post traumatic stress disorder (PTSD), major depressive disorder (MDD), bipolar disorder, and borderline personality disorder (BPD) to name a few (Awad *et al*., 2007; Salzbrenner and Conaway, 2009). While in the case of cancer, the difficulty to diagnose is due to lacking a comprehensive understanding of the variation between cases, as well as symptoms remaining unrecognized until the disease has spiraled out of control (Yong, 2014; Incisive Health, 2014; Al-Azri, 2016); in mood disorders, misdiagnosis is caused in part by inability to perform physical tests on the brain, and an incomplete understanding of what is causing the disorder (Philips and Kupfer, 2018; Manson, 1995; Avasthi and Ghosh, 2014; Rosen *et al*., 2018). Therefore, like in cancer, there has been great interest in identifying the signatures of mood disorders in the blood (Le-Niculescu *et al*., 2009; Brand *et al*., 2015).

There have been multiple research papers published toward the development of blood biomarkers for post-traumatic stress disorder (PTSD) (Rusch *et al*., 2019; Kuan *et al*., 2017), major depressive disorder (MDD) (Leday *et al*., 2017; Spijker *et al*., 2010), bipolar disorder (Le-Niculescu *et al*., 2021; Sagar and Pattanayak, 2017), schizophrenia (Panda *et al*., 2021; He *et al*., 2017), anxiety and stress (Humer *et al*., 2020; Los and Waszkiewicz, 2021). Many of these disorders are comorbid with each other, meaning that if a patient has one, they are more likely to also have another (Kaufman and Charney, 2000; Nabavi *et al*., 2015). Mood disorders have also been shown to be comorbid with some diseases affecting the body beyond the brain as well (Sanna *et al*., 2013; Wisnivesky *et al*., 2021). Little work has been done to determine how many of the

molecules that comprise the blood signature of one of these mood disorders, also appear in their comorbid counterparts; although inflammatory molecules such as C-reactive protein (CRP) appear to be commonly regulated in the blood of multiple mood disorders, and anti-inflammatory medications have been seen to alleviate symptoms from multiple mood disorders (Chang and Chen, 2020) . Just as the blood may be a critical link between gut bacteria and mood disorders, its contents may also be worthy of study in understanding the root of comorbid disorders within the body.

1.6 Purpose and Objectives of the Thesis

This body of work sought to accomplish three separate objectives in its use of omics data of a range of molecular species collected from the blood: 1) to improve the diagnosis and staging of prostate cancer, leading to a more accurate and less invasive clinical experience; 2) to identify the drivers of comorbid mood disorders, which may give insight into future treatments for comorbid patients that better address the root of the problem; and 3) to more comprehensively elucidate the mechanism by which mood-altering bacteria of the gut influence the brain and behavior of mice, ultimately enhancing our understanding of the antidepressant mechanisms of gut microbiota.

To these ends, each of these objectives use differential expression analysis and gene set enrichment analysis on: 1) circulating vesicle derived protein; 2) total circulating mRNA (includes circulating immune cells & EVs); and 3) circulating and hippocampal mRNA.

Chapter 2: The role of blood circulating extracellular vesicle protein in characterization of prostate cancer

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2.1 Abstract

Prostate cancer (PCa) is the fifth leading cause of death in men globally. Prostate specific antigen (PSA) is still considered the gold standard biomarker test for PCa, despite its high rate of false positives and negatives that result in several inappropriate medical responses, including overtreatment. There is a critical need for other biomarkers for PCa. In this study, we collected extracellular vesicles (EV) from the blood plasma of patients with organ-confined, extracapsular-invading, and seminal vesicle-invading tumours, as well as from healthy subjects. We examined the protein content of these EV using Mass Spectrometry (MS). This in-depth proteomic analysis showed 10 distinct groups of proteins that are differently expressed in each group of patients compared to healthy subjects. We have identified a wide range of potential protein biomarker candidates for distinguishing healthy subjects from PCa patients, as well as for discerning vesicle cargo among the different tumour types. In practice, once verified, these biomarkers have the potential to be more accurate diagnostics for PCa than what currently exists, and may go beyond PSA in their potential to determine and monitor disease staging.

2.2 Introduction

Prostate cancer (PCa) has the fourth highest incidence rate among all cancers globally, with 1.1 million cases, and it is the fifth leading cause of cancer mortality in men (307,000 deaths in 2012) [1]. PCa is the second most diagnosed cancer after lung cancer, and it is more prevalent than lung cancer in the developed world (759,000 cases compared to lung cancer's 490,000 in 2012)[1]. In about 70% of patients with advanced PCa, bone metastases occur in multiple sites [2]. These metastases occur most commonly in the femur, skull, pelvis, ribs, spine, sternum, and humerus and can lead to fractures, reduced mobility, hypercalcemia, and severe bone pain [3, 1]. Prostate specific antigen (PSA) has been measured in serum as the standard biomarker test for PCa [4]. The test has high sensitivity and, when performed routinely, leads to reduced mortality. It has low specificity, however, which in practice can lead to many false positives, including the mischaracterization of benign prostate hyperplasia as PCa [5-7]. There are currently no other accepted biomarkers for PCa detection and PSA continues to be used in the clinical setting [4]. When a blood level PSA of >4 ng/mL is detected, a biopsy is recommended, but in 57.7% of these 10-12 core biopsies cancer is not detected [8, 9]. Exceptionally high levels of PSA are thought to reflect an increased risk of bone metastasis, but are a poor measure as these high levels can be detected independently of bone metastasis [4]. Based on these drawbacks, there is a serious need either for the PSA test to be improved or for the identification of new biomarkers capable of both more accurately differentiating between PCa patients and healthy subjects, and specifying metastatic potential.

Extracellular vesicles (EV) are small membrane compartments that are shed from both malignant and normal cells and are known to contain a cargo of proteins, RNAs and DNAs [10]. EV were originally believed to be shed from cells exclusively for the purpose of waste disposal, but have since been shown to be involved in a host of intercellular signalling pathways as well as the intercellular shuttling of functional cargo such as transcription factors [11-13]. We previously reported that EV in the blood of PCa patients contain diagnostic biomarkers for the disease and, therefore, have important potential as replacements for PSA testing in PCa diagnosis [14]. In support of their diagnostic potential, EV have been referred to as biomarker treasure chests for PCa, and have had this potential identified previously for the detection of ovarian and urogenital cancers [15-17]. It has also been reported that proteins found in exosomes collected from the urine of PCa patients are predictably different from those found in the exosomes of healthy urine [18]. Given the major gap in accurately measuring PCa development and metastasis, we examined the contents of extracellular vesicles extracted from patient blood plasma and characterized their protein content. These data were generated from four groups of six subjects, one healthy group, an organ confined PCa (OC) group, an extracapsular extending PCa (EC) group, and a seminal vesicle invading PCa (SI) group. Our data show that blood EV protein can be identified to distinguish between a PCa patient and a healthy male of the same age, and can also be used to discriminate between PCa patients at different stages of the disease. Upon further validation, EV could provide a simple,

non-invasive and high specificity alternative to PSA to diagnose PCa and monitor its progression.

2.3 Results

2.3.1 Nanoparticle analysis

The NanoSight analysis shows that EV circulating in the plasma of both PCa (Figure 1A) and healthy subjects (Figure 1B) are a homogenous population with a size range of 100-200 nm.

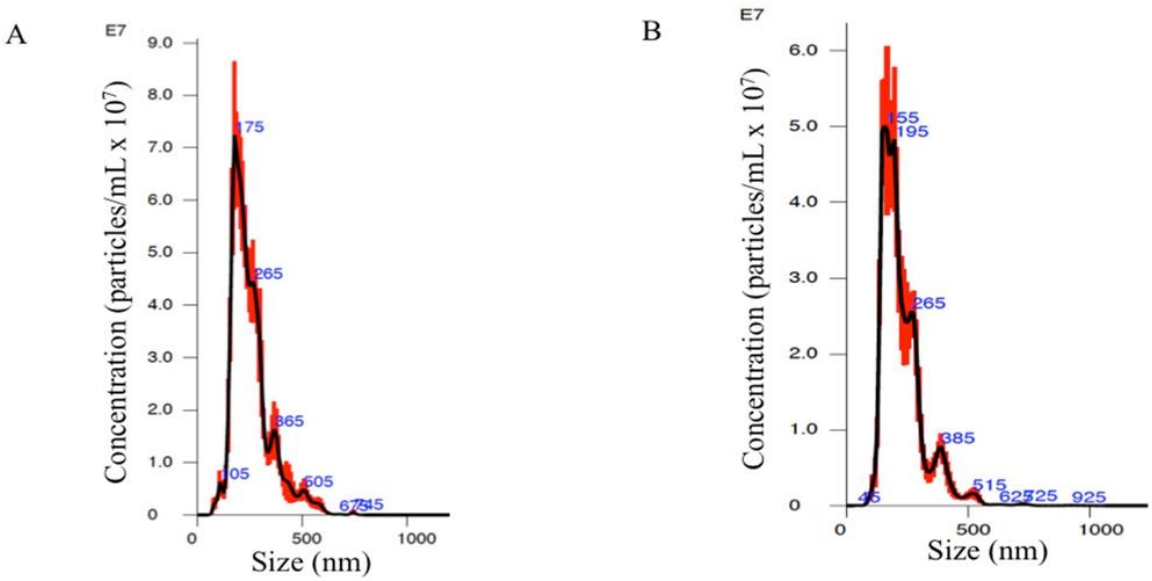

Figure 1. Nanoparticle analysis shows the homogenous population of EV derived from patients and healthy subjects: A) EV from OC PCa patient's plasma. B) EV from plasma of a healthy subject.

2.3.2 Proteomic analysis

Six samples of EV protein from the blood plasma of patients possessing each tumour type (OC, EC, and SI) as well as six samples from healthy males were subjected to proteomic analysis. Healthy males' blood plasma sample donors were of matching

age (50-65) and had no previous history of cancer or benign tumour. A heatmap for the average expression of various EV proteins identified by the proteomic analysis is shown in Figure 2. Ten groups of proteins were identified by proteomics analysis:

A) Group A proteins are those that are more expressed in the blood vesicles of healthy subjects but less so in the vesicles of patients of all tumour types.

B) Group B proteins have mild-to-moderately increased expression in all groups but SI, which shows down-regulation of these proteins.

C) Group C proteins are up-regulated in EC patients but are down-regulated in all other groups.

D) Group D proteins are up-regulated in EC and SI patients but are mildly down-regulated in healthy subjects and moderately down-regulated in OC patients.

E) Group E proteins are up-regulated in SI patients but down-regulated in all other groups.

F) Group F proteins are up-regulated in EC and SI patients but down-regulated (to a larger degree than group D) in healthy subjects and OC patients.

G) Group G proteins are up-regulated in OC and EC patients but down-regulated in SI patients, and to a lesser degree down-regulated in healthy subjects.

H) Group H proteins are up-regulated in OC patients but moderately down-regulated in all other groups.

I) Group I proteins are moderately up-regulated in OC and SI patients, but largely down-regulated in healthy subjects and EC patients.

J) Group J proteins follow the opposite pattern to group A, in that they are moderately up-regulated in patients of all tumour types and moderately down-regulated in healthy subjects.

Information for the protein ID, name and molecular functions of the proteins from each group were summarized in (Table 1). Also, the table contains a comparison between these proteins' expression in EV with their expression level reported in the literature on prostate or other types of cancer. Most of the previous literature shows the expression of these proteins in tumour tissues and not in EV, and very few of them contain an analysis that correlates these proteins with the OC, EC, and SI tumour types. This makes the comparison difficult for all groups but A and J in which the OC, EC, and SI PCa subtypes all show the same relative protein expression level. For example among various proteins in group A plasma EV proteins, which we found to be downregulated in PCa patients compared to healthy subjects, are pericentrin and Myb-binding protein 1A - these proteins play role in microtubule organization during mitosis and meiosis, and engages with specific DNA binding proteins, respectively. The literature shows these proteins to be upregulated in prostate tumour endothelium and in the tumour itself, respectively [20, 21]. Two proteins selected from group B (upregulated in healthy, OC, EC, downregulated in SI), complement factor B (CFB) and retinol-binding protein 4 (RBP4), which respectively are a component of the C3 or C5 convertase, and a mediator of retinol transport in the blood. The literature shows that CFB is upregulated in PCa tumours, and RBP4 was found to be upregulated in PC3 cells of xenographted mice [22, 23]. This is not a perfect comparison due to the absence of tumour staging in the

literature. Of the proteins examined in Table 1, in groups C through I, the following were found to be overexpressed in some form of cancer: integrin-linked protein kinase [25]; glyceraldehyde-3-phosphate dehydrogenase [27]; dynein assembly factor 1, axonemal [30]; WD and tetratricopeptide repeats protein 1 [33]; C-reactive protein [40]; and runt-related transcription factor 1 [42]. Proteins in these groups found by literature to be downregulated in cancer include: LINE-1 type transposase domain-containing protein 1 [31], PR domain zinc finger protein 2 [32], glutathione peroxidase 3 [41], and Inter-alpha-trypsin inhibitor heavy chain H3 [45].

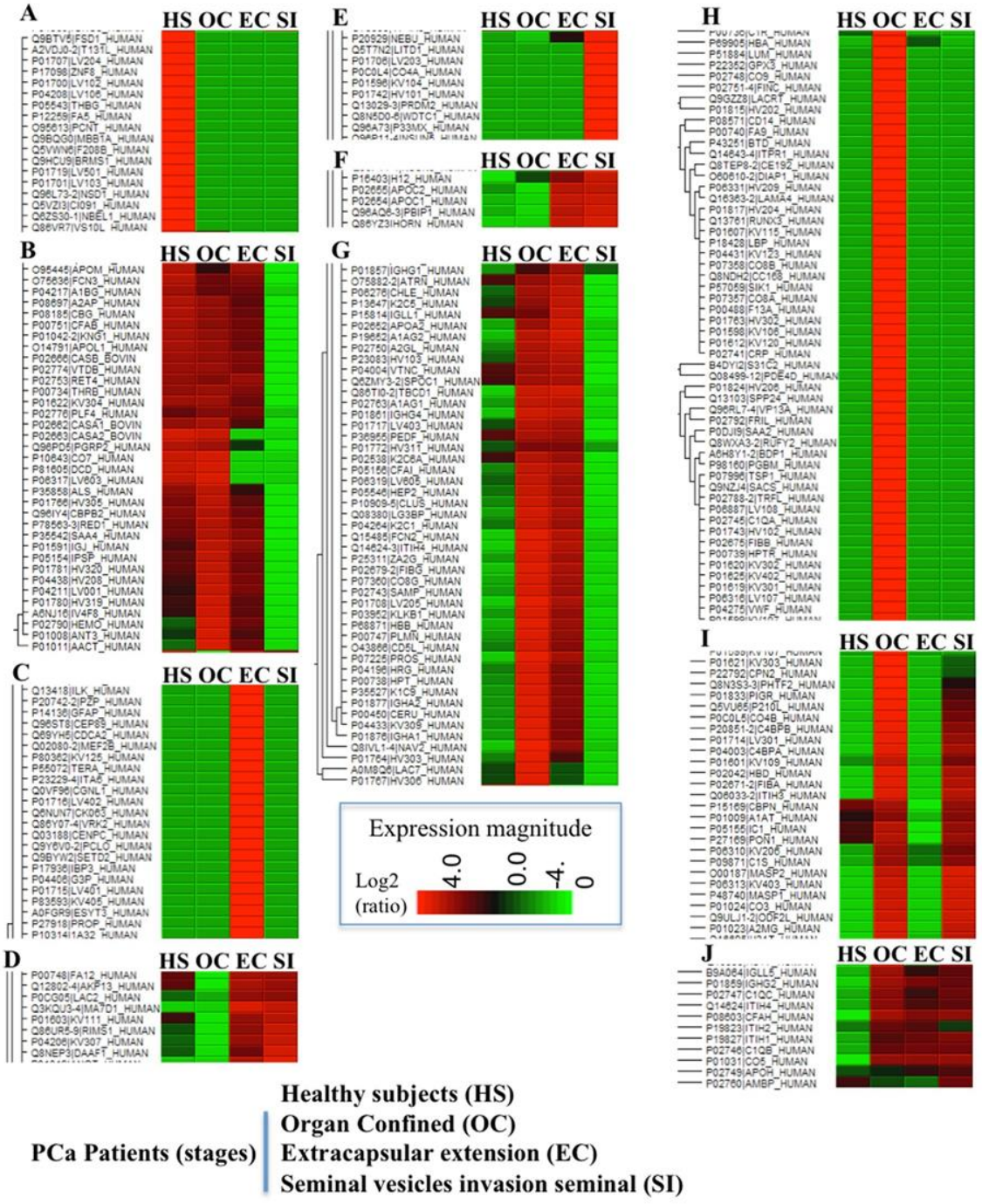

Figure 2. Proteomic analysis from the EV from the plasma of 6 healthy male plasma samples, 6 OC patient plasma samples, 6 EC patient plasma samples, and 6 SI patient plasma samples. 150 µg of protein from the vesicle preparations were used for each group. The heat map shows a distinctive protein profile for each

stage of the disease, compared to healthy subjects. Ten clusters of proteins were identified that could discriminate between prostate cancer and healthy subjects, as well as distinguish the disease stage. The figure shows in principal that EV proteins could be used as biomarkers for each stage of tumour that could aid in diagnostic and prognostic information.

The existence of these distinct groups suggests that EV from blood plasma possess different protein profiles between healthy and cancer subjects and, as such, have valuable potential as biomarkers for PCa diagnosis and progression tracking. To further illustrate this, we have created Venn diagrams that show, for comparative purposes, which proteins are unique to each subject type and which are shared by multiple subject types (Figure 3). When comparing vesicle proteins from healthy subjects and OC patients, there are 72 proteins expressed uniquely in the patients and 29 expressed uniquely in the healthy subjects, with 167 of the analyzed proteins being common to both (Figure 3A). Similarly, when comparing the other PCa sub-groups with healthy subjects, there are some proteins uniquely expressed in the patient vesicles, some uniquely expressed in the healthy vesicles, and a majority shared between them (Figure 3B, C). When vesicle proteins from patients with different types of PCa were compared, we detected 57 proteins expressed uniquely in OC patients, 27 expressed uniquely in EC patients, 13 expressed uniquely in SI patients, and 141 common to all cancer types (Figure 3D).

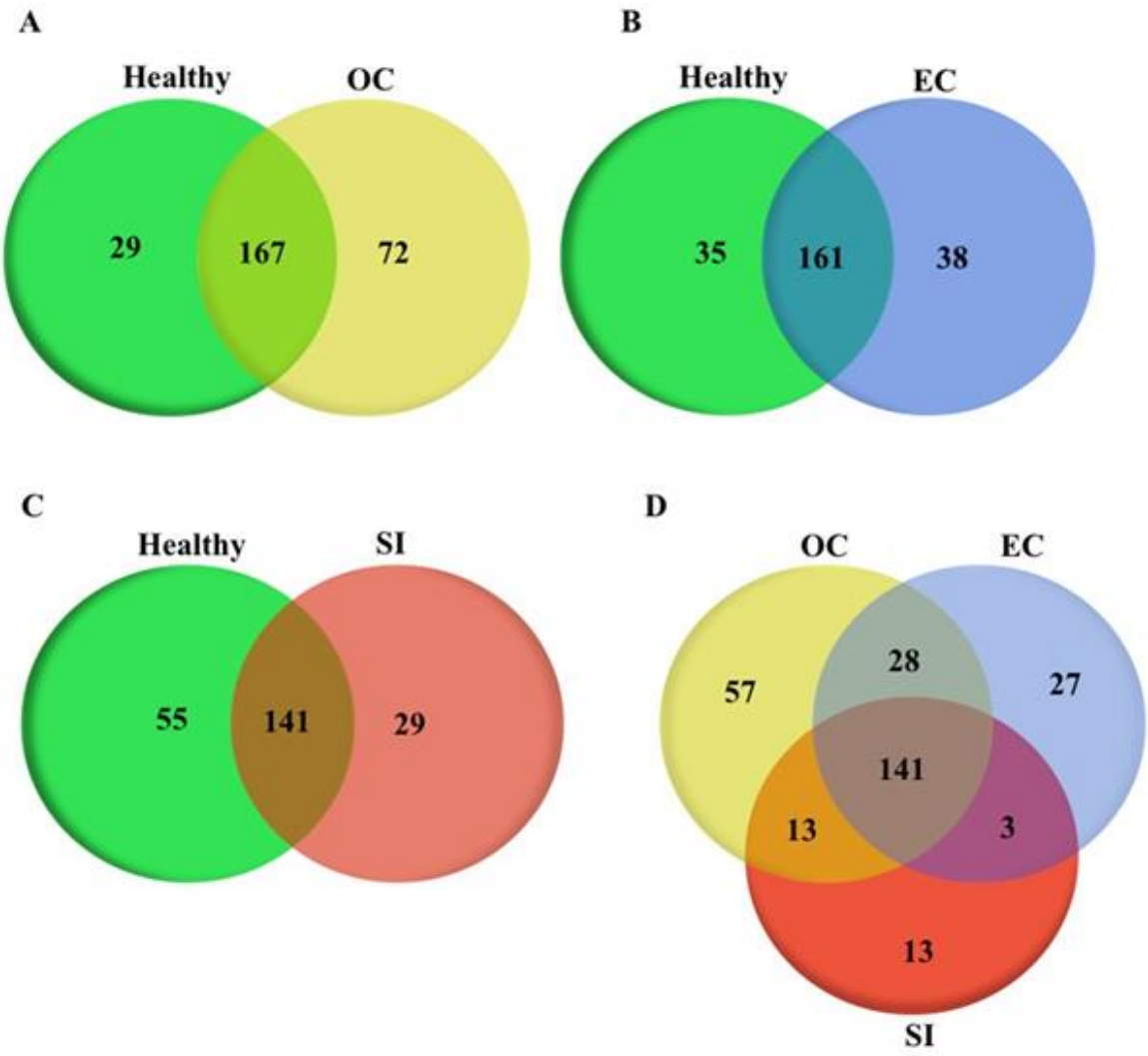

Figure 3. Venn diagrams illustrating the number of proteins unique to each tumour type group compared to healthy vesicle protein expression (A, B, C), and expression common and unique to each tumour type (D). Any part of the circle not overlapping represents the number of proteins that could be used as biomarkers to make a particular distinction (ex. between healthy and SI, or between each tumour type).

We also carried out a protein ontology analysis of protein function, localization preference, and the biological processes associated with all proteins detected across all samples (Figure 4). The three most common processes these

vesicle proteins were involved in were general binding (86 proteins), enzyme regulator activities (45 proteins), and miscellaneous molecular functions (95 proteins) (Figure 4A). These three ‘functions’ alone account for over 80% of all molecular functions of proteins in the subject vesicles. The leading three fractions after the analysis of biological processes that all detected vesicle proteins were involved in are as follows: 100 proteins were involved in general biological processes, 100 in establishment of localization, and 100 in localization (Figure 4B). These three ‘biological processes’ make up a smaller percentage of the total pool - about 36% - due to there being a greater number of biological process categories identified by the software. 100 proteins preferentially localize to a miscellaneous cellular component; 100 preferentially localize to an extracellular region; and 72 preferentially localize to the cytoplasm (Figure 4C). These three preferred targets for localization make up just over 50% of all the detected proteins across all samples.

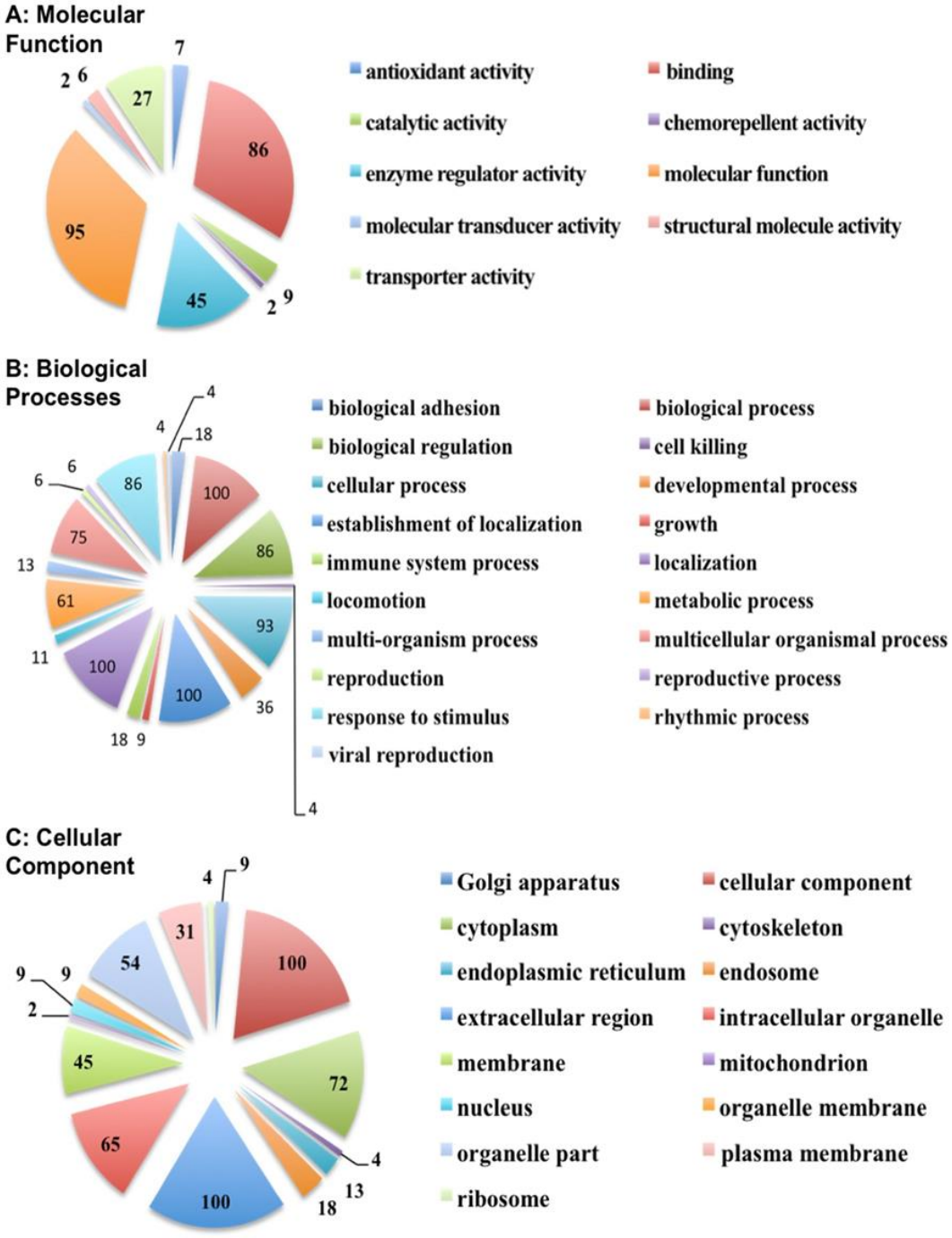

Figure 4. Protein ontology analysis showing the number of EV proteins from all subject groups and categorized according to molecular functions (A), biological processes (B), and cellular components (C).

Protein ID	Name	Group	Function	Prostate Cancer Literature	Ref.
Q9HCU9 BRMS1_HUMAN	Breast cancer metastasis-suppressor 1	A	Represses transcriptional activation by NF-kappa-B.	Acts as a tumour suppressor in prostate cancer	19
O95613 PCNT_HUMAN	Pericentrin	A	Aids in the establishment of organized microtubule arrays during meiosis and mitosis.	Upregulated in tumour endothelium compared to healthy and is associated with angiogenesis.	20
Q9BQG0 MBB1A_HUMAN	Myb-binding protein 1A	A	Interacts with sequence specific DNA-binding proteins.	Upregulated in cancer cells, while negligibly expressed in healthy. Particularly overexpressed in castration resistant tumours. More associated with advanced stages of the disease.	21
P00751 CFAB_HUMAN	Complement factor B	B	Component of the C5 or C3 convertase.	Upregulated in prostate cancer both in inflammatory and non-inflammatory conditions	22
P01042 KNG1_HUMAN	Kininogen-1	B	Kininogens inhibit thiol proteases.	Inhibits migration and invasion of prostate cancer cell lines.	23

P02753 RET4_ HUMA N	Retinol-binding protein 4	B	Mediates the transport of retinol in blood plasma.	Upregulated in Prostate Cancer Cell Line xenographted mice	24
Q13418 ILK_H UMAN	Integrin-linked protein kinase	C	Regulates integrin-mediated signal transduction.	Up regulated as needed by tumours for angiogenesis	25
P23229 ITGA6_ HUMA N	Integrin Alpha 6	C	Laminin receptor on platelets.	Expression is associated with aggressive and invasive phenotype of prostate cancer	26
P04406 G3P_H UMAN	Glyceraldehyd e-3-phosphate dehydrogenase	C	Plays a role in glycolysis and possesses nuclear functions.	Increased in late stage prostate cancer	27
P00748 FA12_H UMAN	Coagulation Factor XII	D	Helps initiate fibrinolysis, blood coagulation, and bradykinin and angiotensin generation.	Coagulation factor XII drives prostate cancer-associated venous thrombosis.	28
F1M3G 7 AKP13_ HUMA N	A-kinase anchor protein 13	D	Assembles signaling complexes downstream of many G protein-coupled receptors.	Interacts with tissue transglutaminase, another protein found to be a good biomarker candidate, in prostate cancer.	29
Q8NEP 3 DAAF1 _HUMA N	Dynein assembly factor 1, axonemal	D	Stabilizes ciliary architecture.	Overexpresion is associated with prostate cancer progression	30

Q5T7N2 L1TD1_ HUMA N	LINE-1 type transposase domain-containing protein 1	E	Binds single stranded RNA.	Gene methylated in non-small cell lung cancer (downregulated)	31
Q13029-3 PRDM2 _HUMA N	PR domain zinc finger protein 2	E	Methylates 'Lys-9' of histone H3. Thought to be tumour suppressor.	Downregulated or deleted in many tumour types: colon, liver, and breast.	32
Q8N5D 0-6 WDTC1 _HUMA N	WD and tetratricopeptid e repeats protein 1	E	CUL4-DDB1 E3 ubiquitin-protein ligase complex substrate receptor.	Overexpressed in neuroblastoma.	33
P02654 APOC1 _HUMA N	Apolipoprotein C-I	F	Low density lipoprotein (LDL) receptor inhibitor.	Not associated with prostate cancer incidence	34
Q96AQ 6-3 PBIP1_ HUMA N	Pre-B-cell leukemia transcription factor-interacting protein 1	F	Pre-B-cell leukemia transcription factor (BPXs) regulator.	Regulates VCP, high levels of which are associated with prostate cancer progression and re-occurrence in patients.	35
Q86YZ3 HORN_ HUMA N	Hornerin	F	Component of epidermal cell envelopes.	Expressed in prostate cancer	36
P19652 A1AG2 _HUMA N	Alpha-1-acid glycoprotein 2	G	Blood stream transport protein.	Correlated with PSA levels in the blood in both cancer and non-cancer cases	37

O75882 ATRN_ HUMA N	Attractin	G	Active in the initial inflammatory response and may regulate chemokine activity.	Complex glycoforms of attractin have been found to be overexpressed in prostate cancer.	38
P25311 ZA2G_ HUMA N	Zinc-alpha-2-glycoprotein	G	Stimulates lipid degradation in adipocytes .	Has previously been identified as a good potential serum biomarker for prostate cancer.	39
P02741 CRP_H UMAN	C-reactive protein	H	Involved in several aspects of host defense.	Higher levels are associated with poorer survival in prostate cancer	40
P22352 GPX3_ HUMA N	Glutathione peroxidase 3	H	Protects against oxidative damage.	Expression is usually down-regulated or halted in prostate cancer as it suppresses growth and metastasis.	41
Q01196 RUNX1 _HUMA N	Runt-related transcription factor 1	H	With CBFB, forms the complex core-binding factor.	Upregulated by fibroblasts in the prostate tumour microenvironment	42
P01023 A2MG_ HUMA N	Alpha-2-macroglobulin	I	Inhibitor of all classes of proteinase.	Is present in the blood of prostate cancer patients and has been found to be inversely correlated with PSA in the blood of patients with more advanced prostate cancer.	43

P27169 PON1_ HUMA N	Serum paraoxonase/ar ylesterase 1	I	Hydrolyzes various organophosphorus insecticide metabolites.	Has been reported to have increased activity in prostate cancer patients.	44
Q06033 ITIH3_ HUMA N	Inter-alpha-trypsin inhibitor heavy chain H3	I	Carries and or aids in binding of hyaluronan.	Downregulated in the majority of prostate cancers.	45
P19827 ITIH1_ HUMA N	Inter-alpha-trypsin inhibitor heavy chain H1	J	Carries and or aids in binding of hyaluronan.	Found by the same study as the other Inter-alpha-trypsin inhibitors to not be differentially expressed in prostate cancer.	45
P19823 ITIH2_ HUMA N	Inter-alpha-trypsin inhibitor heavy chain H2	J	Thought to stimulate phagocytotic cells.	Downregulated in 50% of prostate cancers.	45
Q14624 ITIH4_ HUMA N	Inter-alpha-trypsin inhibitor heavy chain H4	J	Plays a role in trauma indiced inflammatory response.	Downregulated in the majority of prostate cancers.	45
P02760 AMBP_ HUMA N	AMBP	J	Light chain of the inter-alpha-trypsin inhibitor.	Upregulated in prostate cancer.	45

Table 1. Annotated list of proteins identifying their respective roles in prostate or other cancers in the literature. The table contains proteins with various expression levels, as in Figure 2, with information for the function and expression levels from the literature. Groups: A) Upregulated in healthy, and downregulated in all 3 cancer stages. B) Upregulated in healthy, OC, and EC, and downregulated in SI. C) Upregulated in EC, and downregulated in healthy, OC, and SI. D) Upregulated in EC and SI, and Downregulated in OC. H) Upregulated in OC, and downregulated in healthy, EC, and SI. I) Upregulated in OC and SI, and downregulated in healthy and EC. J) Upregulated in all cancer stages, and downregulated in healthy

2.4 Discussion

This research highlights the potential for EV to be collected from the blood as biomarker-containing capsules for both the diagnosis of PCa and for making a determination of the patient's prognosis. EV protein was able to discriminate between healthy subjects and cancer patients, as well as between tumour types. These potential EV biomarkers, after verification, could contend with PSA as the gold standard for PCa detection with a lower incidence of false positives and over-treatment. PSA is said to have the ability to 'detect metastases' in the sense that detecting it in abundance over 4 ng/mL suggests that it is more likely that the patient's cancer has metastasized to bone [4]. A higher PSA level as a test for metastasis is poor because it relies on arbitrary numerical values in PSA measurements, rather than biomarkers that actually reflect the biological change occurring within the cancer and or the body's response to it. There are, in fact, a number of cancer-unrelated conditions and circumstances that are known to cause an abundance of blood PSA, including prostatitis, urinary tract infections, and benign prostate hyperplasia, as mentioned previously [44-48, 5]. While each of these biomarkers is not exclusively associated with cancer, elucidating a profile of vesicular biomarkers offers the most detailed picture of the unique problem. That some of these EV proteins (ex. PBIP1, and APOC2), are present in the vesicles of EC or SI tumour patients, exclusively, suggests that they are able to fulfill that unique role. The potential of these biomarkers becomes even more powerful when one considers that they may be measured together. Measuring multiple biomarkers together will always create a more accurate picture for diagnosis, leading to fewer false positives and negatives and to

pinpointing the nature of the tumour and its progression. Because EV can be isolated from the blood, they are also very valuable as a diagnostic tool due to their collection and analysis being less invasive than biopsy. For this reason, blood plasma EV, along with EV collected from urine as described in [18], have the potential to greatly increase patient comfort and well-being during the diagnostic process.

Kim et al. [49] found pigment epithelium-derived factor (PEDF) and Immunoglobulin joining chain (IGJ) to be upregulated in EV collected from the urine of PCa patients. This is consistent with our finding that both proteins are overexpressed in OC and EC tumours (Figure 2). There are many other proteins from this and other urine EV studies [49, 50, 18] that we did not detect in blood EV, which suggests that EV from the blood plasma have different molecular make up from the EV in the urine.

Table 1 illustrates the discrepancies between the level of expression we find in EV proteins and those previously reported in cells and tumour tissues. We found some EV proteins across all groups that have expression consistent with what was previously reported by others using cancer cells or tumour tissues (ex. AMBP, CFB, RBP4). We found other EV proteins whose expression is in contrast to what the literature describes for the cell (ITIH2, MBB1A, PCNT). Also, for many cases, cell expression of the vesicle protein could not be determined with current literature (ex. RET4). It is difficult to make biological assertions as to why the vesicle expression does or does not match cellular expression of proteins, as it is unknown if the cell is actively or passively loading the EV in each case. Cases where the cell overexpresses a given protein and that protein is found to be overexpressed also in the vesicles (ex. AMBP) may be due to passive loading of the

vesicle. AMBP is the light chain of the inter-alpha trypsin inhibitors (ITI) and we found it, along with the heavy chains ITIH1, ITIH2, and ITIH4, to be contained in group J EV, meaning they tend to be upregulated in all cancer EV, while ITIH3 is associated with the group I EV (upregulated in OC and SI subjects but downregulated in healthy and EC subjects). Hamm et al., 2008 found that ITIH1 was not differentially expressed between PCa and healthy cells, but that while AMBP was upregulated in PCa, ITIH2, ITIH3, and ITIH4 were all downregulated in PCa. The example of this protein family illustrates that there is no homogeneity of expression of certain proteins as between cells or tumours and vesicles (Figure 2).

We found previously that PCa EV have the ability to transport nuclear receptors and other transcription factors (AR and EGFR, respectively) directly to the nucleus of other cells [13]. We have also suggested that this is potentially a mechanism by which PCa loses its sensitivity to androgen deprivation therapy (ADT). With this in mind, it is striking to observe what a large fraction of the proteins examined in this study are involved in localization and the establishment of localization, and to ask whether some large fraction of the vesicles released into the blood by PCa are meant to deliver functional cargo to distal sites in the body. The exact biological role of each of these proteins has not been characterized in this study, which sought to focus on markers, but is interesting and important for the understanding of EV tumour biology.

In conclusion, extracellular vesicles collected from blood have the potential to be a less invasive, highly sensitive and specific source of PCa biomarkers that have the potential to outperform PSA's disease staging ability.

2.5 Materials and Methods

2.5.1 Prostate cancer and healthy subjects

Four groups of six subjects were used for each of the proteomics; the groups included one healthy group, and one group for each of the three different PCa stages. These three tumour types include organ-confined tumors (OC), tumours exhibiting extracapsular extension (EC), and tumours exhibiting seminal vesicle invasion (SI). Tumour subject samples, accompanied by full clinical and demographic information, were obtained from the Ontario Institute of Cancer Research tumor bank. Healthy subject samples were obtained thanks to St. Joseph's Healthcare Hamilton, Ontario, Canada. This study received ethical approval from the Hamilton Integrated Research Ethics Board.

2.5.2. Collection of Extracellular Vesicles from Blood Plasma

Differential centrifugation, as described in (12, 13, 14, 30), was used to purify EV in plasma. In brief, 1 mL of plasma was diluted 1:1 in PBS to reduce viscosity and subjected to differential centrifugation at 2000 x g for 30 minutes at 4˚C, 12,000 x g for 20 mins at 4˚C, and 100,000 x g for 2 hours at 4˚C. Pelleted extracellular vesicles were scraped from ultracentrifuge tubes and resuspended in 200 µL of PBS.

2.5.3 Nanoparticles analysis

EV collected from PCa patients and healthy subjects were subjected to nanoparticle analysis 262 using the NanoSight LM14C with an infusion rate of 80 and after being diluted 1:10.

2.5.4 Proteomics Sample Preparation

EV were lysed in RIPA-buffer and protein concentration was assessed by Bradford assay (Bio-Rad 265 Laboratories Ltd., Mississauga, ON, Canada). Ten percent SDS-PAGE gels were loaded and run with 150 µg of vesicle protein from each subject sample, and stained with Coomassie blue (BioRad). After visualization, sample bands were excised from the gel of each patient and individually subjected to MS-based proteomics. Excised bands were dehydrated in 50% acetonitrile (ACN) and reconstituted in 50 mM ammonium bicarbonate containing 10 mM Tris-2-carboxyethyl phosphine before being vortexed at 37˚C for one hour. Sample alkylation was achieved with chloroacetamide at a final concentration of 55 mM and 1 µL of 0.1 mg/mL trypsin to perform digestion. Peptides were extracted in 90% ACN and were subjected to label-free quantification MS experiments using a Q-Exactive Plus Mass Spectrometer with collision-induced dissociation in a linear ion trap. PEAKS software (Bioinformatics Solutions Inc.) was used to convert MS data to peaks lists, and then to fit the data to the Human1302S database, assuming trypsin to be the digestion enzyme to perform MS/MS spectra analysis. A parent ion mass tolerance of 10.0 PPM (Monoisotopic) and a fragment ion mass tolerance of 0.0100 Da (Monoisotopic) were permitted during the database search. The variable modifications: deamination of asparagine and glutamine; oxidation of methionine; carbamidomethylation of cysteine; and phosphorylation of serine, threonine, and tyrosine were all specified in PEAKS. Proteins' score and expectation values were used to determine protein match probabilities, and resolved protein identities were considered to be correct when they contained four unique peptides 282 and had a

score higher than the identity threshold at $p<0.05$. The proteomics analysis was repeated 3 times for each sample.

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Abbreviations

EV	Extracellular vesicles
PCa	Prostate Cancer
PSA	Prostate Specific Antigen

2.6 References

1. Ferlay, J.; Soerjomataram, I.; Dikshit, R.; Eser, S.; Mathers, C.; Rebelo, M.; Parkin, D. M.; Forman, D.; Bray, F. Cancer incidence and mortality worldwide: sources, methods and major patterns in GLOBOCAN 2012. *Int. J. cancer* **2015**, *136*, E359-86, doi:10.1002/ijc.29210.
2. Coleman, R. E. Skeletal complications of malignancy. *Cancer* **1997**, *80*, 1588–94.
3. Jacobs, S. C. Spread of prostatic cancer to bone. *Urology* **1983**, *21*, 337–44.
4. Lojanapiwat, B.; Anutrakulchai, W.; Chongruksut, W.; Udomphot, C. Correlation and diagnostic performance of the prostate-specific antigen level with the diagnosis, aggressiveness, and bone metastasis of prostate cancer in clinical practice. *Prostate Int.* **2014**, *2*, 133–9, doi:10.12954/PI.14054.
5. Benson, M. C.; Whang, I. S.; Pantuck, A.; Ring, K.; Kaplan, S. A.; Olsson, C. A.; Cooner, W. H. Prostate specific antigen density: a means of distinguishing benign prostatic hypertrophy and prostate cancer. *J. Urol.* **1992**, *147*, 815–6.
6. Schröder, F. H.; Hugosson, J.; Roobol, M. J.; Tammela, T. L. J.; Zappa, M.; Nelen, V.; Kwiatkowski, M.; Lujan, M.; Määttänen, L.; Lilja, H.; Denis, L. J.; Recker, F.; Paez, A.; Bangma, C. H.; Carlsson, S.; Puliti, D.; Villers, A.; Rebillard, X.; Hakama, M.; Stenman, U.-H.; Kujala, P.; Taari, K.; Aus, G.; Huber, A.; van der Kwast, T. H.; van Schaik, R. H. N.; de Koning, H. J.; Moss, S. M.; Auvinen, A.; ERSPC Investigators Screening and prostate cancer mortality: results of the European Randomised Study of Screening for Prostate Cancer (ERSPC) at 13 years of follow-up. *Lancet (London, England)* **2014**, *384*, 2027–35, doi:10.1016/S0140-6736(14)60525-0.
7. Wu, J. T. Assay for prostate specific antigen (PSA): problems and possible solutions. *J. Clin. Lab. Anal.* **1994**, *8*, 51–62.
8. Mistry, K.; Cable, G. Meta-analysis of prostate-specific antigen and digital rectal examination as screening tests for prostate carcinoma. *J. Am. Board Fam. Pract.* **2003**, *16*, 95–101.
9. Obort, A. S.; Ajadi, M. B.; Akinloye, O. Prostate-specific antigen: any successor in sight? *Rev. Urol.* **2013**, *15*, 97–107.
10. van Doormaal, F. F.; Kleinjan, A.; Di Nisio, M.; Büller, H. R.; Nieuwland, R. Cell-derived microvesicles and cancer. *Neth. J. Med.* **2009**, *67*, 266–73.
11. Kalra, H.; Drummen, G. P. C.; Mathivanan, S. Focus on Extracellular Vesicles: Introducing the Next Small Big Thing. *Int. J. Mol. Sci.* **2016**, *17*, 170, doi:10.3390/ijms17020170.
12. Al-Nedawi, K.; Meehan, B.; Micallef, J.; Lhotak, V.; May, L.; Guha, A.; Rak, J. Intercellular transfer of the oncogenic receptor EGFRvIII by microvesicles derived from tumour cells. *Nat. Cell Biol.* **2008**, *10*, 619–24, doi:10.1038/ncb1725.

13. Read, J.; Ingram, A.; Al Saleh, H. A.; Platko, K.; Gabriel, K.; Kapoor, A.; Pinthus, J.; Majeed, F.; Qureshi, T.; Al-Nedawi, K. Nuclear transportation of exogenous epidermal growth factor receptor and androgen receptor via extracellular vesicles. *Eur. J. Cancer.* **2017**, *70*, 62–74, doi:10.1016/j.ejca.2016.10.017.
14. Gabriel, K.; Ingram, A.; Austin, R.; Kapoor, A.; Tang, D.; Majeed, F.; Qureshi, T.; Al-Nedawi, K. Regulation of the tumor suppressor PTEN through exosomes: a diagnostic potential for prostate cancer. *PLoS One* **2013**, *8*, e70047, doi:10.1371/journal.pone.0070047.
15. Duijvesz, D.; Luider, T.; Bangma, C. H.; Jenster, G. Exosomes as biomarker treasure chests for prostate cancer. *Eur. Urol.* **2011**, *59*, 823–31, doi:10.1016/j.eururo.2010.12.031.
16. Nawaz, M.; Fatima, F.; Nazarenko, I.; Ekström, K.; Murtaza, I.; Anees, M.; Sultan, A.; Neder, L.; Camussi, G.; Valadi, H.; Squire, J.A.; Kislinger, T. Extracellular vesicles in ovarian cancer: applications to tumor biology, immunotherapy and biomarker discovery. *Expert. Rev. Proteomic*. **2016**, *13*, 395-409, doi: 10.1586/14789450.2016.1165613
17. Nawaz, M.; Camussi, G.; Valadi, H.; Nazarenko, I.; Ekström, K.; Wang, X.; Principe, S.; Shah, N.; Ashraf, N.M.; Fatima, F.; Neder, L.; Kislinger, T. The emerging role of extracellular vesicles as biomarkers for urogenital cancers. *Nat. Rev. Urol*. **2014**, *11*, 688-701, doi: 10.1038/nrurol.2014.301
18. Wang, L.; Skotland, T.; Berge, V.; Sandvig, K.; Llorente, A. Exosomal proteins as prostate cancer biomarkers in urine: From mass spectrometry discovery to immunoassay-based validation. *Eur. J. Pharm. Sci.* **2017**, *98*, 80–85, doi:10.1016/j.ejps.2016.09.023.
19. Zhang, H.-M.; Qiao, Q.-D.; Xie, H.-F.; Wei, J.-X. Breast cancer metastasis suppressor 1 (BRMS1) suppresses prostate cancer progression by inducing apoptosis and regulating invasion. *Eur. Rev. Med. Pharmacol. Sci.* **2017**, *21*, 68–75.
20. Kim, J.; Choi, Y.-L.; Vallentin, A.; Hunrichs, B. S.; Hellerstein, M. K.; Peehl, D. M.; Mochly-Rosen, D. Centrosomal PKCbetaII and pericentrin are critical for human prostate cancer growth and angiogenesis. *Cancer Res.* **2008**, *68*, 6831–9, doi:10.1158/0008-5472.CAN-07-6195.
21. Srivastava, S. K.; Bhardwaj, A.; Singh, S.; Arora, S.; McClellan, S.; Grizzle, W. E.; Reed, E.; Singh, A. P. Myb overexpression overrides androgen depletion-induced cell cycle arrest and apoptosis in prostate cancer cells, and confers aggressive malignant traits: potential role in castration resistance. *Carcinogenesis* **2012**, *33*, 1149–57, doi:10.1093/carcin/bgs134.

22. Bergamini, S.; Bellei, E.; Reggiani Bonetti, L.; Monari, E.; Cuoghi, A.; Borelli, F.; Sighinolfi, M. C.; Bianchi, G.; Ozben, T.; Tomasi, A. Inflammation: an important parameter in the search of prostate cancer biomarkers. *Proteome Sci.* **2014**, *12*, 32, doi:10.1186/1477-5956-12-32.
23. Liu, Y.; Pixley, R.; Fusaro, M.; Godoy, G.; Kim, E.; Bromberg, M. E.; Colman, R. W. Cleaved high-molecular-weight kininogen and its domain 5 inhibit migration and invasion of human prostate cancer cells through the epidermal growth factor receptor pathway. *Oncogene* **2009**, *28*, 2756–65, doi:10.1038/onc.2009.132.
24. Uehara, H.; Takahashi, T.; Izumi, K. Induction of retinol-binding protein 4 and placenta-specific 8 expression in human prostate cancer cells remaining in bone following osteolytic tumor growth inhibition by osteoprotegerin. *Int. J. Oncol.* **2013**, *43*, 365–74, doi:10.3892/ijo.2013.1954.
25. Tan, C.; Cruet-Hennequart, S.; Troussard, A.; Fazli, L.; Costello, P.; Sutton, K.; Wheeler, J.; Gleave, M.; Sanghera, J.; Dedhar, S. Regulation of tumor angiogenesis by integrin-linked kinase (ILK). *Cancer Cell* **2004**, *5*, 79–90.
26. Rabinovitz, I.; Nagle, R. B.; Cress, A. E. Integrin alpha 6 expression in human prostate carcinoma cells is associated with a migratory and invasive phenotype in vitro and in vivo. *Clin. Exp. Metastasis* **1995**, *13*, 481–91.
27. Rondinelli, R. H.; Epner, D. E.; Tricoli, J. V Increased glyceraldehyde-3-phosphate dehydrogenase gene expression in late pathological stage human prostate cancer. *Prostate Cancer Prostatic Dis.* **1997**, *1*, 66–72, doi:10.1038/sj.pcan.4500208.
28. Nickel, K. F.; Ronquist, G.; Langer, F.; Labberton, L.; Fuchs, T. A.; Bokemeyer, C.; Sauter, G.; Graefen, M.; Mackman, N.; Stavrou, E. X.; Ronquist, G.; Renné, T. The polyphosphate-factor XII pathway drives coagulation in prostate cancer-associated thrombosis. *Blood* **2015**, *126*, 1379–89, doi:10.1182/blood-2015-01-622811.
29. Lewis, T. E.; Milam, T. D.; Klingler, D. W.; Rao, P. S.; Jaggi, M.; Smith, D. J.; Hemstreet, G. P.; Balaji, K. C. Tissue transglutaminase interacts with protein kinase A anchor protein 13 in prostate cancer. *Urol. Oncol.* **2005**, *23*, 407–12, doi:10.1016/j.urolonc.2005.04.002.
30. Wang, Y.; Ledet, R. J.; Imberg-Kazdan, K.; Logan, S. K.; Garabedian, M. J. Dynein axonemal heavy chain 8 promotes androgen receptor activity and associates with prostate cancer progression. *Oncotarget* **2016**, *7*, 49268–49280, doi:10.18632/oncotarget.10284.
31. Altenberger, C.; Heller, G.; Ziegler, B.; Tomasich, E.; Marhold, M.; Topakian, T.; Müllauer, L.; Heffeter, P.; Lang, G.; End-Pfützenreuter, A.; Döme, B.; Arns, B.-M.; Klepetko, W.; Zielinski, C. C.; Zöchbauer-Müller, S. SPAG6 and L1TD1 are

transcriptionally regulated by DNA methylation in non-small cell lung cancers. *Mol. Cancer* **2017**, *16*, 1, doi:10.1186/s12943-016-0568-5.

32. Xie, W.; Li, X.; Chen, X.; Huang, S.; Huang, S. Decreased expression of PRDM2 (RIZ1) and its correlation with risk stratification in patients with myelodysplastic syndrome. *Br. J. Haematol.* **2010**, *150*, 242–4, doi:10.1111/j.1365-2141.2010.08186.x.
33. Mishra, M.; Inoue, N.; Heese, K. Characterizing the novel protein p33MONOX. *Mol. Cell. Biochem.* **2011**, *350*, 127–34, doi:10.1007/s11010-010-0690-4.
34. Borgquist, S.; Butt, T.; Almgren, P.; Shiffman, D.; Stocks, T.; Orho-Melander, M.; Manjer, J.; Melander, O. Apolipoproteins, lipids and risk of cancer. *Int. J. cancer* **2016**, *138*, 2648–56, doi:10.1002/ijc.30013.
35. Qiu, Y.; Tomita, Y.; Zhang, B.; Nakamichi, I.; Morii, E.; Aozasa, K. Pre-B-cell leukemia transcription factor 1 regulates expression of valosin-containing protein, a gene involved in cancer growth. *Am. J. Pathol.* **2007**, *170*, 152–9, doi:10.2353/ajpath.2007.060722.
36. Gutknecht, M. F.; Seaman, M. E.; Ning, B.; Cornejo, D. A.; Mugler, E.; Antkowiak, P. F.; Moskaluk, C. A.; Hu, S.; Epstein, F. H.; Kelly, K. A. Identification of the S100 fused-type protein hornerin as a regulator of tumor vascularity. *Nat. Commun.* **2017**, *8*, 552, doi:10.1038/s41467-017-00488-6.
37. Kuvibidila, S.; Rayford, W. Correlation between serum prostate-specific antigen and alpha-1-antitrypsin in men without and with prostate cancer. *J. Lab. Clin. Med.* **2006**, *147*, 174–81, doi:10.1016/j.lab.2005.11.012.
38. Totten, S. M.; Adusumilli, R.; Kullolli, M.; Tanimoto, C.; Brooks, J. D.; Mallick, P.; Pitteri, S. J. Multi-lectin Affinity Chromatography and Quantitative Proteomic Analysis Reveal Differential Glycoform Levels between Prostate Cancer and Benign Prostatic Hyperplasia Sera. *Sci. Rep.* **2018**, *8*, 6509, doi:10.1038/s41598-018-24270-w.
39. Hale, L. P.; Price, D. T.; Sanchez, L. M.; Demark-Wahnefried, W.; Madden, J. F. Zinc alpha-2-glycoprotein is expressed by malignant prostatic epithelium and may serve as a potential serum marker for prostate cancer. *Clin. Cancer Res.* **2001**, *7*, 846–53.
40. Liu, Z.-Q.; Chu, L.; Fang, J.-M.; Zhang, X.; Zhao, H.-X.; Chen, Y.-J.; Xu, Q. Prognostic role of C-reactive protein in prostate cancer: a systematic review and meta-analysis. *Asian J. Androl.* **2014,** *16*, 467–71, doi:10.4103/1008-682X.123686.
41. Yu, Y. P.; Yu, G.; Tseng, G.; Cieply, K.; Nelson, J.; Defrances, M.; Zarnegar, R.; Michalopoulos, G.; Luo, J.-H. Glutathione peroxidase 3, deleted or methylated in

prostate cancer, suppresses prostate cancer growth and metastasis. *Cancer Res.* **2007**, *67*, 8043–50, doi:10.1158/0008-5472.CAN-07-0648.

42. Coller, H. A. The Runt-related transcription factor 1 in prostate cancer-associated fibroblasts. *Proc. Natl. Acad. Sci. U. S. A.* **2014**, *111*, 16238–9, doi:10.1073/pnas.1418976111.
43. Kostova, M. B.; Brennen, W. N.; Lopez, D.; Anthony, L.; Wang, H.; Platz, E.; Denmeade, S. R. PSA-alpha-2-macroglobulin complex is enzymatically active in the serum of patients with advanced prostate cancer and can degrade circulating peptide hormones. *Prostate* **2018**, *78*, 819–829, doi:10.1002/pros.23539.
44. Eroglu, M.; Yilmaz, N.; Yalcinkaya, S.; Ay, N.; Aydin, O.; Sezer, C. Enhanced HDL-cholesterol-associated anti-oxidant PON-1 activity in prostate cancer patients. *Kaohsiung J. Med. Sci.* **2013**, *29*, 368–73, doi:10.1016/j.kjms.2012.11.004.
45. Hamm, A.; Veeck, J.; Bektas, N.; Wild, P. J.; Hartmann, A.; Heindrichs, U.; Kristiansen, G.; Werbowetski-Ogilvie, T.; Del Maestro, R.; Knuechel, R.; Dahl, E. Frequent expression loss of Inter-alpha-trypsin inhibitor heavy chain (ITIH) genes in multiple human solid tumors: a systematic expression analysis. *BMC Cancer* **2008**, *8*, 25, doi:10.1186/1471-2407-8-25.
46. Al-Nedawi, K.; Szemraj, J.; Cierniewski, C. S. Mast cell-derived exosomes activate endothelial cells to secrete plasminogen activator inhibitor type 1. *Arterioscler. Thromb. Vasc. Biol.* **2005**, *25*, 1744–9, 433 doi:10.1161/01.ATV.0000172007.86541.76.
47. Azab, S.; Osama, A.; Rafaat, M. Does normalizing PSA after successful treatment of chronic 1. Kim, Y.; Jeon, J.; Mejia, S.; Yao, C. Q.; Ignatchenko, V.; Nyalwidhe, J. O.; Gramolini, A. O.; Lance, R. S.; Troyer, D. A.; Drake, R. R.; Boutros, P. C.; Semmes, O. J.; Kislinger, T. Targeted proteomics identifies liquid-biopsy signatures for extracapsular prostate cancer. *Nat. Commun.* **2016**, *7*, 11906, doi:10.1038/ncomms11906.
48. Bell, C. R.; Natale, S.; McInerney, P. D.; Hammonds, J. C. Prostate specific antigen in urinary tract infection. *Br. J. Gen. Pract.* **1998**, *48*, 1005–6.
49. Kim, Y.; Ignatchenko, V.; Yao, C. Q.; Kalatskaya, I.; Nyalwidhe, J. O.; Lance, R. S.; Gramolini, A. O.; Troyer, D. A.; Stein, L. D.; Boutros, P. C.; Medin, J. A.; Semmes, O. J.; Drake, R. R.; Kislinger, T. Identification of differentially expressed proteins in direct expressed prostatic secretions of men with organ-confined versus extracapsular prostate cancer. *Mol. Cell. Proteomics* **2012**, *11*, 1870–84, 444 doi:10.1074/mcp.M112.017889.
50. Kim, Y.; Jeon, J.; Mejia, S.; Yao, C. Q.; Ignatchenko, V.; Nyalwidhe, J. O.; Gramolini, A. O.; Lance, R. S.; Troyer, D. A.; Drake, R. R.; Boutros, P. C.;

Semmes, O. J.; Kislinger, T. Targeted proteomics identifies liquid-biopsy signatures for extracapsular prostate cancer. *Nat. Commun.* **2016**, *7*, 11906, doi:10.1038/ncomms11906.48.

Chapter 3: Severe, but not moderate asthmatics share blood transcriptomic changes with post-traumatic stress disorder and depression.

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3.1 Abstract

Asthma, an inflammatory disorder of the airways, is one of the most common chronic illnesses worldwide and is associated with significant morbidity. There is growing recognition of an association between asthma and mood disorders including post-traumatic stress disorder (PTSD) and major depressive disorder (MDD). Although there are several hypotheses regarding the relationship between asthma and mental health, there is little understanding of underlying mechanisms and causality. In the current study we utilized publicly available datasets of human blood mRNA collected from patients with severe and moderate asthma, MDD, and PTSD. We performed differential expression (DE) analysis and Gene Set Enrichment Analysis (GSEA) on diseased subjects against the healthy subjects from their respective datasets, compared the results between diseases, and validated DE genes and gene sets with 4 more independent datasets. Our analysis revealed that commonalities in blood transcriptomic changes were only found between the severe form of asthma and mood disorders. Gene expression commonly regulated in PTSD and severe asthma, included *ORMDL3* a gene known to be associated with asthma risk and STX8, which is involved in TrkA signaling. We also identified several pathways commonly regulated to both MDD and severe asthma. This study reveals gene and pathway regulation that potentially drives the comorbidity between severe asthma, PTSD, and MDD and may serve as foci for future research aimed at gaining a better understanding of both the relationship between asthma and PTSD, and the pathophysiology of the individual disorders.

3.2 Introduction

Asthma is a chronic inflammatory disease of the airways associated with recurrent episodes of wheezing, shortness of breath, chest tightness, and coughing. Generally, asthma is characterized by reversible constriction of the airways in response to allergen, but it can also be triggered by viral infection, physical activity, stress, or a negative mood [1]. Asthma affects 300 million people worldwide and the World Health Organization has estimated that it is responsible for the loss of 15 million disability-adjusted life years (DALYs) annually [2,3]. Asthma is also the most common chronic disease in children [4].

Epidemiological studies have shown significant association between asthma and mental health disorders, including anxiety, depression, panic attacks, and posttraumatic stress disorder (PTSD) [5,6,7,8,9,10].

MDD, more commonly referred to as ‘depression’ is a mental health disorder characterized by a low self-esteem, mood, and enjoyment of activities [11].
Studies have demonstrated consistent comorbidity between asthma and depression [7] and youth with asthma are close to twice as likely to have anxiety and depressive disorders as those without asthma [8]. The co-occurrence of an anxiety or depressive disorder is associated with poor symptom control, impaired quality of life and increased health care utilization. While many studies have focused on psychosocial factors linking asthma and depression there is evidence that there may be shared pathophysiological factors between the diseases. For example, in a large-scale study in adult twins the association between depression and asthma remained significant after controlling for

genetic and environmental factors [12]. However, the potential mechanisms and causality relating depression and asthma remain unclear [13,14,15].

PTSD is a mental health disorder that usually follows exposure to a traumatic event. The characteristic symptoms of PTSD include intrusive memories and nightmares, negative mood impaired cognition, avoidance behaviors, and changes to arousal behaviors such as increased irritability [16].

Clinical evidence supports a strong link between inflammatory conditions and PTSD with a particularly strong association between asthma and the prevalence and severity of PTSD [17,18]. A twin study of Vietnam war veterans found that those with the top quartile of PTSD scores were 2-fold more likely to have asthma than those in the lower quartile [6]. This association was shown not to be predicted by familial or genetic factors, smoking, depression, or demographic factors [6]. Wisnivesky *et al.*, (2021) [5] found that 19% of world trade center rescue and recovery workers with asthma also had PTSD, 10 times the prevalence in the general population. PTSD is also one of the greatest risk factors for decreased quality-of-life related to asthma [17,18] and these poorer asthma outcomes do not appear to be due to differences in key asthma self-management behaviors [18]. Conversely, individuals with asthma prior to PTSD have been demonstrated to develop more aggravated asthma symptoms after the development of PTSD, while non-asthmatic subjects who develop PTSD have increased risk of adult onset asthma, suggesting a bidirectional relationship between these disorders [17].

An attempt by Jiang *et al*., (2014) [7] to identify a mechanism behind the comorbidity of asthma and MDD suggested immune factors may underlie both disorders.

The investigation of 38 depression studies found that monocyte-derived, and other inflammatory cytokines (IL-1, IL-4, IL-6, and TNF) were significantly overexpressed in individuals with depression, while T cell derived cytokines (IL-10, and INF-γ) were uncorrelated with depression. Data comparing CD4+ T-cell expression in asthmatics with and without depression has also shown that 156 of 1448 total identified genes were differentially expressed in the depressed asthmatics group [19], suggesting that in circulating T-cells there is a unique transcriptomic profile for comorbid asthma and depression.

Genome-wide association studies (GWAS) have identified some shared genetic traits between those with asthma and MDD [20,21]. In a cross-trait meta-analysis, Zhu et al. (2019) [20] identified 10 genomic loci shared between asthma and MDD and mendelian randomization identified a significant causal effect of MDD on asthma. The cross-trait meta-analysis performed by Cao et al., (2021) [21] identified 18 loci jointly associated between MDD and atopic diseases (asthma, eczema, and hay fever). Through Mendelian randomization analysis the investigators found that MDD confers a stronger causal effect on those atopic diseases than they confer on MDD.

Similarly, in a meta-analysis by Nievergelt *et al*., (2019) [22], a pairwise genetic correlation demonstrated a high association between PTSD and asthma. Chronic stress, maternal stress, and more fundamentally, oxidative stress are also associated with severe asthma and increased asthma exacerbations [23,24,25,26]. Yan *et al*. 2021 [24] identified 12 genes methylated in individuals with exposure to chronic stress and violence, that were then shown to be associated with atopic childhood asthma. Although these studies

were not looking at PTSD specifically, it is likely that genes associated with violence and chronic stress exposure would have close ties to those associated with PTSD.
Here, we downloaded 5 publicly available datasets from GEO, each of which compare one of PTSD, MDD, or asthma (a very large dataset which we split randomly into 2 datasets) blood transcription to that of healthy subjects. One dataset of each disease was used to explore genes and gene sets commonly shared between diseased subjects, and the other of each disease dataset was used to validate the genes and sets identified. Prior to conducting the investigation, we were interested in transcription specifically, as it facilitates functional change in the body and therefore we decided to compare the data to the hallmark, and C2 gene sets, which characterize canonical and curated changes in the body. Additionally, we hypothesized that as Jiang *et al.* (2014) [7] found immune factors involved in comorbidity, immune transcriptional changes commonly differentiated in whole blood would delineate the source of comorbidity. Immune factors have also been found partially responsible for cross talk between gut and brain in psycho-active probiotic treated mice exhibiting mood disorder-like symptoms [27,28,29]. For these reasons, we also compared these datasets to the C7 - immune signature gene set.

With a deeper understanding of the established comorbidity between mental health disorders and asthma, may come tangible knowledge on how to combat the root cause of these diseases and an expectation for how treatment of one disorder might affect another. Therefore, the goal of this study was to expand on genome-wide association studies by using publicly available data to characterize transcriptomic similarities

between these disorders through analysis of genes and gene sets commonly differentially expressed between those suffering from the diseases and healthy subjects.

3.3 Results

3.3.1 Exploration of Commonly Differentially Expressed Genes:

The 3 exploration datasets first underwent hierarchical clustering analysis, but there were no distinct clusters formed pertaining to diseased vs healthy subjects or along the lines of any other collected meta data. Principal component analysis was then used to check that no known variables could account for major differences that may arise during DE and GSEA analysis (Fig 1). There was no apparent grouping along PC1 or PC2 for any of the datasets, including for diseased vs healthy subjects (Fig 1). For the PTSD exploration cohort, 40.7% of the variance was accounted for by PC1, and 10.6% by PC2; for MDD, 16.8% of the variance was accounted for by PC1, and 6.2% by PC2; and for asthma, 22.7% of the variance was explained by PC1, and 10.0% by PC2.

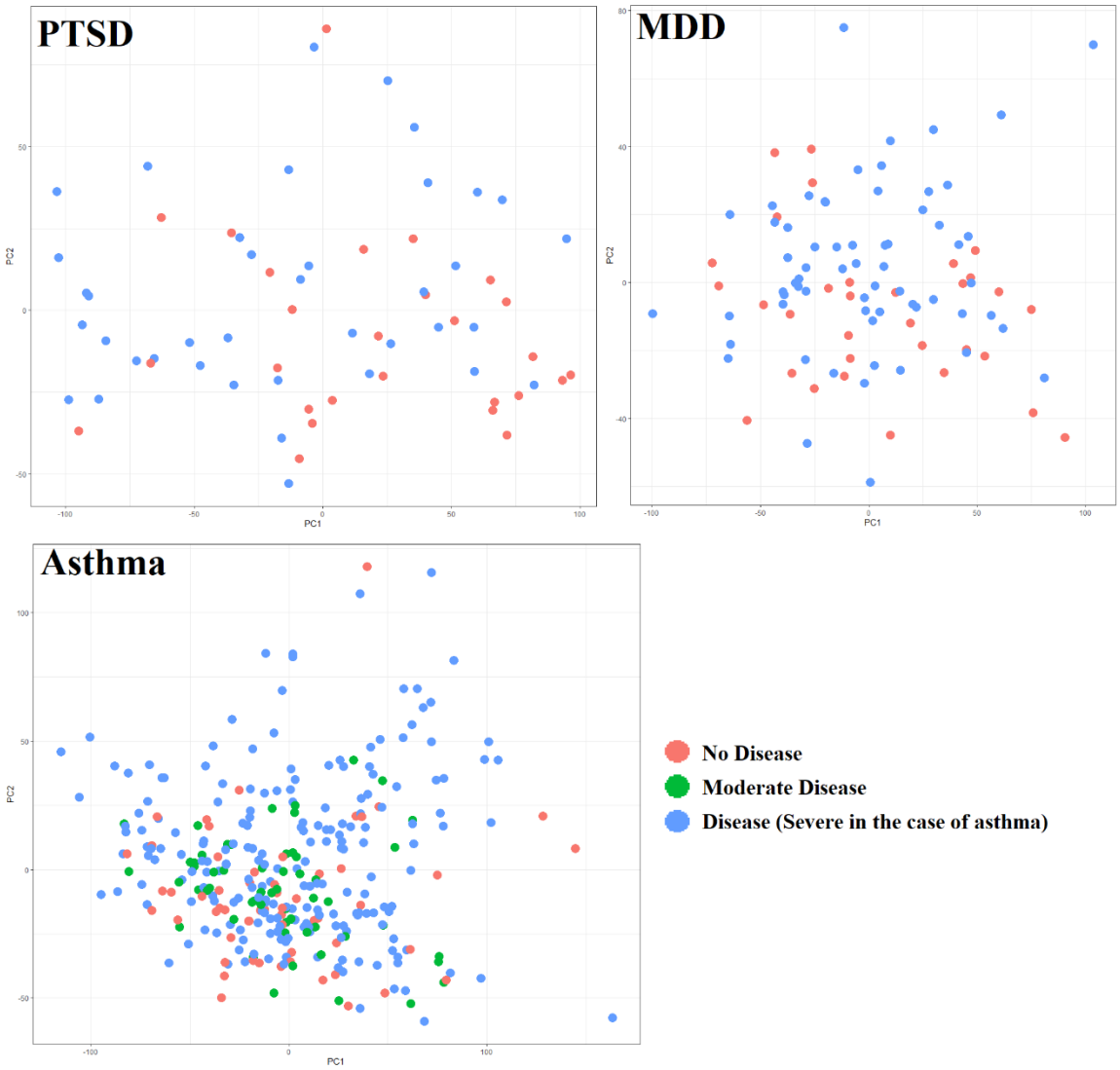


Fig 1. Principal component analysis. (PCA) showing PC1 and PC2 in each of the 3 disease exploration datasets.

Differential expression analysis of each disease to control subjects from their respective datasets reveals significant differences in both genes being up- and downregulated in all diseases (Fig 2). The analysis identified 8,321, 208, 1,736, and 373 genes significantly upregulated (adjusted p-value < 0.05; FC ≥ 1.5) in PTSD, MDD, severe asthma, and moderate asthma respectively, as compared to the corresponding

controls. 7,062, 294, 2,735, and 901 genes were found to be significantly downregulated (adjusted p-value < 0.05; FC ≤ -1.5) in the same comparisons respectively.

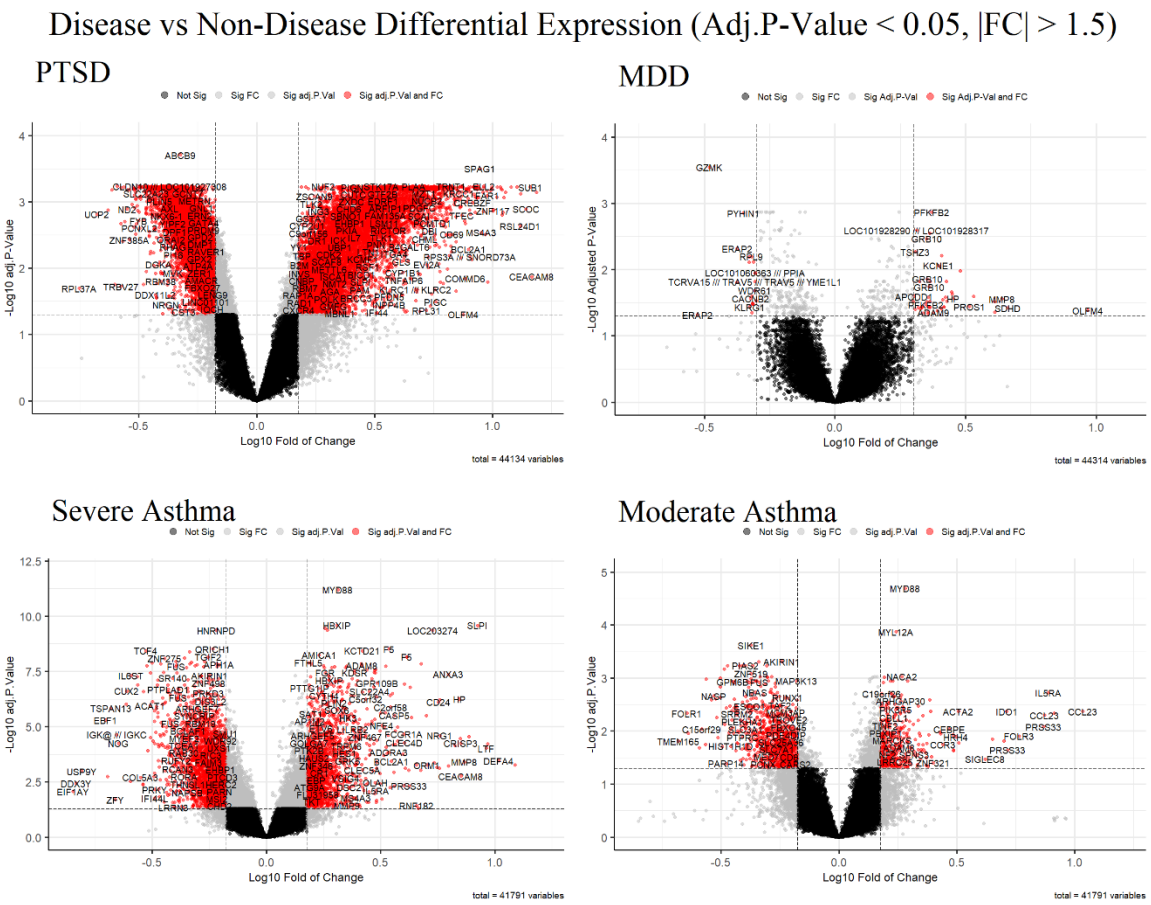


Fig 2. mRNA from the blood of subjects with a disease (PTSD, MDD, severe asthma, and moderate asthma) were compared to blood mRNA from non-diseased subjects for each exploration cohort dataset. The vertical threshold denotes genes or transcripts that are statistically significant (adjusted p-value < 0.05) while the horizontal threshold denotes genes or transcripts with an absolute fold change greater than 1.5. Genes or transcripts that meet none of these criteria are black, one of these criteria are grey, and both are red. The red genes, found to be significant, are also shown next to their symbols.

Significantly regulated (adjusted P-value < 0.05, |FC| ≥1.5) genes were compared between the exploration datasets for each disease. Genes found commonly to be regulated

in the same direction in patients relatively to the healthy controls for multiple diseases were plotted in (Fig 3).

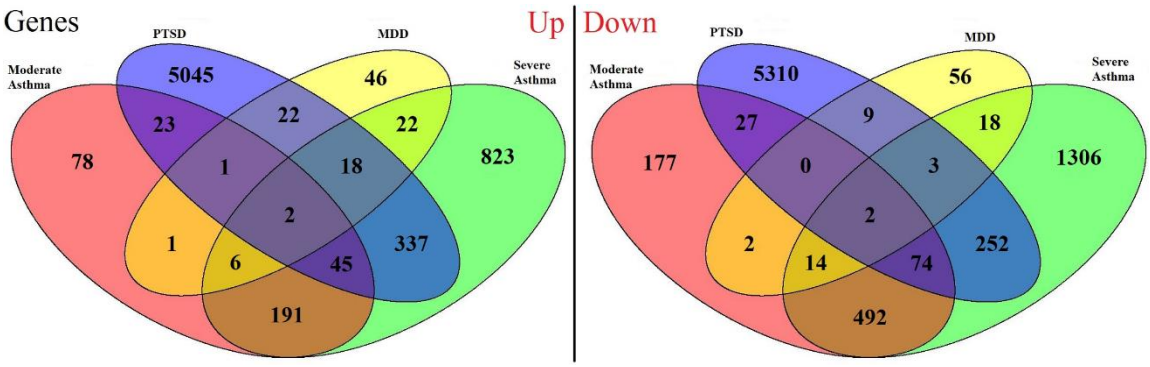


Fig 3. The number of genes differentially expressed from healthy subjects in the same direction between different diseases. The numbers within the different overlaps of the venn diagram are the number of genes significantly (adjusted P-value < 0.05) differentially expressed in both exploration datasets. For example, in the left 'up' panel, there are 22 genes in the PTSD and MDD exploration sets that are similarly significantly overexpressed, and in the right 'down' panel, there are 2 genes commonly underexpressed in all disease exploration datasets compared to their respective healthy controls.

3.3.2 Exploration of Commonly Regulated Gene Sets:

To detect the biological effect of more nuanced changes in all disease groups, Gene Set Enrichment Analysis (GSEA) was performed. GSEA compared expression of selected lists of genes (here termed "gene sets") between diseased and healthy subjects in each dataset (Fig 4). Gene sets from the Hallmark, C2, and C7 collections were compared against. Hallmark gene sets are sets of genes that comprise 50 of the best studied signaling pathways in the body. The C2 gene sets, or curated gene sets, in addition to the well understood and mapped 'KEGG pathways', include other sets of genes found previously to be differentially expressed in literature. C7 gene sets are immune signature gene sets found previously to be differentially expressed in literature.

No Hallmark gene sets were enriched in the same direction between all 4 datasets. 3 C2 gene sets were found to be upregulated in all 4 groups: REN_ALVEOLAR_RHABDOMYOSARCOMA_DN, JISON_SICKLE_CELL_DISEASE_UP, TAKEDA_TARGETS_OF_NUP98_HOXA9_FUSION_8D_DN, and REACTOME_NEUTROPHIL_DEGRANULATION. No C2 gene sets were commonly downregulated in all 4 groups. 65 C7 gene sets were commonly upregulated in all 4 groups, but nothing was commonly downregulated in all 4 of those groups.

3.3.3 Validation of Differentially Expressed Genes in independent transcriptomic datasets:

To challenge these findings, the 'validation' datasets for each of: MDD, PTSD, severe asthma, and moderate asthma underwent DE analysis with limma. No genes were found to be significantly regulated (adjusted P-value < 0.05) in the same directions for all 4 sets as no individual genes were significantly differentially expressed in the MDD validation dataset. 2 genes were validated as upregulated in PTSD and severe asthma: *STX8* (Adjusted p-values in PTSD exploration, PTSD validation, severe asthma exploration, severe asthma validation were: 1.6E-3, 1.8E-2, 2.6E-3, 3.7E-4) and *ARHGAP24* (1.4E-2, 1.6E-2, 3.9E-2, 3.2E-2). Commonly downregulated to PTSD and severe asthma were *ORMDL3* (2.2E-2, 1.9E-3, 2.7E-3, 3.9E-3), *PTP4A3* (2.6E-3, 2.3E-2, 4.5E-3, 5.2E-3), *SHISA4* (1.1E-2, 4.4E-2, 9.8E-3, 6.2E-3), and *TPPP3* (2.2E-2, 1.1E-2, 3.1E-3, 2.7E-2). No differentially expressed genes were validated between PTSD and

moderate asthma in either direction, however. 582 genes were validated as significantly downregulated between moderate and severe asthma while no upregulated genes could be validated.

3.3.4 Validation of Regulated Pathways in independent transcriptomic datasets:

The same datasets used to validate differentially expressed genes were used to validate gene sets and pathways identified as being commonly regulated in either direction in the exploration datasets. Interestingly, despite no genes being significantly differentially expressed in MDD patients vs healthy controls in the validation dataset, there were pathways identified as being significantly altered in severe asthma patients as compared to their corresponding controls (Table 1).

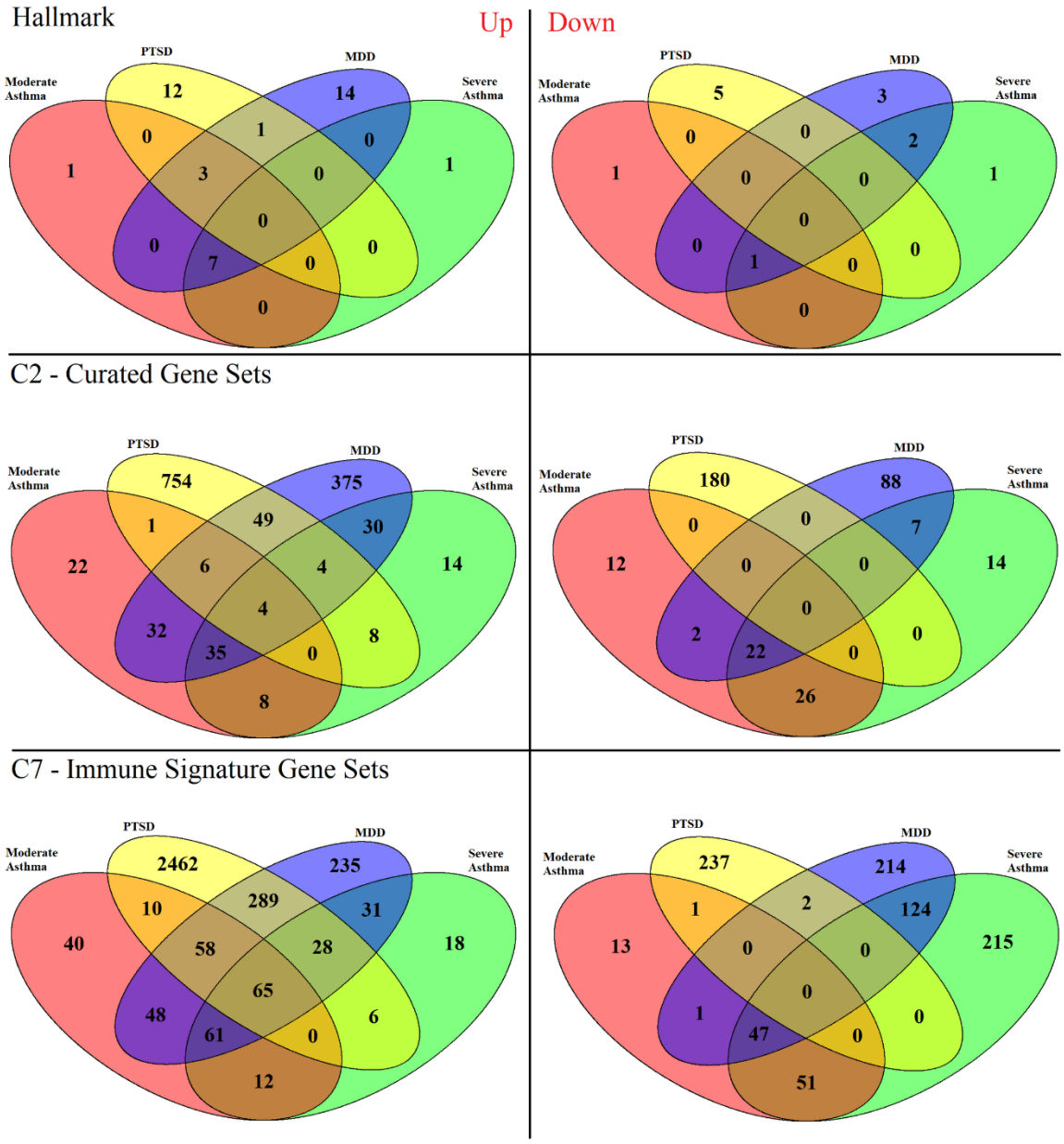


Fig 4. Gene set enrichment analysis. (GSEA) showing significantly (adjusted P-value < 0.05) modified pathways between all 4 exploration datasets. Labels identify the gene sets being compared to and in which direction (left = upregulated, right = downregulated).

Table 1: Directionally validated pathway comparisons in the Hallmark, C2, and C7 collections

Direction	Comparison	Enriched Gene Set	Adjusted P-Values
Up	MDD and Severe Asthma C7	GSE4748_CYANOBACTERIUM_LPSLIKE_VS_LPS_AND_CYANOBACTERIUM_LPSLIKE_STIM_DC_3H_DN	MDD1 - 1.58e-11, MDD2 - 1.30e-2, S.Asthma1 - 5.49e-14, S.Asthma2 - 3.42e-12
		GSE34205_HEALTHY_VS_RSV_INF_INFANT_PBMC_DN	MDD1 - 7.69e-12, MDD2 - 1.16e-2, S.Asthma1 - 1.79e-10, S.Asthma2 - 6.29e-10
Down	MDD and Severe Asthma C7	GSE22886_NAIVE_BCELL_VS_NEUTROPHIL_UP	MDD1 - 1.30e-10, MDD2 - 2.61e-3, S.Asthma1 - 4.41e-7, S.Asthma2 - 2.27e-9
		GSE34205_HEALTHY_VS_FLU_INF_INFANT_PBMC_UP	MDD1 - 4.69e-3, MDD2 - 2.26e-2, S.Asthma1 - 4.47e-3, S.Asthma2 - 9.99e-6
		GSE22886_NEUTROPHIL_VS_MONOCYTE_DN	MDD1 - 3.37e-2, MDD2 - 7.46e-8, S.Asthma1 - 3.74e-2, S.Asthma2 - 3.40e-3
Down	MDD and Severe Asthma C2	JISON_SICKLE_CELL_DISEASE_DN	MDD1 - 1.37e-8, MDD2 - 4.42e-2, S.Asthma1 - 4.44e-3, S.Asthma2 - 9.13e-5

Directionally validated pathway comparisons in the Hallmark, C2, and C7 collections, following GSEA excluding comparison between severe and moderate asthma.

As may be expected, many pathways were found to be commonly modified between moderate and severe asthma when comparing against the C2 and C7 gene sets and although they are not the focus of this study on comorbidity, can be found listed in supplementary information (S1 Table). Barcode plots showing a more detailed cross-

section of gene expression from the sets in Table 1 can be found in supplementary information (S1-S6).

Finally, we pooled all genes from each significantly differentially expressed set common to MDD and severe asthma (Table 1) and performed a STRING cluster analysis for proteins to determine if any other functional networks emerged. Two networks were examined, grouping genes enriched in both MDD and severe asthma compared to healthy subjects, as well as genes enriched in healthy subjects compared to MDD and severe asthma (Fig 5). Among many other associations, STRING analysis found that proteins encoded by the disease-enriched genes of the "GSE4748_CYANOBACTERIUM_LPSLIKE_VS_LPS_AND_CYANOBACTERIUM_LPSLIKE_STIM_DC_3H_DN," and "GSE34205_HEALTHY_VS_RSV_INF_INFANT_PBMC_DN" gene sets have been previously identified in literature in various roles - including modulation of immune function, cancer involvement, and more (Fig 5A)..

Likewise, proteins encoded by the healthy subject-enriched genes of the "GSE22886_NAIVE_BCELL_VS_NEUTROPHIL_UP," "GSE34205_HEALTHY_VS_FLU_INF_INFANT_PBMC_UP," "GSE22886_NEUTROPHIL_VS_MONOCYTE_DN," and "JISON_SICKLE_CELL_DISEASE_DN" gene sets have been shown involved in several functional enrichments including several facets of ribosome regulation, and MHC class II activity.

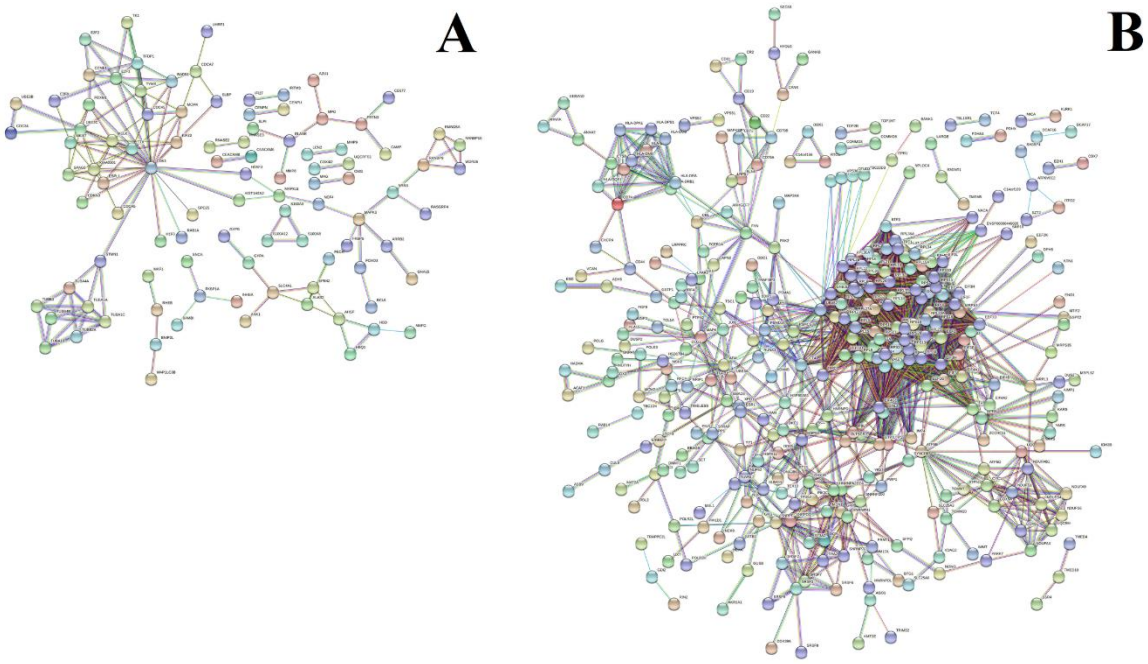


Fig 5. STRING analysis for proteins coded by genes. found A) enriched in the peripheral whole blood of both MDD and severe asthma patients compared to healthy subjects, and B), enriched in healthy subjects compared to both MDD and severe asthma patients. Only high confidence interactions between proteins are shown, and proteins that are not involved in a high confidence interaction do not appear.

3.4 Discussion

While it is widely accepted that psychosocial factors affect asthma pathobiology in children and adults, there is little understanding of potential common biological pathways underlying comorbidity between asthma and mental health disorders. Previous reports based on GWAS studies were focusing on determination of shared genetic traits between Asthma, MDD and PTSD or on circulating levels of specific inflammatory cytokines to explore potential shared pathophysiology of these disorders. In an attempt to provide further insight into the comorbidity of these conditions and to identify target pathways for further investigation, we utilized publicly available data to assess similarities between asthma, MDD and PTSD at the transcriptomic level.

As expected, asthma, MDD and PTSD were associated with many differentially expressed genes and gene sets, and, in comparing exploration cohorts, a number of these genes and gene sets were significantly regulated in the same direction in all diseases. Upon validation, commonalities in transcriptomic changes were restricted to comparisons between severe asthma and MDD or PTSD.

In keeping with literature indicating a close association with regards to comorbidity and reciprocal enhancement of symptom severity [17,18], our cross-disease comparisons found the greatest transcriptomic level similarities between severe asthma and PTSD.

With regard to commonly differentially expressed genes we found *ORMDL3* to be downregulated in the blood of both PTSD and severe asthma subjects. *ORMDL3* codes for a protein called “ORMDL sphingolipid biosynthesis regulator 3” which resides in the

endoplasmic reticulum and is a regulator of sphingolipid synthesis [30]. ORMDL3 requires precise expression to function correctly - under normal conditions it inhibits the rate limiting enzyme of sphingolipid biosynthesis, serine palmitoyl transferase (SPT) [30]. Downstream of uninhibited SPT activity, ceramide - the central sphingolipid metabolite - is produced and transported to the golgi [31]. Therefore, a knockdown of ORMDL3 can result in an abundance of ceramide [32]. When slightly overexpressed, ORMDL3 leads to a dearth of ceramide, however, when highly overexpressed, ORMDL3 increases ceramide biosynthesis through the alternate, recycling/salvage pathway [33,34].

Numerous GWAS have identified ORMDL3 as a potential susceptibility gene for asthma and polymorphisms controlling ORMDL3 expression have been associated with both asthma occurrence and exacerbation [34,35,36,37,38,39,40,41].

However, the mechanistic contribution of ORMDL3 to the pathogenesis of asthma remains unclear and experimental evidence suggests the relationship between ORMDL3 and asthma is complex. Studies in animal models of allergic airway inflammation have indicated that overexpression of ORMDL3 leads to increased ceramide levels and the accompanying ER stress leads to characteristic features of asthma including increased mucus production, an exacerbated inflammatory response, and airway hyperresponsiveness. Correspondingly, downregulation of ORMDL3 expression, and decreased ceramide levels, were demonstrated to significantly ameliorate asthmatic symptoms in a mouse model [42,43,44,45,46,47]. Furthermore, the expression of ORMDL3 in eosinophils seems to play a role in recruitment, attachment and activation of eosinophils in asthma [48]. However, seemingly conflicting evidence

suggests that decreased expression of ORMDL3 can also promote asthma symptoms. Selective knockdown of ORMDL3 in lung epithelial cells leads to airway hyperresponsiveness [49], while downregulation of ORMDL3 in mast cells, cells key to asthma pathogenesis, enhances antigen mediated expression of proinflammatory cytokines and production of prostaglandin D2 and promotes mast cell driven inflammation in vivo [50].

While, to our knowledge there have been no studies associating ORMDL3 and PTSD, ceramide is a precursor for complex sphingolipids that are highly abundant in neural cellular membranes and are regulators of brain homeostasis [51]. Ceramide has also been shown to promote stress-induced depression-like behavior in mice, and intervention with drugs that reduce hippocampal ceramide (amitriptyline and fluoxetine) rescued those behaviours [52,53].

Upregulated in the blood of severe asthma and PTSD subjects were mRNA encoding Syntaxin 8 (STX8), and Rho GTPase Activating Protein 24 (ARHGAP24). STX8 is a t-SNARE protein (target soluble N-ethylmaleimide-sensitive factor attachment protein receptor) involved in diverse vesicle docking and membrane fusion events. STX8 has been demonstrated to regulate the function of receptors and ion channels, including TrkA and CFTR. The TrkA receptor is transported from the golgi to the plasma membrane by STX8, a process which with nerve growth factor (NGF) stimulation promotes downstream TrkA signaling [54]. Interestingly, higher levels of TrkA expression have been identified in patients with allergic asthma [55], and although its role in asthma has not been fully elucidated, there are several proposed mechanisms by which

neurotrophin signaling exacerbates asthma [56]. Some evidence suggests neurotrophin signaling may modulate airway hyperactivity and bronchoconstrictor release, enhancement of airway contractility, as well as airway remodeling [56,57,58]. TrkA has also been previously implicated in PTSD, as NGF signaling via TrkA alleviated stress induced PTSD-like symptoms in mice [59]. In contrast to enhancing TrkA signaling, STX8 also interacts with CFTR to inhibit function and trafficking to the cell surface [60]. CFTR is largely studied in relation to cystic fibrosis, however, impaired function of this ion channel has been associated with more severe or difficult to treat asthma [61,62,63]. While to our knowledge there has been no suggested relationship between CFTR and PTSD, the ion channel is expressed throughout the central nervous system [64].

ARHGAP24 converts the Rac-type GTPase into its inactive GDP-bound state which, downstream of Rho, suppresses actin remodelling [65]. Increased activation of RhoA/Rho-kinase is associated with airway hyper-responsiveness and smooth muscle contraction in asthma [66]. Cerebral RhoA activation is known to enhance fear memory which may have implications for PTSD [67]. So, in both asthma and PTSD, increased Rho activity is associated with increased pathology. It is curious then, that we find an inhibitor of its downstream activity differentially overexpressed in the blood of the diseased subjects. The reason for this would have to be elucidated by further research.

Other genes commonly downregulated in severe asthma and PTSD were Protein Tyrosine Phosphatase 4A3 (PTP4A3), known for its role in stimulating progression from G1 to S phase in mitosis [68]; Shisa Family Member 4 (SHISA4), a transmembrane scaffold/adaptor protein [69]; and Tubulin Polymerization Promoting Protein Family

Member 3 (TPPP3), a regulator of microtubule dynamics [70]. To our knowledge, none of these proteins have previously been associated with asthma or PTSD and their identification here may warrant further investigation.

Neither ORMDL3, STX8, nor ARHGAP24 are discussed by Bigler *et al*., (2017) [71] in relation to the asthma datasets; nor are they identified in the PTSD dataset by Rusch *et al*., (2019) [72]. ARHGAP24 is discussed briefly in regards to PTSD in the validation data set, (Kuan *et al*., 2017) [73] as being a member of the PTSD-associated actin cytoskeleton pathway.

One of the gene sets "GSE34205_HEALTHY_VS_RSV_INF_INFANT_PBMC_DN" refers to a list of genes found to be more highly expressed in peripheral blood mononuclear cells (PBMC) of infants with RSV (Respiratory syncytial virus) bronchiolitis [70] when compared to those of healthy subjects. We also found that "GSE34205_HEALTHY_VS_FLU_INF_INFANT_PBMC_UP," a list of genes with decreased in expression infants with acute influenza compared to PBMCs of healthy subjects, was downregulated in both MDD and severe asthma [74]. These 2 congruent pieces of evidence suggest that the immune signature to respiratory infection in infants is similar to the immune signature of both asthma and MDD whole blood. In human airway epithelial cells Ioannidis et al. (2012) [74] found that comparing both influenza and RSV treatment to control exhibited DE reminiscent of a type I interferon immune signature and genes downstream of IFN-α/β were expressed abundantly in infected cells. Type I interferon signaling is known to be a contributing factor in some cases of both depression

and asthma [75,76,77,78].

Two additional gene sets we found downregulated in both MDD and severe asthma: “GSE22886_NAIVE_BCELL_VS_NEUTROPHIL_UP,” and “GSE22886_NEUTROPHIL_VS_MONOCYTE_DN" were both compiled by Abbas *et al*. (2005) [79] to identify patterns in immune cell-specific expression in order to identify states of activation. The gene sets we identified as being underexpressed in MDD and severe asthma can be congruently explained by a reduction of neutrophil specific gene expression, or by an increase in naive B-cell and monocyte specific gene expression. The latter is perhaps more likely as neutrophils have been demonstrated to be activated in patients with MDD and asthma [80,81]. Furthermore, B cell homeostasis is altered in individuals with MDD and B cells play a crucial role in regulating the hyperactivity of airways in asthma [82,83,84,85]. Likewise, there is generally increased activity and larger numbers of monocytes in MDD and asthma compared to healthy subjects [86,87,88,89]. This highlights the possibility of enhanced B cell and monocyte activity playing a key role in comorbid asthma and MDD.

JISON_SICKLE_CELL_DISEASE_DN, found downregulated in the blood for both MDD and severe asthma, are genes previously found to be downregulated in peripheral blood mononuclear cells (PBMCs) in sickle-cell disease patients compared with non-diseased counterparts. Asthma is common in children with sickle cell disease and this comorbidity is becoming increasingly well documented [90]. In sickle cell, nitric oxide consumption mediated by plasma hemoglobin, ischemia-reperfusion injury, and the generation of free radicals activate an inflammatory stress response [90]. Jison *et al*.

(2004) [91], who discovered the gene set, found many of the genes differentially expressed within PBMCs were linked to inflammatory stress as well. To find these same genes underexpressed in two comorbid conditions suggests that the inflammatory stress response itself could be a driver behind comorbidity for sickle cell disease, MDD, and severe asthma.

The STRING analysis for proteins translated from the individual genes in the gene sets commonly regulated between MDD and severe asthma show that the genes upregulated in each of these diseases have several functional associations. By combining curated gene sets enriched in both diseases we gave the string analysis a more complete picture of all the systems that may be modified downstream of these blood transcriptional changes. In addition to basic biological processes, cellular compartments, molecular functions, and pathways, several smaller literature-backed gene sets were found in common. Examining the top 5 in descending order of strength our genes enriched in MDD and severe asthma, we observed matches to biomarkers for severe influenza infection (Adj. P-value = 2.2E-5) [92], genes associated with arthritis (Adj. P-value = 1.5E-3) [93], respiratory distress syndrome phenotypes (Adj. P-value = 1.2E-2) [94], lung epithelial function in sepsis (Adj. P-value = 5.6E-6) [95], and myocardial infarction and neutrophil degranulation (Adj. P-value = 2.6E-4) [96]. Looking at the top 5 for genes enriched in healthy subjects compared to MDD and severe asthma we identified many matches associated with ribosomal regulation, and to a lesser extent immune function and anemia (Adj. P-values = 3.1E-9, 2.0E-4, 2.0E-4, 1.3E-3, 6.5E-3) [97,98,99,100,101]. This could suggest that there is less ribosomal regulation in MDD and severe asthma, and

further suggests that immune involvement could drive the relationship between these disorders.

Despite MDD being a major comorbidity in PTSD, and 440 immune signature gene sets commonly upregulated between the exploration datasets, no genes or gene sets were validated in this study when comparing MDD and PTSD. However, the neurobiology of the link between PTSD and MDD is unclear and it is entirely feasible that similarities in gene expression between the disorders is restricted to the CNS and are undetectable in the blood.

It is notable that there were no validated genes or gene sets in common between mild/moderate asthma and either of the mental health disorders. This finding is consistent with the phenomenon that mental health disorders such as PTSD and MDD are correlated with more severe disease outcomes [102]. It may be that activation of specific genes or pathways that are involved in MDD or PTSD are also factors that contribute to the development of more severe asthma. In this regard, there is evidence to suggest that antidepressant treatment improved asthma symptoms in severe but not mild asthmatics with co-morbid depression.

Overall, with six parallel DGE analyses and GSEA on whole blood gene expression, we identified genes and gene set expression that potentially links severe asthma to both PTSD and MDD. The gene sets commonly regulated between asthma and MDD, support previously suggested links between inflammation related immune factors and the two disorders [7]. Epidemiological evidence indicating that PTSD has a stronger association with asthma than other chronic inflammatory diseases [103,104] suggests that

the relationship is driven by more than common immune factors. Here we identify 6 genes (2 upregulated in disease and 4 downregulated) being differentially expressed in both PTSD and asthma. Of particular note, our results identify mechanisms involving ceramide biosynthesis and SNARE regulated signaling pathways as potential targets for future research aimed at understanding both the relationship between PTSD and asthma and the pathophysiology of the individual disorders.

3.5 Methods

3.5.1 Obtaining and Preprocessing Datasets:

Data were downloaded from the Gene Expression Omnibus (GEO) repository and preprocessed using the methods described by the respective authors associated with each dataset (Table 2). Specific blood RNA datasets were chosen over others on GEO due to there being among the few datasets on GEO that met the specific criteria of whole blood (rather than PBMCs, or biopsy), the specific diseases in question, and focused on mRNA (rather than total RNA or miRNA). Any remaining appropriate datasets on GEO were on different platforms. We decided against pooling these datasets since attempts to correct for technical variation forces data modification that can confound and obscure the true biological variation of interest, and increase the likelihood of generating erroneous results. Therefore, we preferred to select the largest available datasets that did not require pooling for a classic exploration and validation analysis.

Rusch *et al*., (2019) [72] (preprocessed and raw data available at: https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE81761) measured blood mRNA military service members, with and without PTSD. Only samples from the first time-point collection, rather than the follow up collection, were selected for analysis. Other information collected on the subjects included sex (63 male, 3 female), age (22-49), and race. Kuan *et al*., (2017) [73] (preprocessed and raw data available at: https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE97356) measured blood mRNA in World Trade Center responders with PTSD currently, never, and in the past. Samples collected from subjects who never had PTSD or had PTSD at the time of the

collection were selected for further analysis. No other sample information was supplied with the dataset. Leday *et al*., (2017) [105] (preprocessed and raw data available at: https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE98793) pooled human blood mRNA data from two depression studies: the "Janssen–Brain Resource Company "study, and the "GlaxoSmithKline–High-Throughput Disease-specific target Identification Program" study into subjects with MDD, and without. Batch 1 and batch 2 were originally found to generate distinct groups in principal component analysis (PCA), and were batch corrected with the 'removeBatchEffect' function in limma package (Ritchie et al., 2015 [106]) in R. This dataset contained additional information, such as including gender (144 female, 48 male), age (31-72), and anxiety status (128 no, 64 yes). Spijker *et al*., (2010) [107] (preprocessed and raw data available at: https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE19738) collected blood from subjects with and without MDD prior to and following stimulation with lipopolysaccharide (LPS), data which we excluded. Additional information in the dataset was age (21-63), gender (41 female, 26 male), and smoking status (20 non-smoking, 18 quit smoking, 29 smoking). The Unbiased Biomarkers for the Prediction of Respiratory Disease Outcomes (U-BIOPRED) study dataset (Bigler *et al*., 2017) [71] (preprocessed and raw data available at: https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE69683) measured blood mRNA in subjects with moderate (lung function tests are 60-80% of expected value), severe (lung function tests are <60% of expected value), and no asthma. The dataset also contained information on gender of the patients (275 female, 223 male), smoking or non-

smoking (410 non-smoking, 88 smoking). We randomly divided this dataset into an exploration and a validation cohort at a 2:1 ratio. Low expressed genes were filtered out prior to trimmed mean of M-values (TMM) normalization of the RNAseq dataset as it is more sensitive due to its single nucleotide resolution [108,109]. This was performed using the edgeR packages 'filterByExpr' function [110]. Data on race was only available in the Rusch *et al*., (2019), and Bigler *et al*., (2017) datasets and both studies had predominantly white caucasian participants (66% and 90%, respectively).

Seeing as not all datasets contained the same background information on their respective subjects, and because the purpose of this study was to detect commonalities between comorbid diseases that may exist robustly in a particular disease regardless of other variables, demographic information such as age, race, gender, and smoking status was not taken into consideration.

Table 2: List of datasets used in this paper

GSE #	Platform (GPL)	Source and Species	Normalization Method	Purpose	Associated Publication	# Samples	# Genes / Variants
GSE81761	GPL570 (Array)	Human whole blood mRNA	RMA*	PTSD Exploration	Rusch *et al*., 2019	27 - PTSD, 39 - No PTSD	44,134
GSE97356	GPL11154 (RNAseq)	Human whole blood mRNA	TMM	PTSD Validation	Kuan *et al*., 2017	82 - PTSD, 201 - No PTSD	15,112
GSE98793	GPL570 (Array)	Human whole blood RNA	RMA	MDD Exploration	Leday *et al*., 2018	64 - MDD, 32 - No MDD	44,134

GSE 19738	GPL6848 (Array)	Human whole blood RNA	Quantile	MDD Validation	Spijker *et al*., 2010	33 - MDD, 34 - No MDD	12,816
GSE 69683	GPL13158 (Array)	Human whole blood RNA	RMA	Asthma Exploration and Validation	Bigler *et al*., 2017	After Split: Exploration: 58 - Healthy, 58 - Moderate, 216 - Severe. Validation: 28 - Healthy, 20 - Moderate, 128 - Severe	41,791

List of datasets used in this paper with a description of data type, preprocessing, number of genes and gene variants remaining in the dataset following preprocessing, and associated publications. * Robust multichip average (RMA) normalization.

Principal component analysis (PCA) was done in base R using the prcomp function, and after log transforming any RNAseq data before visualizing in ggplot2 [111]. Venn diagrams were generated using the VennDiagram R package [112].

3.5.2 Differential Gene Expression:

Each dataset, including the split asthma datasets for both severe and moderate asthma, underwent differential gene expression analysis individually, comparing their disease to the respective control group (the non-disease group) from the same study. Analysis was performed using the limma package with multiple hypothesis correction and Benjamini-Hochberg FDR applied. Genes were considered to be differentially expressed with an adjusted p-value < 0.05 and $|FC| \geq 1.5$.

3.5.3 Gene Set Enrichment Analysis:

3 MSigDB collections of gene sets (v7.4) were downloaded from the GSEA website (https://www.gsea-msigdb.org/gsea/msigdb/index.jsp): Hallmark - well-defined biological states or processes, C2 - curated gene sets from PubMed publications and online pathway databases (including KEGG), and C7 - immunologic signature gene sets representative of immune and cell states.

Fold change values generated by the differential expression analysis of diseased subjects vs healthy subjects were compared to each of the 3 collections via their entrez gene IDs using the Gage package in R [113]. Gage uses the differential expression output of all genes, not just those with significant fold change or p-value. Barcode plots were generated using barcodeplot() function (limma package). Volcano plots were generated using the R package 'EnhancedVolcano' [114].

3.6 References

1. Janssens T, Ritz T. Perceived triggers of asthma: key to symptom perception and management. Clin Exp Allergy [Internet]. 2013 Sep;43(9):1000–8. Available from: https://onlinelibrary.wiley.com/doi/10.1111/cea.12138
2. Masoli M, Fabian D, Holt S, Beasley R. The global burden of asthma: executive summary of the GINA Dissemination Committee Report. Allergy [Internet]. 2004 May;59(5):469–78. Available from: https://onlinelibrary.wiley.com/doi/10.1111/j.1398-9995.2004.00526.x
3. Vos T, Lim SS, Abbafati C, Abbas KM, Abbasi M, Abbasifard M, et al. Global burden of 369 diseases and injuries in 204 countries and territories, 1990–2019: a systematic analysis for the Global Burden of Disease Study 2019. Lancet [Internet]. 2020 Oct;396(10258):1204–22. Available from: https://linkinghub.elsevier.com/retrieve/pii/S0140673620309259
4. Centers for Disease Control and Prevention NC for HS. 2011-2012 National Survey of Children's Health Frequently Asked Questions [Internet]. 2013. Available from: https://www.cdc.gov/nchs/slaits/nsch.htm
5. Wisnivesky JP, Markowitz SB, James S, Stone K, Dickens B, Busse P, et al. Comorbid posttraumatic stress disorder and major depressive disorder are associated with asthma morbidity among World Trade Center workers. Ann Allergy, Asthma Immunol [Internet]. 2021 Mar;126(3):278–83. Available from: https://linkinghub.elsevier.com/retrieve/pii/S1081120620311078
6. Goodwin RD, Fischer ME, Goldberg J. A Twin Study of Post–Traumatic Stress Disorder Symptoms and Asthma. Am J Respir Crit Care Med [Internet]. 2007 Nov 15;176(10):983–7. Available from: http://www.atsjournals.org/doi/abs/10.1164/rccm.200610-1467OC
7. Jiang M, Qin P, Yang X. Comorbidity between depression and asthma via immune-inflammatory pathways: A meta-analysis. J Affect Disord [Internet]. 2014 Sep;166:22–9. Available from: https://linkinghub.elsevier.com/retrieve/pii/S0165032714002110
8. Katon W, Lozano P, Russo J, McCauley E, Richardson L, Bush T. The Prevalence of DSM-IV Anxiety and Depressive Disorders in Youth with Asthma Compared with Controls. J Adolesc Heal [Internet]. 2007 Nov;41(5):455–63. Available from: https://linkinghub.elsevier.com/retrieve/pii/S1054139X07002443
9. Caramanica K, Brackbill RM, Liao T, Stellman SD. Comorbidity of 9/11-Related PTSD and Depression in the World Trade Center Health Registry 10-11 Years Postdisaster. J Trauma Stress [Internet]. 2014 Dec;27(6):680–8. Available from: https://onlinelibrary.wiley.com/doi/10.1002/jts.21972
10. Rytwinski NK, Scur MD, Feeny NC, Youngstrom EA. The Co-Occurrence of Major Depressive Disorder Among Individuals With Posttraumatic Stress Disorder: A Meta-Analysis. J Trauma Stress [Internet]. 2013 Jun;26(3):299–309. Available from: https://onlinelibrary.wiley.com/doi/10.1002/jts.21814

11. Kennedy SH. Core symptoms of major depressive disorder: relevance to diagnosis and treatment. Dialogues Clin Neurosci [Internet]. 2008 Sep 30;10(3):271–7. Available from: https://www.tandfonline.com/doi/full/10.31887/DCNS.2008.10.3/shkennedy
12. Tedner SG, Lundholm C, Olsson H, Almqvist C. Depression or anxiety in adult twins is associated with asthma diagnosis but not with offspring asthma. Clin Exp Allergy [Internet]. 2016 Jun;46(6):803–12. Available from: https://onlinelibrary.wiley.com/doi/10.1111/cea.12714
13. Hasler G, Gergen PJ, Kleinbaum DG, Ajdacic V, Gamma A, Eich D, et al. Asthma and Panic in Young Adults. Am J Respir Crit Care Med [Internet]. 2005 Jun;171(11):1224–30. Available from: http://www.atsjournals.org/doi/abs/10.1164/rccm.200412-1669OC
14. To T, Ryckman K, Zhu J, Williams D, Feldman LY, Larsen K, et al. Mental Health Services Claims and Adult Onset Asthma in Ontario, Canada. J Allergy Clin Immunol Pract [Internet]. 2017 Sep;5(5):1388-1393.e3. Available from: https://linkinghub.elsevier.com/retrieve/pii/S2213219817301137
15. Vargas PA. Spreading the Word: Comorbidity of Asthma and Depression Is Not Just the Product of a Vulnerable Personality. J Allergy Clin Immunol Pract [Internet]. 2020 Jan;8(1):208–9. Available from: https://linkinghub.elsevier.com/retrieve/pii/S2213219819306415
16. Lancaster C, Teeters J, Gros D, Back S. Posttraumatic Stress Disorder: Overview of Evidence-Based Assessment and Treatment. J Clin Med [Internet]. 2016 Nov 22;5(11):105. Available from: http://www.mdpi.com/2077-0383/5/11/105
17. Xu KY, Goodman E, Goswami R, Crane M, Crowley L, Busse P, et al. Determinants of asthma morbidity in World Trade Center rescue and recovery workers. Ann Allergy, Asthma Immunol [Internet]. 2016 Nov;117(5):568–70. Available from: https://linkinghub.elsevier.com/retrieve/pii/S108112061630624X
18. Chung MC, Rudd H, Wall N. Posttraumatic stress disorder following asthma attack (post-asthma attack PTSD) and psychiatric co-morbidity: The impact of alexithymia and coping. Psychiatry Res [Internet]. 2012 May;197(3):246–52. Available from: https://linkinghub.elsevier.com/retrieve/pii/S0165178112000194
19. Wang T, Ji Y-L, Yang Y-Y, Xiong X-Y, Wang I-M, Sandford AJ, et al. Transcriptomic profiling of peripheral blood CD4+ T-cells in asthmatics with and without depression. Gene [Internet]. 2015 July;565(2):282-87.
20. Zhu Z, Zhu X, Liu C-L, Shi H, Shen S, Yang Y, et al. Shared genetics of asthma and mental health disorders: a large-scale genome-wide cross-trait analysis. Eur Respir J [Internet]. 2019 Dec;54(6):1901507. Available from: http://erj.ersjournals.com/lookup/doi/10.1183/13993003.01507-2019
21. Cao H, Li S, Baranova A, Zhang F. Shared Genetic Liability Between Major Depressive Disorder and Atopic Diseases. Front Immunol [Internet]. 2021 Sep 8;12. Available from: https://www.frontiersin.org/articles/10.3389/fimmu.2021.665160/full

22. Nievergelt CM, Maihofer AX, Klengel T, Atkinson EG, Chen C-Y, Choi KW, et al. International meta-analysis of PTSD genome-wide association studies identifies sex- and ancestry-specific genetic risk loci. Nat Commun [Internet]. 2019 Dec 8;10(1):4558. Available from: http://www.nature.com/articles/s41467-019-12576-w
23. Gautam, Y., Johansson, E., & Mersha, T. B. Multi-Omics Profiling Approach to Asthma: An Evolving Paradigm. J. pers. Med [Internet]. 2022:*12*(1), 66. Available from: https://doi.org/10.3390/jpm12010066
24. Yan, Q., Forno, E., Cardenas, A., Qi, C., Han, Y. Y., Acosta-Pérez, E., Kim, S., Zhang, R., Boutaoui, N., Canino, G., Vonk, J. M., Xu, C. J., Chen, W., Marsland, A., Oken, E., Gold, D. R., Koppelman, G. H., & Celedón, J. C. Exposure to violence, chronic stress, nasal DNA methylation, and atopic asthma in children. Pediatr. Pulmonol. [Internet]. 2021 Mar:*56*(7), 1896–1905. Available from: https://doi.org/10.1002/ppul.25372
25. Trump, S., Bieg, M., Gu, Z., Thürmann, L., Bauer, T., Bauer, M., Ishaque, N., Röder, S., Gu, L., Herberth, G., Lawerenz, C., Borte, M., Schlesner, M., Plass, C., Diessl, N., Eszlinger, M., Mücke, O., Elvers, H. D., Wissenbach, D. K., von Bergen, M., … Eils, R. Prenatal maternal stress and wheeze in children: novel insights into epigenetic regulation. Sci. rep. 2016:6, 28616. Available from: https://doi.org/10.1038/srep28616
26. Fitzpatrick, A. M., Park, Y., Brown, L. A., & Jones, D. P. Children with severe asthma have unique oxidative stress-associated metabolomic profiles. J. Allergy Clin. Immunol. 2014:133(1), 258–61.e618. Available from: https://doi.org/10.1016/j.jaci.2013.10.012
27. Bravo, J. A., Forsythe, P., Chew, M. V., Escaravage, E., Savignac, H. M., Dinan, T. G., Bienenstock, J., & Cryan, J. F. (2011). Ingestion of Lactobacillus strain regulates emotional behavior and central GABA receptor expression in a mouse via the vagus nerve. *Proceedings of the National Academy of Sciences of the United States of America*, *108*(38), 16050–16055. https://doi.org/10.1073/pnas.1102999108
28. Bharwani, A., Mian, M. F., Surette, M. G., Bienenstock, J., & Forsythe, P. (2017). Oral treatment with Lactobacillus rhamnosus attenuates behavioural deficits and immune changes in chronic social stress. *BMC medicine*, *15*(1), 7. https://doi.org/10.1186/s12916-016-0771-7
29. Liu, Y., Mian, M. F., McVey Neufeld, K. A., & Forsythe, P. (2020). $CD4^{+}CD25^{+}$ T Cells are Essential for Behavioral Effects of Lactobacillus rhamnosus JB-1 in Male BALB/c mice. *Brain, behavior, and immunity*, *88*, 451–460. https://doi.org/10.1016/j.bbi.2020.04.014
30. Breslow DK, Collins SR, Bodenmiller B, Aebersold R, Simons K, Shevchenko A, et al. Orm family proteins mediate sphingolipid homeostasis. Nature [Internet]. 2010 Feb;463(7284):1048–53. Available from: http://www.nature.com/articles/nature08787

31. James B, Milstien S, Spiegel S. ORMDL3 and allergic asthma: From physiology to pathology. J Allergy Clin Immunol [Internet]. 2019 Sep;144(3):634–40. Available from: https://linkinghub.elsevier.com/retrieve/pii/S0091674919309728
32. Cai L, Oyeniran C, Biswas DD, Allegood J, Milstien S, Kordula T, et al. ORMDL proteins regulate ceramide levels during sterile inflammation. J Lipid Res [Internet]. 2016 Aug;57(8):1412–22. Available from: https://linkinghub.elsevier.com/retrieve/pii/S0022227520352135
33. Oyeniran C, Sturgill JL, Hait NC, Huang W-C, Avni D, Maceyka M, et al. Aberrant ORM (yeast)–like protein isoform 3 (ORMDL3) expression dysregulates ceramide homeostasis in cells and ceramide exacerbates allergic asthma in mice. J Allergy Clin Immunol [Internet]. 2015 Oct;136(4):1035-1046.e6. Available from: https://linkinghub.elsevier.com/retrieve/pii/S0091674915003334
34. Ferreira MAR, McRae AF, Medland SE, Nyholt DR, Gordon SD, Wright MJ, et al. Association between ORMDL3, IL1RL1 and a deletion on chromosome 17q21 with asthma risk in Australia. Eur J Hum Genet [Internet]. 2011 Apr 8;19(4):458–64. Available from: http://www.nature.com/articles/ejhg2010191
35. Moffatt MF, Kabesch M, Liang L, Dixon AL, Strachan D, Heath S, et al. Genetic variants regulating ORMDL3 expression contribute to the risk of childhood asthma. Nature [Internet]. 2007 Jul 4;448(7152):470–3. Available from: http://www.nature.com/articles/nature06014
36. Choudhry S, Taub M, Mei R, Rodriguez-Santana J, Rodriguez-Cintron W, Shriver MD, et al. Genome-wide screen for asthma in Puerto Ricans: evidence for association with 5q23 region. Hum Genet [Internet]. 2008 Jun 10;123(5):455–68. Available from: http://link.springer.com/10.1007/s00439-008-0495-7
37. Himes BE, Hunninghake GM, Baurley JW, Rafaels NM, Sleiman P, Strachan DP, et al. Genome-wide Association Analysis Identifies PDE4D as an Asthma-Susceptibility Gene. Am J Hum Genet [Internet]. 2009 May;84(5):581–93. Available from: https://linkinghub.elsevier.com/retrieve/pii/S0002929709001487
38. Hancock DB, Romieu I, Shi M, Sienra-Monge J-J, Wu H, Chiu GY, et al. Genome-Wide Association Study Implicates Chromosome 9q21.31 as a Susceptibility Locus for Asthma in Mexican Children. Gibson G, editor. PLoS Genet [Internet]. 2009 Aug 28;5(8):e1000623. Available from: https://dx.plos.org/10.1371/journal.pgen.1000623
39. Sleiman PMA, Flory J, Imielinski M, Bradfield JP, Annaiah K, Willis-Owen SAG, et al. Variants of DENND1B Associated with Asthma in Children. N Engl J Med [Internet]. 2010 Jan 7;362(1):36–44. Available from: http://www.nejm.org/doi/abs/10.1056/NEJMoa0901867
40. Mathias RA, Grant A V., Rafaels N, Hand T, Gao L, Vergara C, et al. A genome-wide association study on African-ancestry populations for asthma. J Allergy Clin Immunol [Internet]. 2010 Feb;125(2):336-346.e4. Available from: https://linkinghub.elsevier.com/retrieve/pii/S0091674909013165
41. Li X, Howard TD, Zheng SL, Haselkorn T, Peters SP, Meyers DA, et al. Genome-wide association study of asthma identifies RAD50-IL13 and HLA-DR/DQ

regions. J Allergy Clin Immunol [Internet]. 2010 Feb;125(2):328-335.e11. Available from: https://linkinghub.elsevier.com/retrieve/pii/S0091674909017357
42. Oyeniran C, Sturgill JL, Hait NC, Huang W-C, Avni D, Maceyka M, et al. Aberrant ORM (yeast)–like protein isoform 3 (ORMDL3) expression dysregulates ceramide homeostasis in cells and ceramide exacerbates allergic asthma in mice. J Allergy Clin Immunol [Internet]. 2015 Oct;136(4):1035-1046.e6. Available from: https://linkinghub.elsevier.com/retrieve/pii/S0091674915003334
43. Masini E, Giannini L, Nistri S, Cinci L, Mastroianni R, Xu W, et al. Ceramide: a Key Signaling Molecule in a Guinea Pig Model of Allergic Asthmatic Response and Airway Inflammation. J Pharmacol Exp Ther [Internet]. 2008 Feb;324(2):548–57. Available from: http://jpet.aspetjournals.org/lookup/doi/10.1124/jpet.107.131565
44. Petrache I, Kamocki K, Poirier C, Pewzner-Jung Y, Laviad EL, Schweitzer KS, et al. Ceramide Synthases Expression and Role of Ceramide Synthase-2 in the Lung: Insight from Human Lung Cells and Mouse Models. Chu HW, editor. PLoS One [Internet]. 2013 May 14;8(5):e62968. Available from: https://dx.plos.org/10.1371/journal.pone.0062968
45. Spassieva SD, Mullen TD, Townsend DM, Obeid LM. Disruption of ceramide synthesis by CerS2 down-regulation leads to autophagy and the unfolded protein response. Biochem J [Internet]. 2009 Dec 1;424(2):273–83. Available from: https://portlandpress.com/biochemj/article/424/2/273/79924/Disruption-of-ceramide-synthesis-by-CerS2-down
46. Epstein S, Kirkpatrick CL, Castillon GA, Muñiz M, Riezman I, David FPA, et al. Activation of the unfolded protein response pathway causes ceramide accumulation in yeast and INS-1E insulinoma cells. J Lipid Res [Internet]. 2012 Mar;53(3):412–20. Available from: https://linkinghub.elsevier.com/retrieve/pii/S0022227520413562
47. Choi S, Snider JM, Olakkengil N, Lambert JM, Anderson AK, Ross-Evans JS, et al. Myristate-induced endoplasmic reticulum stress requires ceramide synthases 5/6 and generation of C14-ceramide in intestinal epithelial cells. FASEB J [Internet]. 2018 Oct 16;32(10):5724–36. Available from: https://onlinelibrary.wiley.com/doi/abs/10.1096/fj.201800141R
48. Ha SG, Ge XN, Bahaie NS, Kang BN, Rao A, Rao SP, et al. ORMDL3 promotes eosinophil trafficking and activation via regulation of integrins and CD48. Nat Commun [Internet]. 2013 Dec 23;4(1):2479. Available from: http://www.nature.com/articles/ncomms3479
49. Miller M, Tam AB, Mueller JL, Rosenthal P, Beppu A, Gordillo R, et al. Cutting Edge: Targeting Epithelial ORMDL3 Increases, Rather than Reduces, Airway Responsiveness and Is Associated with Increased Sphingosine-1-Phosphate. J Immunol [Internet]. 2017 Apr 15;198(8):3017–22. Available from: http://www.jimmunol.org/lookup/doi/10.4049/jimmunol.1601848
50. Bugajev V, Halova I, Draberova L, Bambouskova M, Potuckova L, Draberova H, et al. Negative regulatory roles of ORMDL3 in the FcεRI-triggered expression of

proinflammatory mediators and chemotactic response in murine mast cells. Cell Mol Life Sci [Internet]. 2016 Mar 25;73(6):1265–85. Available from: http://link.springer.com/10.1007/s00018-015-2047-3

51. Mencarelli C, Martinez–Martinez P. Ceramide function in the brain: when a slight tilt is enough. Cell Mol Life Sci [Internet]. 2013 Jan 24;70(2):181–203. Available from: http://link.springer.com/10.1007/s00018-012-1038-x
52. Kornhuber J, Gulbins E. New Molecular Targets for Antidepressant Drugs. Pharmaceuticals [Internet]. 2021 Sep 2;14(9):894. Available from: https://www.mdpi.com/1424-8247/14/9/894
53. Gulbins E, Palmada M, Reichel M, Lüth A, Böhmer C, Amato D, et al. Acid sphingomyelinase–ceramide system mediates effects of antidepressant drugs. Nat Med [Internet]. 2013 Jul 16;19(7):934–8. Available from: http://www.nature.com/articles/nm.3214
54. Chen B, Zhao L, Li X, Ji Y-S, Li N, Xu X-F, et al. Syntaxin 8 Modulates the Post-synthetic Trafficking of the TrkA Receptor and Inflammatory Pain Transmission*. J Biol Chem [Internet]. 2014 Jul;289(28):19556–69. Available from: https://linkinghub.elsevier.com/retrieve/pii/S0021925820477208
55. Piedimonte G. Contribution of neuroimmune mechanisms to airway inflammation and remodeling during and after respiratory syncytial virus infection. Pediatr Infect Dis J [Internet]. 2003 Feb;22(Supplement):S66–75. Available from: http://journals.lww.com/00006454-200302001-00010
56. Prakash Y, Thompson MA, Meuchel L, Pabelick CM, Mantilla CB, Zaidi S, et al. Neurotrophins in lung health and disease. Expert Rev Respir Med [Internet]. 2010 Jun 9;4(3):395–411. Available from: http://www.tandfonline.com/doi/full/10.1586/ers.10.29
57. Prakash YS, Thompson MA, Pabelick CM. Brain-Derived Neurotrophic Factor in TNF-α Modulation of Ca 2+ in Human Airway Smooth Muscle. Am J Respir Cell Mol Biol [Internet]. 2009 Nov;41(5):603–11. Available from: http://www.atsjournals.org/doi/abs/10.1165/rcmb.2008-0151OC
58. Micera A, Vigneti E, Pickholtz D, Reich R, Pappo O, Bonini S, et al. Nerve growth factor displays stimulatory effects on human skin and lung fibroblasts, demonstrating a direct role for this factor in tissue repair. Proc Natl Acad Sci [Internet]. 2001 May 22;98(11):6162–7. Available from: https://pnas.org/doi/full/10.1073/pnas.101130898
59. Feng D-Y, Guo B-L, Liu G-H, Xu K, Yang J, Tao K, et al. Nerve growth factor against PTSD symptoms: Preventing the impaired hippocampal cytoarchitectures. Prog Neurobiol [Internet]. 2020 Jan;184:101721. Available from: https://linkinghub.elsevier.com/retrieve/pii/S0301008219303399
60. Bilan F, Thoreau V, Nacfer M, Dérand R, Norez C, Cantereau A, et al. Syntaxin 8 impairs trafficking of cystic fibrosis transmembrane conductance regulator (CFTR) and inhibits its channel activity. J Cell Sci [Internet]. 2004 Apr 15;117(10):1923–35. Available from:

https://journals.biologists.com/jcs/article/117/10/1923/27738/Syntaxin-8-impairs-trafficking-of-cystic-fibrosis
61. Dahl M, Tybærg-Hansen A, Lange P, Nordestgaard BG. ΔF508 heterozygosity in cystic fibrosis and susceptibility to asthma. Lancet [Internet]. 1998 Jun;351(9120):1911–3. Available from: https://linkinghub.elsevier.com/retrieve/pii/S0140673697114192
62. Dahl M, Nordestgaard BG, Lange P, Tybjaerg-Hansen A. Fifteen-year follow-up of pulmonary function in individuals heterozygous for the cystic fibrosis phenylalanine-508 deletion. J Allergy Clin Immunol [Internet]. 2001 May;107(5):818–23. Available from: https://linkinghub.elsevier.com/retrieve/pii/S0091674901141187
63. Douros K, Loukou I, Doudounakis S, Tzetis M, Priftis KN, Kanavakis E. Asthma and pulmonary function abnormalities in heterozygotes for cystic fibrosis transmembrane regulator gene mutations. Int J Clin Exp Med [Internet]. 2008;1(4):345–9. Available from: http://www.ncbi.nlm.nih.gov/pubmed/19079680
64. Reznikov LR. Cystic Fibrosis and the Nervous System. Chest [Internet]. 2017 May;151(5):1147–55. Available from: https://linkinghub.elsevier.com/retrieve/pii/S0012369216623565
65. Lavelin I, Geiger B. Characterization of a Novel GTPase-activating Protein Associated with Focal Adhesions and the Actin Cytoskeleton. J Biol Chem [Internet]. 2005 Feb;280(8):7178–85. Available from: https://linkinghub.elsevier.com/retrieve/pii/S002192581962836X
66. Chiba Y, Ueno A, Shinozaki K, Takeyama H, Nakazawa S, Sakai H, et al. Involvement of RhoA-mediated Ca2+ sensitization in antigen-induced bronchial smooth muscle hyperresponsiveness in mice. Respir Res [Internet]. 2005 Dec 8;6(1):4. Available from: http://respiratory-research.biomedcentral.com/articles/10.1186/1465-9921-6-4
67. Diana G, Valentini G, Travaglione S, Falzano L, Pieri M, Zona C, et al. Enhancement of learning and memory after activation of cerebral Rho GTPases. Proc Natl Acad Sci [Internet]. 2007 Jan 9;104(2):636–41. Available from: https://pnas.org/doi/full/10.1073/pnas.0610059104
68. den Hollander P, Rawls K, Tsimelzon A, Shepherd J, Mazumdar A, Hill J, et al. Phosphatase PTP4A3 Promotes Triple-Negative Breast Cancer Growth and Predicts Poor Patient Survival. Cancer Res [Internet]. 2016 Apr 1;76(7):1942–53. Available from: http://cancerres.aacrjournals.org/lookup/doi/10.1158/0008-5472.CAN-14-0673
69. Pei J, Grishin N V. Unexpected diversity in Shisa-like proteins suggests the importance of their roles as transmembrane adaptors. Cell Signal [Internet]. 2012 Mar 1;24(3):758–69. Available from: http://www.ncbi.nlm.nih.gov/pubmed/26921331
70. Oláh J, Lehotzky A, Szunyogh S, Szénási T, Orosz F, Ovádi J. Microtubule-Associated Proteins with Regulatory Functions by Day and Pathological Potency

at Night. Cells [Internet]. 2020 Feb 4;9(2):357. Available from: https://www.mdpi.com/2073-4409/9/2/357
71. Bigler J, Boedigheimer M, Schofield JPR, Skipp PJ, Corfield J, Rowe A, et al. A Severe Asthma Disease Signature from Gene Expression Profiling of Peripheral Blood from U-BIOPRED Cohorts. Am J Respir Crit Care Med [Internet]. 2017 May 15;195(10):1311–20. Available from: http://www.atsjournals.org/doi/10.1164/rccm.201604-0866OC
72. Rusch HL, Robinson J, Yun S, Osier ND, Martin C, Brewin CR, et al. Gene expression differences in PTSD are uniquely related to the intrusion symptom cluster: A transcriptome-wide analysis in military service members. Brain Behav Immun [Internet]. 2019 Aug;80:904–8. Available from: https://linkinghub.elsevier.com/retrieve/pii/S0889159119301540
73. Kuan P-F, Waszczuk MA, Kotov R, Clouston S, Yang X, Singh PK, et al. Gene expression associated with PTSD in World Trade Center responders: An RNA sequencing study. Transl Psychiatry [Internet]. 2017 Dec 18;7(12):1297. Available from: http://www.nature.com/articles/s41398-017-0050-1
74. Ioannidis I, McNally B, Willette M, Peeples ME, Chaussabel D, Durbin JE, et al. Plasticity and Virus Specificity of the Airway Epithelial Cell Immune Response during Respiratory Virus Infection. J Virol [Internet]. 2012 May 15;86(10):5422–36. Available from: https://journals.asm.org/doi/10.1128/JVI.06757-11
75. Franscina Pinto E, Andrade C. Interferon-Related Depression: A Primer on Mechanisms, Treatment, and Prevention of a Common Clinical Problem. Curr Neuropharmacol [Internet]. 2016 Aug 26;14(7):743–8. Available from: http://www.eurekaselect.com/openurl/content.php?genre=article&issn=1570-159X&volume=14&issue=7&spage=743
76. Zheng L-S, Hitoshi S, Kaneko N, Takao K, Miyakawa T, Tanaka Y, et al. Mechanisms for Interferon-α-Induced Depression and Neural Stem Cell Dysfunction. Stem Cell Reports [Internet]. 2014 Jul;3(1):73–84. Available from: https://linkinghub.elsevier.com/retrieve/pii/S2213671114001568
77. Rich HE, Antos D, Melton NR, Alcorn JF, Manni ML. Insights Into Type I and III Interferons in Asthma and Exacerbations. Front Immunol [Internet]. 2020;11:574027. Available from: http://www.ncbi.nlm.nih.gov/pubmed/33101299
78. Gomez JL, Diaz MP, Nino G, Britto CJ. Impaired type I interferon regulation in the blood transcriptome of recurrent asthma exacerbations. BMC Med Genomics [Internet]. 2018 Dec 27;11(1):21. Available from: https://bmcmedgenomics.biomedcentral.com/articles/10.1186/s12920-018-0340-3
79. Abbas AR, Baldwin D, Ma Y, Ouyang W, Gurney A, Martin F, et al. Immune response in silico (IRIS): immune-specific genes identified from a compendium of microarray expression data. Genes Immun [Internet]. 2005 Jun 1;6(4):319–31. Available from: https://www.nature.com/articles/6364173
80. Atli A, Demir S, Bulut M, Okan İbiloğlu A, Güneş M, Kaya M, et al. Neutrophil–lymphocyte ratio in patients with major depressive disorder undergoing no pharmacological therapy. Neuropsychiatr Dis Treat

[Internet]. 2015 Aug;2253. Available from: http://www.dovepress.com/neutrophilndashlymphocyte-ratio-in-patients-with-major-depressive-diso-peer-reviewed-article-NDT
81. Gao H, Ying S, Dai Y. Pathological Roles of Neutrophil-Mediated Inflammation in Asthma and Its Potential for Therapy as a Target. J Immunol Res [Internet]. 2017;2017:1–12. Available from: https://www.hindawi.com/journals/jir/2017/3743048/
82. Ahmetspahic D, Schwarte K, Ambrée O, Bürger C, Falcone V, Seiler K, et al. Altered B Cell Homeostasis in Patients with Major Depressive Disorder and Normalization of CD5 Surface Expression on Regulatory B Cells in Treatment Responders. J Neuroimmune Pharmacol [Internet]. 2018 Mar 13;13(1):90–9. Available from: http://link.springer.com/10.1007/s11481-017-9763-4
83. Habener A, Happle C, Grychtol R, Skuljec J, Busse M, Dalüge K, et al. Regulatory B cells control airway hyperreactivity and lung remodeling in a murine asthma model. J Allergy Clin Immunol [Internet]. 2021 Jun;147(6):2281-2294.e7. Available from: https://linkinghub.elsevier.com/retrieve/pii/S0091674920316341
84. Ghosh S, Hoselton SA, Asbach S V, Steffan BN, Wanjara SB, Dorsam GP, et al. B lymphocytes regulate airway granulocytic inflammation and cytokine production in a murine model of fungal allergic asthma. Cell Mol Immunol [Internet]. 2015 Mar 3;12(2):202–12. Available from: http://www.nature.com/articles/cmi2014103
85. De Vooght V, Carlier V, Devos FC, Haenen S, Verbeken E, Nemery B, et al. B-lymphocytes as Key Players in Chemical-Induced Asthma. Shiku H, editor. PLoS One [Internet]. 2013 Dec 13;8(12):e83228. Available from: https://dx.plos.org/10.1371/journal.pone.0083228
86. Alvarez-Mon MA, Gómez AM, Orozco A, Lahera G, Sosa MD, Diaz D, et al. Abnormal Distribution and Function of Circulating Monocytes and Enhanced Bacterial Translocation in Major Depressive Disorder. Front Psychiatry [Internet]. 2019 Nov 15;10. Available from: https://www.frontiersin.org/article/10.3389/fpsyt.2019.00812/full
87. Seidel A, Arolt V, Hunstiger M, Rink L, Behnisch A, Kirchner H. Major depressive disorder is associated with elevated monocyte counts. Acta Psychiatr Scand [Internet]. 1996 Sep;94(3):198–204. Available from: https://onlinelibrary.wiley.com/doi/10.1111/j.1600-0447.1996.tb09849.x
88. Niessen NM, Baines KJ, Simpson JL, Scott HA, Qin L, Gibson PG, et al. Neutrophilic asthma features increased airway classical monocytes. Clin Exp Allergy [Internet]. 2021 Feb;51(2):305–17. Available from: https://onlinelibrary.wiley.com/doi/10.1111/cea.13811
89. Tanizaki Y, Hosokawa M, Goda Y, Akagi K, Takeyama H, Kimura I. Numerical changes in blood monocytes in bronchial asthma. Acta Med Okayama [Internet]. 1982 Oct;36(5):341–8. Available from: http://www.ncbi.nlm.nih.gov/pubmed/7180570
90. DeBaun MR, Strunk RC. The intersection between asthma and acute chest syndrome in children with sickle-cell anaemia. Lancet [Internet]. 2016

Jun;387(10037):2545–53. Available from: https://linkinghub.elsevier.com/retrieve/pii/S0140673616001458

91. Jison ML, Munson PJ, Barb JJ, Suffredini AF, Talwar S, Logun C, et al. Blood mononuclear cell gene expression profiles characterize the oxidant, hemolytic, and inflammatory stress of sickle cell disease. Blood [Internet]. 2004 Jul 1;104(1):270–80. Available from: https://ashpublications.org/blood/article/104/1/270/114489/Blood-mononuclear-cell-gene-expression-profiles
92. Liu, S., Huang, Z., Deng, X., Zou, X., Li, H., Mu, S., & Cao, B. (2021). Identification of key candidate biomarkers for severe influenza infection by integrated bioinformatical analysis and initial clinical validation. *Journal of cellular and molecular medicine*, *25*(3), 1725–1738. https://doi.org/10.1111/jcmm.16275
93. Yang, Z., Ni, J., Kuang, L., Gao, Y., & Tao, S. (2020). Identification of genes and pathways associated with subchondral bone in osteoarthritis via bioinformatic analysis. *Medicine*, *99*(37), e22142. https://doi.org/10.1097/MD.0000000000022142
94. Sinha, P., & Calfee, C. S. (2019). Peeking under the Hood of Acute Respiratory Distress Syndrome Phenotypes: Deeper Insights into Biological Heterogeneity. *American journal of respiratory and critical care medicine*, *200*(1), 4–6. https://doi.org/10.1164/rccm.201901-0195ED
95. Gong, F., Li, R., Zheng, X., Chen, W., Zheng, Y., Yang, Z., Chen, Y., Qu, H., Mao, E., & Chen, E. (2021). OLFM4 Regulates Lung Epithelial Cell Function in Sepsis-Associated ARDS/ALI via LDHA-Mediated NF-κB Signaling. *Journal of inflammation research*, *14*, 7035–7051. https://doi.org/10.2147/JIR.S335915
96. Zhang, N., Aiyasiding, X., Li, W. J., Liao, H. H., & Tang, Q. Z. (2022). Neutrophil degranulation and myocardial infarction. *Cell communication and signaling : CCS*, *20*(1), 50. https://doi.org/10.1186/s12964-022-00824-4
97. Robledo, S., Idol, R. A., Crimmins, D. L., Ladenson, J. H., Mason, P. J., & Bessler, M. (2008). The role of human ribosomal proteins in the maturation of rRNA and ribosome production. *RNA (New York, N.Y.)*, *14*(9), 1918–1929. https://doi.org/10.1261/rna.1132008
98. Gan, S. J., Ye, B., Qian, S. X., Zhang, C., Mao, J. Q., Li, K., & Tang, J. D. (2015). Immune- and ribosome-related genes were associated with systemic vasculitis. *Scandinavian journal of immunology*, *81*(2), 96–101. https://doi.org/10.1111/sji.12252
99. Quarello, P., Garelli, E., Carando, A., Brusco, A., Calabrese, R., Dufour, C., Longoni, D., Misuraca, A., Vinti, L., Aspesi, A., Biondini, L., Loreni, F., Dianzani, I., & Ramenghi, U. (2010). Diamond-Blackfan anemia: genotype-phenotype correlations in Italian patients with RPL5 and RPL11 mutations. *Haematologica*, *95*(2), 206–213. https://doi.org/10.3324/haematol.2009.011783

100. Konno, Y., Toki, T., Tandai, S., Xu, G., Wang, R., Terui, K., Ohga, S., Hara, T., Hama, A., Kojima, S., Hasegawa, D., Kosaka, Y., Yanagisawa, R., Koike, K., Kanai, R., Imai, T., Hongo, T., Park, M. J., Sugita, K., & Ito, E. (2010). Mutations in the ribosomal protein genes in Japanese patients with Diamond-Blackfan anemia. *Haematologica*, *95*(8), 1293–1299. https://doi.org/10.3324/haematol.2009.020826
101. Ahamad, J., Ojha, S., Srivastava, A., Bhattacharya, A., & Bhattacharya, S. (2015). Post-transcriptional regulation of ribosomal protein genes during serum starvation in Entamoeba histolytica. *Molecular and biochemical parasitology*, *201*(2), 146–152. https://doi.org/10.1016/j.molbiopara.2015.07.006
102. Kewalramani, A., Bollinger, M. E., & Postolache, T. T. (2008). Asthma and Mood Disorders. *International journal of child health and human development : IJCHD*, *1*(2), 115–123.
103. O'Toole BI, Catts S V. Trauma, PTSD, and physical health: An epidemiological study of Australian Vietnam veterans. J Psychosom Res [Internet]. 2008 Jan;64(1):33–40. Available from: https://linkinghub.elsevier.com/retrieve/pii/S0022399907002899
104. Allgire E, McAlees JW, Lewkowich IP, Sah R. Asthma and posttraumatic stress disorder (PTSD): Emerging links, potential models and mechanisms. Brain Behav Immun [Internet]. 2021 Oct;97:275–85. Available from: https://linkinghub.elsevier.com/retrieve/pii/S0889159121002324
105. Leday GGR, Vértes PE, Richardson S, Greene JR, Regan T, Khan S, et al. Replicable and Coupled Changes in Innate and Adaptive Immune Gene Expression in Two Case-Control Studies of Blood Microarrays in Major Depressive Disorder. Biol Psychiatry [Internet]. 2018 Jan;83(1):70–80. Available from: https://linkinghub.elsevier.com/retrieve/pii/S0006322317316177
106. Ritchie ME, Phipson B, Wu D, Hu Y, Law CW, Shi W, et al. limma powers differential expression analyses for RNA-sequencing and microarray studies. Nucleic Acids Res [Internet]. 2015 Apr 20;43(7):e47–e47. Available from: http://academic.oup.com/nar/article/43/7/e47/2414268/limma-powers-differential-expression-analyses-for
107. Spijker S, Van Zanten JS, De Jong S, Penninx BWJH, van Dyck R, Zitman FG, et al. Stimulated Gene Expression Profiles as a Blood Marker of Major Depressive Disorder. Biol Psychiatry [Internet]. 2010 Jul;68(2):179–86. Available from: https://linkinghub.elsevier.com/retrieve/pii/S0006322310002465
108. Wilhelm BT, Marguerat S, Watt S, Schubert F, Wood V, Goodhead I, et al. Dynamic repertoire of a eukaryotic transcriptome surveyed at single-nucleotide resolution. Nature [Internet]. 2008 Jun 18;453(7199):1239–43. Available from: http://www.nature.com/articles/nature07002
109. Xu X, Zhang Y, Williams J, Antoniou E, McCombie WR, Wu S, et al. Parallel comparison of Illumina RNA-Seq and Affymetrix microarray platforms on transcriptomic profiles generated from 5-aza-deoxy-cytidine treated HT-29 colon cancer cells and simulated datasets. BMC Bioinformatics [Internet]. 2013 Jun

28;14(S9):S1. Available from: https://bmcbioinformatics.biomedcentral.com/articles/10.1186/1471-2105-14-S9-S1

110. Robinson MD, McCarthy DJ, Smyth GK. edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. Bioinformatics [Internet]. 2010 Jan 1;26(1):139–40. Available from: https://academic.oup.com/bioinformatics/article-lookup/doi/10.1093/bioinformatics/btp616
111. Wickham H. ggplot2 [Internet]. New York, NY: Springer New York; 2009. Available from: http://link.springer.com/10.1007/978-0-387-98141-3
112. Chen H, Boutros PC. VennDiagram: a package for the generation of highly-customizable Venn and Euler diagrams in R. BMC Bioinformatics [Internet]. 2011 Dec 26;12(1):35. Available from: https://bmcbioinformatics.biomedcentral.com/articles/10.1186/1471-2105-12-35
113. Luo W, Friedman MS, Shedden K, Hankenson KD, Woolf PJ. GAGE: generally applicable gene set enrichment for pathway analysis. BMC Bioinformatics [Internet]. 2009 Dec 27;10(1):161. Available from: https://bmcbioinformatics.biomedcentral.com/articles/10.1186/1471-2105-10-161
114. Blighe K, Rana S, Lewis M. EnhancedVolcano: Publication-ready volcano plots with enhanced colouring and labeling [Internet]. 2018. Available from: https://github.com/kevinblighe/EnhancedVolcano.

3.7 Supplementary Information:

S1 Table. Pathways commonly differentially regulated between severe and moderate asthma

		Q. Explore S	Q. Validate S	Q. Explore M	Q. Validate M
C2 Up					
	TAKEDA_TARGETS_OF_NUP98_HOXA9_FUSION_10D_DN	3.65E-4	1.06E-3	1.79E-2	1.80E-2
	ALTEMEIER_RESPONSE_TO_LPS_WITH_MECHANICAL_VENTILATION	5.54E-3	4.43E-6	2.08E-4	4.84E-2
	VERHAAK_AML_WITH_NPM1_MUTATED_UP	8.48E-7	1.42E-8	1.36E-6	8.29E-4
	SMIRNOV_CIRCULATING_ENDOTHELIOCYTES_IN_CANCER_UP	1.99E-6	1.72E-3	1.00E-4	1.75E-2
	REN_ALVEOLAR_RHABDOMYOSARCOMA_DN	3.09E-3	3.23E-2	9.36E-3	2.12E-2
	JISON_SICKLE_CELL_DISEASE_UP	1.44E-3	1.15E-6	7.58E-5	1.35E-2
	REACTOME_NEUTROPHIL_DEGRANULATION	7.23E-20	1.20E-13	3.05E-7	1.54E-3
C7 Up					
	GSE10325_CD4_TCELL_VS_MYELOID_DN	2.24E-5	2.24E-4	7.93E-7	1.61E-4
	GSE10325_BCELL_VS_MYELOID_DN	3.83E-12	6.76E-10	1.15E-10	1.34E-3

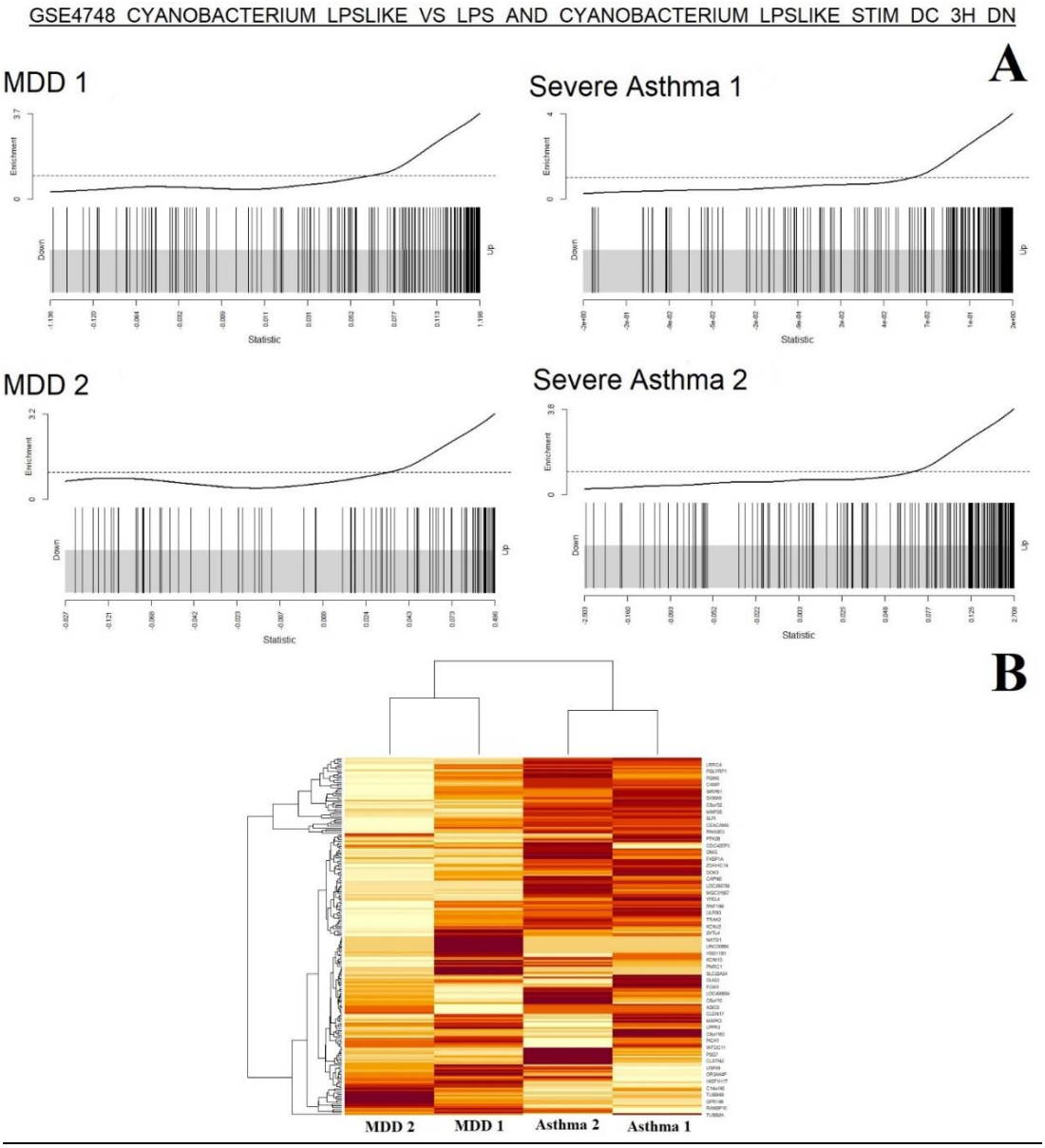
	GSE10325_LUPUS_CD4_TCELL_VS_LUPUS_MYELOID_DN	4.13E-13	1.03E-8	3.27E-16	2.30E-2
	GSE11057_NAIVE_CD4_VS_PBMC_CD4_TCELL_DN	1.98E-4	2.12E-3	1.05E-5	8.84E-5
	GSE11057_CD4_EFF_MEM_VS_PBMC_DN	2.22E-3	4.71E-3	4.06E-5	8.84E-5
	GSE11057_CD4_CENT_MEM_VS_PBMC_DN	8.59E-6	3.92E-2	8.42E-9	3.65E-3
	GSE11057_PBMC_VS_MEM_CD4_TCELL_UP	2.00E-5	6.57E-3	8.51E-11	2.81E-4
	GSE22886_NAIVE_TCELL_VS_DC_DN	1.13E-2	3.63E-2	1.26E-2	2.26E-4
	GSE22886_NAIVE_TCELL_VS_MONOCYTE_DN	4.13E-13	5.98E-10	5.20E-8	6.88E-4
	GSE22886_NAIVE_CD8_TCELL_VS_MONOCYTE_DN	9.19E-12	7.10E-9	8.61E-8	2.50E-3
	GSE22886_NAIVE_CD4_TCELL_VS_MONOCYTE_DN	4.99E-11	5.03E-11	1.35E-5	1.17E-4
	GSE24634_TREG_VS_TCONV_POST_DAY3_IL4_CONVERSION_DN	5.67E-3	9.80E-3	4.45E-3	4.52E-3
	GSE24634_TREG_VS_TCONV_POST_DAY10_IL4_CONVERSION_DN	6.69E-5	1.16E-3	2.71E-5	4.65E-3
	GSE24634_IL4_VS_CTRL_TREATED_NAIVE_CD4_TCELL_DAY10_DN	5.34E-3	1.69E-4	1.59E-4	7.05E-3
	GSE29618_BCELL_VS_MONOCYTE_DN	4.00E-9	1.50E-6	6.46E-6	1.50E-4
	GSE29618_BCELL_VS_MDC_DN	1.67E-3	5.11E-3	2.63E-4	4.29E-3
	GSE29618_MONOCYTE_VS_PDC_	8.75E-12	1.23E-9	2.20E-8	2.27E-3

	UP				
	GSE29618_MONOCYTE_VS_MDC_UP	1.72E-14	6.29E-11	3.69E-8	4.12E-4
	GSE29618_PDC_VS_MDC_DN	6.04E-7	2.72E-6	1.83E-5	1.50E-2
	GSE29618_BCELL_VS_MONOCYTE_DAY7_FLU_VACCINE_DN	4.91E-13	2.42E-7	9.08E-9	1.56E-2
	GSE29618_BCELL_VS_MDC_DAY7_FLU_VACCINE_DN	2.37E-5	1.39E-3	4.42E-4	1.50E-2
	GSE29618_MONOCYTE_VS_PDC_DAY7_FLU_VACCINE_UP	1.72E-14	4.18E-11	1.19E-9	1.82E-2
	GSE29618_MONOCYTE_VS_MDC_DAY7_FLU_VACCINE_UP	2.15E-13	3.46E-10	5.79E-6	2.81E-4
	GSE29618_PDC_VS_MDC_DAY7_FLU_VACCINE_DN	3.32E-6	3.48E-4	2.58E-6	6.48E-3
	GSE3982_EOSINOPHIL_VS_EFF_MEMORY_CD4_TCELL_UP	3.59E-6	1.18E-4	3.00E-5	6.60E-4
	GSE3982_EOSINOPHIL_VS_CENT_MEMORY_CD4_TCELL_UP	6.39E-8	1.51E-5	9.46E-6	2.67E-4
	GSE3982_EOSINOPHIL_VS_NKCELL_UP	3.41E-4	1.18E-4	3.46E-4	6.88E-4
	GSE3982_BASOPHIL_VS_CENT_MEMORY_CD4_TCELL_UP	2.31E-5	4.40E-6	2.46E-2	6.97E-3
	GSE34156_UNTREATED_VS_6H_TLR1_TLR2_LIGAND_TREATED_MONOCYTE_UP	1.15E-7	6.63E-7	5.01E-7	2.50E-3
	GSE34156_UNTREATED_VS_24H_NOD2_LIGAND_TREATED_MONOCYTE_DN	6.95E-6	3.01E-5	8.21E-7	1.90E-3
	SCHERER_PBMC_APSV_WETVA	1.36E-4	3.46E-10	6.67E-9	4.38E-3

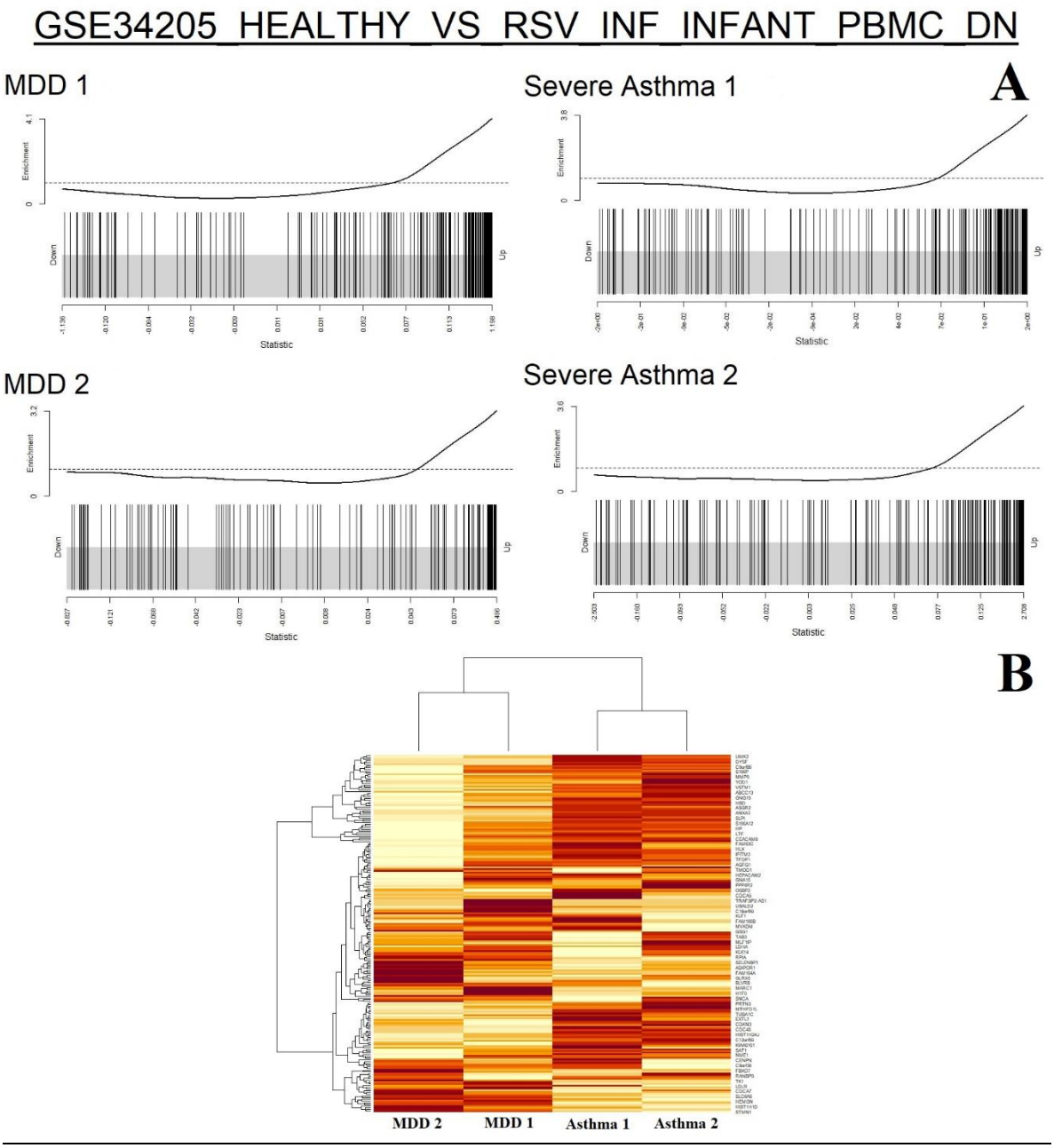
	X_AGE_18_32YO_5_TO_7DY_UP				
	HOWARD_PBMC_INACT_MONOV_INFLUENZA_A_INDONESIA_05_2005_H5N1_AGE_19_39YO_AS03_ADJUVANT_VS_BUFFER_1DY_UP	2.23E-11	4.50E-24	5.33E-17	2.06E-3
	NAKAYA_PBMC_FLUARIX_FLUVIRIN_AGE_18_50YO_CORRELATED_WITH_HAI_28DY_RESPONSE_AT_3DY_POSITIVE	4.10E-15	1.14E-7	1.05E-9	1.03E-2
	GSE22886_NAIVE_TCELL_VS_NEUTROPHIL_DN	1.72E-14	1.24E-12	1.31E-8	4.74E-2
	GSE6269_HEALTHY_VS_STAPH_PNEUMO_INF_PBMC_DN	4.13E-13	1.49E-10	5.32E-6	3.51E-2
	GSE34156_TLR1_TLR2_LIGAND_VS_NOD2_AND_TLR1_TLR2_LIGAND_24H_TREATED_MONOCYTE_UP	9.01E-9	3.16E-8	2.42E-8	6.97E-3
	GSE34156_NOD2_LIGAND_VS_TLR1_TLR2_LIGAND_6H_TREATED_MONOCYTE_DN	6.29E-10	2.20E-7	1.57E-7	3.46E-2
	NAKAYA_PBMC_IMUVAC_MALE_AGE_14_27YO_1D_POSTBOOST_VS_0DY_PREIMM_TIV_UP	1.36E-3	4.56E-4	2.25E-2	2.52E-2
	FLETCHER_PBMC_BCG_10W_INFANT_PPD_STIMULATED_VS_UNSTIMULATED_10W_DN	9.57E-4	5.64E-3	1.85E-3	2.52E-2
	GSE3982_EOSINOPHIL_VS_DC_UP	1.29E-2	1.36E-2	2.99E-2	2.52E-2
	GSE3982_EOSINOPHIL_VS_TH2_UP	2.19E-2	3.79E-3	3.87E-2	7.22E-3
C7					

Down					
	GSE11057_CD4_CENT_MEM_VS_PBMC_UP	3.20E-5	3.24E-12	2.63E-2	1.62E-4
	GSE11057_PBMC_VS_MEM_CD4_TCELL_DN	1.05E-4	1.72E-10	1.27E-3	8.02E-3
	GSE22886_NAIVE_TCELL_VS_MONOCYTE_UP	2.83E-14	1.91E-19	2.00E-4	4.00E-2
	GSE22886_NAIVE_CD8_TCELL_VS_DC_UP	1.54E-3	4.35E-6	3.71E-2	2.22E-2
	GSE22886_NAIVE_CD8_TCELL_VS_MONOCYTE_UP	4.24E-12	7.60E-11	1.68E-4	3.33E-2
	GSE22886_NAIVE_CD4_TCELL_VS_MONOCYTE_UP	2.30E-9	1.95E-11	2.29E-4	2.22E-2

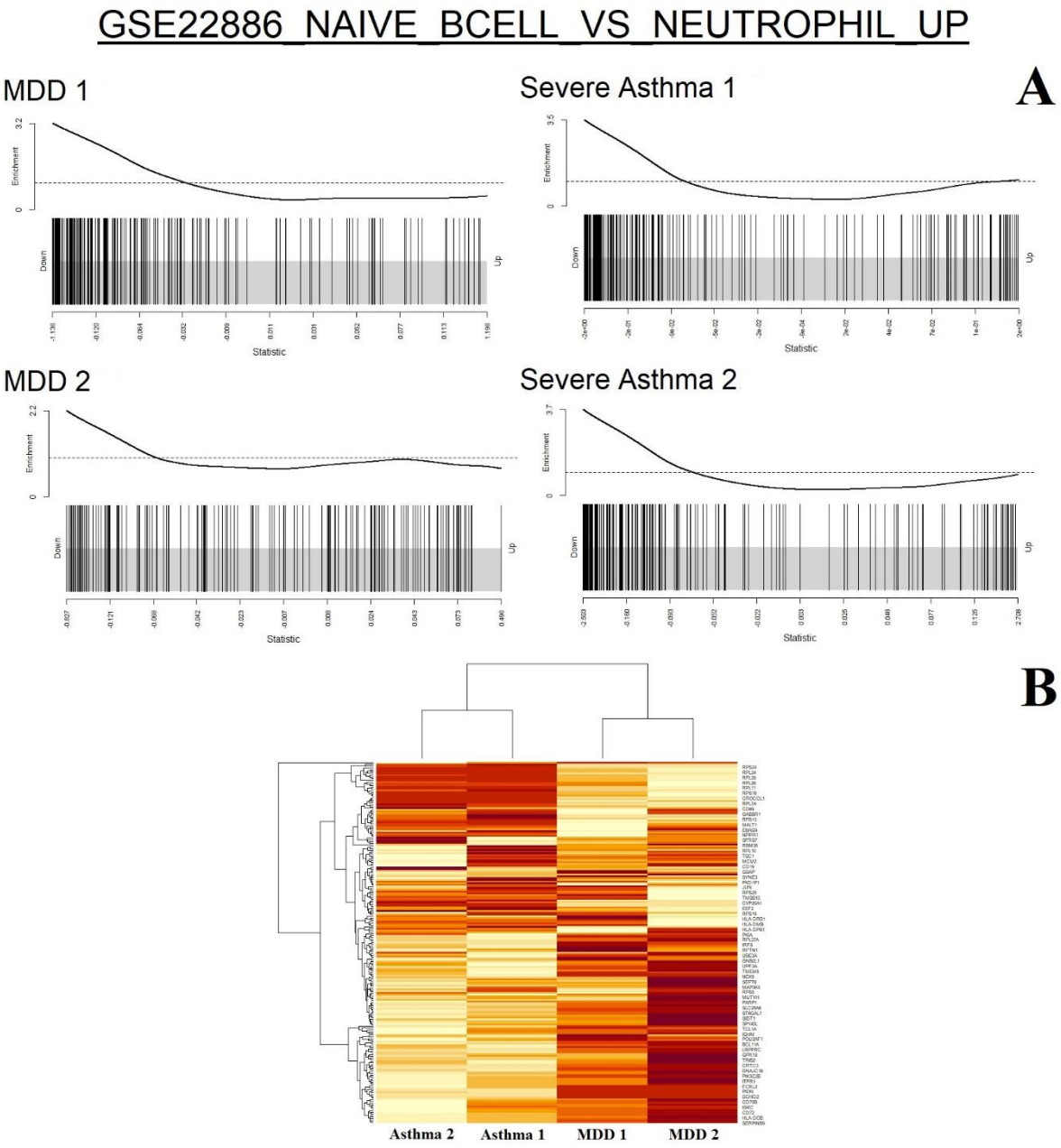
Pathways commonly differentially regulated between severe and moderate asthma and adjusted P (Q) values for: severe asthma (S) and moderate asthma (M) exploration and validation cohorts.



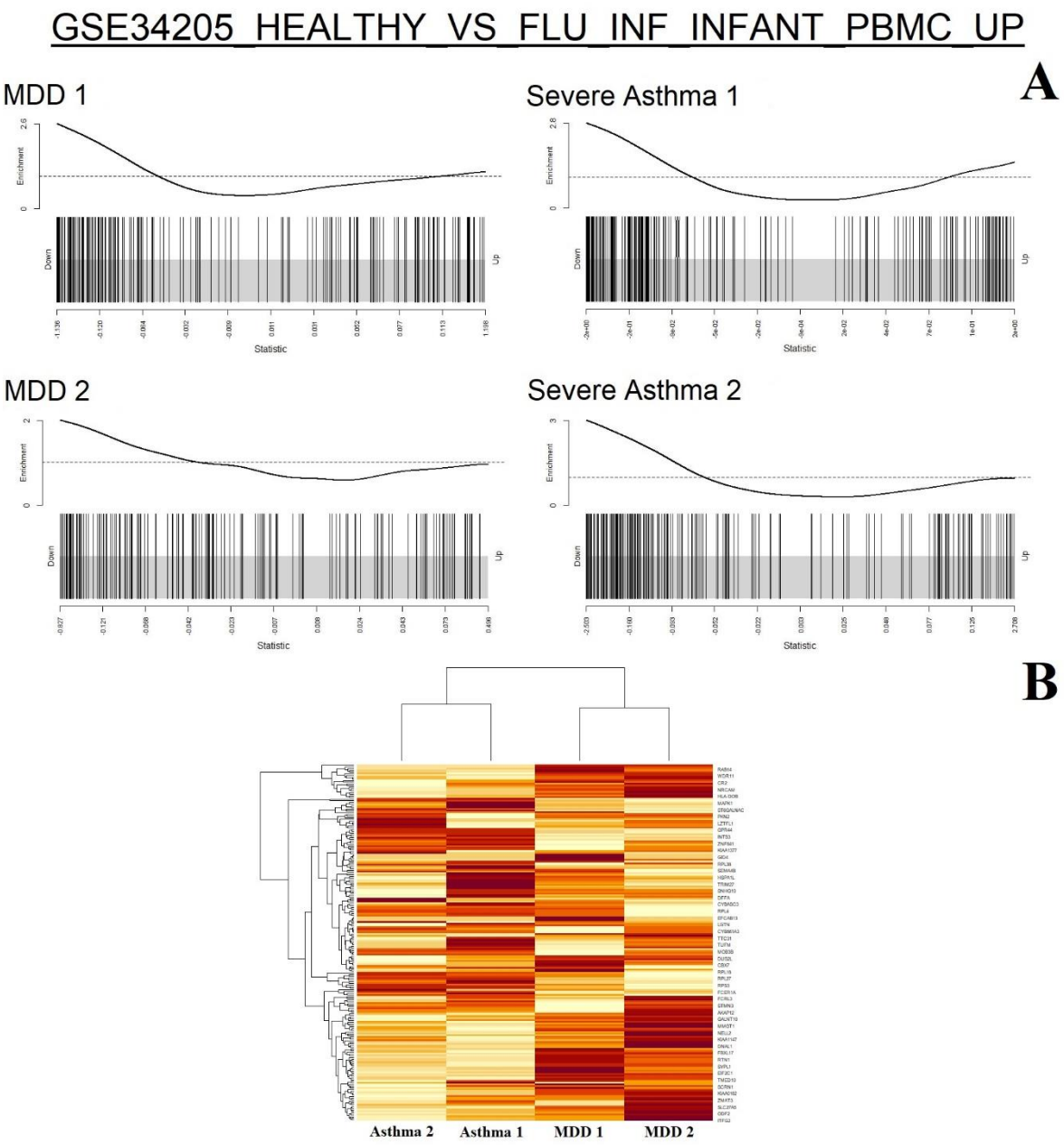
S1 Figure. Validated gene set expression in "GSE4748_CYANOBACTERIUM_LPSLIKE_VS_LPS_AND_CYANOBACTERIUM_LPSLIKE_STIM_DC_3H_DN." A) Barcode plots showing cumulative individual genes in the gene set (as bars) for the exploration and validation datasets of MDD and severe asthma. Clusters of bars on one end represent individual genes that are differentially expressed in one direction or another within the gene set. B) A heatmap of differential expression of individual genes within the set for each of the exploration and validation MDD and severe asthma datasets.



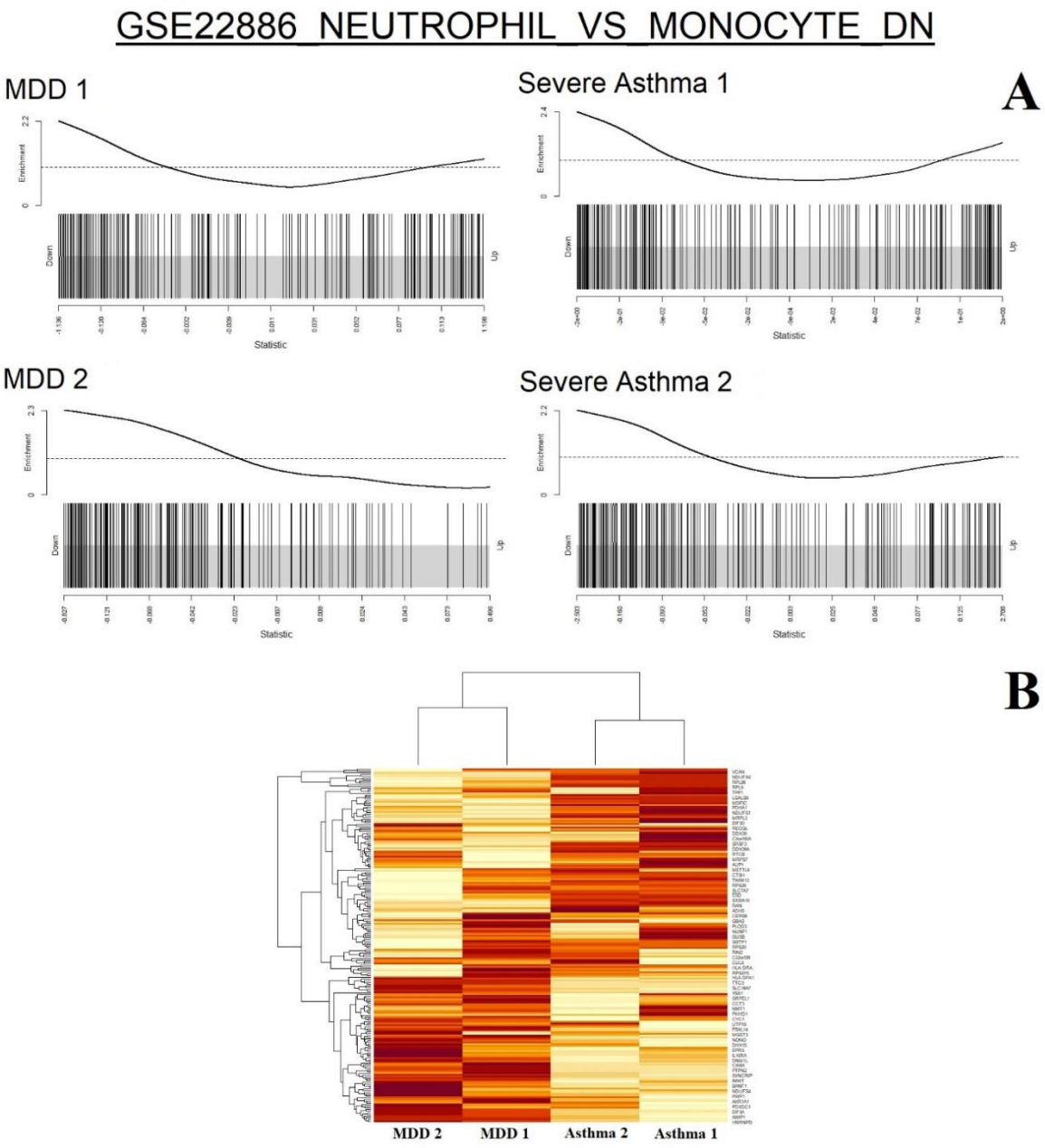
S2 Figure. Validated gene set expression in "GSE34205_HEALTHY_VS_RSV_INF_INFANT_PBMC_DN." A) Barcode plots showing cumulative individual genes in the gene set (as bars) for the exploration and validation datasets of MDD and severe asthma. Clusters of bars on one end represent individual genes that are differentially expressed in one direction or another within the gene set. B) A heatmap of differential expression of individual genes within the set for each of the exploration and validation MDD and severe asthma datasets.



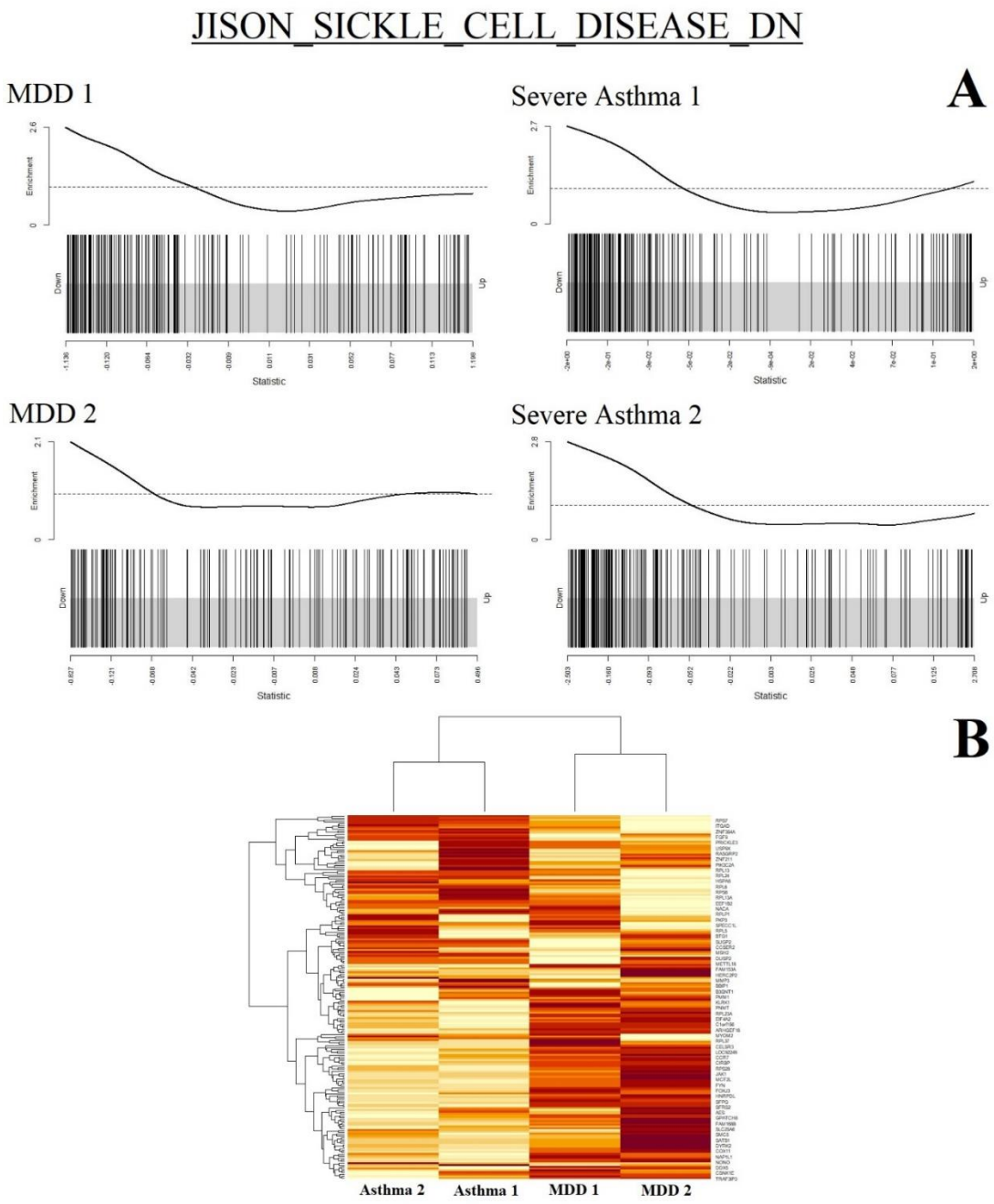
S3 Figure. Validated gene set expression in "GSE22886_NAIVE_BCELL_VS_NEUTROPHIL_UP." A) Barcode plots showing cumulative individual genes in the gene set (as bars) for the exploration and validation datasets of MDD and severe asthma. Clusters of bars on one end represent individual genes that are differentially expressed in one direction or another within the gene set. B) A heatmap of differential expression of individual genes within the set for each of the exploration and validation MDD and severe asthma datasets.



S4 Figure. Validated gene set expression in "GSE34205_HEALTHY_VS_FLU_INF_INFANT_PBMC_UP." A) Barcode plots showing cumulative individual genes in the gene set (as bars) for the exploration and validation datasets of MDD and severe asthma. Clusters of bars on one end represent individual genes that are differentially expressed in one direction or another within the gene set. B) A heatmap of differential expression of individual genes within the set for each of the exploration and validation MDD and severe asthma datasets.



S5 Figure. Validated gene set expression in "GSE22886_NEUTROPHIL_VS_MONOCYTE_DN." A) Barcode plots showing cumulative individual genes in the gene set (as bars) for the exploration and validation datasets of MDD and severe asthma. Clusters of bars on one end represent individual genes that are differentially expressed in one direction or another within the gene set. B) A heatmap of differential expression of individual genes within the set for each of the exploration and validation MDD and severe asthma datasets.



S6 Figure. Validated gene set expression in "JISON_SICKLE_CELL_DISEASE_DN." A) Barcode plots showing cumulative individual genes in the gene set (as bars) for the exploration and validation datasets of MDD and severe asthma. Clusters of bars on one end represent individual genes that are differentially expressed in one direction or another within the gene set. B) A heatmap of differential expression of individual genes within the set for each of the exploration and validation MDD and severe asthma datasets.

Chapter 4: Effects of Two Distinct Psychoactive Microbes, *Lacticaseibacillus rhamnosus* JB-1 and *Limosilactobacillus reuteri* 6475, on Circulating and Hippocampal mRNA in Male Mice

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4.1 Abstract:

Discovery of the microbiota-gut–brain axis has led to proposed microbe-based therapeutic strategies in mental health, including the use of mood-altering bacterial species, termed psychobiotics. However, we still have limited understanding of the key signaling pathways engaged by specific organisms in modulating brain function, and evidence suggests that bacteria with broadly similar neuroactive and immunomodulatory actions can drive different behavioral outcomes. We sought to identify pathways distinguishing two psychoactive bacterial strains that seemingly engage similar gut–brain signaling pathways but have distinct effects on behaviour. We used RNAseq to identify mRNAs differentially expressed in the blood and hippocampus of mice following *Lacticaseibacillus rhamnosus* JB-1, and *Limosilactobacillus reuteri* 6475 treatment and performed Gene Set Enrichment Analysis (GSEA) to identify enrichment in pathway activity. *L. rhamnosus*, but not *L. reuteri* treatment altered several pathways in the blood and hippocampus, and the *rhamnosus* could be clearly distinguished based on mRNA profile. In particular, *L. rhamnosus* treatment modulated the activity of interferon signaling, JAK/STAT, and TNF-alpha via NF-KB pathways. Our results highlight that psychobiotics can induce complex changes in host gene expression, and in understanding these changes, we may help fine-tune selection of psychobiotics for treating mood disorders.

Keywords: depression; stress; gut–brain-axis; JB-1; psychobiotics; mRNA; hippocampus; blood; microbiota

4.2 Introduction

Anxiety and depression are two of the most common mood disorders in the western world becoming increasingly prevalent in millennials and adolescents [1,2]. The COVID-19 pandemic only exacerbated the problem. Among adults in the UK the reported rate of depression symptoms nearly doubled from pre-to-post pandemic (10% to 19%), and in the US it nearly quadrupled (11% to 42%) [3]. Much attention has been given to the gut–brain-axis in recent years as it is beginning to revolutionize our understanding and treatment of mental health disorders [4].

Numerous direct and indirect interactions between bacteria endemic to the gut, and the central nervous system characterize what is known as the microbiota-gut–brain axis [5]. The introduction of certain bacteria to the gut that modulate brain function, termed psychoactive-probiotics or psychobiotics, have been demonstrated to influence behaviour in animal models and mood/anxiety in humans [6,7]. One such potential psychobiotic is *Lacticaseibacillus rhamnosus* JB-1, which has previously been shown to reduce anxiety and depression-like behaviours in mice [8–10].

It is incompletely understood how JB-1 facilitates these cognitive and behavioural changes, although both the peripheral nervous system and the immune system are critical mediators [8,9,11]. Specifically, feeding of JB-1 was only able to alleviate the depression and anxiety-like behaviours of mice when the vagus nerve was intact [9]. Feeding of JB-1 also results in modulation of the immune system and induces regulatory T cells, which have been demonstrated to be both necessary and sufficient to mediate the behavioral effects of the bacteria [8,11]. Other psychobiotics have been suggested to modulate

behaviour and cognition via the endocrine system, the release of soluble metabolites including neurotransmitters into circulation, and the release of bacterial membrane vesicles (MV) carrying similar metabolites and RNAs into circulation [12–15]. Up to this point however these mechanisms have not been demonstrated to be involved in the behavioural changes induced by *L. rhamnosus* JB-1.

Limosilactobacillus reuteri 6475 (LR6475) is a probiotic bacteria that has previously been shown to modulate social behaviours; rescuing autism-spectrum-disorder-like social deficits induced by a maternal high-fat diet in mice [16]. Mechanistically, LR6475 achieves this in a vagus-dependant manner and by boosting oxytocin levels [17,18]. LR6475 has also been shown to have efficacy in treating irritable bowel syndrome and increase bone density via T-lymphocyte regulation [19,20]. However, despite both JB-1 and LR6475 engaging the vagus nerve and regulatory immune responses the bacteria have some distinct actions on behaviour with JB-1, but not LR6475, having antidepressant-like effects in mice [21]. The reasons for the distinct behavioral effects of the bacteria are unclear.

Here, in an attempt to identify potential pathways distinguishing two psychoactive bacterial strains that seemingly engage similar gut–brain signaling pathways but have distinct effects on behaviour, we compare transcriptomic changes in blood and hippocampus, a region of the brain responsible for memory and emotion and closely linked with depression [22–24], following feeding with JB-1 and LR6475.

4.3 Results

4.3.1 Many mRNAs and Gene Sets Are Altered in the Blood of JB-1-Fed Mice, but Not LR6475-Fed:

Principal component analysis (PCA) of normalized, filtered mRNA in the blood of mice shows no distinct groups between PBS and LR6475 fed mice; however, JB-1 fed mice differ greatly from the cluster PBS and LR6475-fed mice form along both PC 1 and 2 (adonis *p*-value = 0.049) (Figure 1A). As the PCA indicates, many genes were found to be differentially expressed when comparing JB-1 to PBS-fed mice (Figure 1B) and a few- when comparing JB-1 to LR6475 (Figure 1D); however, no genes were significantly differentially expressed between LR6475 and PBS-fed mice. FKBP1A was among the genes upregulated in the blood of JB-1-fed mice in both comparisons.

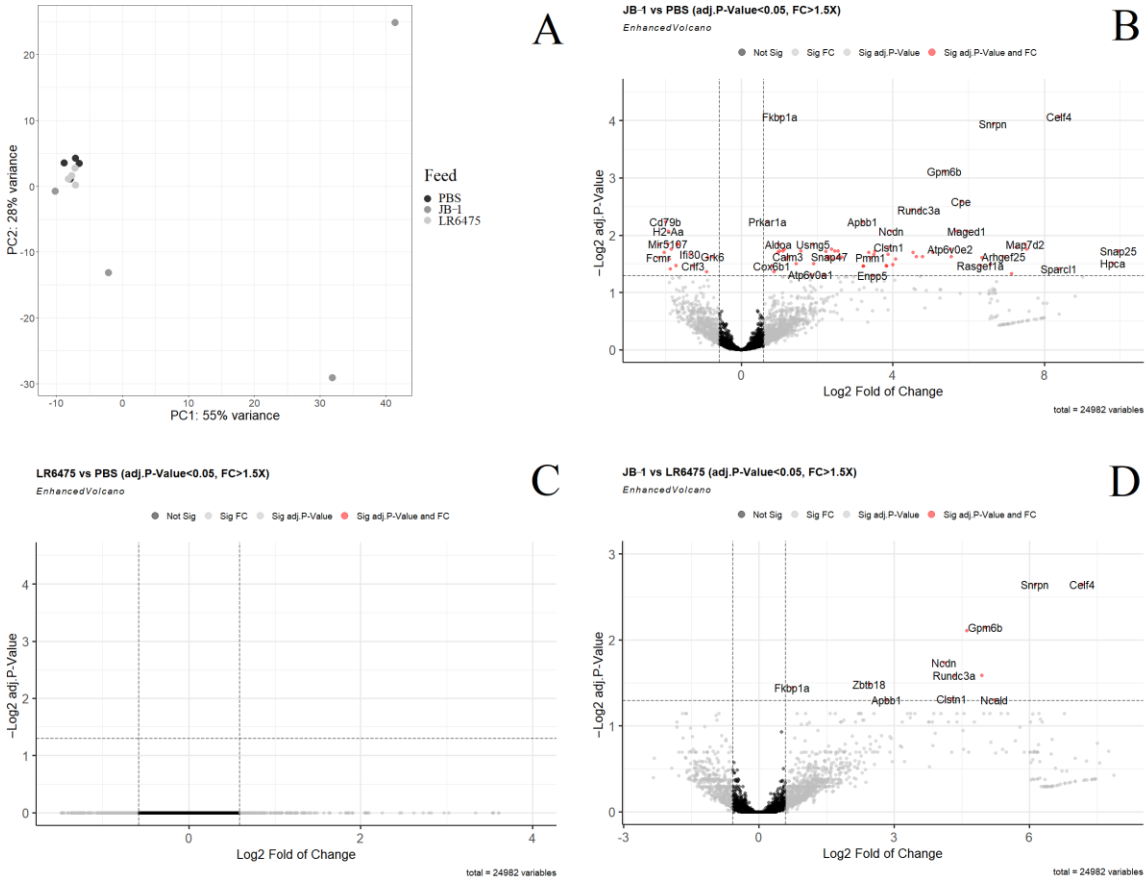

Figure 1. Differential expression analysis of mRNA measured by RNAseq in the blood of mice fed either *Lacticaseibacillus rhamnosus* JB-1, *Limosilactobacillus reuteri* 6475, or PBS. (**A**) Principal component analysis of all three treatment groups. (**B**) Volcano plot showing individual differentially expressed genes in a comparison of JB-1 vs. PBS-fed mice. (**C**) Volcano plot showing individual differentially expressed genes in a comparison of LR6475 vs. PBS-fed mice. (**D**) Volcano plot showing individual differentially expressed genes in a comparison of JB-1 vs. LR6475-fed mice. Genes with a positive log fold change are more highly expressed.

To elucidate additional sources of grouping along PC1 and 2 in the blood, Gene Set Enrichment Analysis was performed, comparing KEGG and Hallmark gene set expression in JB-1-fed mice to LR6475 and to PBS-fed independently. LR6475 and PBS-fed mice were not compared as their groups were not distinct in PCA. Many pathways from both KEGG and Hallmark were found differentially expressed in both directions, in both JB-1 comparisons, and are summarized in (Table 1). All of the pathways enriched in JB-1 vs.

PBS were common to the JB-1 vs. LR6475 comparison, with the exception of KEGG_LEUKOCYTE_TRANSENDOTHELIAL_MIGRATION, and KEGG_NATURAL_KILLER_CELL_MEDIATED_CYTOTOXICITY which were enriched in PBS compared to JB-1 but did not appear significant in the JB-1 vs. LR6475 comparison. 1 Hallmark pathway and 4 KEGG pathways were commonly enriched in JB-1 for both comparisons, and 6 Hallmark and 8 KEGG pathways were commonly enriched in LR6475, and PBS compared to JB-1. Mouse gene names converted to human orthologs were used to visualize KEGG pathways in pathview - these figures were created for the blood mRNA JB-1 vs. PBS comparison and can be found in the supplementary figures (Supplementary Figure S1–S18).

Table 1. Enriched pathways in JB-1 compared with PBS and compared with LR6475. The left column shows the relative direction of expression in JB-1 for each gene set and the right column shows the adjusted *p*-Value for each. Both JB-1 comparisons had nearly identical pathways and corresponding directions of expression, except for the last two entries on the table, which were significant only in the JB-1 vs. PBS comparison.

Enriched In	Pathway Name	Adj.*p*-Val (JvC, JvR)
JB-1	HALLMARK_OXIDATIVE_PHOSPHORYLATION	$3.4 \times 10\text{-}^{4}$, 3.4×10^{-4}
JB-1	KEGG_OXIDATIVE_PHOSPHORYLATION	3.7×10^{-3}, 9.9×10^{-3}
JB-1	KEGG_PARKINSONS_DISEASE	1.3×10^{-2}, 2.3×10^{-2}
JB-1	KEGG_HUNTINGTONS_DISEASE	1.3×10^{-2}, 2.3×10^{-2}
JB-1	KEGG_ALZHEIMERS_DISEASE	2.1×10^{-2}, 3.7×10^{-2}
PBS	HALLMARK_ALLOGRAFT_REJECTION	1.5×10^{-6}, 2.7×10^{-6}
PBS	HALLMARK_INTERFERON_GAMMA_RESPONSE	5.4×10^{-6}, 8.9×10^{-6}

PBS	HALLMARK_INFLAMMATORY_RESPONSE	4.6×10^{-3}, 9.4×10^{-3}
PBS	HALLMARK_INTERFERON_ALPHA_RESPONSE	7.7×10^{-3}, 8.3×10^{-3}
PBS	HALLMARK_TNFA_SIGNALLING_VIA_NFKB	3.2×10^{-2}, 3.1×10^{-2}
PBS	HALLMARK_IL6_JAK_STAT3_SIGNALLING	3.2×10^{-2}, 2.5×10^{-2}
PBS	KEGG_PRIMARY_IMMUNODEFICIENCY	1.5×10^{-3}, 3.6×10^{-3}
PBS	KEGG_CYTOKINE_CYTOKINE_RECEPTOR_INTERACTION	4.9×10^{-3}, 9.2×10^{-3}
PBS	KEGG_JAK_STAT_SIGNALLING_PATHWAY	4.9×10^{-3}, 8.0×10^{-3}
PBS	KEGG_CHEMOKINE_SIGNALLING_PATHWAY	1.8×10^{-2}, 1.0×10^{-2}
PBS	KEGG_HEMATOPOIETIC_CELL_LINEAGE	1.9×10^{-2}, 1.0×10^{-2}
PBS	KEGG_B_CELL_RECEPTOR_SIGNALLING_PATHWAY	1.9×10^{-2}, 2.8×10^{-2}
PBS	KEGG_RIBOSOME	2.1×10^{-2}, 6.7×10^{-3}
PBS	KEGG_T_CELL_RECEPTOR_SIGNALLING_PATHWAY	2.2×10^{-2}, 2.8×10^{-2}
PBS	KEGG_LEUKOCYTE_TRANSENDOTHELIAL_MIGRATION	3.8×10^{-2}
PBS	KEGG_NATURAL_KILLER_CELL_MEDIATED_CYTOTOXICITY	4.5×10^{-2}

4.3.2 Few mRNAs and Gene Sets Are Altered in the Hippocampus of Psychobiotic-Fed Mice:

In the principal component analysis of normalized, filtered mRNA in the hippocampus of mice, JB-1 and LR6475-fed do not form distinct clusters from one another; however, both JB-1 and LR6475-fed mice differ from the PBS cluster formed along both PC1 and 2 (adonis *p*-value = 0.17) (Figure 2A). Only 2 genes, TPPP3 and SGK1 were

found to be differentially expressed when comparing JB-1 to PBS-fed mice (Figure 2B). When comparing JB-1 to LR6475, and LR6475 to PBS-fed mice (Figure 2C,D); however, no genes were found to be significantly differentially expressed. In order to identify sources of the group distinction for both PBS comparisons, Gene Set Enrichment Analysis was performed.

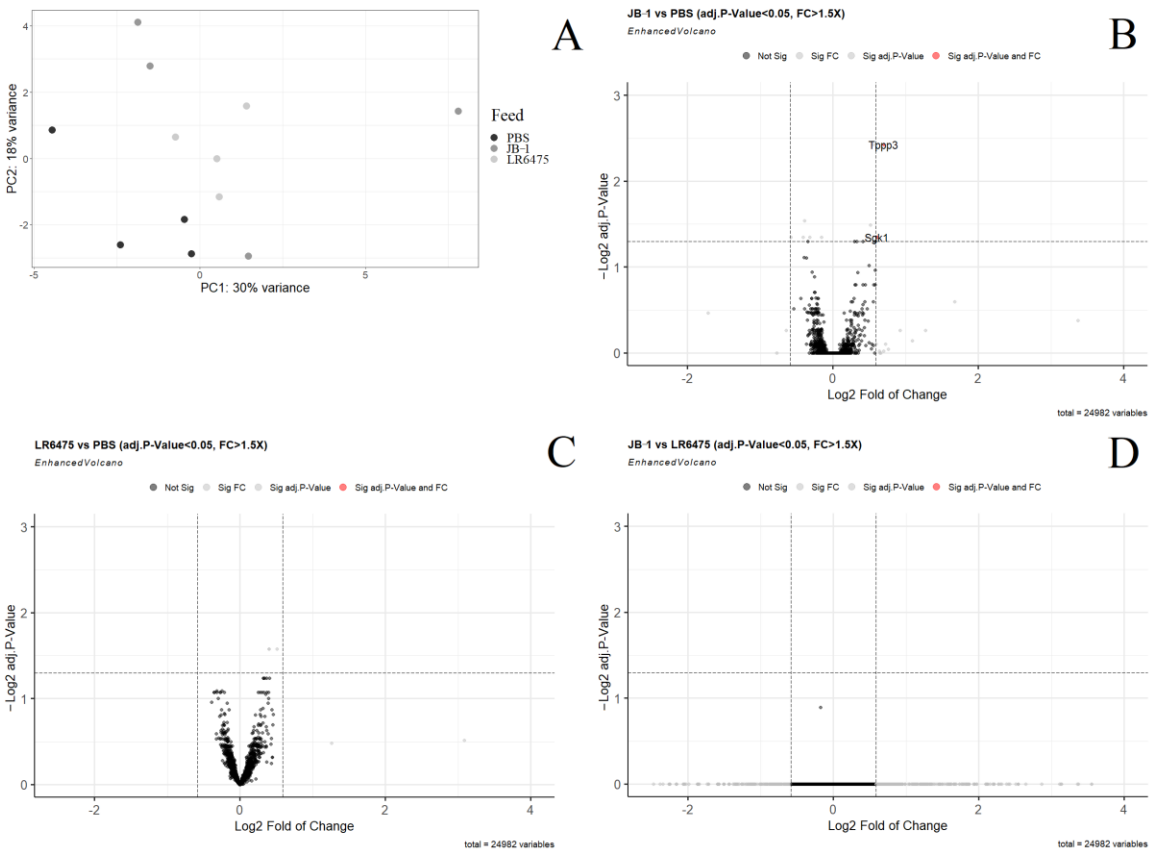

Figure 2. Differential expression analysis of mRNA measured by RNAseq in the hippocampus of mice fed either *Lacticaseibacillus rhamnosus* JB-1, *Limosilactobacillus reuteri* 6475, or PBS. (**A**) Principal component analysis of all three treatment groups. (**B**) Volcano plot showing individual differentially expressed genes in a comparison of JB-1 vs. PBS-fed mice. (**C**) Volcano plot showing individual differentially expressed genes in a comparison of LR6475 vs. PBS-fed mice. (**D**) Volcano plot showing individual differentially expressed genes in a comparison of JB-1 vs. LR6475-fed mice. Genes with a positive log fold change are more highly expressed.

Enrichment analysis of hippocampal mRNA following treatment was also poorly able to identify the source of PCA group distinction. No Hallmark pathways were found differentially expressed in any of the three comparisons. KEGG_RIBOSOME was enriched in the hippocampi of both JB-1 (adj.*p*-value = 1.3×10^{-4}) and LR6475-fed mice (adj.*p*-value = 2.4×10^{-7}) compared to PBS-fed mice, and KEGG_VIBRO_CHOLERAE_INFECTION was enriched in PBS compared to LR6475 (adj.*p*-value = 1.6×10^{-2}). With so few discerning features between individual gene expression and enrichment analysis, it remains unclear what is driving the distinction seen between PBS and the other treatment groups in the PCA. Additional PCs were checked (up to PC12) but none alone explain the distinction.

4.3.3 Weighted Correlation Network Analysis Confirms Differences in Blood Expression between Feeding Groups:

As a second line of evidence that JB-1, but not LR6475 has a unique impact on blood and hippocampal mRNA expression, we performed weighted correlation network analysis (WGCNA) which relies on unsupervised clustering of genes to construct a network with modules of commonly co-expressed genes.

24 groups of genes were identified and mapped to the cluster dendogram (Figure 3A). There were several strong relationships identified when comparing these gene modules to the feed groups (traits) including statistically significant differences between JB-1 and PBS-fed mouse blood in the ‘black’ and ‘light green’ groups (Figure 3B). None of the

modules had a significant relationship with LR6475-fed mice, making it statistically indiscernible from PBS, which is consistent with the PCA (Figure 1A).

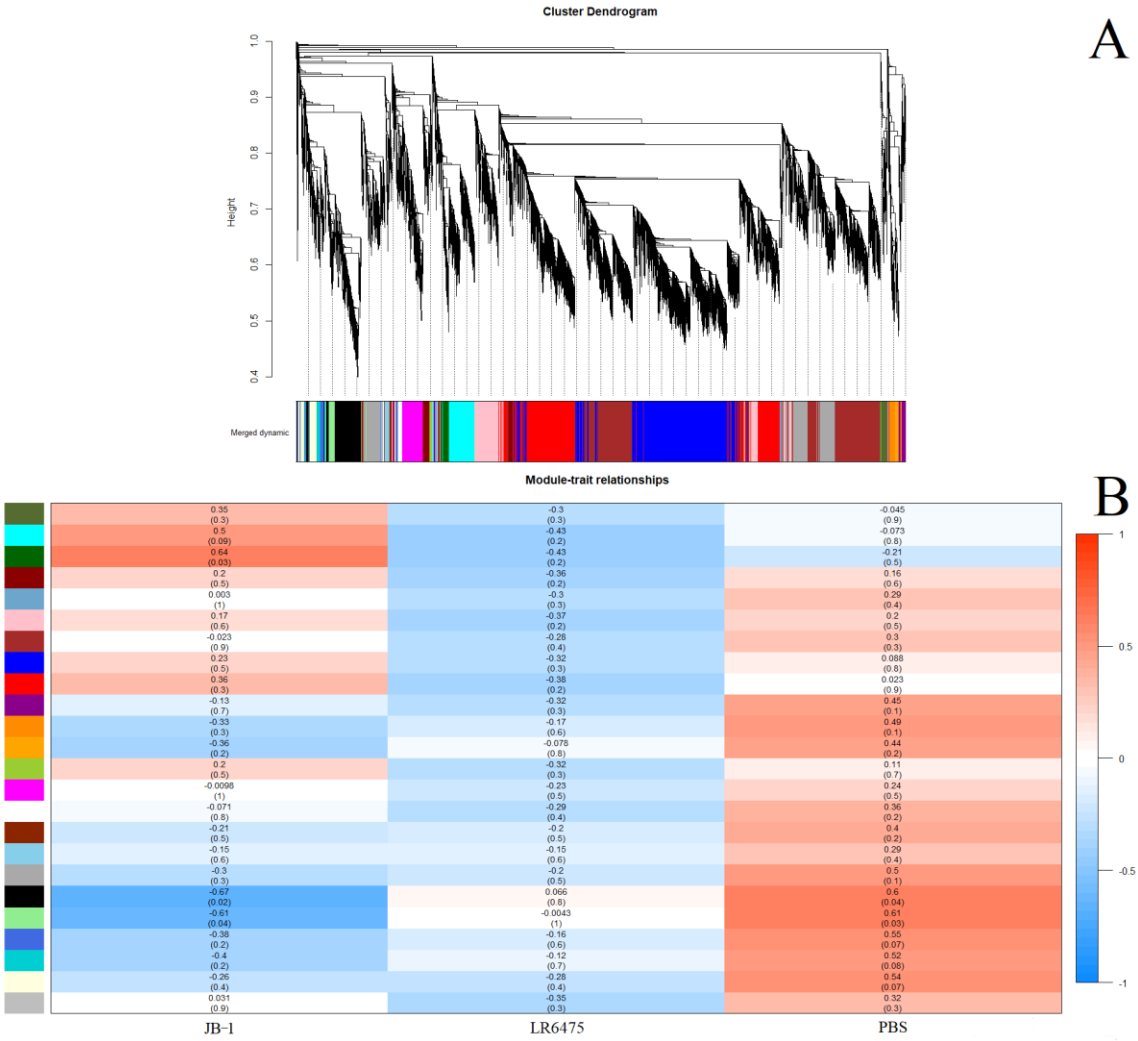

Figure 3. Weighted correlation network analysis (WGCNA) of blood mRNA from mice fed one of JB-1, LR6475, or PBS. (**A**) Unsupervised cluster dendogram of commonly co-expressed genes shown grouped into modules by colour. (**B**) Gene module and feed group relationships shown in a heatmap with relative expression level and *p*-value in brackets under it for each relationship.

For hippocampal mRNA, 9 groups of genes were identified and mapped to a cluster dendrogram (Figure 4A). No statistically significant relationships between gene modules and feed group pairs were found (Figure 4B), which is consistent with the PCA (Figure

2A). Additionally, consistent with the PCA in Figure 2A is that JB-1 and LR6475 appear harder to distinguish on the heatmap, while PBS-fed mice appear distinct. There were also statistically significant associations between individual feed groups and gene modules: JB-1 was associated with pink, while black, magenta, and green were associated with control. Again, LR6475 was not significantly associated in either direction with either gene module.

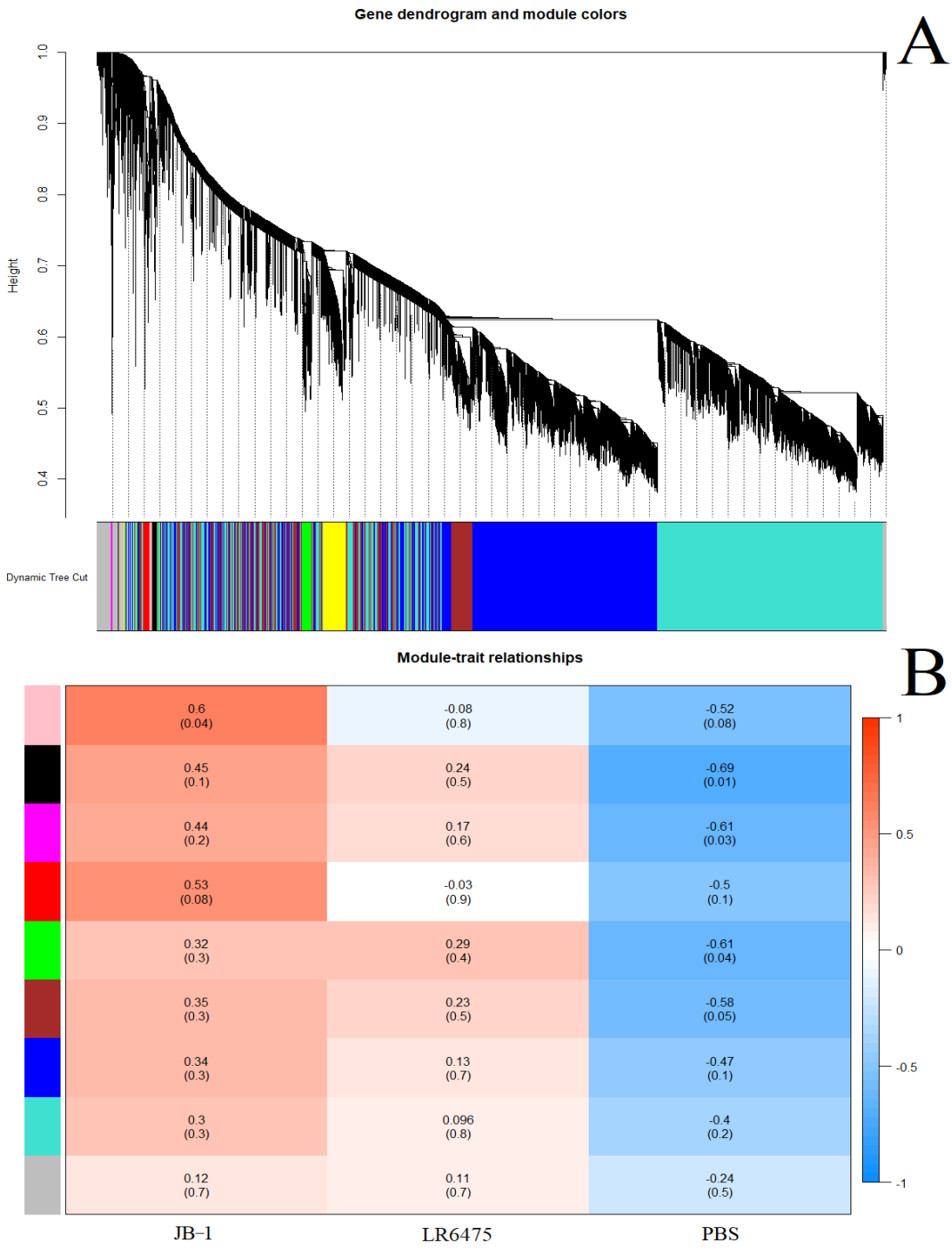

Figure 4. Weighted correlation network analysis (WGCNA) of hippocampal mRNA from mice fed one of JB-1, LR6475, or PBS. (**A**) Unsupervised cluster dendogram of commonly co-expressed genes shown grouped into modules by colour. (**B**) Gene module and feed group relationships shown in a heatmap with relative expression level and *p*-value in brackets under it for each relationship.

4.4 Discussion

Here, we examined blood and hippocampal transcriptional changes induced by two lactobacillus species that have previously been demonstrated to have distinct effects on behaviour in mice [8–10,16,21]. This study identified clear transcriptomic changes in the blood, and to a lesser extent, the hippocampus following feeding with JB-1, but not LR6475.

4.4.1 Inflammatory Response:

Immunomodulatory actions have been described for both JB-1 and LR6475 and in the case of JB-1 these have been demonstrated to mediate effects on behaviour [8–10,16]. The current study identified transcriptomic changes reflective of immunomodulation. In particular, several genes involved in antigen presentation were enriched in the PBS and LR6475 vs. JB-1 (Figure 1B,D). Histocompatibility 2, class II antigen A, alpha (H2-Aa), a subunit of the major histocompatibility complex II (MHCII) enables peptide antigen binding activity, and it participates in the interferon-γ response [25]. Additionally, enriched in the blood of PBS and LR6475 vs. JB-1 was gamma-interferon-inducible lysosomal thiol reductase (IFI30) an enzyme that reduces endocytic disulphide bonds to bring about production of MHC class II-restricted epitopes [26,27]. B-cell antigen receptor complex-associated protein alpha chain and beta chain (CD79a and CD79b) were both enriched in the blood of PBS and LR6475 compared to JB-1-fed mice. These proteins cooperate, and are required for antigen presentation on B cells, as they facilitate the signal transduction cascade activated by an antigen binding to the B cell antigen receptor complex [28,29].

There are several lines of evidence suggesting JB-1 has physiological effects similar to selective serotonin reuptake inhibitors (SSRIs) [10,21,30]. It is therefore interesting to note that SSRIs such as fluoxetine have also been shown to modulate antigen presentation, reducing co-stimulatory marker expression on dendritic cells and subsequent antigen induced T cell response [31].

We also observed marked changes in mRNA related interferon signaling with significant diminution of HALLMARK_INTERFERON_ALPHA_RESPONSE, HALLMARK_INTERFERON_GAMMA_RESPONSE, and HALLMARK_INFLAMMATORY_RESPONSE in the blood of mice treated with JB-1 in comparison to both PBS and reuteri 6475. The Interferons are known to play a role in the link between the immune system and mood disorders. INF-α is used to treat hepatitis C and is associated with a 30–70% increased risk of emergent depression [32]. Interferon gamma (INF-γ) is also indicated to play a role in depression [33]. Patients with MDD demonstrate higher levels of INF-γ production by peripheral blood mononuclear cells [34] and successful antidepressant treatment decreases levels of this inflammatory cytokine while increasing regulatory IL-10 [35]. Furthermore INF-γ −/− mice demonstrate decreased anxiety- and depressive-like behaviors. More broadly, inflammatory cytokines including INF-γ are upregulated as part of the stress response, which in turn leads to activation of the microglia, hypothalamic-pituitary-adrenal (HPA) axis, and the sympathetic nervous system (SNS) [33]. Our observation of decreased INF-α, INF-γ, and inflammatory pathway activity in the blood, indicates a broad anti-inflammatory effect of JB-1 and is consistent with our previous findings of increased regulatory T cells and inhibition of mast cell

degranulation [8,36,37]. Additional evidence for a general anti-inflammatory response to JB-1 is indicated by the decreased expression of the KEGG_T_CELL_RECEPTOR_SIGNALLING_PATHWAY in the blood of JB-1 treated mice compared to both LR6475 treated and control animals. The T cell receptor signaling pathway is critical for the activation of T lymphocytes (CD25+) which have previously been found elevated in the blood of depressed individuals [38]. The JB-1 associated differences in circulating gene expression of interferon signaling pathways was not observed in the hippocampus.

HALLMARK_TNFA_SIGNALLING_VIA_NFKB was underexpressed in both JB1 blood comparisons. These are genes that are regulated by NF-KB in response to TNF-$\boldsymbol{\alpha}$. TNF-$\boldsymbol{\alpha}$ signaling through NF-KB has previously been shown to activate microglia and increase neuroinflammation in mice showing depression-like behaviour [39]. Mice instilled with depressive-like behaviour via chronic unpredictable mild stress also showed heightened levels of inflammatory cytokines, and NF-KB in the prefrontal cortex and hippocampus, the signaling of which was associated with greater risk of depressive symptoms [40].

Finally, FKBP12, one of the genes increased in expression in the blood of JB-1-fed animals compared to the other treatment groups, is a known inhibitor of mTOR signaling [41]. This may be part of the anti-inflammatory response to JB-1, as mTOR controls immune cell activity as well through assisting the differentiation of T cells, and by regulating translation, modulating cytokine responses, macrophage migration and polarization, and antigen presentation [41–44].

4.4.2 Cerebral Cortical Signaling:

The Janus kinase/signal transducers and activators of transcription (JAK/STAT) signaling pathway controls several processes in the cerebral cortex and hippocampus, including microglial activation, synaptic plasticity, gliogenesis, and neurogenesis [45–47]. Here, we found it downregulated in the blood mRNA for both the Hallmark and KEGG gene sets (HALLMARK_IL6_JAK_STAT3_SIGNALING, KEGG_JAK_STAT_SIGNALING_PATHWAY). Al-Samhari et al. (2016) [48] found that treating rats with anti-oxidant precursor, N-acetylcysteine, inhibited STAT3 protein activation and led to reduced depression-like symptoms in rats (increased locomotor activity). This is one example of why it has been proposed that JAK/STAT pathway inhibitors could serve as good candidates for antidepressants [48,47]. The reduced expression of the JAK/STAT pathway in the blood of mice after JB1 feeding suggests this may be a previously unrecognized mechanism contributing to the antidepressant-like effects of the bacteria.

Within all comparisons made, only two genes were significantly overexpressed in the hippocampus-TPPP3 and SGK1 which were altered in the JB-1 vs. PBS-fed comparison. Tubulin polymerization-promoting protein family member 3 (TPPP3) is a protein that regulates microtubule dynamics [49], and Serine/threonine-protein kinase Sg1k (SGK1) is a kinase that regulates a variety of ion channels, transcription factors, cellular enzymes, cell growth, membrane transporters, and is known to play a significant role in the stress response [50–53]. SGK1, is a negative regulator of VEGF and BDNF, has been shown to interact with NF-KB, RAN, mTOR, FOXO3A, and is increased in a human

hippocampal progenitor cell line during MDD, and decreased in the prefrontal cortex of PTSD patients [54–56]. Licznerski et al. (2015) [56] also found that in the hippocampus of foot-shock stressed rats, SGK1 mRNA was underexpressed, but the amount of hippocampal protein remained unchanged. Zhang et al. (2016) [57] found that in a chronic corticosterone (CORT) mouse model for anxiety and depression, glucocorticoid receptor levels were diminished, leading to an insufficient hippocampal neurogenesis. By treating mice with baicalin, they were able to restore hippocampal neurogenesis and reverse depression-like behaviours [57]. In their model, baicalin is thought to be undoing phosphorylation of SGK1, allowing it to phosphorylate the glucocorticoid receptor, encouraging translation to the nucleus, where it may promote neurogenesis.

4.4.3 JB-1-Modulated Genes in the Blood:

Several mRNAs were upregulated in the blood of JB1-fed mice compared to both LR6475 and PBS-fed mice. They were: FKBP1A, SNRPN, CELF4, GPM6B, APBB1, NCDN, RUNDC3A, CPE, and CLSTN1.

Neuronal membrane glycoprotein gene (GPM6B) codes for a protein involved in bone formation and osteoblast function, as well as being a binding partner of the serotonin transporter (SERT) [58,59]. It has been suggested that GPM6B could play a role in regulating SERT cellular trafficking and activity, which could potentially have a broad impact on mood disorders [59,60]. It has also been found that GPM6B expression is severely reduced in the hippocampus of depressed suicides, and mechanistically, it has

been proposed that this lack of GPM6B in the hippocamus alters oligodendrocyte function to contribute to MDD [61,62].

Carboxypeptidase E (CPE) codes for an exopeptidase that removes C-terminal lysine or arginine acids from peptides [63]. Rodriguiz et al. (2013) [63] found that a point mutation to the CPE gene induced anxiety-like behaviours in older mice, and depression-like behaviours in mice of all ages. Anxiety-like behaviours were reversed following acute treatment with fluoxetine or diazapam, while depression-like behaviours were reversed with acute reboxetine administration, or prolonged treatment with bupropion or fluoxetine [63]. A similar mutation was discovered in an Alzheimer's patient by Cheng et al. (2016) [64] and replicating the mutation in mice led to decreased neurogenesis in the hippocampus, decreased dendrites, impaired memory, and depression-like behaviour.

Calsyntenin-1 (CLSTN1) codes for a protein that encourages vesicle association with KLC1 in axonal anterograde transport [65]. CLSTN1 has previously been found to be differentially hypermethylated in the blood of MDD patients (n = 118) compared to healthy subjects (n = 236) [66]. The mechanism by which CLSTN1 affects depression has yet to be elucidated, however Li et al. (2021) [67] confirmed that overexpression of CLSTN1 in the hippocampus of mice and rats increased anxiety and depression-like phenotypes. We did not find that JB1 altered hippocampal expression of CLSTN1, and it is interesting that we see it increased in the blood. As the differentially methylated DNA samples in Davies et al. (2014) [66] were collected from the blood, this suggests that a dearth of CLSTN1 expression in the body is implicated in depression.

4.5 Materials and Methods

4.5.1 Feeding and Tissue Collection:

7–9-week-old male balb/c mice from Charles River Laboratories were orally gavaged with 200 µL of either *Lacticaseibacillus rhamnosus JB*-1 (2×10^9), *Limosilactobacillus reuteri* 6475 (2×10^9), or PBS (n = 5, 5, 5). Mice were gavaged once per day for 2 weeks and sacrificed 3 h after the last gavage. Trunk blood was collected and whole brains were flash frozen and stored. Later, hippocampi of alternating side half brains were isolated and stored for RNA isolation.

4.5.2 RNA Isolation and Analysis:

Total RNA was isolated from fresh whole blood using a PureLink RNA mini kit for total RNA isolation and using the manufacturer-recommended protocol for whole blood extraction.

Total RNA from hippocampi was isolated first by homogenizing the tissue with mortar and pestle in lysis buffer, followed by up-down pipetting through a 27-gauge syringe. After the tissue was completely homogenized, the same PureLink RNA mini kit was used to extract total RNA using the manufacturer-recommended protocol for tissue extraction.

Four samples from each gavage group, totaling at 12 samples for blood and hippocampus, underwent paired-end RNAseq (P3, 2 × 50 bp) on an illumina NextSeq for mRNA discovery and analysis.

4.5.3 Data Preprocessing and Differential Expression:

Raw RNAseq data were adaptor trimmed and aligned using the 'RNA-Seq Alignment' app in Illumina BaseSpace which uses the Spliced Transcripts Alignment to a Reference (STAR) alignment method with the USCS mm10 refseq gene annotation file [68]. Next transcript expression of the annotation data was quantified by Salmon to produce count data [69] within the 'RNA-Seq Alignment' app, variant calling was performed by Strelka Variant caller [70], quality control metrics were performed by picard. Raw count data were downloaded, and differential expression was performed by using 'DESeq2′ package in R [71], which automatically filters low expressed genes and normalizes the data by the geometric mean. Individual mRNAs were considered to be differentially expressed when they exhibited adjusted p-value < 0.05, and an |FC| > 1.5. p-values were adjusted using Benjamini–Hochberg method [72].

4.5.4 PCA:

Percent variables and principal components were calculated using DESeq2′s plotPCA function on the list of internally normalized and filtered genes, and then graphed using ggplot2 [73].

4.5.5 Enrichment Analysis:

Fold change and adjusted p-values values generated by DESeq2′s 'results' function were used by the generally applicable *gage* (gene-set enrichment for pathway analysis) package in R to generate enrichment results to the KEGG and Hallmark pathway gene sets [74]. KEGG pathway visualization was performed using the 'pathview' R package [75]

and shows relative expression of each gene in each differentially expressed pathway (determined by gage). Enrichment of a certain pathway was considered significant at Benjamini–Hochberg adjusted *p*-value < 0.05.

4.5.6 Weighted Correlation Network Analysis:

To support the findings of the PCAs we conducted a weighted correlation network analysis using the 'WGCNA' package in R [76]. We used a power of 13 for blood RNA and merged close gene modules at a threshold of 0.05; and for hippocampal RNA, a power of 30 and merged close gene modules at a threshold of 0.04. Differences between groups of clustered genes were visualized in respect to each of the feeding groups using the 'labeledHeatmap' function within the WGCNA package.

4.6 Conclusions

Here, we identified several pathways and genes that may be associated with JB-1, treatment that may plausibly be related to the effects of these organisms on behavior, summarized in (Tables 2 and 3). Some are likely related to the previously described immunomodulatory effects of these organisms. In particular, the role interferon signaling pathways in mediating gut–brain signaling warrants further exploration. The lack of any significant modified pathways or genes following LR6475 is surprising. However, this study examined the direct effect of JB-1 and LR6475 on normal BALB/c mice, while this mouse strain has high trait anxiety it may be that the effects of LR6475 would only be observable following a stress challenge or in a pathological mood disorder model.

Table 2. Differential expression of pathways relevant to depression, in depression, after established antidepressant treatment, and following JB-1 treatment in mice. The up and down arrows represent the direction of change in pathway expression in our findings and in literature.

Pathway	INF-γ/INF-α	JAK/STAT	TNF-α Via NF-KB
Change in Depression	↑ generally increased, but suboptimal expression has also been associated with depression [33,77,78]	↑ activation in stress and depression in mice [79]	↑ in prefrontal cortex and hippocampus of mice [40]
Change in MDD Treatment	↓ after treatment with either sertraline, clomipramine, or trazodone in human blood [35]	↓ phosphorylation (activation) of Jak-3 returned to normal in mice following Amitriptyline treatment [79]	↓ SSRIs such as imipramine reduce TNF-α levels in rats [80]
Blood Change Following JB-1	↓ mRNA (5.4×10^{-6}) (7.7×10^{-3})	↓ mRNA (4.9×10^{-3})	↓ mRNA (3.2×10^{-2})

Table 3. Differential expression of genes relevant to depression, in depression, after established antidepressant treatment, and following JB-1 treatment in mice. The up and down arrows represent the direction of change in pathway expression in our findings and in literature.

Gene	SGK1	GPM6B	NCDN	CLSTN1
Change in Depression	↓ in hippocampus of rats [56]	↓ in the hippocampus of humans [60]	↓ in CNS of mice [81]	↓ in blood of humans [66], ↑ in hippocampus of rats and mice [67]
Change in Depression Treatment	↑ mRNA in hippocampus and prefrontal cortex of rats following icariin or baicalin treatment [82,83]	N/A	↓ in hippocampus after ketamine treatment in rats [84]	N/A
Change Following JB-1	↑ mRNA in hippocampus of mice (4.5×10^{-2})	↑ mRNA in blood of mice (7.6×10^{-4})	↑ mRNA in blood of mice (8.5×10^{-3})	↑ mRNA in blood of mice (1.6×10^{-2})

There are certain limitations to the current study. The brains used in the study were not perfused following collection, and therefore could contain a small amount of blood from within capillaries in the hippocampus. Furthermore, only male mice were used, there is evidence that the outcome of gut–brain signaling can be sex dependent and thus sex comparisons would be worthy of examination in the future. This is particularly pertinent as in humans mental health disorders disproportionately affect women. Finally, WGCNA recommends 15 samples minimum for analysis and we performed the analysis with 12, which may have reduced the precision of the results.

Overall, our results highlight that microbes labeled as psychobiotics, or potential psychobiotics, induce complex changes in systemic gene expression which are far from uniform between organisms. A better understanding of the many pathways impacted by

individual organisms may help develop more tailored microbe-based approaches to specific mental health issues.

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4.7 References

1. Wittchen, H.U.; Jacobi, F.; Rehm, J.; Gustavsson, A.; Svensson, M.; Jönsson, B.; Olesen, J.; Allgulander, C.; Alonso, J.; Faravelli, C.; et al. The Size and Burden of Mental Disorders and Other Disorders of the Brain in Europe 2010. *Eur. Neuropsychopharmacol.* **2011**, *21*, 655–679. https://doi.org/10.1016/j.euroneuro.2011.07.018.
2. Patalay, P.; Gage, S.H. Changes in Millennial Adolescent Mental Health and Health-Related Behaviours over 10 Years: A Population Cohort Comparison Study. *Int. J. Epidemiol.* **2019**, *48*, 1650–1664. https://doi.org/10.1093/ije/dyz006.
3. Abbott, A. COVID's Mental-Health Toll: How Scientists Are Tracking a Surge in Depression. *Nature* **2021**, *590*, 194–195. https://doi.org/10.1038/d41586-021-00175-z.
4. Long-Smith, C.; O'Riordan, K.J.; Clarke, G.; Stanton, C.; Dinan, T.G.; Cryan, J.F. Microbiota-Gut-Brain Axis: New Therapeutic Opportunities. *Annu. Rev. Pharmacol. Toxicol.* **2020**, *60*, 477–502. https://doi.org/10.1146/annurev-pharmtox-010919-023628.
5. Carabotti, M.; Scirocco, A.; Maselli, M.A.; Severi, C. The Gut-Brain Axis: Interactions between Enteric Microbiota, Central and Enteric Nervous Systems. *Ann. Gastroenterol.* **2015**, *28*, 203–209.
6. Sarkar, A.; Lehto, S.M.; Harty, S.; Dinan, T.G.; Cryan, J.F.; Burnet, P.W.J. Psychobiotics and the Manipulation of Bacteria–Gut–Brain Signals. *Trends Neurosci.* **2016**, *39*, 763–781. https://doi.org/10.1016/j.tins.2016.09.002.
7. Wallace, C.J.K.; Milev, R. The Effects of Probiotics on Depressive Symptoms in Humans: A Systematic Review. *Ann. Gen. Psychiatry* **2017**, *16*, 14. https://doi.org/10.1186/s12991-017-0138-2.
8. Bharwani, A.; Mian, M.F.; Surette, M.G.; Bienenstock, J.; Forsythe, P. Oral Treatment with Lactobacillus Rhamnosus Attenuates Behavioural Deficits and Immune Changes in Chronic Social Stress. *BMC Med.* **2017**, *15*, 7. https://doi.org/10.1186/s12916-016-0771-7.
9. Bravo, J.A.; Forsythe, P.; Chew, M.V.; Escaravage, E.; Savignac, H.M.; Dinan, T.G.; Bienenstock, J.; Cryan, J.F. Ingestion of Lactobacillus Strain Regulates Emotional Behavior and Central GABA Receptor Expression in a Mouse via the Vagus Nerve. *Proc. Natl. Acad. Sci. USA* **2011**, *108*, 16050–16055. https://doi.org/10.1073/pnas.1102999108.
10. McVey Neufeld, K.-A.; Kay, S.; Bienenstock, J. Mouse Strain Affects Behavioral and Neuroendocrine Stress Responses Following Administration of Probiotic Lactobacillus Rhamnosus JB-1 or Traditional Antidepressant Fluoxetine. *Front. Neurosci.* **2018**, *12*, 294. https://doi.org/10.3389/fnins.2018.00294.
11. Liu, Y.; Mian, M.F.; McVey Neufeld, K.-A.; Forsythe, P. $CD4^+CD25^+$ T Cells Are Essential for Behavioral Effects of Lactobacillus Rhamnosus JB-1 in Male BALB/c Mice. *Brain. Behav. Immun.* **2020**, *88*, 451–460. https://doi.org/10.1016/j.bbi.2020.04.014.
12. Martin, A.M.; Sun, E.W.; Rogers, G.B.; Keating, D.J. The Influence of the Gut Microbiome on Host Metabolism Through the Regulation of Gut Hormone Release. *Front. Physiol.* **2019**, *10*, 428. https://doi.org/10.3389/fphys.2019.00428.

13. Dinan, T.G.; Stanton, C.; Cryan, J.F. Psychobiotics: A Novel Class of Psychotropic. *Biol. Psychiatry* **2013**, *74*, 720–726. https://doi.org/10.1016/j.biopsych.2013.05.001.
14. Choi, J.; Kim, Y.-K.; Han, P.-L. Extracellular Vesicles Derived from Lactobacillus Plantarum Increase BDNF Expression in Cultured Hippocampal Neurons and Produce Antidepressant-like Effects in Mice. *Exp. Neurobiol.* **2019**, *28*, 158–171. https://doi.org/10.5607/en.2019.28.2.158.
15. Haas-Neill, S.; Forsythe, P. A Budding Relationship: Bacterial Extracellular Vesicles in the Microbiota-Gut-Brain Axis. *Int. J. Mol. Sci.* **2020**, *21*, 8899. https://doi.org/10.3390/ijms21238899.
16. Sgritta, M.; Dooling, S.W.; Buffington, S.A.; Momin, E.N.; Francis, M.B.; Britton, R.A.; Costa-Mattioli, M. Mechanisms Underlying Microbial-Mediated Changes in Social Behavior in Mouse Models of Autism Spectrum Disorder. *Neuron* **2019**, *101*, 246–259.e6. https://doi.org/10.1016/j.neuron.2018.11.018.
17. Buffington, S.A.; Di Prisco, G.V.; Auchtung, T.A.; Ajami, N.J.; Petrosino, J.F.; Costa-Mattioli, M. Microbial Reconstitution Reverses Maternal Diet-Induced Social and Synaptic Deficits in Offspring. *Cell* **2016**, *165*, 1762–1775. https://doi.org/10.1016/j.cell.2016.06.001.
18. Poutahidis, T.; Kleinewietfeld, M.; Smillie, C.; Levkovich, T.; Perrotta, A.; Bhela, S.; Varian, B.J.; Ibrahim, Y.M.; Lakritz, J.R.; Kearney, S.M.; et al. Microbial Reprogramming Inhibits Western Diet-Associated Obesity. *PLoS ONE* **2013**, *8*, e68596. https://doi.org/10.1371/journal.pone.0068596.
19. Medicine, N.L. L. Reuteri DSM 17938 and L. Reuteri ATCC PTA 6475 in Moderate to Severe Irritable Bowel in Adults (Reuteri-IBS). 2019. Available from: https://clinicaltrials.gov/ct2/show/NCT04037826 (accessed on 28 Feburary 2022).
20. Collins, N.; Han, S.-J.; Enamorado, M.; Link, V.M.; Huang, B.; Moseman, E.A.; Kishton, R.J.; Shannon, J.P.; Dixit, D.; Schwab, S.R.; et al. The Bone Marrow Protects and Optimizes Immunological Memory during Dietary Restriction. *Cell* **2019**, *178*, 1088–1101.e15. https://doi.org/10.1016/j.cell.2019.07.049.
21. West, C.L.; McVey Neufeld, K.-A.; Mao, Y.-K.; Stanisz, A.M.; Forsythe, P.; Bienenstock, J.; Barbut, D.; Zasloff, M.; Kunze, W.A. Identification of SSRI-Evoked Antidepressant Sensory Signals by Decoding Vagus Nerve Activity. *Sci. Rep.* **2021**, *11*, 21130. https://doi.org/10.1038/s41598-021-00615-w.
22. Campbell, S.; Macqueen, G. The Role of the Hippocampus in the Pathophysiology of Major Depression. *J. Psychiatry Neurosci.* **2004**, *29*, 417–426.
23. MacQueen, G.; Frodl, T. The Hippocampus in Major Depression: Evidence for the Convergence of the Bench and Bedside in Psychiatric Research? *Mol. Psychiatry* **2011**, *16*, 252–264. https://doi.org/10.1038/mp.2010.80.
24. Roddy, D.W.; Farrell, C.; Doolin, K.; Roman, E.; Tozzi, L.; Frodl, T.; O'Keane, V.; O'Hanlon, E. The Hippocampus in Depression: More Than the Sum of Its Parts? Advanced Hippocampal Substructure Segmentation in Depression. *Biol. Psychiatry* **2019**, *85*, 487–497. https://doi.org/10.1016/j.biopsych.2018.08.021.
25. Knapp, B.; Omasits, U.; Schreiner, W.; Epstein, M.M. A Comparative Approach Linking Molecular Dynamics of Altered Peptide Ligands and MHC with In Vivo Immune Responses. *PLoS ONE* **2010**, *5*, e11653. https://doi.org/10.1371/journal.pone.0011653.

26. Maric, M.; Arunachalam, B.; Phan, U.T.; Dong, C.; Garrett, W.S.; Cannon, K.S.; Alfonso, C.; Karlsson, L.; Flavell, R.A.; Cresswell, P. Defective Antigen Processing in GILT-Free Mice. *Science* **2001**, *294*, 1361–1365. https://doi.org/10.1126/science.1065500.
27. Singh, R.; Cresswell, P. Defective Cross-Presentation of Viral Antigens in GILT-Free Mice. *Science* **2010**, *328*, 1394–1398. https://doi.org/10.1126/science.1189176.
28. Fuentes-Pananá, E.M.; Bannish, G.; van der Voort, D.; King, L.B.; Monroe, J.G. Igα/Igβ Complexes Generate Signals for B Cell Development Independent of Selective Plasma Membrane Compartmentalization. *J. Immunol.* **2005**, *174*, 1245–1252. https://doi.org/10.4049/jimmunol.174.3.1245.
29. Siemasko, K.; Eisfelder, B.J.; Stebbins, C.; Kabak, S.; Sant, A.J.; Song, W.; Clark, M.R. Ig Alpha and Ig Beta Are Required for Efficient Trafficking to Late Endosomes and to Enhance Antigen Presentation. *J. Immunol.* **1999**, *162*, 6518–6525.
30. Liu, Y.; Steinhausen, K.; Bharwani, A.; Mian, M.F.; McVey Neufeld, K.-A.; Forsythe, P. Increased Persistence of Avoidance Behaviour and Social Deficits with L.Rhamnosus JB-1 or Selective Serotonin Reuptake Inhibitor Treatment Following Social Defeat. *Sci. Rep.* **2020**, *10*, 13485. https://doi.org/10.1038/s41598-020-69968-y.
31. Branco-de-Almeida, L.S.; Kajiya, M.; Cardoso, C.R.; Silva, M.J.B.; Ohta, K.; Rosalen, P.L.; Franco, G.C.N.; Han, X.; Taubman, M.A.; Kawai, T. Selective Serotonin Reuptake Inhibitors Attenuate the Antigen Presentation from Dendritic Cells to Effector T Lymphocytes. *FEMS Immunol. Med. Microbiol.* **2011**, *62*, 283–294. https://doi.org/10.1111/j.1574-695X.2011.00816.x.
32. Pinto, F.E.; Andrade, C. Interferon-Related Depression: A Primer on Mechanisms, Treatment, and Prevention of a Common Clinical Problem. *Curr. Neuropharmacol.* **2016**, *14*, 743–748. https://doi.org/10.2174/1570159X14666160106155129.
33. Inserra, A.; Mastronardi, C.A.; Rogers, G.; Licinio, J.; Wong, M.-L. Neuroimmunomodulation in Major Depressive Disorder: Focus on Caspase 1, Inducible Nitric Oxide Synthase, and Interferon-Gamma. *Mol. Neurobiol.* **2019**, *56*, 4288–4305. https://doi.org/10.1007/s12035-018-1359-3.
34. Maes, M.; Scharpé, S.; Meltzer, H.Y.; Okayli, G.; Bosmans, E.; D'Hondt, P.; Vanden Bossche, B.; Cosyns, P. Increased Neopterin and Interferon-Gamma Secretion and Lower Availability of L-Tryptophan in Major Depression: Further Evidence for an Immune Response. *Psychiatry Res.* **1994**, *54*, 143–160. https://doi.org/10.1016/0165-1781(94)90003-5.
35. Maes, M. Negative Immunoregulatory Effects of Antidepressants Inhibition of Interferon-γ and Stimulation of Interleukin-10 Secretion. *Neuropsychopharmacology* **1999**, *20*, 370–379. https://doi.org/10.1016/S0893-133X(98)00088-8.
36. Karimi, K.; Kandiah, N.; Chau, J.; Bienenstock, J.; Forsythe, P. A Lactobacillus Rhamnosus Strain Induces a Heme Oxygenase Dependent Increase in Foxp3+ Regulatory T Cells. *PLoS ONE* **2012**, *7*, e47556. https://doi.org/10.1371/journal.pone.0047556.
37. Forsythe, P.; Wang, B.; Khambati, I.; Kunze, W.A. Systemic Effects of Ingested Lactobacillus Rhamnosus: Inhibition of Mast Cell Membrane Potassium (IKCa) Current and Degranulation. *PLoS ONE* **2012**, *7*, e41234. https://doi.org/10.1371/journal.pone.0041234.

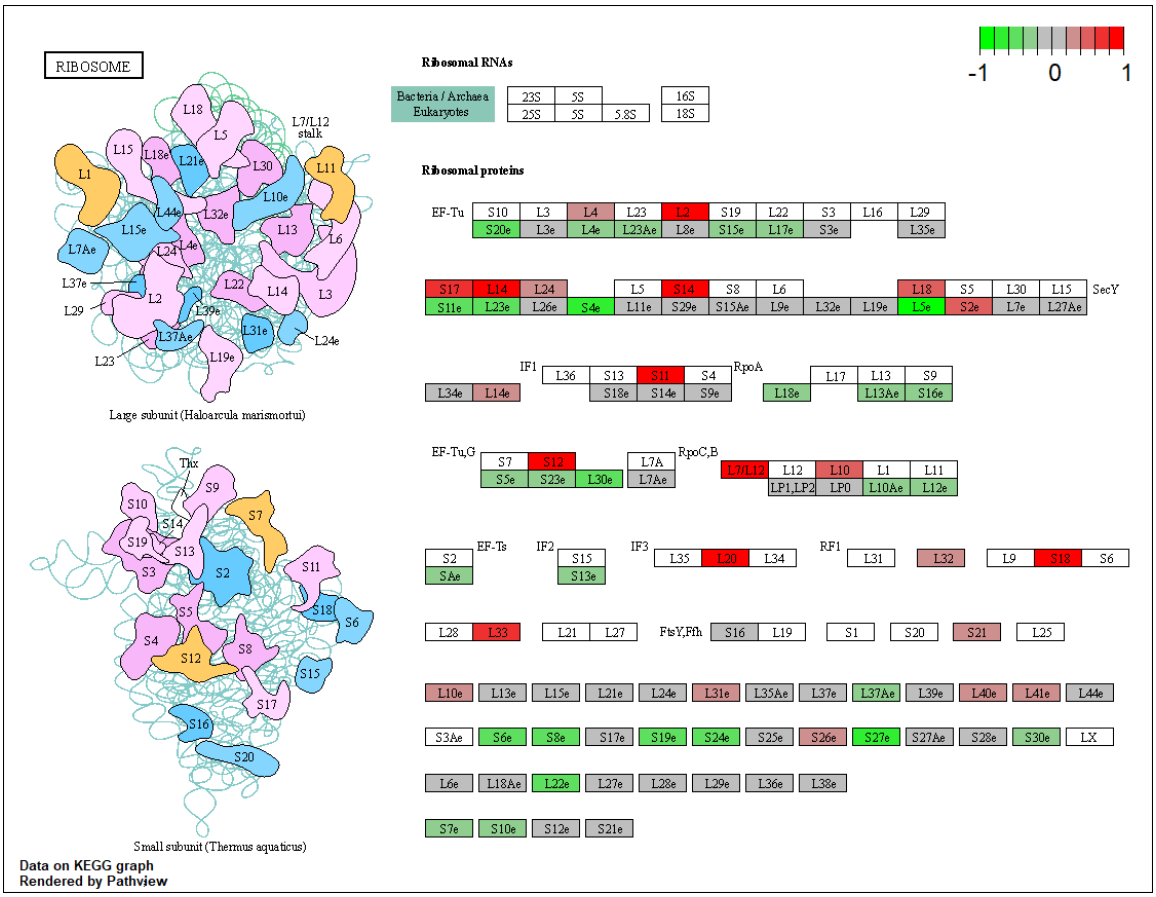
38. Maes, M.; Lambrechts, J.; Bosmans, E.; Jacobs, J.; Suy, E.; Vandervorst, C.; De Jonckheere, C.; Minner, B.; Raus, J. Evidence for a Systemic Immune Activation during Depression: Results of Leukocyte Enumeration by Flow Cytometry in Conjunction with Monoclonal Antibody Staining. *Psychol. Med.* **1992**, *22*, 45–53. https://doi.org/10.1017/S0033291700032712.
39. Kong, X.; Zhang, Z.; Fu, T.; Ji, J.; Yang, J.; Gu, Z. TNF-α Regulates Microglial Activation via the NF-KB Signaling Pathway in Systemic Lupus Erythematosus with Depression. *Int. J. Biol. Macromol.* **2019**, *125*, 892–900. https://doi.org/10.1016/j.ijbiomac.2018.12.146.
40. Wang, Y.; Xu, J.; Liu, Y.; Li, Z.; Li, X. TLR4-NF-κ B Signal Involved in Depressive-Like Behaviors and Cytokine Expression of Frontal Cortex and Hippocampus in Stressed C57BL/6 and Ob/Ob Mice. *Neural Plast.* **2018**, *2018*, 7254016. https://doi.org/10.1155/2018/7254016.
41. Yang, H.; Rudge, D.G.; Koos, J.D.; Vaidialingam, B.; Yang, H.J.; Pavletich, N.P. mTOR Kinase Structure, Mechanism and Regulation. *Nature* **2013**, *497*, 217–223. https://doi.org/10.1038/nature12122.
42. Hu, L.; Chen, F.; Wu, C.; Wang, J.; Chen, S.; Li, X.; Wang, J.; Wu, L.; Ding, J.; Wang, J.; et al. Rapamycin Recruits SIRT2 for FKBP12 Deacetylation during MTOR Activity Modulation in Innate Immunity. *iScience* **2021**, *24*, 103177. https://doi.org/10.1016/j.isci.2021.103177.
43. Chi, H. Regulation and Function of MTOR Signalling in T Cell Fate Decisions. *Nat. Rev. Immunol.* **2012**, *12*, 325–338. https://doi.org/10.1038/nri3198.8.
44. Weichhart, T.; Hengstschläger, M.; Linke, M. Regulation of Innate Immune Cell Function by mTOR. *Nat. Rev. Immunol.* **2015**, *15*, 599–614. https://doi.org/10.1038/nri3901.
45. Takeda, K.; Akira, S. STAT Family of Transcription Factors in Cytokine-Mediated Biological Responses. *Cytokine Growth Factor Rev.* **2000**, *11*, 199–207. https://doi.org/10.1016/S1359-6101(00)00005-8.
46. Jankovic, D.; Trinchieri, G. IL-10 or Not IL-10: That Is the Question. *Nat. Immunol.* **2007**, *8*, 1281–1283. https://doi.org/10.1038/ni1207-1281.
47. Shariq, A.S.; Brietzke, E.; Rosenblat, J.D.; Pan, Z.; Rong, C.; Ragguett, R.-M.; Park, C.; McIntyre, R.S. Therapeutic Potential of JAK/STAT Pathway Modulation in Mood Disorders. *Rev. Neurosci.* **2018**, *30*, 1–7. https://doi.org/10.1515/revneuro-2018-0027.
48. Al-Samhari, M.M.; Al-Rasheed, N.M.; Al-Rejaie, S.; Al-Rasheed, N.M.; Hasan, I.H.; Mahmoud, A.M.; Dzimiri, N. Possible Involvement of the JAK/STAT Signaling Pathway in N-Acetylcysteine-Mediated Antidepressant-like Effects. *Exp. Biol. Med.* **2016**, *241*, 509–518. https://doi.org/10.1177/1535370215619707.
49. Vincze, O.; Tökési, N.; Oláh, J.; Hlavanda, E.; Zotter, Á.; Horváth, I.; Lehotzky, A.; Tirián, L.; Medzihradszky, K.F.; Kovács, J.; et al. Tubulin Polymerization Promoting Proteins (TPPPs): Members of a New Family with Distinct Structures and Functions. *Biochemistry* **2006**, *45*, 13818–13826. https://doi.org/10.1021/bi061305e.
50. Leong, M.L.L.; Maiyar, A.C.; Kim, B.; O'Keeffe, B.A.; Firestone, G.L. Expression of the Serum- and Glucocorticoid-Inducible Protein Kinase, Sgk, Is a Cell Survival Response to Multiple Types of Environmental Stress Stimuli in Mammary Epithelial Cells. *J. Biol. Chem.* **2003**, *278*, 5871–5882. https://doi.org/10.1074/jbc.M211649200.

51. Yoo, D.; Kim, B.Y.; Campo, C.; Nance, L.; King, A.; Maouyo, D.; Welling, P.A. Cell Surface Expression of the ROMK (Kir 1.1) Channel Is Regulated by the Aldosterone-Induced Kinase, SGK-1, and Protein Kinase A. *J. Biol. Chem.* **2003**, *278*, 23066–23075. https://doi.org/10.1074/jbc.M212301200.
52. Eylenstein, A.; Gehring, E.; Heise, N.; Shumilina, E.; Schmidt, S.; Szteyn, K.; Münzer, P.; Nurbaeva, M.K.; Eichenmüller, M.; Tyan, L.; et al. Stimulation of Ca^{2+}-channel Orai1/STIM1 by Serum-and Glucocorticoid-inducible Kinase 1 (SGK1). *FASEB J.* **2011**, *25*, 2012–2021. https://doi.org/10.1096/fj.10-178210.
53. Mo, J.-S.; Ann, E.-J.; Yoon, J.-H.; Jung, J.; Choi, Y.-H.; Kim, H.-Y.; Ahn, J.-S.; Kim, S.-M.; Kim, M.-Y.; Hong, J.-A.; et al. Serum- and Glucocorticoid-Inducible Kinase 1 (SGK1) Controls Notch1 Signaling by Downregulation of Protein Stability through Fbw7 Ubiquitin Ligase. *J. Cell Sci.* **2011**, *124*, 100–112. https://doi.org/10.1242/jcs.073924.
54. Dattilo, V.; Amato, R.; Perrotti, N.; Gennarelli, M. The Emerging Role of SGK1 (Serum- and Glucocorticoid-Regulated Kinase 1) in Major Depressive Disorder: Hypothesis and Mechanisms. *Front. Genet.* **2020**, *11*, 826. https://doi.org/10.3389/fgene.2020.00826.
55. Anacker, C.; Cattaneo, A.; Musaelyan, K.; Zunszain, P.A.; Horowitz, M.; Molteni, R.; Luoni, A.; Calabrese, F.; Tansey, K.; Gennarelli, M.; et al. Role for the Kinase SGK1 in Stress, Depression, and Glucocorticoid Effects on Hippocampal Neurogenesis. *Proc. Natl. Acad. Sci. USA* **2013**, *110*, 8708–8713. https://doi.org/10.1073/pnas.1300886110.
56. Licznerski, P.; Duric, V.; Banasr, M.; Alavian, K.N.; Ota, K.T.; Kang, H.J.; Jonas, E.A.; Ursano, R.; Krystal, J.H.; Duman, R.S. Decreased SGK1 Expression and Function Contributes to Behavioral Deficits Induced by Traumatic Stress. *PLoS Biol.* **2015**, *13*, e1002282. https://doi.org/10.1371/journal.pbio.1002282.
57. Zhang, K.; Pan, X.; Wang, F.; Ma, J.; Su, G.; Dong, Y.; Yang, J.; Wu, C. Baicalin Promotes Hippocampal Neurogenesis via SGK1- and FKBP5-Mediated Glucocorticoid Receptor Phosphorylation in a Neuroendocrine Mouse Model of Anxiety/Depression. *Sci. Rep.* **2016**, *6*, 30951. https://doi.org/10.1038/srep30951.
58. Drabek, K.; van de Peppel, J.; Eijken, M.; van Leeuwen, J.P. GPM6B Regulates Osteoblast Function and Induction of Mineralization by Controlling Cytoskeleton and Matrix Vesicle Release. *J. Bone Miner. Res.* **2011**, *26*, 2045–2051. https://doi.org/10.1002/jbmr.435.
59. Fjorback, A.W.; Müller, H.K.; Wiborg, O. Membrane Glycoprotein M6B Interacts with the Human Serotonin Transporter. *J. Mol. Neurosci.* **2009**, *37*, 191–200. https://doi.org/10.1007/s12031-008-9092-4.
60. Haase, J.; Grudzinska-Goebel, J.; Müller, H.K.; Münster-Wandowski, A.; Chow, E.; Wynne, K.; Farsi, Z.; Zander, J.-F.; Ahnert-Hilger, G. Serotonin Transporter Associated Protein Complexes Are Enriched in Synaptic Vesicle Proteins and Proteins Involved in Energy Metabolism and Ion Homeostasis. *ACS Chem. Neurosci.* **2017**, *8*, 1101–1116. https://doi.org/10.1021/acschemneuro.6b00437.
61. Fuchsova, B.; Alvarez Juliá, A.; Rizavi, H.S.; Frasch, A.C.; Pandey, G.N. Altered Expression of Neuroplasticity-Related Genes in the Brain of Depressed Suicides. *Neuroscience* **2015**, *299*, 1–17. https://doi.org/10.1016/j.neuroscience.2015.04.057.

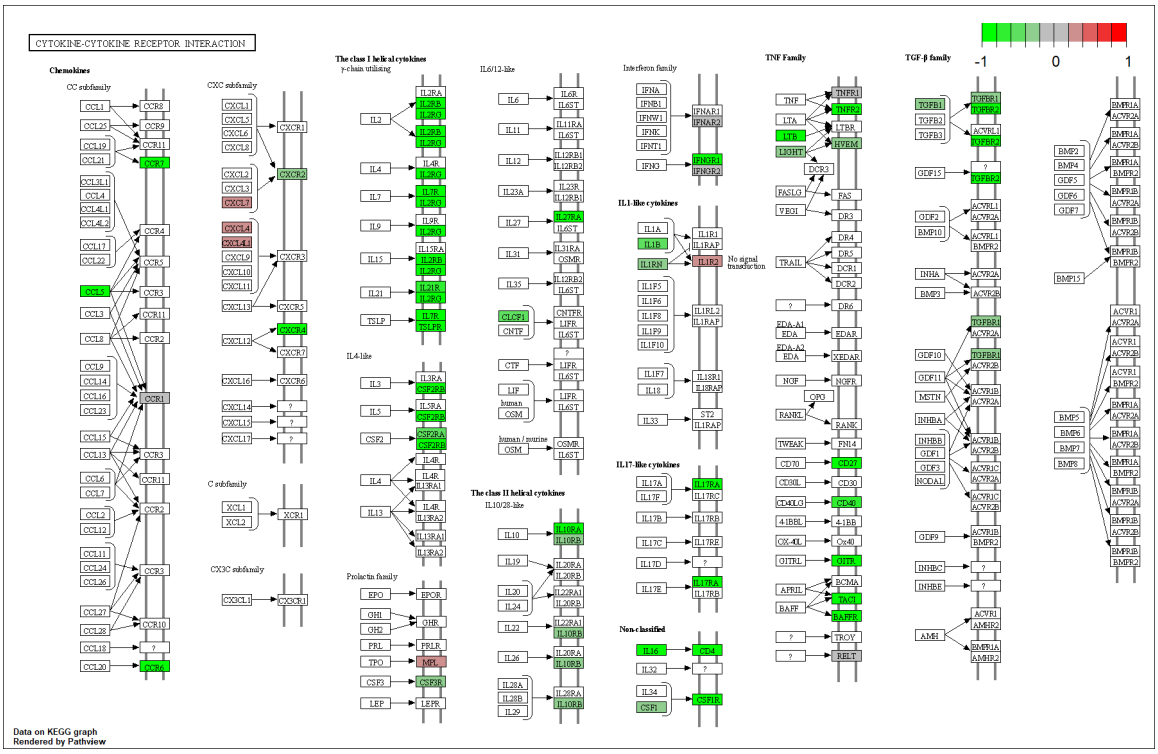
62. Mariani, N.; Cattane, N.; Pariante, C.; Cattaneo, A. Gene Expression Studies in Depression Development and Treatment: An Overview of the Underlying Molecular Mechanisms and Biological Processes to Identify Biomarkers. *Transl. Psychiatry* **2021**, *11*, 354. https://doi.org/10.1038/s41398-021-01469-6.
63. Rodriguiz, R.M.; Wilkins, J.J.; Creson, T.K.; Biswas, R.; Berezniuk, I.; Fricker, A.D.; Fricker, L.D.; Wetsel, W.C. Emergence of Anxiety-like Behaviours in Depressive-like Cpefat/Fat Mice. *Int. J. Neuropsychopharmacol.* **2013**, *16*, 1623–1634. https://doi.org/10.1017/S1461145713000059.
64. Cheng, Y.; Cawley, N.X.; Yanik, T.; Murthy, S.R.K.; Liu, C.; Kasikci, F.; Abebe, D.; Loh, Y.P. A Human Carboxypeptidase E/NF-A1 Gene Mutation in an Alzheimer's Disease Patient Leads to Dementia and Depression in Mice. *Transl. Psychiatry* **2016**, *6*, e973–e973. https://doi.org/10.1038/tp.2016.237.
65. Araki, Y.; Kawano, T.; Taru, H.; Saito, Y.; Wada, S.; Miyamoto, K.; Kobayashi, H.; Ishikawa, H.O.; Ohsugi, Y.; Yamamoto, T.; et al. The Novel Cargo Alcadein Induces Vesicle Association of Kinesin-1 Motor Components and Activates Axonal Transport. *EMBO J.* **2007**, *26*, 1475–1486. https://doi.org/10.1038/sj.emboj.7601609.
66. Davies, M.N.; Krause, L.; Bell, J.T.; Gao, F.; Ward, K.J.; Wu, H.; Lu, H.; Liu, Y.; Tsai, P.-C.; Collier, D.A.; et al. Hypermethylation in the ZBTB20 Gene Is Associated with Major Depressive Disorder. *Genome Biol.* **2014**, *15*, R56. https://doi.org/10.1186/gb-2014-15-4-r56.
67. Li, S.; Cao, W.; Zhou, S.; Ma, M.; Zhang, W.; Li, F.; Li, C. Expression of Cntn1 Is Regulated by Stress and Associated with Anxiety and Depression Phenotypes. *Brain. Behav. Immun.* **2021**, *95*, 142–153. https://doi.org/10.1016/j.bbi.2021.03.012.
68. Dobin, A.; Davis, C.A.; Schlesinger, F.; Drenkow, J.; Zaleski, C.; Jha, S.; Batut, P.; Chaisson, M.; Gingeras, T.R. STAR: Ultrafast Universal RNA-Seq Aligner. *Bioinformatics* **2013**, *29*, 15–21. https://doi.org/10.1093/bioinformatics/bts635.
69. Patro, R.; Duggal, G.; Love, M.I.; Irizarry, R.A.; Kingsford, C. Salmon Provides Fast and Bias-Aware Quantification of Transcript Expression. *Nat. Methods* **2017**, *14*, 417–419. https://doi.org/10.1038/nmeth.4197.
70. Saunders, C.T.; Wong, W.S.W.; Swamy, S.; Becq, J.; Murray, L.J.; Cheetham, R.K. Strelka: Accurate Somatic Small-Variant Calling from Sequenced Tumor–Normal Sample Pairs. *Bioinformatics* **2012**, *28*, 1811–1817. https://doi.org/10.1093/bioinformatics/bts271.
71. Love, M.I.; Huber, W.; Anders, S. Moderated Estimation of Fold Change and Dispersion for RNA-Seq Data with DESeq2. *Genome Biol.* **2014**, *15*, 550.
72. Benjamini, Y.; Hochberg, Y. Controlling the False Discovery Rate: A Practical and Powerful Approach to Multiple Testing. *J. R. Stat. Soc. Ser. B* **1995**, *57*, 289–300. https://doi.org/10.1111/j.2517-6161.1995.tb02031.x.
73. Wickham, H. *Ggplot2: Elegant Graphics for Data Analysis*; Springer: New York, NY, USA, 2016; ISBN 978-3-319-24277-4.
74. Luo, W.; Friedman, M.S.; Shedden, K.; Hankenson, K.D.; Woolf, P.J. GAGE: Generally Applicable Gene Set Enrichment for Pathway Analysis. *BMC Bioinform.* **2009**, *10*, 161. https://doi.org/10.1186/1471-2105-10-161.
75. Luo, W.; Brouwer, C. Pathview: An R/Bioconductor Package for Pathway-Based Data Integration and Visualization. *Bioinformatics* **2013**, *29*, 1830–1831. https://doi.org/10.1093/bioinformatics/btt285.

76. Langfelder, P., & Horvath, S. (2008). WGCNA: an R package for weighted correlation network analysis. *BMC bioinformatics, 9*, 559. https://doi.org/10.1186/1471-2105-9-559
77. Litteljohn, D.; Nelson, E.; Hayley, S. IFN-Y Differentially Modulates Memory-Related Processes under Basal and Chronic Stressor Conditions. *Front. Cell. Neurosci.* **2014**, *8*, 391. https://doi.org/10.3389/fncel.2014.00391.
78. Lambert, G.; Johansson, M.; Ågren, H.; Friberg, P. Reduced Brain Norepinephrine and Dopamine Release in Treatment-Refractory Depressive Illness. *Arch. Gen. Psychiatry* **2000**, *57*, 787. https://doi.org/10.1001/archpsyc.57.8.787.
79. Gulbins, A.; Grassmé, H.; Hoehn, R.; Kohnen, M.; Edwards, M.J.; Kornhuber, J.; Gulbins, E. Role of Janus-Kinases in Major Depressive Disorder. *Neurosignals* **2016**, 71–80. https://doi.org/10.1159/000442613.
80. Kubera, M.; Basta-Kaim, A.; Papp, M. The Effect of Chronic Treatment with Imipramine on the Immunoreactivity of Animals Subjected to a Chronic Mild Stress Model of Depression. *Immunopharmacology* **1995**, *30*, 225–230. https://doi.org/10.1016/0162-3109(95)00026-P.
81. Fatima, A.; Hoeber, J.; Schuster, J.; Koshimizu, E.; Maya-Gonzalez, C.; Keren, B.; Mignot, C.; Akram, T.; Ali, Z.; Miyatake, S.; et al. Monoallelic and Bi-Allelic Variants in NCDN Cause Neurodevelopmental Delay, Intellectual Disability, and Epilepsy. *Am. J. Hum. Genet.* **2021**, *108*, 739–748. https://doi.org/10.1016/j.ajhg.2021.02.015.
82. Pan, Y.; Zhang, W.-Y.; Xia, X.; Kong, L.-D. Effects of Icariin on Hypothalamic-Pituitary-Adrenal Axis Action and Cytokine Levels in Stressed Sprague-Dawley Rats. *Biol. Pharm. Bull.* **2006**, *29*, 2399–2403. https://doi.org/10.1248/bpb.29.2399.
83. Wei, K.; Xu, Y.; Zhao, Z.; Wu, X.; Du, Y.; Sun, J.; Yi, T.; Dong, J.; Liu, B. Icariin Alters the Expression of Glucocorticoid Receptor, FKBP5 and SGK1 in Rat Brains Following Exposure to Chronic Mild Stress. *Int. J. Mol. Med.* **2016**, *38*, 337–344. https://doi.org/10.3892/ijmm.2016.2591.
84. Wesseling, H.; Rahmoune, H.; Tricklebank, M.; Guest, P.C.; Bahn, S. A Targeted Multiplexed Proteomic Investigation Identifies Ketamine-Induced Changes in Immune Markers in Rat Serum and Expression Changes in Protein Kinases/Phosphatases in Rat Brain. *J. Proteome Res.* **2015**, *14*, 411–421. https://doi.org/10.1021/pr5009493.

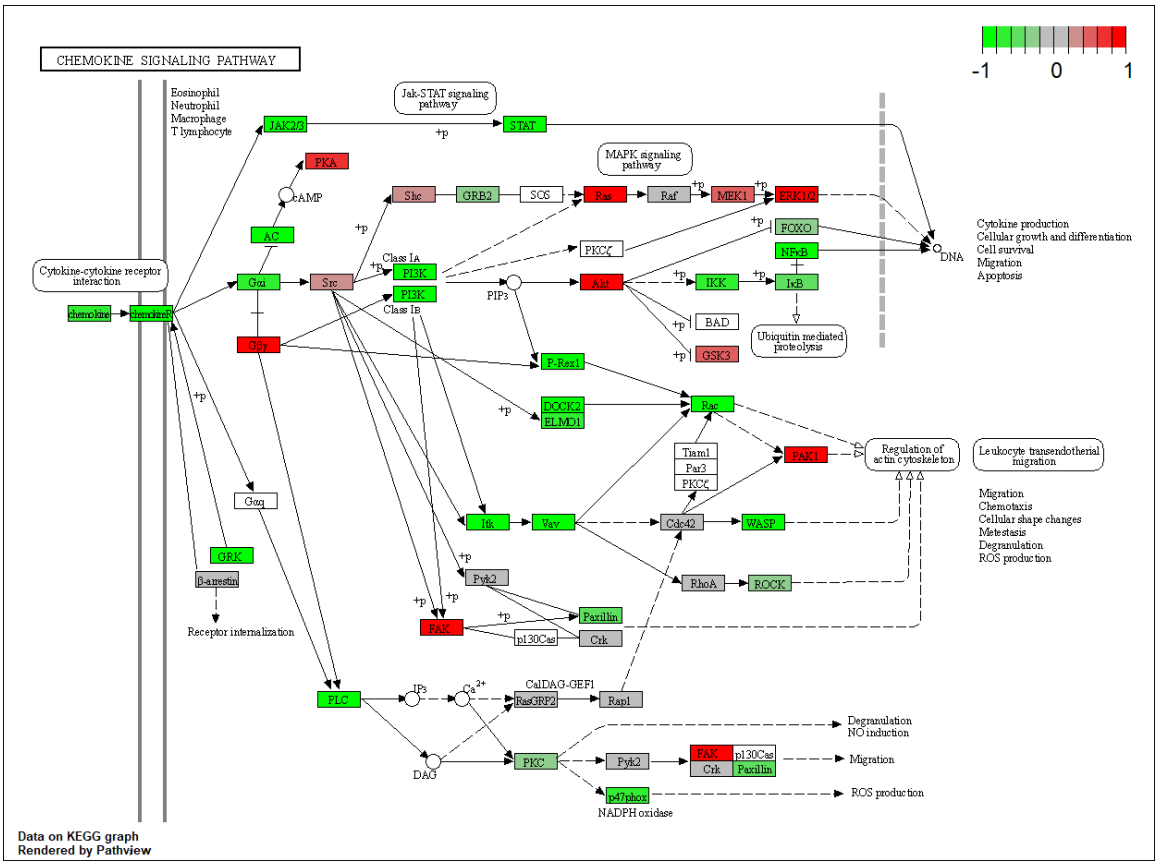
4.8 Supplementary Information



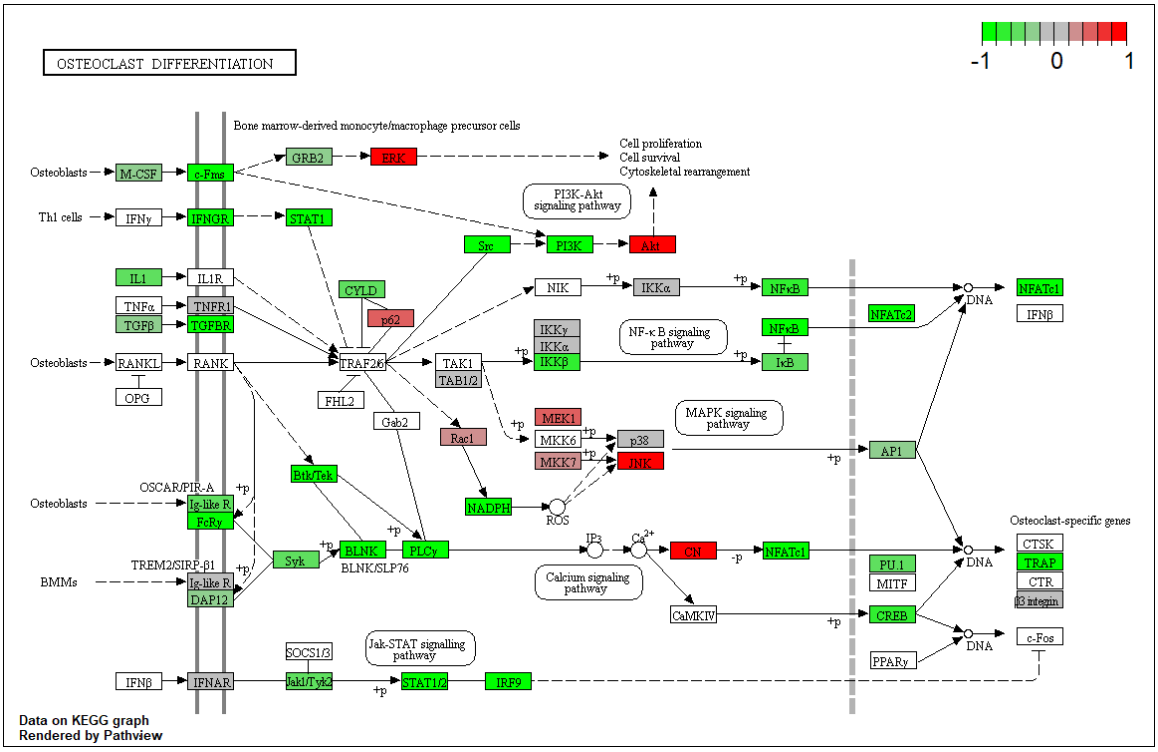
Supplementary Figure 1: Differentially expressed genes in the KEGG_RIBOSOME pathway in the blood of mice fed JB-1 compared to control.



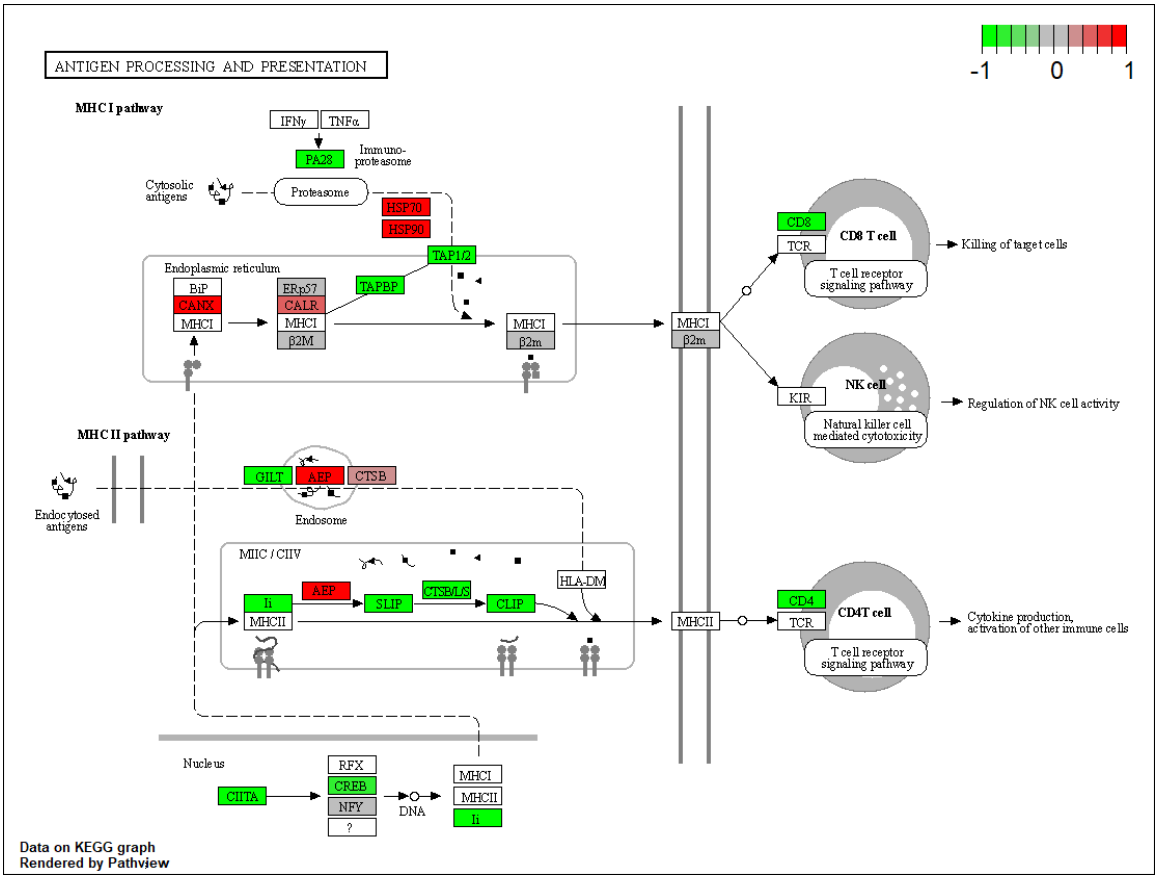
Supplementary Figure 2: Differentially expressed genes in the KEGG_CYTOKINE_CYTOKINE_RECEPTOR_INTERACTION pathway in the blood of mice fed JB-1 compared to control.



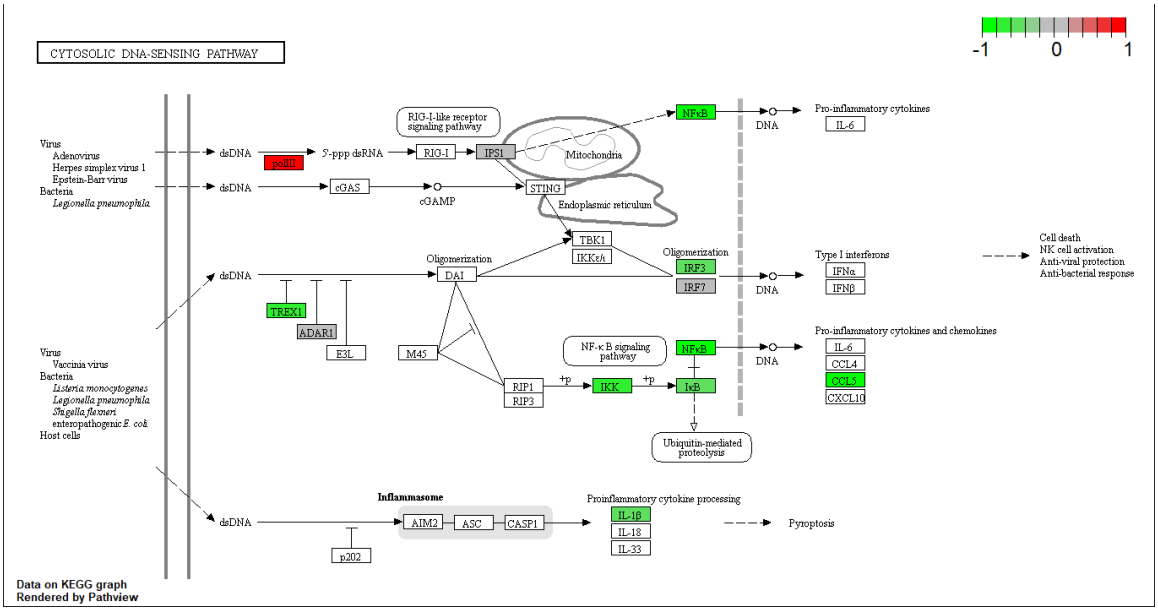
Supplementary Figure 3: Differentially expressed genes in the KEGG_CHEMOKINE_SIGNALING pathway in the blood of mice fed JB-1 compared to control.



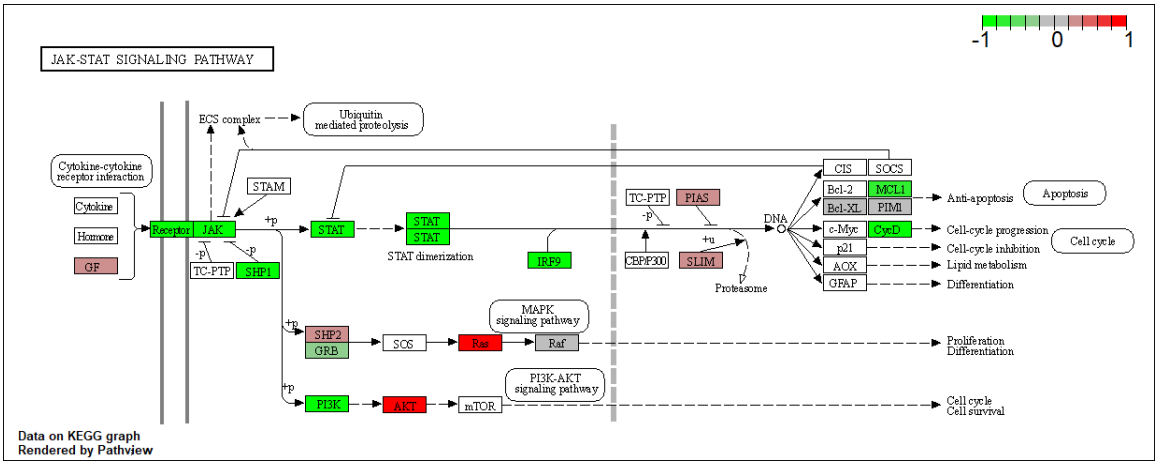
Supplementary Figure 4: Differentially expressed genes in the KEGG_OSTEOBLAST_DIFFERENTIATION pathway in the blood of mice fed JB-1 compared to control.



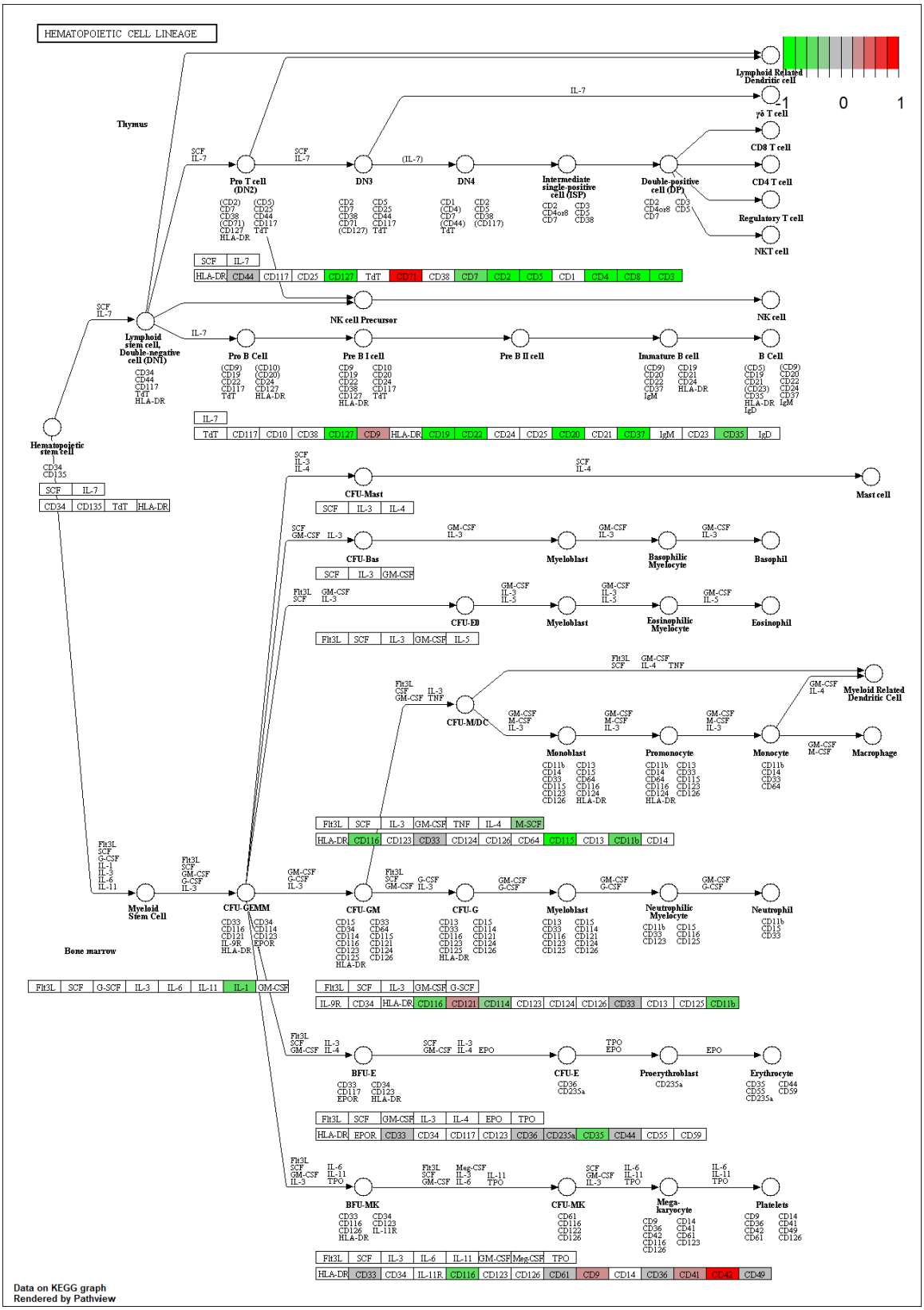
Supplementary Figure 5: Differentially expressed genes in the KEGG_ANTIGEN_PROCESSING_AND_PRESENTATION pathway in the blood of mice fed JB-1 compared to control.



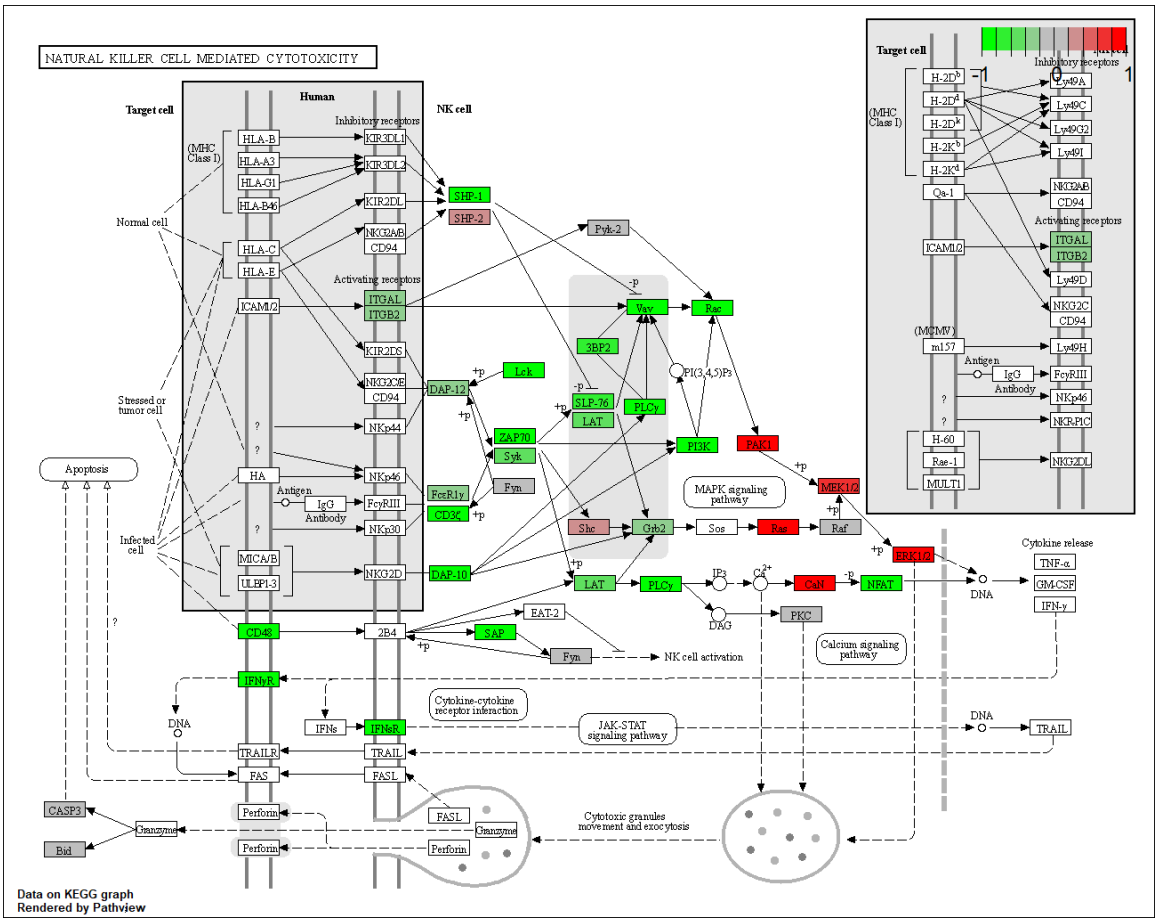
Supplementary Figure 6: Differentially expressed genes in the KEGG_CYTOSOLIC_DNA_SENSING pathway in the blood of mice fed JB-1 compared to control.



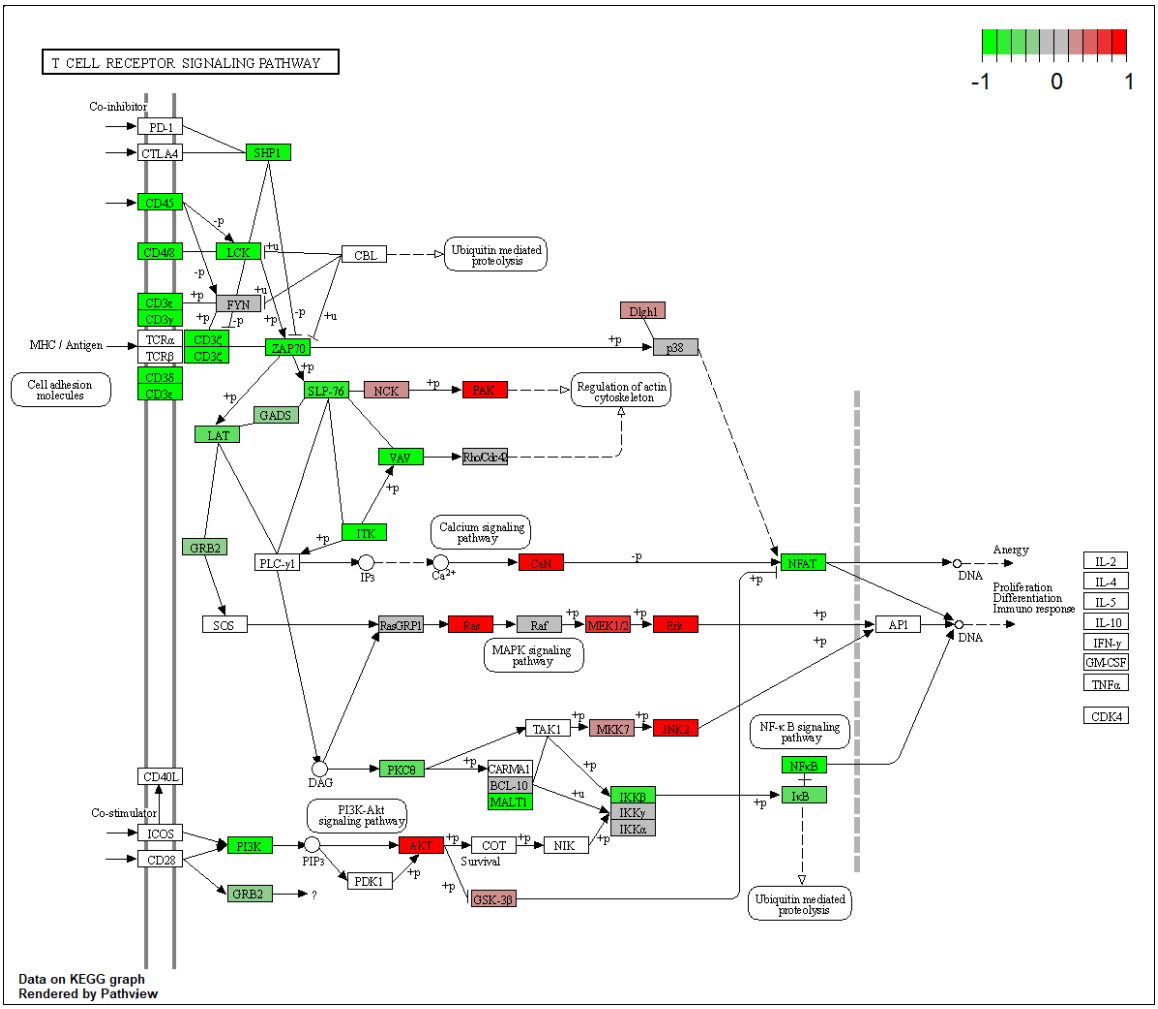
Supplementary Figure 7: Differentially expressed genes in the KEGG_JAK_STAT_SIGNALING pathway in the blood of mice fed JB-1 compared to control.


HEMATOPOIETIC CELL LINEAGE
-1
0
1
Lymphoid Related Dendritic cell
γδ T cell
CD8 T cell
CD4 T cell
Regulatory T cell
NKT cell
NK cell
Thymus
Pro T cell (DN2)
DN3
DN4
Intermediate single positive cell (ISP)
Double-positive cell (DP)
NK cell Precursor
Lymphoid stem cell, Double-negative cell (DN1)
Pro B Cell
Pre B I cell
Pre B II cell
Immature B cell
B Cell
Hematopoietic stem cell
CFU-Mast
Mast cell
CFU-Bas
Myeloblast
Basophilic Myelocyte
Basophil
CFU-Eo
Eosinophilic Myelocyte
Eosinophil
CFU-M/DC
Myeloid Related Dendritic Cell
Monoblast
Promonocyte
Monocyte
Macrophage
Myeloid Stem Cell
CFU-GEMM
CFU-GM
CFU-G
Neutrophilic Myelocyte
Neutrophil
Bone marrow
BFU-E
CFU-E
Proerythroblast
Erythrocyte
BFU-MK
CFU-MK
Mega-karyocyte
Platelets
Data on KEGG graph
Rendered by Pathview

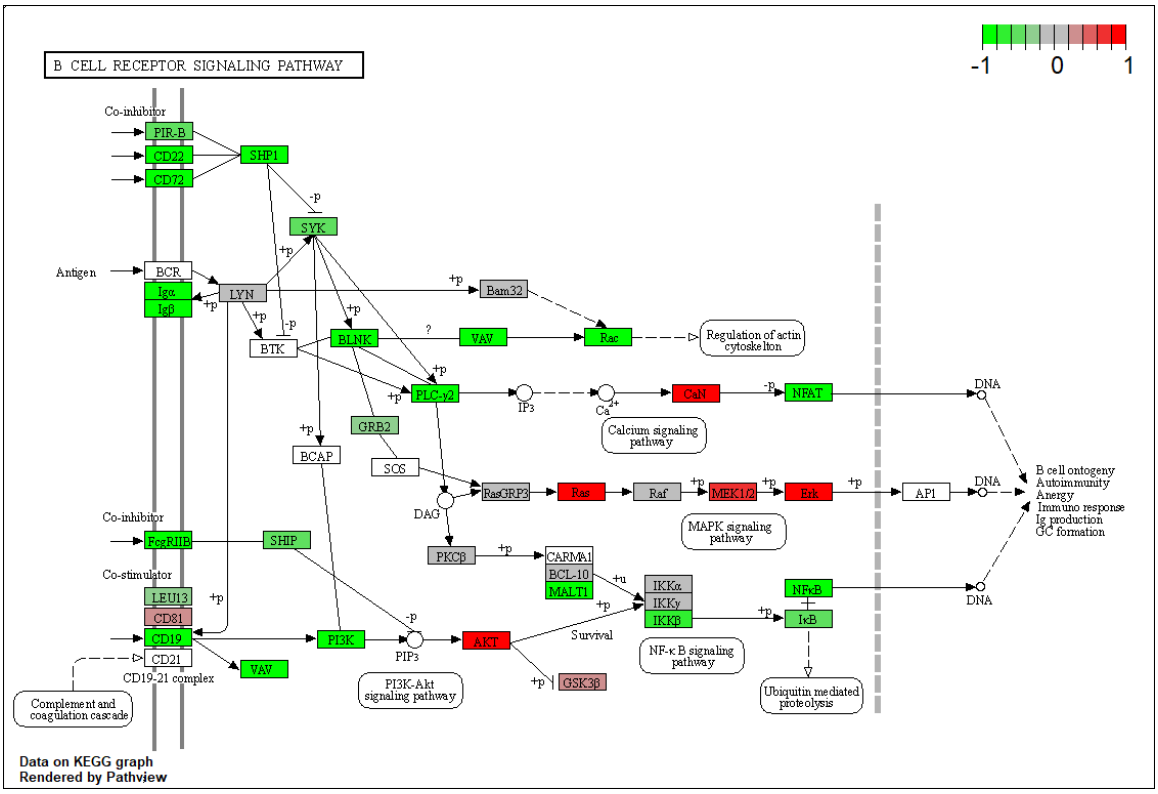
Supplementary Figure 8: Differentially expressed genes in the KEGG_HEMATOPOETIC_CELL_LINEAGE pathway in the blood of mice fed JB-1 compared to control.



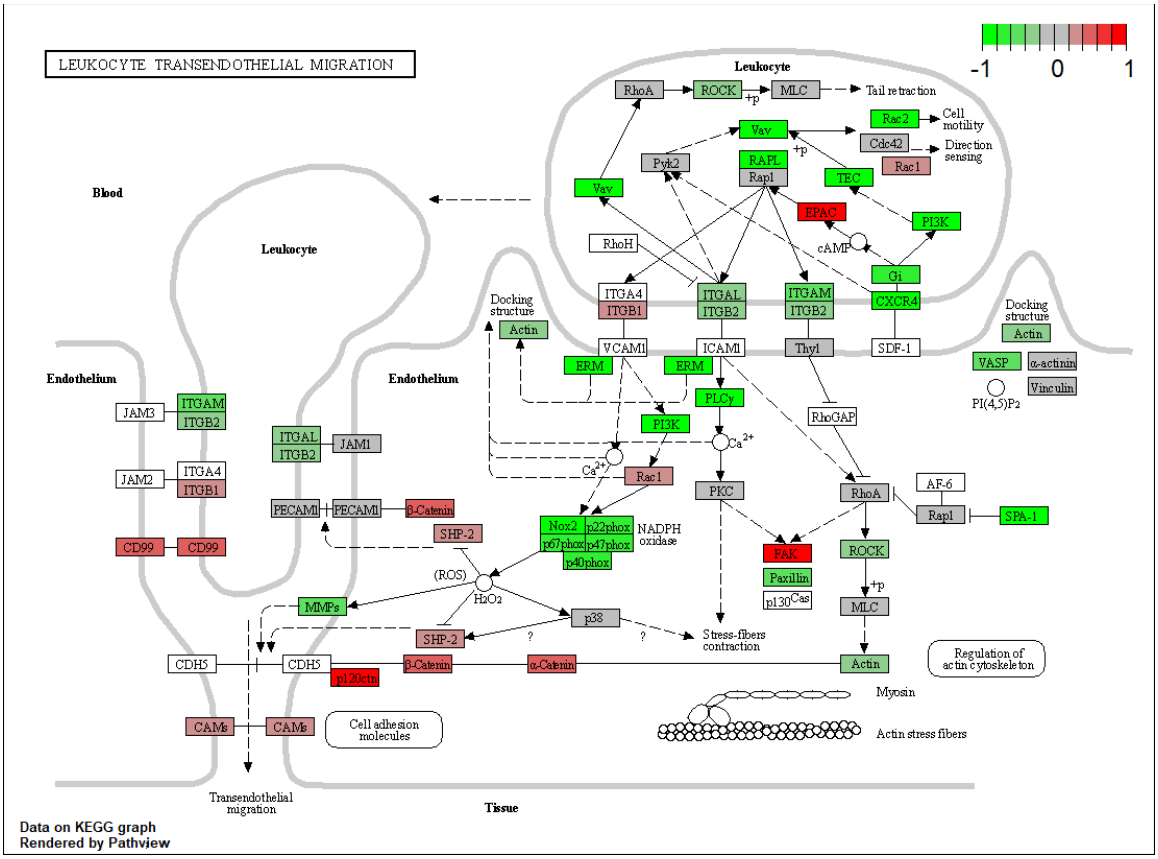
Supplementary Figure 9: Differentially expressed genes in the KEGG_NATURAL_KILLER_CELL_MEDIATED_CYTOTOXICITY pathway in the blood of mice fed JB-1 compared to control.



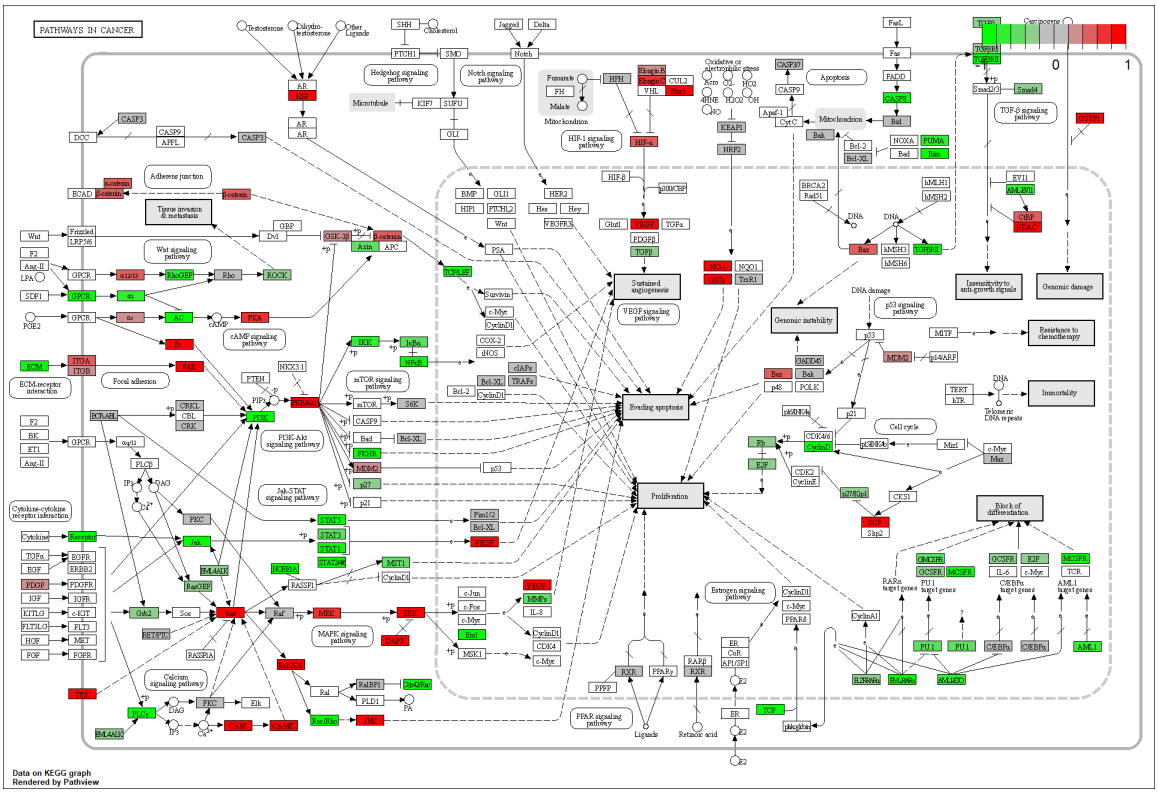
Supplementary Figure 10: Differentially expressed genes in the KEGG_T_CELL_RECEPTOR_SIGNALING pathway in the blood of mice fed JB-1 compared to control.



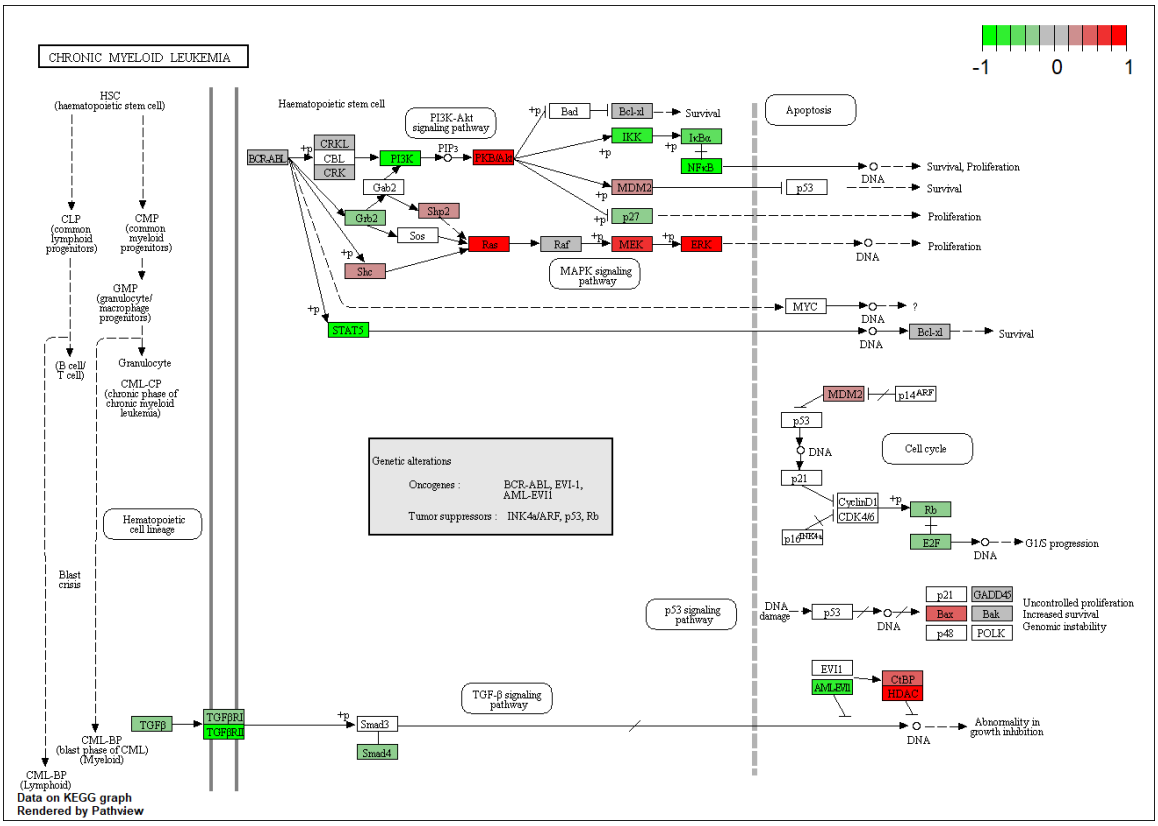
Supplementary Figure 11: Differentially expressed genes in the KEGG_B_CELL_RECEPTOR_SIGNALING pathway in the blood of mice fed JB-1 compared to control.



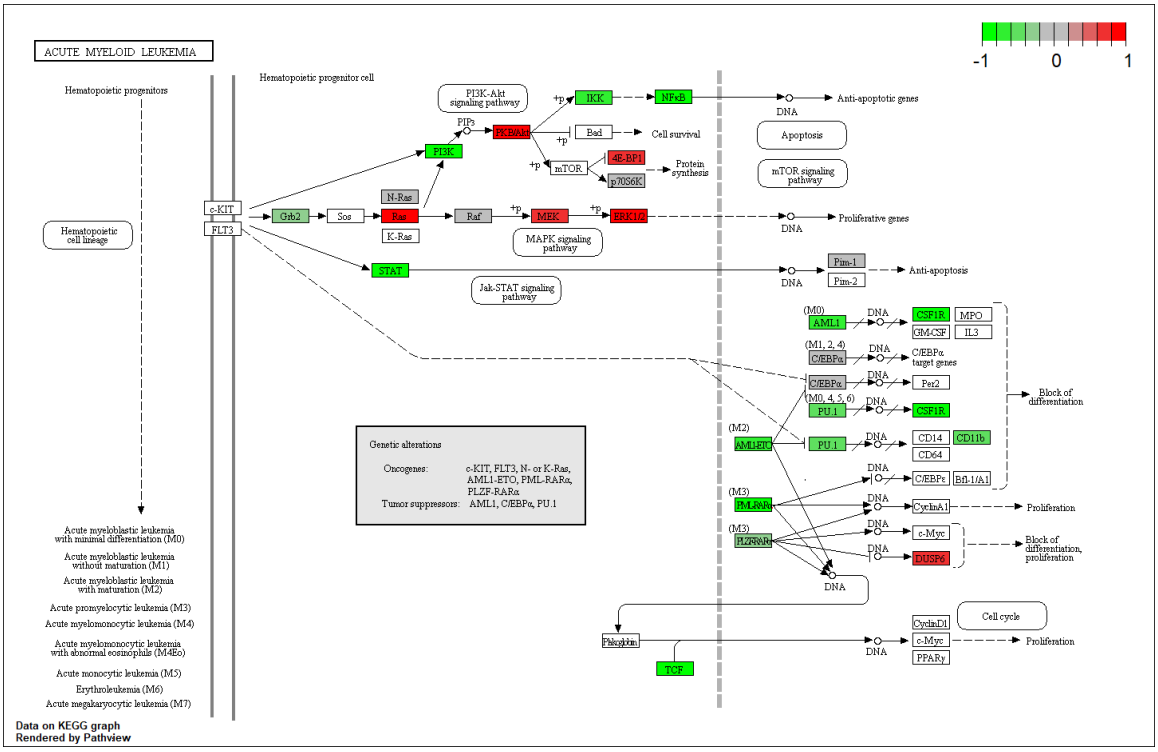
Supplementary Figure 12: Differentially expressed genes in the KEGG_LEUKOCYTE_TRANSENDOTHELIAL_MIGRATION pathway in the blood of mice fed JB-1 compared to control.



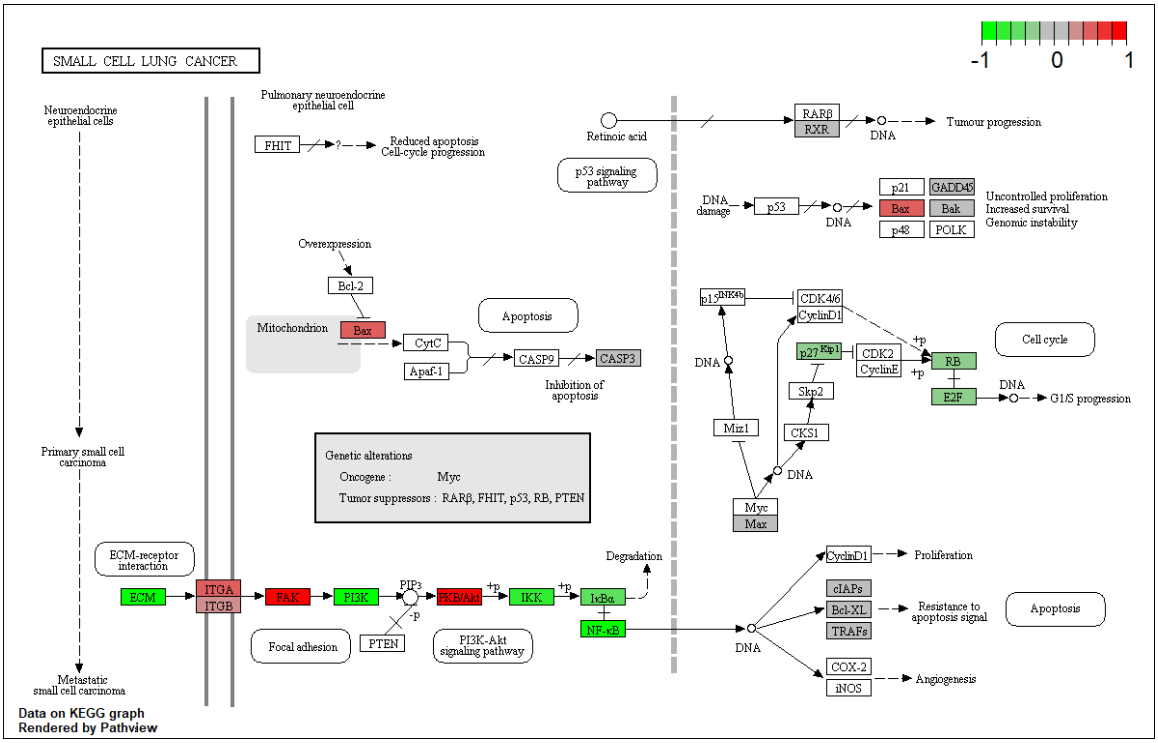
Supplementary Figure 13: Differentially expressed genes in the KEGG PATHWAYS IN CANCER in the blood of mice fed JB-1 compared to control.



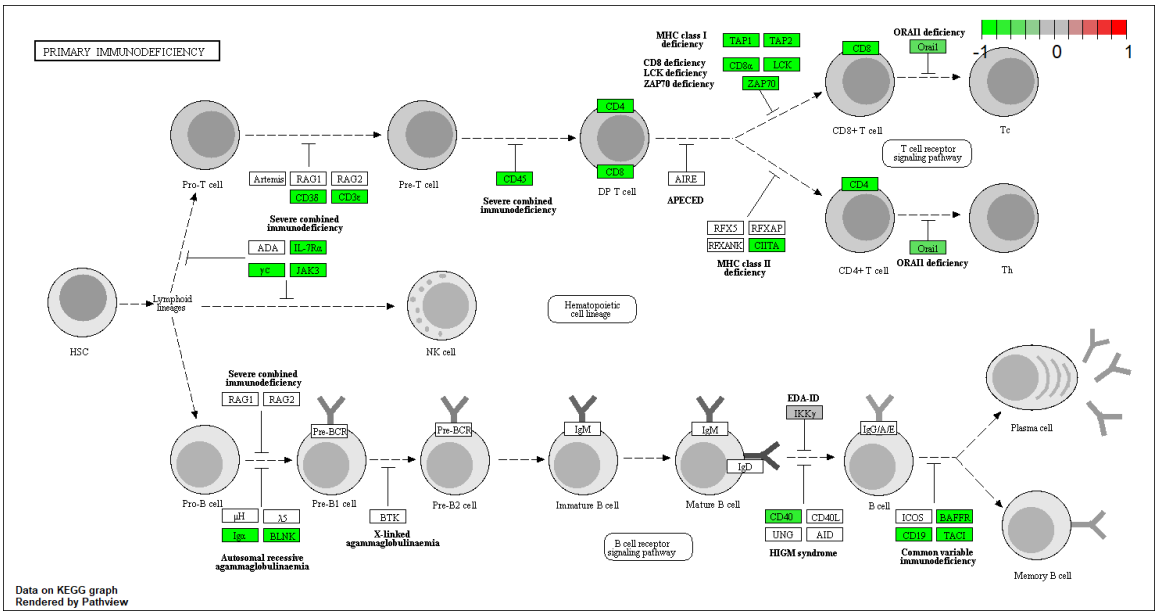
Supplementary Figure 14: Differentially expressed genes in the KEGG_CHRONIC_MYELOID_LEUKEMIA pathway in the blood of mice fed JB-1 compared to control.



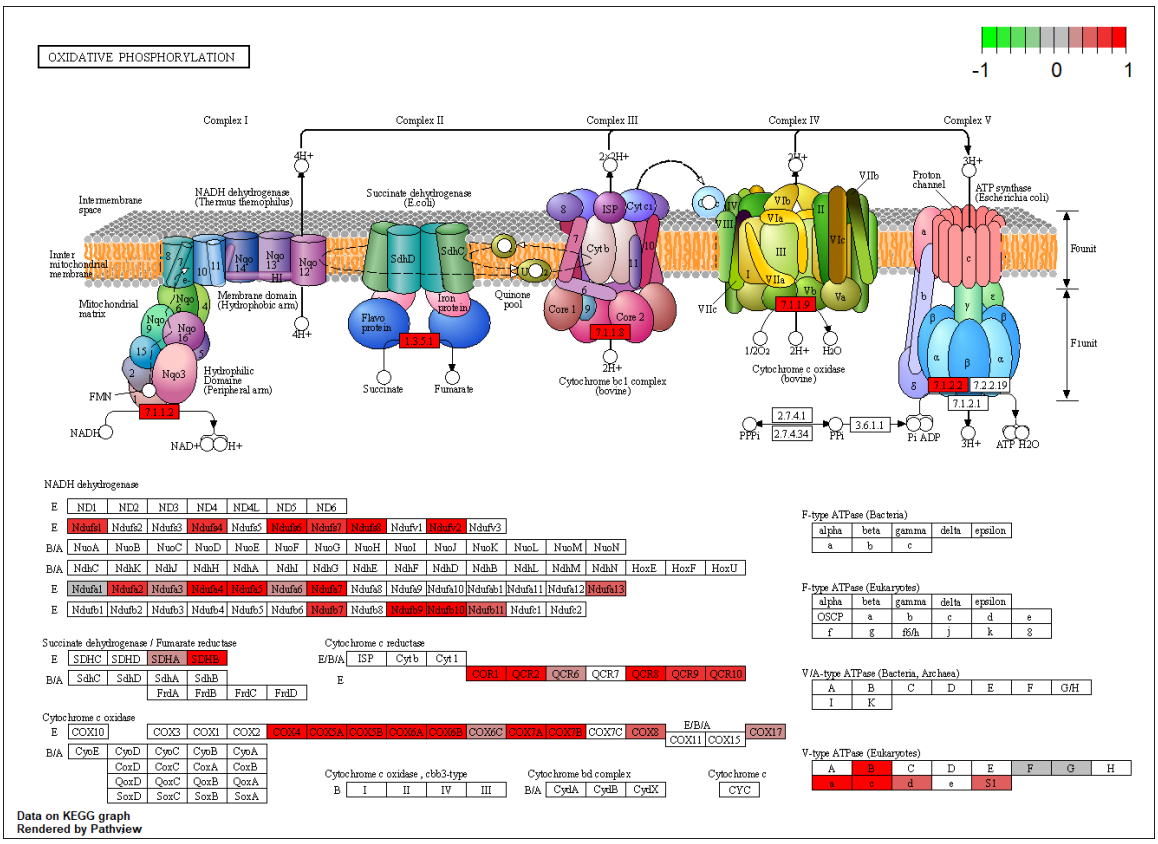
Supplementary Figure 15: Differentially expressed genes in the KEGG_ACUTE_MYELOID_LEUKEMIA pathway in the blood of mice fed JB-1 compared to control.



Supplementary Figure 16: Differentially expressed genes in the KEGG_SMALL_CELL_LUNG_CANCER pathway in the blood of mice fed JB-1 compared to control.



Supplementary Figure 17: Differentially expressed genes in the KEGG_PRIMARY_IMMUNODEFICIENCY pathway in the blood of mice fed JB-1 compared to control.



Supplementary Figure 18: Differentially expressed genes in the KEGG_OXIDATIVE_PHOSPHORYLATION pathway in the blood of mice fed JB-1 compared to control.

Chapter 5: Discussion and Conclusion:

These three separate studies were conducted examining blood 1) as a source of biomarkers, 2) to determine commonalities between comorbid diseases, and 3) in order to gain mechanistic insights. In each study differential expression and gene set enrichment analyses were performed on omics data from the blood or its components, illustrating the value of these types of analysis across different fields, as well as the seemingly universal utility of the blood for addressing challenges within medical science. Below, the results of each study are summarized, explaining the significance of the finding in the context of the study, and the contributions of each study to their fields are discussed.

5.1 Circulating EV-Derived Prostate Cancer Biomarkers

5.1.1 Summary of Results:

First, we identified that the majority of circulating EV in both prostate cancer patients, and healthy subjects were exosomes, while there were still some microvesicles. This is merely to show that there are no glaring differences between the types or quantity of EV isolated from the blood of cancer patients or healthy subjects. Within the circulating EV of prostate cancer patients, we identified 141 proteins that have the potential to act as biomarkers for prostate cancer, as they were found significantly more abundantly expressed in cancer patients but not in healthy subjects. Additionally we identified several proteins that appeared to be abundant exclusively within circulating EV of each of patients with organ confined tumours (57 proteins), extracapsular extending tumours (27 proteins), and seminal vesicle invading tumours (13 proteins). This suggests that circulating EV derived protein could also be used beyond a general diagnostic, to

further classify the stage of the prostate cancer. For general knowledge, a protein ontology analysis to assess the molecular function, biological process, and cellular component associated with all vesicular proteins, identified binding, localization, and extracellular localization, respectively, being the most abundant features, perhaps unsurprising for EV protein. Overall, many proteins were identified with the potential to act as biomarkers.

5.1.2 Contribution to the Field:

In 2008, exosomes derived from VCaP and PC346 prostate cancer cell lines were isolated by Jansen *et al* (2008). They identified several proteins known to be upregulated in prostate cancer, and first proposed that prostate tumour derived exosomes could be valuable sources of biomarkers for the disease (Jansen *et al*., 2008). In 2009, EVs isolated from the urine of prostate cancer patients were shown to contain prostate cancer RNA biomarkers, TMPRSS2:ERG, and PCA3 (Nilsson *et al*., 2009). Several body fluids have since been found to contain prostate derived EV due to the prostates proximity to the bladder, and its role in reproductive function (Drake and Kislinger, 2014; Ramirez-Garrastacho *et al*., 2022). These include blood, semen, and urine (Drake and Kislinger, 2014; Ramirez-Garrastacho *et al*., 2022), and in the last 12 years each have been tested as a potential source for vesicular biomarkers for prostate cancer (Drake *et al*., 2010; Ramirez-Garrastacho *et al*., 2022). Of the three fluids, semen contains the highest prostate-derived EV, however its collection is fairly invasive and, in older men with prostatic disease, collection can be extremely challenging if not impossible (Drake *et al*., 2010). Urine is the fluid least invasive to collect, however urine is highly dynamic, and

its concentration and composition vary in relation to diet, and fitness, which complicates the discovery and validation of biomarkers (Ramirez-Garrastacho *et al*., 2022). Blood is the fluid thought best suited to detecting metastatic prostate cancers via EVs (Ramirez-Garrastacho *et al*., 2022), and it is from blood the majority of urinary EVs have been shown to originate (Erdbrugger *et al*., 2021). Blood collection is more invasive than urine collection however, and it contains cell debris and other non-EV particles that can be difficult to remove during EV isolation (Ramirez-Garrastacho *et al*., 2022). 13 prostate cancer EV biomarker papers with 50 patients or more were published from 2010 to 2017, and in 30% of them, blood was the selected biofluid, compared with 70% for urine (Campos-Fernandez *et al*., 2019). This suggests that urine is the most popular biofluid, however, we chose to work with blood EVs because, along with its other advantages, we were interested in being able to detect differences between the stages of prostate cancer, leading to metastasis.

There were several biomarker searches conducted from 2012 to 2017 (when our work was done) that investigated circulating vesicle protein. Khan et al. (2012) identified the prostate cancer associated protein, 'survivin,' significantly increased in EV derived from cancer patients when compared to those from healthy subjects. PTEN was also found within blood EVs of prostate cancer patients, but not in those of healthy subjects (Gabriel *et al*., 2013). Some preliminary work had also been done to examine the differences in circulating EV protein that may be associated with prostate cancer progression: Park *et al*. (2016) showed that circulating EV PSMA was in higher abundance in accordance with gleason score. Our work however was the first to compare

circulating vesicle protein stage of the prostate cancer, as our samples came from patients with T2A, tumour confined to one lobe of the prostate; T3A, the tumour is expanding into the extracellular space; and T3B, the tumour has invaded the seminal vesicles. Similar studies on stage have been done looking at urine-derived EV protein, and found the same potential to differentiate between stages of prostate cancer, as well as metastatic vs non-metastatic (Sequeiros *et al*., 2017; Fujita *et al*., 2017; Bijnsdorp *et al*., 2013), but to this day our work is the only that has used blood-derived EV protein to attempt to biomark the stage of non-metastatic cancers.

Due to meta-analysis in the modern age, studies that repeat an experiment involving high-throughput data can also, still be beneficial to the field. With this in mind, the comparison between each tumour stage and healthy subjects is also valuable, albeit not novel. Another reason blood could be seen as more valuable than urine for collection and isolation of EV biomarkers, is that it can be used for many other types of cancers beyond prostate cancer. As discussed in the introduction, a lack of standardized collection methods for EVs is one of the major factors preventing their widespread use as biomarker vehicles. Blood vesicles have the unique potential to meet a standard across all cancer biomarkers, even if somewhat more invasive than urine collection. Perhaps in the future, our understanding of circulating vesicular biomarkers will be comprehensive enough that clinicians will prescribe an annual general cancer screen to detect multiple cancers with a single blood test.

5.2 mRNA Markers of Comorbidity Between Asthma, PTSD, and MDD

5.2.1 Summary of Results:

For each of the asthma, MDD, and PTSD datasets, a PCA was performed revealing no distinct groups between any groups of variables, suggesting that any differences between diseased patients and healthy subjects in each dataset were not glaring, and also that there was no variance along any other groups from metadata. Differential expression analysis of exploration datasets revealed differentially expressed genes for each disease between disordered patients and healthy subjects. Many of these genes were found to be differentially expressed in the same direction in multiple diseases including every combination of severe asthma, moderate asthma, PTSD, MDD, as well as every combination of these diseases. This meant that they had the potential to be drivers of comorbidity between diseases, and were genes of interest for that reason.

However, aside from genes commonly expressed in the blood of the asthma severities, only 6 genes could be validated, including: STX8, and ARHGAP24 upregulated in the blood of patients with PTSD and severe asthma; and *ORMDL3*, *PTP4A3*, *SHISA4*, and *TPPP3*, which were downregulated in the blood of patients with either PTSD or severe asthma. A gene set enrichment analysis uncovered a number of gene sets, particularly from the C7- immune signature gene set catalog, commonly upregulated in several of the diseases, including 65 C7 gene sets shared between all diseases. 5 immune signature gene sets and 1 curated gene set were validated as being either up or downregulated in the blood of both MDD and severe asthma patients, including:

GSE4748_CYANOBACTERIUM_LPSLIKE_VS_LPS_AND_CYANOBACTERIUM_LPSLIKE_STIM_DC_3H_DN, GSE34205_HEALTHY_VS_RSV_INF_INFANT_PBMC_DN, both more abundantly expressed, and GSE22886_NAIVE_BCELL_VS_NEUTROPHIL_UP, GSE34205_HEALTHY_VS_FLU_INF_INFANT_PBMC_UP, GSE22886_NEUTROPHIL_VS_MONOCYTE_DN, JISON_SICKLE_CELL_DISEASE_DN, all less abundantly expressed. These validated genes and gene sets could be drivers of, or associated with the comorbidity between diseases. STRING analysis was performed to identify networks of proteins coded by the genes in the differentially regulated gene sets between MDD and severe asthma. The analysis suggested multiple systems including translation and immune function may be altered differentially in MDD and severe asthma, which serves as an additional layer of evidence of their modulation in comorbidity.

5.2.2 Contribution to the Field:

The scientific community has known of the comorbidity between asthma and mental health disorders for decades (Goodwin *et al*., 2007; Katon *et al*., 2007), but there is still lacking a comprehensive understanding of what drives this comorbidity. As mentioned above, there is some evidence that cytokine dysregulation (overexpression of protein) may be involved (Jiang *et al*., 2014), and that there may be some underlying genetic factors determined by GWAS (Zhu *et al*., 2019). In addition to these possibilities, based on epidemiological data, early life stress, glucocorticoid resistance, and autonomic nervous system dysregulation have been suggested as drivers of comorbidity between

asthma and MDD (Van Lieshout *et al*., 2009). Our study takes advantage of data gathered to examine circulating mRNA biomarkers measured in whole blood, to build on the next logical step after GWAS studies - to look at transcription underlying the comorbidity, rather than polymorphisms of the genes themselves. This type of exploration and validation analysis between independent datasets has never been used previously to assess the transcriptional similarities between these diseases, and many of the results corroborate known malfunctioning or misexpressed proteins in other literature (ex. ORMDL3 and ceramide)(Kornhuber and Gulbins, 2021; Miller *et al*., 2017). Many other genes and gene sets we found differentially expressed appear to be entirely novel findings, worthy of further investigation.

Most differentially expressed gene sets were from the immune signature (C7) collection of gene sets, which also supports the notion that immune involvement is critical to the development of comorbid asthma and mood disorders. Transcriptomic signatures in the blood differ enough to potentially act as biomarkers for mood disorders (Rusch *et al*., 2019; Leday *et al*., 2018). Therefore, immune cells circulating between blood and brain may not only be the carriers of these unique transcriptomic signatures, but may also be the mediators of asthma and mood disorder comorbidity.

5.3 Blood and Hippocampal Expression of RNAs Following Psychobiotic Treatment

5.3.1 Summary of Results:

A PCA of circulating mRNA in our mice showed PBS and LR6475-fed mice clustered together, and JB1 fed mice scattered far from them. To determine why blood expression in JB1 mice differed so greatly from the other feed groups, a differential

expression analysis was performed. As the PCA would suggest, dozens of individual mRNAs were found significantly different from PBS-fed mice, and close to a dozen mRNAs were also found differentially upregulated in JB1-fed mice compared to LR6475-fed. Many of these were upregulated in JB-fed mice in both comparisons, including: FKBP1A, SNRPN, CELF4, GPM6B, APBB1, NCDN, RUNDC3A, CPE, and CLSTN1. As anticipated, there were no individually expressed circulating mRNAs between LR6475 and PBS-fed mice. JB1-fed mice became further differentiated from the other feed groups after performing a gene set enrichment analysis, which revealed several differentially expressed Hallmark and KEGG gene sets, expressed in both directions and in most cases in both JB1 feed group comparisons.

A PCA was performed on mRNA expression data in the hippocampus of these same mice, revealing a separation between PBS and psychobiotic fed mice clusters, with no clear distinction between LR6475 and JB1 fed mice. Some of the differences between PBS and JB1-fed mice could be explained by differential expression analysis of individual hippocampal genes, as two were found significantly upregulated in JB1-fed mice: TPPP3, and SGK1. Gene set enrichment analysis elucidated a bit more of the difference, as KEGG_RIBOSOME was enriched in the hippocampuses of both psychobiotic-fed mice compared to PBS-fed, and KEGG_VIBRO_CHOLERAE_INFECTION was also enriched in LR6475 vs PBS. This snapshot of ribonucleic acid expression in the blood and hippocampus of mice fed bacteria have helped to uncover some of the mechanisms behind how mood-altering bacteria may function.

Finally, a separate weighted correlation network analysis was conducted for both blood and hippocampal mRNA. In the blood, this revealed significant associations in two particular eigengenes with both JB1 and PBS, however no eigengenes were significantly associated with LR6475. In the hippocampus, JB1 and PBS had significant associations with eigengenes, but not the same ones. As in the blood, LR6475 was not significantly associated with any eigengenes. This analysis serves as a separate confirmation of differences between groups seen in the PCAs for blood and hippocampus, and is further justification for the results from the differential expression and enrichment analyses.

5.3.2 Contribution to the Field:

In previous studies done by our group that identified the vagus nerve, and immune system as necessary for the behavioral effects of JB1, a small amount of expression data from the hippocampus was also collected (Bravo *et al.*, 2011; Bharwani *et al*., 2017; Liu *et al*., 2020). This data revealed altered levels of GABA in different brain regions (Bravo *et al.*, 2011). As this data was collected via qPCR, however, a limited number of interesting potentially differentially expressed genes went unobserved. This work is both the first, and most thorough analysis of transcription in the hippocampus of mice fed JB1, as well as the blood. It is a detailed look at what transcriptional changes occur system-wide that may contribute to the anti-depressant and anti-anxiolytic effects seen after JB1 feeding.

Some differential expression analysis studies have been performed examining the effects of LR6475 feeding in relation to bone density (Collins et al., 2016; Quach *et al*., 2019). One study fed LR6475 to female mice, and examined differential transcript

expression in bone marrow and intestinal tissue (Collins *et al*., 2016). Quach *et al*. (2019) examined the differential expression of differentiating osteoclast cell line, RAW264.7 in the presence of LR6475. LR6475 has also been shown to regulate immune modulation and histamine production via expression of its gene rsiR (Hemarajata *et al*., 2013). Little is known about altered transcription induced by LR6475 feeding in the brain, although consumption has been shown to upregulate oxytocin in the hypothalamus (Buffington *et al*., 2016), which is thought to promote social behaviour by stimulating the mesolimbic dopamine reward system. Our work is the most thorough examination of LR6475 altered mRNA expression in the hippocampus and blood to date, and suggests that changes in social behaviour caused by LR6475 are not due to changes in transcription.

This comprehensive expression data, and detailed analysis of mRNA in the blood and hippocampus of mice fed two differently functioning psychobiotics serves the general understanding of the gut-brain axis as well, although it was not the focus of the study. Being able to compare the gene expression in mice after feeding mood-altering bacteria could prove valuable in future meta-analyses that seek to understand the brain, gut, and blood - through which they can communicate.

5.4 Concluding Remarks

Here, I, with the help of my colleagues: identified protein biomarkers for prostate cancer stages within EV circulating in the blood; assessed potential drivers of comorbidity between asthma, PTSD, and MDD, via an examination of transcription within the blood; and characterized many differentially expressed transcripts that may play a role in the mood-altering of psychiobiotics, within the hippocampus and blood of

mice. This body of work spans several fields, but together, it illustrates the utility of high-throughput omics data, the depth, and breadth of information that is contained within blood, and the versatility of that information for addressing manifold challenges within medical science.

Chapter 6: Thesis References:

Adkins, J. N., Varnum, S. M., Auberry, K. J., Moore, R. J., Angell, N. H., Smith, R. D., Springer, D. L., & Pounds, J. G. (2002). Toward a human blood serum proteome: analysis by multidimensional separation coupled with mass spectrometry. *Molecular & cellular proteomics : MCP*, *1*(12), 947–955. https://doi.org/10.1074/mcp.m200066-mcp200

Al-Azri M. H. (2016). Delay in Cancer Diagnosis: Causes and Possible Solutions. *Oman medical journal*, *31*(5), 325–326. https://doi.org/10.5001/omj.2016.65

Alberts B, Johnson A, Lewis J, et al. Molecular Biology of the Cell. 4th edition. New York: Garland Science; 2002. Chapter 24, The Adaptive Immune System. Available from: https://www.ncbi.nlm.nih.gov/books/NBK21070/

Al-Nedawi, K., Haas-Neill, S., Gangji, A., Ribic, C. M., Kapoor, A., & Margetts, P. (2019). Circulating microvesicle protein is associated with renal transplant outcome. *Transplant immunology*, *55*, 101210. https://doi.org/10.1016/j.trim.2019.06.002

Al-Nedawi, K., Mian, M. F., Hossain, N., Karimi, K., Mao, Y. K., Forsythe, P., Min, K.K.,Stanisz, A.M., Kunze, W.A., Bienenstock, J. (2015). Gut commensal microvesicles reproduce parent bacterial signals to host immune and enteric nervous systems. *FASEB Journal*, *29*(2), 684–695.

Al-Nedawi, K., Meehan, B., Micallef, J., Lhotak, V., May, L., Guha, A., & Rak, J. (2008). Intercellular transfer of the oncogenic receptor EGFRvIII by microvesicles derived from tumour cells. *Nature cell biology*, *10*(5), 619–624. https://doi.org/10.1038/ncb1725

Al Saleh, H. A., Haas-Neill, S., Al-Hashimi, A., Kapoor, A., Shayegan, B., Austin, R. C., & Al-Nedawi, K. (2018). Thrombotic characteristics of extracellular vesicles derived from prostate cancer cells. *The Prostate*, *78*(13), 953–961. https://doi.org/10.1002/pros.23653

Alvarez C, Gimenez R, Canas M, Vera R, Diaz-Garrido N, Badia J, and Baldoma L. (2019). Extracellular vesicles and soluble factors secreted by Escherichia coli Nissle 1917 and ECOR63 protect against enteropathogenic E. coli-induced intestinal epithelial barrier dysfunction. *BMC Microbiology* **19**(166).

Anfossi, N., André, P., Guia, S., Falk, C. S., Roetynck, S., Stewart, C. A., Breso, V., Frassati, C., Reviron, D., Middleton, D., Romagné, F., Ugolini, S., & Vivier, E. (2006). Human NK cell education by inhibitory receptors for MHC class I. *Immunity*, *25*(2), 331–342. https://doi.org/10.1016/j.immuni.2006.06.013

Avasthi, A., & Ghosh, A. (2014). Depression in primary care: challenges and controversies. *The Indian journal of medical research*, *139*(2), 188–190.

Awad, A. G., Rajagopalan, K., Bolge, S. C., and McDonnell, D. D. (2007). Quality of life among bipolar disorder patients misdiagnosed with major depressive disorder. *Primary care companion to the Journal of clinical psychiatry*. **9**(3):195–202.

Baranyai, T., Herczeg, K., Onódi, Z., Voszka, I., Módos, K., Marton, N., Nagy, G.,

Mäger, I., Wood, M. J., El Andaloussi, S., Pálinkás, Z., Kumar, V., Nagy, P., Kittel, Á., Buzás, E. I., Ferdinandy, P., & Giricz, Z. (2015). Isolation of Exosomes from Blood Plasma: Qualitative and Quantitative Comparison of Ultracentrifugation and Size Exclusion Chromatography Methods. *PloS one*, *10*(12), e0145686. https://doi.org/10.1371/journal.pone.0145686

Benjamini, Y., & Hochberg, Y. (1995). Controlling the False Discovery Rate: A Practical and Powerful Approach to Multiple Testing. *Journal of the Royal Statistical Society. Series B (Methodological)*, *57*(1), 289–300. http://www.jstor.org/stable/2346101

Bercik P, Denou E, Collins J, Jackson W, Lu J, Jury J, Deng Y, Blennerhassett P, Macri J, McCoy KD, Verdu EF, and Collins SM. (2011). The intestinal microbiota affect central levels of brain derived neurotrophic factor and behavior in mice. *Gastroenterology* **141**:599–609.

Bettegowda, C., Sausen, M., Leary, R. J., Kinde, I., Wang, Y., Agrawal, N., Bartlett, B. R., Wang, H., Luber, B., Alani, R. M., Antonarakis, E. S., Azad, N. S., Bardelli, A., Brem, H., Cameron, J. L., Lee, C. C., Fecher, L. A., Gallia, G. L., Gibbs, P., Le, D., … Diaz, L. A., Jr (2014). Detection of circulating tumor DNA in early- and late-stage human malignancies. *Science translational medicine*, *6*(224), 224ra24. https://doi.org/10.1126/scitranslmed.3007094

Betts, J. Gordon (2013). *Anatomy & physiology*. pp. 787–846. ISBN 978-1-938168-13-0.

Bezbradica, J. S., Gordy, L. E., Stanic, A. K., Dragovic, S., Hill, T., Hawiger, J., Unutmaz, D., Van Kaer, L., & Joyce, S. (2006). Granulocyte-macrophage colony-stimulating factor regulates effector differentiation of invariant natural killer T cells during thymic ontogeny. *Immunity*, *25*(3), 487–497. https://doi.org/10.1016/j.immuni.2006.06.017

Bharwani, A., Mian, M. F., Surette, M. G., Bienenstock, J., & Forsythe, P. (2017). Oral treatment with Lactobacillus rhamnosus attenuates behavioural deficits and immune changes in chronic social stress. *BMC medicine*, *15*(1), 7. https://doi.org/10.1186/s12916-016-0771-7

Bijnsdorp, I. V., Geldof, A. A., Lavaei, M., Piersma, S. R., van Moorselaar, R. J., & Jimenez, C. R. (2013). Exosomal ITGA3 interferes with non-cancerous prostate cell functions and is increased in urine exosomes of metastatic prostate cancer patients. *Journal of extracellular vesicles*, *2*, 10.3402/jev.v2i0.22097. https://doi.org/10.3402/jev.v2i0.22097

Brand, S. J., Moller, M., & Harvey, B. H. (2015). A Review of Biomarkers in Mood and Psychotic Disorders: A Dissection of Clinical vs. Preclinical Correlates. *Current neuropharmacology*, *13*(3), 324–368. https://doi.org/10.2174/1570159x13666150307004545

Bravo, J. A., Forsythe, P., Chew, M. V., Escaravage, E., Savignac, H. M., Dinan, T. G., Bienenstock, J., & Cryan, J. F. (2011). Ingestion of Lactobacillus strain regulates emotional behavior and central GABA receptor expression in a mouse via the vagus nerve. *Proceedings of the National Academy of Sciences of the United*

States of America, *108*(38), 16050–16055. https://doi.org/10.1073/pnas.1102999108

Buffington, S. A., Di Prisco, G. V., Auchtung, T. A., Ajami, N. J., Petrosino, J. F., & Costa-Mattioli, M. (2016). Microbial Reconstitution Reverses Maternal Diet-Induced Social and Synaptic Deficits in Offspring. *Cell*, *165*(7), 1762–1775. https://doi.org/10.1016/j.cell.2016.06.001

Bull MJ, Plummer NT. (2014). Part 1: The human gut microbiome in health and disease. *Integrative Medicine (Encinitas)* **13**:17-22.

Campos-Fernández, E., Barcelos, L. S., de Souza, A. G., Goulart, L. R., & Alonso-Goulart, V. (2019). Research landscape of liquid biopsies in prostate cancer. *American journal of cancer research*, *9*(7), 1309–1328.

Cantaert, T., Baeten, D., Tak, P. P., & van Baarsen, L. G. (2010). Type I IFN and TNFα cross-regulation in immune-mediated inflammatory disease: basic concepts and clinical relevance. *Arthritis research & therapy*, *12*(5), 219. https://doi.org/10.1186/ar3150

Chang, H. H., & Chen, P. S. (2020). Inflammatory Biomarkers for Mood Disorders - A Brief Narrative Review. *Current pharmaceutical design*, *26*(2), 236–243. https://doi.org/10.2174/1381612826666200115100726

Chen, K., Xu, W., Wilson, M., He, B., Miller, N. W., Bengtén, E., Edholm, E. S., Santini, P. A., Rath, P., Chiu, A., Cattalini, M., Litzman, J., B Bussel, J., Huang, B., Meini, A., Riesbeck, K., Cunningham-Rundles, C., Plebani, A., & Cerutti, A. (2009). Immunoglobulin D enhances immune surveillance by activating antimicrobial, proinflammatory and B cell-stimulating programs in basophils. *Nature immunology*, *10*(8), 889–898. https://doi.org/10.1038/ni.1748

Choi, J., Kim, Y. K., Han, P. L. (2019). Extracellular vesicles derived from Lactobacillus plantarum increase BDNF expression in cultured hippocampal neurons and produce antidepressant-like effects in mice. *Experimental Neurobiology*, *28*(2), 158–171.

Collins, F. L., Irwin, R., Bierhalter, H., Schepper, J., Britton, R. A., Parameswaran, N., & McCabe, L. R. (2016). Lactobacillus reuteri 6475 Increases Bone Density in Intact Females Only under an Inflammatory Setting. *PloS one*, *11*(4), e0153180. https://doi.org/10.1371/journal.pone.0153180

Coumans, F., Brisson, A. R., Buzas, E. I., Dignat-George, F., Drees, E., El-Andaloussi, S., Emanueli, C., Gasecka, A., Hendrix, A., Hill, A. F., Lacroix, R., Lee, Y., van Leeuwen, T. G., Mackman, N., Mäger, I., Nolan, J. P., van der Pol, E., Pegtel, D. M., Sahoo, S., Siljander, P., … Nieuwland, R. (2017). Methodological Guidelines to Study Extracellular Vesicles. *Circulation research*, *120*(10), 1632–1648. https://doi.org/10.1161/CIRCRESAHA.117.309417

Cullen, S. P., & Martin, S. J. (2008). Mechanisms of granule-dependent killing. *Cell death and differentiation*, *15*(2), 251–262. https://doi.org/10.1038/sj.cdd.4402244

Cvjetkovic, A., Jang, S., Konečná, B., Hoog, J.L., Sihlbom, C., Lasser, C., & Lotvall, J.

(2016). Detailed Analysis of Protein Topology of Extracellular Vesicles–Evidence of Unconventional Membrane Protein Orientation. *Scientific Reports, 6,* 36338.https://doi.org/10.1038/srep36338

Danne, C., Ryzhakov, G., Martínez-López, M., Ilott, N. E., Franchini, F., Cuskin, F., Lowe, E. C., Bullers, S. J., Arthur, J., & Powrie, F. (2017). A Large Polysaccharide Produced by Helicobacter hepaticus Induces an Anti-inflammatory Gene Signature in Macrophages. *Cell host & microbe*, *22*(6), 733–745.e5. https://doi.org/10.1016/j.chom.2017.11.002

Dean L. Blood Groups and Red Cell Antigens [Internet]. Bethesda (MD): National Center for Biotechnology Information (US); 2005. Chapter 1, Blood and the cells it contains. Available from: https://www.ncbi.nlm.nih.gov/books/NBK2263/

Deng, Z., Zhang, Q., Zhao, Z., Li, Y., Chen, X., Lin, Z., Deng, Z., Liu, J., Duan, L., Wang, D., & Li, W. (2021). Crosstalk between immune cells and bone cells or chondrocytes. *International immunopharmacology*, *101*(Pt A), 108179. https://doi.org/10.1016/j.intimp.2021.108179

Devarakonda, S., & Govindan, R. (2019). Biomarker-Driven Staging-Are We There Yet?. *JAMA network open*, *2*(12), e1917052. https://doi.org/10.1001/jamanetworkopen.2019.17052

Di Gioia, D., Blankenburg, I., Nagel, D., Heinemann, V., & Stieber, P. (2016). Tumor markers in the early detection of tumor recurrence in breast cancer patients: CA 125, CYFRA 21-1, HER2 shed antigen, LDH and CRP in combination with CEA and CA 15-3. *Clinica chimica acta; international journal of clinical chemistry*, *461*, 1–7. https://doi.org/10.1016/j.cca.2016.07.014

Drake, R. R., Elschenbroich, S., Lopez-Perez, O., Kim, Y., Ignatchenko, V., Ignatchenko, A., Nyalwidhe, J. O., Basu, G., Wilkins, C. E., Gjurich, B., Lance, R. S., Semmes, O. J., Medin, J. A., & Kislinger, T. (2010). In-depth proteomic analyses of direct expressed prostatic secretions. *Journal of proteome research*, *9*(5), 2109–2116. https://doi.org/10.1021/pr1001498

Drake, R. R., & Kislinger, T. (2014). The proteomics of prostate cancer exosomes. *Expert review of proteomics*, *11*(2), 167–177. https://doi.org/10.1586/14789450.2014.890894

Duffy M. J. (2001). Clinical uses of tumor markers: a critical review. *Critical reviews in clinical laboratory sciences*, *38*(3), 225–262. https://doi.org/10.1080/20014091084218

Duffy M. J. (2013). Tumor markers in clinical practice: a review focusing on common solid cancers. *Medical principles and practice : international journal of the Kuwait University, Health Science Centre*, *22*(1), 4–11. https://doi.org/10.1159/000338393

Duffy, M. J., McDermott, E. W., & Crown, J. (2018). Blood-based biomarkers in breast cancer: From proteins to circulating tumor cells to circulating tumor DNA. *Tumour biology : the journal of the International Society for Oncodevelopmental Biology and Medicine*, *40*(5), 1010428318776169. https://doi.org/10.1177/1010428318776169

Elmentaite, R., Teichmann, S.A., & Madissoon, E. (2019). Studying immune to non-immune cell cross-talk using single-cell technologies. Current Opinion in Systems Biology, 18, 87-94. https://doi.org/10.1016/j.coisb.2019.10.005

Enelund, L., Nielsen, L. N., & Cirera, S. (2017). Evaluation of microRNA Stability in Plasma and Serum from Healthy Dogs. *MicroRNA (Shariqah, United Arab Emirates)*, *6*(1), 42–52. https://doi.org/10.2174/2211536606666170113124114

Erdbrügger, U., Blijdorp, C. J., Bijnsdorp, I. V., Borràs, F. E., Burger, D., Bussolati, B., Byrd, J. B., Clayton, A., Dear, J. W., Falcón-Pérez, J. M., Grange, C., Hill, A. F., Holthöfer, H., Hoorn, E. J., Jenster, G., Jimenez, C. R., Junker, K., Klein, J., Knepper, M. A., Koritzinsky, E. H., … Martens-Uzunova, E. S. (2021). Urinary extracellular vesicles: A position paper by the Urine Task Force of the International Society for Extracellular Vesicles. *Journal of extracellular vesicles*, *10*(7), e12093. https://doi.org/10.1002/jev2.12093

Fond, A. M., & Ravichandran, K. S. (2016). Clearance of Dying Cells by Phagocytes: Mechanisms and Implications for Disease Pathogenesis. *Advances in experimental medicine and biology*, *930*, 25–49. https://doi.org/10.1007/978-3-319-39406-0_2

Fu, F., Jiang, W., Zhou, L., & Chen, Z. (2018). Circulating Exosomal miR-17-5p and miR-92a-3p Predict Pathologic Stage and Grade of Colorectal Cancer. *Translational oncology*, *11*(2), 221–232. https://doi.org/10.1016/j.tranon.2017.12.012

Fujita, K., Kume, H., Matsuzaki, K., Kawashima, A., Ujike, T., Nagahara, A., Uemura, M., Miyagawa, Y., Tomonaga, T., & Nonomura, N. (2017). Proteomic analysis of urinary extracellular vesicles from high Gleason score prostate cancer. *Scientific reports*, *7*, 42961. https://doi.org/10.1038/srep42961

Gabriel, K., Ingram, A., Austin, R., Kapoor, A., Tang, D., Majeed, F., Qureshi, T., & Al-Nedawi, K. (2013). Regulation of the tumor suppressor PTEN through exosomes: a diagnostic potential for prostate cancer. *PloS one*, *8*(7), e70047. https://doi.org/10.1371/journal.pone.0070047

Gaffen S. L. (2009). Structure and signalling in the IL-17 receptor family. *Nature reviews. Immunology*, *9*(8), 556–567. https://doi.org/10.1038/nri2586

Gerlinger, M., Rowan, A. J., Horswell, S., Math, M., Larkin, J., Endesfelder, D., Gronroos, E., Martinez, P., Matthews, N., Stewart, A., Tarpey, P., Varela, I., Phillimore, B., Begum, S., McDonald, N. Q., Butler, A., Jones, D., Raine, K., Latimer, C., Santos, C. R., … Swanton, C. (2012). Intratumor heterogeneity and branched evolution revealed by multiregion sequencing. *The New England journal of medicine*, *366*(10), 883–892. https://doi.org/10.1056/NEJMoa1113205

Goodrich JK, Waters JL, Poole AC, Sutter JL, Koren O, Blekhman R, Beaumont M, Van Treuren W, Knight R, Bell JT, Spector TD, Clark AG, and Ley RE (2014). Human genetics shape the gut microbiome. *Cell* **159**:789-99.

Goodwin, R. D., Fischer, M. E., & Goldberg, J. (2007). A twin study of post-traumatic stress disorder symptoms and asthma. *American journal of respiratory and critical care medicine*, *176*(10), 983–987. https://doi.org/10.1164/rccm.200610-1467OC

Govorukhina, N. I., Keizer-Gunnink, A., van der Zee, A. G., de Jong, S., de Bruijn, H. W., & Bischoff, R. (2003). Sample preparation of human serum for the analysis of tumor markers. Comparison of different approaches for albumin and gamma-globulin depletion. *Journal of chromatography. A*, *1009*(1-2), 171–178. https://doi.org/10.1016/s0021-9673(03)00921-x

Haas-Neill, S., and Forsythe, P. (2020). A budding relationship: Bacterial Extracellular Vesicles in the Microbiota-Gut-Brain Axis. *International Journal of Molecular Sciences*. **21**(23):8899.

Hagai, T., Chen, X., Miragaia, R. J., Rostom, R., Gomes, T., Kunowska, N., Henriksson, J., Park, J. E., Proserpio, V., Donati, G., Bossini-Castillo, L., Vieira Braga, F. A., Naamati, G., Fletcher, J., Stephenson, E., Vegh, P., Trynka, G., Kondova, I., Dennis, M., Haniffa, M., … Teichmann, S. A. (2018). Gene expression variability across cells and species shapes innate immunity. *Nature*, *563*(7730), 197–202. https://doi.org/10.1038/s41586-018-0657-2

Hamilton, W., Stapley, S., Campbell, C., Lyratzopoulos, G., Rubin, G., & Neal, R. D. (2015). For which cancers might patients benefit most from expedited symptomatic diagnosis? Construction of a ranking order by a modified Delphi technique. *BMC cancer*, *15*, 820. https://doi.org/10.1186/s12885-015-1865-x

Hampel, H., Frank, R., Broich, K., Teipel, S. J., Katz, R. G., Hardy, J., Herholz, K., Bokde, A. L., Jessen, F., Hoessler, Y. C., Sanhai, W. R., Zetterberg, H., Woodcock, J., & Blennow, K. (2010). Biomarkers for Alzheimer's disease: academic, industry and regulatory perspectives. *Nature reviews. Drug discovery*, *9*(7), 560–574. https://doi.org/10.1038/nrd3115

Han M, Pang B, Zhou C, Li X, Wang Q, Jiang J, Li Y. (2022). Liquid biopsy of extracellular vesicle biomarkers for prostate cancer personalized treatment decision. *Extracell Vesicles Circ Nucleic Acids* 3:3-9. http://dx.doi.org/10.20517/evcna.2021.20

Hartwick, J., Barki, H. (1994). Research Report—Hypothesis Testing and Hypothesis Generating Research: An Example from the User Participation Literature. *Information Systems Research, 5*(4), 446-449. https://doi.org/10.1287/isre.5.4.446

He, K., Guo, C., He, L., & Shi, Y. (2017). MiRNAs of peripheral blood as the biomarker of schizophrenia. *Hereditas*, *155*, 9. https://doi.org/10.1186/s41065-017-0044-2

Henry, N. L., & Hayes, D. F. (2012). Cancer biomarkers. *Molecular oncology*, *6*(2), 140–146. https://doi.org/10.1016/j.molonc.2012.01.010

Hemarajata, P., Gao, C., Pflughoeft, K. J., Thomas, C. M., Saulnier, D. M., Spinler, J. K., & Versalovic, J. (2013). Lactobacillus reuteri-specific immunoregulatory gene rsiR modulates histamine production and immunomodulation by Lactobacillus reuteri. *Journal of bacteriology*, *195*(24), 5567–5576. https://doi.org/10.1128/JB.00261-13

Holford, N. C., Sandhu, H. S., Thakkar, H., Butt, A. N., & Swaminathan, R. (2008). Stability of beta-actin mRNA in plasma. *Annals of the New York Academy of Sciences*, *1137*, 108–111. https://doi.org/10.1196/annals.1448.006

Hornick, N. I., Huan, J., Doron, B., Goloviznina, N. A., Lapidus, J., Chang, B. H., &

Kurre, P. (2015). Serum Exosome MicroRNA as a Minimally-Invasive Early Biomarker of AML. *Scientific reports*, *5*, 11295. https://doi.org/10.1038/srep11295

Hoshino, A., Costa-Silva, B., Shen, T. L., Rodrigues, G., Hashimoto, A., Tesic Mark, M., Molina, H., Kohsaka, S., Di Giannatale, A., Ceder, S., Singh, S., Williams, C., Soplop, N., Uryu, K., Pharmer, L., King, T., Bojmar, L., Davies, A. E., Ararso, Y., Zhang, T., … Lyden, D. (2015). Tumour exosome integrins determine organotropic metastasis. *Nature*, *527*(7578), 329–335. https://doi.org/10.1038/nature15756

Huang, L. H., Lin, P. H., Tsai, K. W., Wang, L. J., Huang, Y. H., Kuo, H. C., & Li, S. C. (2017). The effects of storage temperature and duration of blood samples on DNA and RNA qualities. *PloS one*, *12*(9), e0184692. https://doi.org/10.1371/journal.pone.0184692

Humer, E., Pieh, C., & Probst, T. (2020). Metabolomic Biomarkers in Anxiety Disorders. *International journal of molecular sciences*, *21*(13), 4784. https://doi.org/10.3390/ijms21134784

Incisive Health. (2014). *Saving lives, averting costs: An analysis of the financial implications of achieving earlier diagnosis of colorectal, lung and ovarian cancer*. Cancer Research UK. https://www.cancerresearchuk.org/sites/default/files/saving_lives_averting_costs.pdf

Jacobson, A., Yang, D., Vella, M., & Chiu, I. M. (2021). The intestinal neuro-immune axis: crosstalk between neurons, immune cells, and microbes. *Mucosal immunology*, *14*(3), 555–565. https://doi.org/10.1038/s41385-020-00368-1

Janeway CA Jr, Travers P, Walport M, et al. Immunobiology: The Immune System in Health and Disease. 5th edition. New York: Garland Science; 2001. T cell-mediated cytotoxicity. Available from: https://www.ncbi.nlm.nih.gov/books/NBK27101/

Jansen, F.H., Krijgsveld, J., Willemsen, R., Luider, T.M., Van Rijswijk, A., and Jenster, G. (2008). Prostate cancer-derived exosomes as novel biomarker treasure chests. European Urology Supplements, 7(3), 255. https://doi.org/10.1016/S1569-9056(08)60733-2

Jiang, M., Qin, P., & Yang, X. (2014). Comorbidity between depression and asthma via immune-inflammatory pathways: a meta-analysis. *Journal of affective disorders*, *166*, 22–29. https://doi.org/10.1016/j.jad.2014.04.027

Johansen, F. E., Pekna, M., Norderhaug, I. N., Haneberg, B., Hietala, M. A., Krajci, P., Betsholtz, C., & Brandtzaeg, P. (1999). Absence of epithelial immunoglobulin A transport, with increased mucosal leakiness, in polymeric immunoglobulin receptor/secretory component-deficient mice. *The Journal of experimental medicine*, *190*(7), 915–922. https://doi.org/10.1084/jem.190.7.915

Justiz Vaillant, A. A., & Qurie, A. (2021). Interleukin. In *StatPearls*. StatPearls Publishing.

Kalra, H., Drummen, G. P., & Mathivanan, S. (2016). Focus on Extracellular Vesicles:

Introducing the Next Small Big Thing. *International journal of molecular sciences*, *17*(2), 170. https://doi.org/10.3390/ijms17020170

Kandasamy S, Vlasova AN, Fischer D, Kumar A, Chattha KS, Rauf A, Shao L, Langel SN, Rajashekara G, and Saif LJ. (2016). Differential Effects of Escherichia coli Nissle and Lactobacillus rhamnosus Strain GG on Human Rotavirus Binding, Infection, and B Cell Immunity. *Journal of Immunology* **196**(4):1780-9.

Kaparakis, M., Turnbull, L., Carneiro, L., Firth, S., Coleman, H.A., Parkington, H.C., Le Bourhis, L., Karrar, A., Viala, J., Mak, J., Hutton, M.L., Davies, J.K., Crack, P.J., Hertzog, P.J., Philpott, D.J., Girardin, S.E., Whitchurch, C.B., Ferrero, R.L. (2010). Bacterial membrane vesicles deliver peptidoglycan to NOD1 in epithelial cells. Cellular Microbiology, 12(3), 372-385.

Karsten, E., Breen, E., & Herbert, B. R. (2018). Red blood cells are dynamic reservoirs of cytokines. *Scientific reports*, *8*(1), 3101. https://doi.org/10.1038/s41598-018-21387-w

Katon, W., Lozano, P., Russo, J., McCauley, E., Richardson, L., & Bush, T. (2007). The prevalence of DSM-IV anxiety and depressive disorders in youth with asthma compared with controls. *The Journal of adolescent health : official publication of the Society for Adolescent Medicine*, *41*(5), 455–463. https://doi.org/10.1016/j.jadohealth.2007.05.023

Kaufman, J., & Charney, D. (2000). Comorbidity of mood and anxiety disorders. *Depression and anxiety*, *12 Suppl 1*, 69–76. https://doi.org/10.1002/1520-6394(2000)12:1+<69::AID-DA9>3.0.CO;2-K

Kawasaki, T., & Kawai, T. (2014). Toll-like receptor signaling pathways. *Frontiers in immunology*, *5*, 461. https://doi.org/10.3389/fimmu.2014.00461

Khan, S., Jutzy, J. M., Valenzuela, M. M., Turay, D., Aspe, J. R., Ashok, A., Mirshahidi, S., Mercola, D., Lilly, M. B., & Wall, N. R. (2012). Plasma-derived exosomal survivin, a plausible biomarker for early detection of prostate cancer. *PloS one*, *7*(10), e46737. https://doi.org/10.1371/journal.pone.0046737

Kornhuber, J., & Gulbins, E. (2021). New Molecular Targets for Antidepressant Drugs. *Pharmaceuticals (Basel, Switzerland)*, *14*(9), 894. https://doi.org/10.3390/ph14090894

Kuan, P. F., Waszczuk, M. A., Kotov, R., Clouston, S., Yang, X., Singh, P. K., Glenn, S. T., Cortes Gomez, E., Wang, J., Bromet, E., & Luft, B. J. (2017). Gene expression associated with PTSD in World Trade Center responders: An RNA sequencing study. *Translational psychiatry*, *7*(12), 1297.

Kvietys PR. The Gastrointestinal Circulation. San Rafael (CA): Morgan & Claypool Life Sciences; 2010. Chapter 2, Anatomy. Available from: https://www.ncbi.nlm.nih.gov/books/NBK53099/

Lane, R. E., Korbie, D., Hill, M. M., & Trau, M. (2018). Extracellular vesicles as circulating cancer biomarkers: opportunities and challenges. *Clinical and translational medicine*, *7*(1), 14. https://doi.org/10.1186/s40169-018-0192-7

Leday, G., Vértes, P. E., Richardson, S., Greene, J. R., Regan, T., Khan, S.,

Henderson, R., Freeman, T. C., Pariante, C. M., Harrison, N. A., MRC Immunopsychiatry Consortium, Perry, V. H., Drevets, W. C., Wittenberg, G. M., & Bullmore, E. T. (2018). Replicable and Coupled Changes in Innate and Adaptive Immune Gene Expression in Two Case-Control Studies of Blood Microarrays in Major Depressive Disorder. *Biological psychiatry*, *83*(1), 70–80.

Lee, A. J., & Ashkar, A. A. (2018). The Dual Nature of Type I and Type II Interferons. *Frontiers in immunology*, *9*, 2061. https://doi.org/10.3389/fimmu.2018.02061

Le-Niculescu, H., Kurian, S. M., Yehyawi, N., Dike, C., Patel, S. D., Edenberg, H. J., Tsuang, M. T., Salomon, D. R., Nurnberger, J. I., Jr, & Niculescu, A. B. (2009). Identifying blood biomarkers for mood disorders using convergent functional genomics. *Molecular psychiatry*, *14*(2), 156–174. https://doi.org/10.1038/mp.2008.11

Le-Niculescu, H., Roseberry, K., Gill, S. S., Levey, D. F., Phalen, P. L., Mullen, J., Williams, A., Bhairo, S., Voegtline, T., Davis, H., Shekhar, A., Kurian, S. M., & Niculescu, A. B. (2021). Precision medicine for mood disorders: objective assessment, risk prediction, pharmacogenomics, and repurposed drugs. *Molecular psychiatry*, *26*(7), 2776–2804. https://doi.org/10.1038/s41380-021-01061-w

Leon, S. A., Shapiro, B., Sklaroff, D. M., & Yaros, M. J. (1977). Free DNA in the serum of cancer patients and the effect of therapy. *Cancer research*, *37*(3), 646–650.

Lennon, A. M., Buchanan, A. H., Kinde, I., Warren, A., Honushefsky, A., Cohain, A. T., Ledbetter, D. H., Sanfilippo, F., Sheridan, K., Rosica, D., Adonizio, C. S., Hwang, H. J., Lahouel, K., Cohen, J. D., Douville, C., Patel, A. A., Hagmann, L. N., Rolston, D. D., Malani, N., Zhou, S., … Papadopoulos, N. (2020). Feasibility of blood testing combined with PET-CT to screen for cancer and guide intervention. *Science (New York, N.Y.)*, *369*(6499), eabb9601. https://doi.org/10.1126/science.abb9601

Liu, Y., Mian, M. F., McVey Neufeld, K. A., & Forsythe, P. (2020). $CD4^+CD25^+$ T Cells are Essential for Behavioral Effects of Lactobacillus rhamnosus JB-1 in Male BALB/c mice. *Brain, behavior, and immunity*, *88*, 451–460. https://doi.org/10.1016/j.bbi.2020.04.014

Łoś, K., & Waszkiewicz, N. (2021). Biological Markers in Anxiety Disorders. *Journal of clinical medicine*, *10*(8), 1744. https://doi.org/10.3390/jcm10081744

Lötvall, J., Hill, A. F., Hochberg, F., Buzás, E. I., Di Vizio, D., Gardiner, C., Gho, Y. S., Kurochkin, I. V., Mathivanan, S., Quesenberry, P., Sahoo, S., Tahara, H., Wauben, M. H., Witwer, K. W., & Théry, C. (2014). Minimal experimental requirements for definition of extracellular vesicles and their functions: a position statement from the International Society for Extracellular Vesicles. *Journal of extracellular vesicles*, *3*, 26913. https://doi.org/10.3402/jev.v3.26913

Luckheeram, R.V, Zhou, R., Verma, A.D., & Xia B. (2012). $CD4^+$T Cells: Differentiation and Functions. *Journal of Immunology Research*, 2012(925135), 12. https://doi.org/10.1155/2012/925135

Lyons, Y. A., Wu, S. Y., Overwijk, W. W., Baggerly, K. A., & Sood, A. K. (2017).

Immune cell profiling in cancer: molecular approaches to cell-specific identification. *NPJ precision oncology*, *1*(1), 26. https://doi.org/10.1038/s41698-017-0031-0

Lyte, M. (2011). Probiotics function mechanistically as delivery vehicles for neuroactive Compounds: Microbial endocrinology in the design and use of probiotics. *BioEssays*, *33*(8), 574–581.

Mehta, A. K., Gracias, D. T., & Croft, M. (2018). TNF activity and T cells. Cytokine, 101, 14–18. https://doi.org/10.1016/j.cyto.2016.08.003

Mak, T.W., & Saunders, M.E. (2006). The Immune Response: Basic and Clinical Principles. Elsevier. https://doi.org/10.1016/B978-012088451-3.50007-7

Manson S. M. (1995). Culture and major depression. Current challenges in the diagnosis of mood disorders. *The Psychiatric clinics of North America*, *18*(3), 487–501.

Matsumoto, J., Stewart, T., Sheng, L., Li, N., Bullock, K., Song, N., Shi, M., Banks, W. A., & Zhang, J. (2017). Transmission of α-synuclein-containing erythrocyte-derived extracellular vesicles across the blood-brain barrier via adsorptive mediated transcytosis: another mechanism for initiation and progression of Parkinson's disease?. *Acta neuropathologica communications*, *5*(1), 71. https://doi.org/10.1186/s40478-017-0470-4

Meganathan, V., Moyana, R., Natarajan, K., Kujur, W., Kusampudi, S., Mulik, S., Boggaram, V. (2020). Bacterial extracellular vesicles isolated from organic dust induce neutrophilic inflammation in the lung. *American Journal of Physiology-Lung Cellular and Molecular Physiology.*

Mellman, I., & Steinman, R. M. (2001). Dendritic cells: specialized and regulated antigen processing machines. *Cell*, *106*(3), 255–258. https://doi.org/10.1016/s0092-8674(01)00449-4

Melo, S. A., Luecke, L. B., Kahlert, C., Fernandez, A. F., Gammon, S. T., Kaye, J., LeBleu, V. S., Mittendorf, E. A., Weitz, J., Rahbari, N., Reissfelder, C., Pilarsky, C., Fraga, M. F., Piwnica-Worms, D., & Kalluri, R. (2015). Glypican-1 identifies cancer exosomes and detects early pancreatic cancer. *Nature*, *523*(7559), 177–182. https://doi.org/10.1038/nature14581

Meurs, E., Chong, K., Galabru, J., Thomas, N. S., Kerr, I. M., Williams, B. R., & Hovanessian, A. G. (1990). Molecular cloning and characterization of the human double-stranded RNA-activated protein kinase induced by interferon. *Cell*, *62*(2), 379–390. https://doi.org/10.1016/0092-8674(90)90374-n

Miller, M., Tam, A. B., Mueller, J. L., Rosenthal, P., Beppu, A., Gordillo, R., McGeough, M. D., Vuong, C., Doherty, T. A., Hoffman, H. M., Niwa, M., & Broide, D. H. (2017). Cutting Edge: Targeting Epithelial ORMDL3 Increases, Rather than Reduces, Airway Responsiveness and Is Associated with Increased Sphingosine-1-Phosphate. *Journal of immunology (Baltimore, Md. : 1950)*, *198*(8), 3017–3022. https://doi.org/10.4049/jimmunol.1601848

Mir, B., & Goettsch, C. (2020). Extracellular Vesicles as Delivery Vehicles of Specific Cellular Cargo. *Cells*, *9*(7), 1601. https://doi.org/10.3390/cells9071601

Mohsin M, Guenther S, Schierack P, Tedin K, and Wieler LH. (2015). Probiotic

Escherichia Coli Nissle 1917 Reduces Growth, Shiga Toxin Expression, Release and Thus Cytotoxicity of Enterohemorrhagic Escherichia Coli. *International Journal of Medical Microbiology* **305**(1):20- 26.

Mukai, K., Tsai, M., Starkl, P., Marichal, T., & Galli, S. J. (2016). IgE and mast cells in host defense against parasites and venoms. *Seminars in immunopathology*, *38*(5), 581–603. https://doi.org/10.1007/s00281-016-0565-1

Murphy, D. E., de Jong, O. G., Brouwer, M., Wood, M. J., Lavieu, G., Schiffelers, R. M., & Vader, P. (2019). Extracellular vesicle-based therapeutics: natural versus engineered targeting and trafficking. *Experimental & molecular medicine*, *51*(3), 1–12. https://doi.org/10.1038/s12276-019-0223-5

Nabavi, B., Mitchell, A. J., & Nutt, D. (2015). A Lifetime Prevalence of Comorbidity Between Bipolar Affective Disorder and Anxiety Disorders: A Meta-analysis of 52 Interview-based Studies of Psychiatric Population. *EBioMedicine*, *2*(10), 1405–1419. https://doi.org/10.1016/j.ebiom.2015.09.006

Nilsson, J., Skog, J., Nordstrand, A., Baranov, V., Mincheva-Nilsson, L., Breakefield, X. O., & Widmark, A. (2009). Prostate cancer-derived urine exosomes: a novel approach to biomarkers for prostate cancer. *British journal of cancer*, *100*(10), 1603–1607. https://doi.org/10.1038/sj.bjc.6605058

Olsen Saraiva Camara, N., Lepique, A. P., & Basso, A. S. (2012). Lymphocyte differentiation and effector functions. *Clinical & developmental immunology*, *2012*, 510603. https://doi.org/10.1155/2012/510603

Oo, Y. H., & Adams, D. H. (2010). The role of chemokines in the recruitment of lymphocytes to the liver. *Journal of autoimmunity*, *34*(1), 45–54. https://doi.org/10.1016/j.jaut.2009.07.011

Panda, T. S., Lalremmawia, H., & Tiwary, B. K. (2021). Blood genomic biomarkers for early diagnosis of schizophrenia. *Asian journal of psychiatry*, *59*, 102638. https://doi.org/10.1016/j.ajp.2021.102638

Pang, B., Zhu, Y., Ni, J., Thompson, J., Malouf, D., Bucci, J., Graham, P., & Li, Y. (2020). Extracellular vesicles: the next generation of biomarkers for liquid biopsy-based prostate cancer diagnosis. *Theranostics*, *10*(5), 2309–2326. https://doi.org/10.7150/thno.39486

Park, Y. H., Shin, H. W., Jung, A. R., Kwon, O. S., Choi, Y. J., Park, J., & Lee, J. Y. (2016). Prostate-specific extracellular vesicles as a novel biomarker in human prostate cancer. *Scientific reports*, *6*, 30386. https://doi.org/10.1038/srep30386

Pessoa, L. S., Heringer, M., & Ferrer, V. P. (2020). ctDNA as a cancer biomarker: A broad overview. *Critical reviews in oncology/hematology*, *155*, 103109. https://doi.org/10.1016/j.critrevonc.2020.103109

Phillips, M. L., & Kupfer, D. J. (2013). Bipolar disorder diagnosis: challenges and future directions. *Lancet (London, England)*, *381*(9878), 1663–1671. https://doi.org/10.1016/S0140-6736(13)60989-7

Pittman RN. Regulation of Tissue Oxygenation. San Rafael (CA): Morgan & Claypool Life Sciences; 2011. Chapter 2, The Circulatory System and Oxygen Transport. Available from: https://www.ncbi.nlm.nih.gov/books/NBK54112/

Popi, A. F., Longo-Maugéri, I. M., & Mariano, M. (2016). An Overview of B-1 Cells as Antigen-Presenting Cells. *Frontiers in immunology*, *7*, 138. https://doi.org/10.3389/fimmu.2016.00138

Del Prete G. (1992). Human Th1 and Th2 lymphocytes: their role in the pathophysiology of atopy. *Allergy*, *47*(5), 450–455. https://doi.org/10.1111/j.1398-9995.1992.tb00662.x

Quach, D., Parameswaran, N., McCabe, L., & Britton, R. A. (2019). Characterizing how probiotic *Lactobacillus reuteri* 6475 and lactobacillic acid mediate suppression of osteoclast differentiation. *Bone reports*, *11*, 100227. https://doi.org/10.1016/j.bonr.2019.100227

Ramirez-Garrastacho, M., Bajo-Santos, C., Line, A., Martens-Uzunova, E. S., de la Fuente, J. M., Moros, M., Soekmadji, C., Tasken, K. A., & Llorente, A. (2022). Extracellular vesicles as a source of prostate cancer biomarkers in liquid biopsies: a decade of research. *British journal of cancer*, *126*(3), 331–350. https://doi.org/10.1038/s41416-021-01610-8

Rath CM, Dorrestein PC. (2012). The bacterial chemical repertoire mediates metabolic exchange within gut microbiomes. *Current Opinion in Microbiology* 15:147-54.

Rivera, A., Chen, C. C., Ron, N., Dougherty, J. P., & Ron, Y. (2001). Role of B cells as antigen-presenting cells in vivo revisited: antigen-specific B cells are essential for T cell expansion in lymph nodes and for systemic T cell responses to low antigen concentrations. *International immunology*, *13*(12), 1583–1593. https://doi.org/10.1093/intimm/13.12.1583

Roos, A., Xu, W., Castellano, G., Nauta, A. J., Garred, P., Daha, M. R., & van Kooten, C. (2004). Mini-review: A pivotal role for innate immunity in the clearance of apoptotic cells. *European journal of immunology*, *34*(4), 921–929. https://doi.org/10.1002/eji.200424904

Rosales C. (2018). Neutrophil: A Cell with Many Roles in Inflammation or Several Cell Types?. *Frontiers in physiology*, *9*, 113. https://doi.org/10.3389/fphys.2018.00113

Rosen, G. M., Spitzer, R. L., & McHugh, P. R. (2008). Problems with the post-traumatic stress disorder diagnosis and its future in DSM V. *The British journal of psychiatry : the journal of mental science*, *192*(1), 3–4. https://doi.org/10.1192/bjp.bp.107.043083

Roshchina, V. V. (2010). Evolutionary Considerations of Neurotransmitters in Microbial, Plant, and Animal Cells. In M. Lyte & P. P. E. Freestone (Eds.), *Microbial Endocrinology: Interkingdom Signaling in Infectious Disease and Health*, 17–52.

Ross, M.H. & Pawlina, W. 2003. *Histology: A Text and Atlas, 4th Edition.* Lippincott Williams & Wilkins, Philadelphia.

Rothschild D, Weissbrod O, Barkan E, Kurilshikov A, Korem T, Zeevi D, Costea PI, Godneva A, Kalka IN, Bar N, Shilo S, Lador D, Vila AV, Zmora N, Pevsner-Fischer M, Israeli D, Kosower N, Malka G, Wolf BC, Avnit-Sagi T, Lotan-Pompan M, Weinberger A, Halpern Z, Carmi S, Fu J, Wijmenga C, Zhernakova A, Elinav E, and Segal E. (2018). Environment dominates over host genetics in shaping human gut microbiota. *Nature* **555**:210-5.

Rusch, H. L., Robinson, J., Yun, S., Osier, N. D., Martin, C., Brewin, C. R., & Gill, J. M. (2019). Gene expression differences in PTSD are uniquely related to the intrusion symptom cluster: A transcriptome-wide analysis in military service members. *Brain, behavior, and immunity*, *80*, 904–908.

Sagar, R., & Pattanayak, R. D. (2017). Potential biomarkers for bipolar disorder: Where do we stand?. *The Indian journal of medical research*, *145*(1), 7–16. https://doi.org/10.4103/ijmr.IJMR_1386_16

Saleh, R., Sasidharan Nair, V., Toor, S. M., Taha, R. Z., Murshed, K., Al-Dhaheri, M., Khawar, M., Petkar, M. A., Abu Nada, M., Al-Ejeh, F., & Elkord, E. (2020). Differential gene expression of tumor-infiltrating $CD8^+$ T cells in advanced versus early-stage colorectal cancer and identification of a gene signature of poor prognosis. *Journal for immunotherapy of cancer*, *8*(2), e001294. https://doi.org/10.1136/jitc-2020-001294

Salzbrenner, S., and Conaway, E. (2009). Misdiagnosed Bipolar Disorder Reveals Itself to be Posttraumatic Stress Disorder with Comorbid Pseudotumor Cerebri: A Case Report. *Psychiatry (Edgmont (Pa. : Township))*. **6**(8):29–32.

Sanna, L., Stuart, A. L., Pasco, J. A., Kotowicz, M. A., Berk, M., Girardi, P., Brennan, S. L., & Williams, L. J. (2013). Physical comorbidities in men with mood and anxiety disorders: a population-based study. *BMC medicine*, *11*, 110. https://doi.org/10.1186/1741-7015-11-110

Schroeder, H. W., Jr, & Cavacini, L. (2010). Structure and function of immunoglobulins. *The Journal of allergy and clinical immunology*, *125*(2 Suppl 2), S41–S52. https://doi.org/10.1016/j.jaci.2009.09.046

Sequeiros, T., Rigau, M., Chiva, C., Montes, M., Garcia-Grau, I., Garcia, M., Diaz, S., Celma, A., Bijnsdorp, I., Campos, A., Di Mauro, P., Borrós, S., Reventós, J., Doll, A., Paciucci, R., Pegtel, M., de Torres, I., Sabidó, E., Morote, J., & Olivan, M. (2017). Targeted proteomics in urinary extracellular vesicles identifies biomarkers for diagnosis and prognosis of prostate cancer. *Oncotarget*, *8*(3), 4960–4976. https://doi.org/10.18632/oncotarget.13634

Sharp, T. H., Boyle, A. L., Diebolder, C. A., Kros, A., Koster, A. J., & Gros, P. (2019). Insights into IgM-mediated complement activation based on in situ structures of IgM-C1-C4b. *Proceedings of the National Academy of Sciences of the United States of America*, *116*(24), 11900–11905. https://doi.org/10.1073/pnas.1901841116

Sherwin E, Sandhu KV, Dinan TG, and Cryan JF. (2016). May the force be with you: the light and dark sides of the microbiota-gut-brain axis in neuropsychiatry. *CNS Drugs* **30**:1019-1041.

Sokol, C. L., Chu, N. Q., Yu, S., Nish, S. A., Laufer, T. M., & Medzhitov, R. (2009). Basophils function as antigen-presenting cells for an allergen-induced T helper type 2 response. *Nature immunology*, *10*(7), 713–720. https://doi.org/10.1038/ni.1738

Sorenson, G. D., Pribish, D. M., Valone, F. H., Memoli, V. A., Bzik, D. J., & Yao, S. L.

(1994). Soluble normal and mutated DNA sequences from single-copy genes in human blood. *Cancer epidemiology, biomarkers & prevention : a publication of the American Association for Cancer Research, cosponsored by the American Society of Preventive Oncology*, *3*(1), 67–71.

Spijker, S., Van Zanten, J. S., De Jong, S., Penninx, B. W., van Dyck, R., Zitman, F. G., Smit, J. H., Ylstra, B., Smit, A. B., & Hoogendijk, W. J. (2010). Stimulated gene expression profiles as a blood marker of major depressive disorder. *Biological psychiatry*, *68*(2), 179–186.

Stone, K. D., Prussin, C., & Metcalfe, D. D. (2010). IgE, mast cells, basophils, and eosinophils. *The Journal of allergy and clinical immunology*, *125*(2 Suppl 2), S73–S80. https://doi.org/10.1016/j.jaci.2009.11.017

Strimbu, K., & Tavel, J. A. (2010). What are biomarkers?. *Current opinion in HIV and AIDS*, *5*(6), 463–466. https://doi.org/10.1097/COH.0b013e32833ed177

Thursby E, and Juge N. (2017). Introduction to the human gut microbiota. *Biochemical Journal* **474**:1823-1836.

Uhlén, M., Fagerberg, L., Hallström, B. M., Lindskog, C., Oksvold, P., Mardinoglu, A., Sivertsson, Å., Kampf, C., Sjöstedt, E., Asplund, A., Olsson, I., Edlund, K., Lundberg, E., Navani, S., Szigyarto, C. A., Odeberg, J., Djureinovic, D., Takanen, J. O., Hober, S., Alm, T., … Pontén, F. (2015). Proteomics. Tissue-based map of the human proteome. *Science (New York, N.Y.)*, *347*(6220), 1260419. https://doi.org/10.1126/science.1260419

Valdez AM, Walter J, Segal E, and Spector TD. (2018). Role of the gut microbiota in nutrition and health. *The BMJ* 361:k2179.

van de Guchte M, Blottiere HM, and Dore J. (2018). Humans as holobionts: implications for prevention and therapy. *Microbiome* **6**(81).

Van Lieshout, R. J., Bienenstock, J., & MacQueen, G. M. (2009). A review of candidate pathways underlying the association between asthma and major depressive disorder. *Psychosomatic medicine*, *71*(2), 187–195. https://doi.org/10.1097/PSY.0b013e3181907012

Vivier, E., Tomasello, E., Baratin, M., Walzer, T., & Ugolini, S. (2008). Functions of natural killer cells. *Nature immunology*, *9*(5), 503–510. https://doi.org/10.1038/ni1582

Vyas U, Ranganathan N. (2012). Probiotics, prebiotics, and synbiotics: gut and beyond. *Gastroenterology Research and Practice* 2012:872716.

Wang, J., Li, D., Cang, H., & Guo, B. (2019). Crosstalk between cancer and immune cells: Role of tumor-associated macrophages in the tumor microenvironment. *Cancer medicine*, *8*(10), 4709–4721. https://doi.org/10.1002/cam4.2327

Wan, J., Massie, C., Garcia-Corbacho, J., Mouliere, F., Brenton, J. D., Caldas, C., Pacey, S., Baird, R., & Rosenfeld, N. (2017). Liquid biopsies come of age: towards implementation of circulating tumour DNA. *Nature reviews. Cancer*, *17*(4), 223–238. https://doi.org/10.1038/nrc.2017.7

Wang, Y., Li, Z., Song, G., & Wang, J. (2020). Potential of Immune-Related Genes as

Biomarkers for Diagnosis and Subtype Classification of Preeclampsia. *Frontiers in genetics*, *11*, 579709. https://doi.org/10.3389/fgene.2020.579709

Wilgus, T. A., Roy, S., & McDaniel, J. C. (2013). Neutrophils and Wound Repair: Positive Actions and Negative Reactions. *Advances in wound care*, *2*(7), 379–388. https://doi.org/10.1089/wound.2012.0383

Wisnivesky, J. P., Markowitz, S. B., James, S., Stone, K., Dickens, B., Busse, P., Crowley, L., Federman, A., Katz, C., & Gonzalez, A. (2021). Comorbid posttraumatic stress disorder and major depressive disorder are associated with asthma morbidity among World Trade Center workers. *Annals of allergy, asthma & immunology : official publication of the American College of Allergy, Asthma, & Immunology*, *126*(3), 278–283.

Witwer, K. W., Buzás, E. I., Bemis, L. T., Bora, A., Lässer, C., Lötvall, J., Nolte-'t Hoen, E. N., Piper, M. G., Sivaraman, S., Skog, J., Théry, C., Wauben, M. H., & Hochberg, F. (2013). Standardization of sample collection, isolation and analysis methods in extracellular vesicle research. Journal of extracellular vesicles, 2, 10.3402/jev.v2i0.20360. https://doi.org/10.3402/jev.v2i0.20360

Wu ZA, and Wang HX. (2018). A Systematic Review of the Interaction Between Gut Microbiota and Host Health from a Symbiotic Perspective. *SN Comprehensive Clinical Medicine* **1**:224-235.

Xu, R., Greening, D. W., Zhu, H. J., Takahashi, N., & Simpson, R. J. (2016). Extracellular vesicle isolation and characterization: toward clinical application. The Journal of clinical investigation, 126(4), 1152–1162. https://doi.org/10.1172/JCI81129

Yang, T., Martin, P., Fogarty, B., Brown, A., Schurman, K., Phipps, R., Yin, V. P., Lockman, P., & Bai, S. (2015). Exosome delivered anticancer drugs across the blood-brain barrier for brain cancer therapy in Danio rerio. *Pharmaceutical research*, *32*(6), 2003–2014. https://doi.org/10.1007/s11095-014-1593-y

Yong, E. (2014). Cancer biomarkers: Written in blood. *Nature*, *511***,** 524–526. https://doi.org/10.1038/511524a

Yong JY, Tong T, Chew J, and Lim WL. (2020). Antidepressive Mechanisms of Probiotics and Their Therapeutic Potential. *Frontiers in Neuroscience* **13**(1261).

Zakharzhevskaya, N. B., Vanyushkina, A. A., Altukhov, I. A., Shavarda, A. L., Butenko, I. O., Rakitina, D. V., Nikitina, A.S., Manolov, A.I., Egorova, A.N., Kulikov, E.E., Vishnyakov, I.E., Fisunov, G.Y., Govorun, V. M. (2017). Outer membrane vesicles secreted by pathogenic and nonpathogenic Bacteroides fragilis represent different metabolic activities. *Scientific Reports*, *7*(1), 5008.

Zhang, J. M., & An, J. (2007). Cytokines, inflammation, and pain. *International anesthesiology clinics*, *45*(2), 27–37. https://doi.org/10.1097/AIA.0b013e318034194e

Zhao, Z., Fan, J., Hsu, Y. S., Lyon, C. J., Ning, B., & Hu, T. Y. (2019). Extracellular vesicles as cancer liquid biopsies: from discovery, validation, to clinical application. *Lab on a chip*, *19*(7), 1114–1140. https://doi.org/10.1039/c8lc01123k

Zhu, Z., Zhu, X., Liu, C. L., Shi, H., Shen, S., Yang, Y., Hasegawa, K., Camargo, C. A., Jr, & Liang, L. (2019). Shared genetics of asthma and mental health disorders: a large-scale genome-wide cross-trait analysis. The European respiratory journal, 54(6), 1901507. DOI: 10.1183/13993003.01507-2019

Zimmerman, L.J. (2010, March 24-25). *Assessment of Protein Stability in Whole Blood* [Conference presentation]. Biospecimen Research Network Symposium, Bethesda, MD, United States. https://www.biospecimens.cancer.gov/meeting/brnsymposium/2010/