8-2023

Biomarkers in Borrelia burgdorferi infected triple-negative breast cancer cells

Sambuddha Paul

UNIVERSITY OF NEW HAVEN
Department of Biology and Environmental Science

Biomarkers

in *Borrelia burgdorferi* infected triple-negative breast cancer cells

A THESIS

Submitted in partial fulfillment
of the requirements for the degree of
MASTER OF SCIENCES IN CELLULAR AND MOLECULAR BIOLOGY

Sambuddha Paul, MS candidate

August 2023

ADVISOR:

EVA SAPI, Ph.D.
Professor
Department of Biology and Environmental Sciences
University of New Haven
1211 Campbell Avenue,
Charger Plaza LL16
West Haven, CT 06516
(203) 479-4552
esapi@newhaven.edu

CO-ADVISORs:

ANTHONY MELILLO, MS
amelillo@newhaven.edu

ALIREZA G. SENEJANI, Ph.D.
asenejani@newhaven.edu

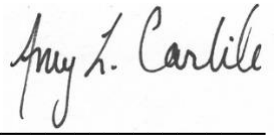
CHRISTINA ZITO, Ph.D.
czito@newhaven.edu

Biomarkers

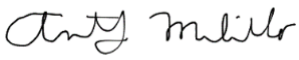
in *Borrelia burgdorferi* infected triple-negative breast cancer cells

APPROVED BY

EVA SAPI, Ph.D.
Thesis Advisor



AMY L. CARLILE, Ph.D.
Chair, Biology and Environmental Science



ANTHONY MELILLO, MS
Thesis Committee Member

ALIREZA G. SENEJANI, Ph.D.
Thesis Committee Member

Christina Zito

CHRISTINA ZITO, Ph.D.
Thesis Committee Member

Shaily Menon

SHAILY MENON, Ph.D.
Dean, College of Arts and Sciences

NANCY ORTINS SAVAGE, Ph.D.
Interim Provost and Vice President of Academic Affairs

TABLE OF CONTENTS

ACKNOWLEDGMENTS

I want to express my deepest gratitude to my esteemed thesis advisor, Dr. Eva Sapi, for her invaluable mentorship, support, and tutelage throughout the course of my master's degree at the University of New Haven. Dr. Sapi has been an amazing mentor and a constant support system who would always encourage me to expand my limits and be my best self. Because of her constant help and guidance, I was able to accomplish this research study. It has been an honor to be a part of the Lyme Disease Research Lab.

I especially want to thank my thesis committee members, Dr. Alireza Senejani, Dr. Christina Zito and Prof. Anthony Melillo, for their valuable support and generous suggestions regarding my research study. I would like to extend my sincere thanks and appreciation to Prof. Anthony Melillo who has helped me throughout my research study.

I would also like to thank the staff and faculties of the Cellular and Molecular Biology Graduate Program of the University of New Haven. Their kind help and support have made my academic pursuits a wonderful experience.

Additionally, I sincerely thank Pink Clover Foundation for greatly supporting my research financially and providing great emotional support.

I am indebted to my peers Sahaja Thippani, Niraj Patel, Vishwa Khatri and Jasmine Jathan, who have been an amazing group of friends. I would like to extend my most profound gratitude to the past and present Lyme Disease Research Group members at the University of New Haven.

Lastly, but most importantly, I express my gratitude towards my family, who have been my greatest source of strength, my pillars, and the most important reason behind my all my successes.

ABSTRACT

Triple-negative breast cancer (TNBC) is an aggressive subtype of breast cancer with limited treatment options and a poor prognosis. Although some studies have attempted to identify new biomarkers and therapies, only a few have shown promise in clinical trials. In the context of cancer development, infectious agents rank as the third major risk factor, contributing to approximately 15-20% of cancer cases, according to the American Cancer Society. Hence, the identification of biomarkers associated with infection-originated cancer is crucial for enhancing cancer prevention, early detection, diagnosis, and treatment. *Borrelia burgdorferi*, the causative agent of Lyme disease, has been linked to TNBC and its aggressive characteristics. Therefore, investigating prognostic or diagnostic markers in *B. burgdorferi*-infected TNBC cells holds great potential for shedding light on the mechanisms underlying this complex association. In this study, we employed RNA-Sequencing (RNA-Seq) technology to examine changes in gene expression induced by *B. burgdorferi* infection in TNBC cell line MDA-MB-231 and a normal breast epithelial cell line (MCF10A). Our analysis identified common biomarkers, including CXCL-1, CXCL-8, C3, PTGS2 (COX2), and SAA1, in *B. burgdorferi* -infected TNBC cells. The genes identified for MCF10A included members of the CXCL family (CXCL10, CXCL8, CXCL3, and CXCL2), CCL20, and IFIT1. Furthermore, pathway analysis using KEGG and REACTOME databases revealed the involvement of the IL-4, 10, 13 & 17 signaling, TNF, NOD like receptor, TLR, NF-kB and Chemokine-signaling pathways. The most enriched signaling pathways in MCF10A included the IL-10 & 17, chemokine, cytokine and TNF signaling pathways. Common infectious diseases pathway associated in TNBC cells included COVID-19, Legionellosis, Prion disease, Influenza-A, Pertussis, Measles, Kaposi-sarcoma-associated herpesvirus, and Chagas disease, among others with several of the above identified genes in

function. The most common infectious disease associated pathways in MCF10A cells included Amoebiasis, Legionellosis, Hepatitis-C and Influenza-A. There were common metabolic pathways observed for MCF10A from the KEGG and REACTOME servers which include fatty acid metabolism, glycolipid metabolism and steroid metabolism. Also, certain biosynthesis pathways such as steroid and terpenoid backbone biosynthesis pathways were observed common from the KEGG and REACTOME servers for MCF10A.These findings highlight the significance of exploring these potential biomarkers as therapeutic targets in *B. burgdorferi* -associated TNBC. Our study provides a promising starting point for future research endeavors aimed at improving diagnostic and treatment strategies for this challenging infection-associated cancer subtype.

LIST OF FIGURES

Figure 6: Venn diagram showing the common up-regulated DEGs in the RNA Sequencing data of MDA-MB-231 Run 1 and 2. Genes with a differential expression ≥ 3-fold and p-value < 0.01 in MDA-MB-231 cells for both the runs were considered.

Figure 7: Venn diagram showing the common up-regulated DEGs in the RNA Sequencing data of MDA-MB-231 Run 1+2 and MCF10A. Genes with a differential expression ≥ 1.5-fold and p-value < 0.01 in MDA-MB-231 cells for both the runs were considered.

Figure 8: Heatmap depicting the expression of top 12 upregulated genes and their fold changes associated with KEGG inflammation pathways from B. burgdorferi -infected MDA-MB-231 (1+2) Runs.

Figure 9: Heatmap depicting the expression of top 12 upregulated genes and their fold changes associated with KEGG infection pathways from B. burgdorferi -infected MDA-MB-231 (1+2) Runs.

Figure 10: Heatmap depicting the expression of top 12 upregulated genes and their fold changes associated with (A) REACTOME inflammation pathways, (B) REACTOME infection pathways and (C) REACTOME metabolic pathways from B. burgdorferi -infected MDA-MB-231 (1+2) Runs.

Figure 11: Heatmap depicting the expression of top 12 upregulated genes and their fold changes associated with (A) KEGG inflammation pathways, (B) KEGG infection pathways and (C) KEGG metabolic pathways from B. burgdorferi -infected MCF10A cells.

Figure 12: Heatmap depicting the expression of top 12 upregulated genes and their fold changes associated with (A) REACTOME inflammation pathways, (B) REACTOME metabolic pathways from B. burgdorferi -infected MCF10A cells.

INTRODUCTION

Breast cancer (BC) accounts for the leading cause of death due to cancer among women, second only to lung cancer, with a 1 in 39 chance of fatality [1]. Among the various sub-types of breast cancer, triple-negative breast cancer (TNBC), which tests negative for estrogen, progesterone, and human epidermal growth factor receptor (HER2), presents with a shorter survival and higher mortality rate [2]. TNBC is highly invasive with a higher risk of metastasis and recurrence [2]. Unlike other breast cancer subtypes, TNBC lacks targeted therapies, making it challenging to treat effectively [3]. Although some progress has been made in the treatment and drug research of breast cancer, studies have shown that due to the lack of understanding of the pathogenesis of this complex disease, there is currently no effective treatment, and the recurrence and death of breast cancer patients are still not effectively controlled [4, 5]

Previously, the use of bioinformatics methods to explore differentially expressed genes has made significant progress in pancreatic cancer [6], gastric cancer [7-9], colorectal cancer [10, 11], prostate cancer [12] and other diseases [13-17]. The occurrence and development of breast cancer is a process involving a synergistic action of multiple genes in multiple stages [18]. Therefore, the differential change of gene expression profile has always been a recent topic in breast cancer research [19]. At present, some studies have explored new biomarkers and relative therapies in TNBC, but only few of these have proven useful in clinical trials [20]. The identification of a germline BRCA1/2 mutations impacted cancer screening and prevention

practices in this subgroup of patients and their relatives. Later, the knowledge of the pathological mechanisms of these mutations, led to the development of new therapeutic approaches, such as poly (ADP-ribose) polymerase (PARP) inhibitors, selectively directed on BRCA 1 or BRCA 2 deficient cells [21]. Multiple anti-HER2 vaccines are also under evaluation in HER2-low BC, with some displaying favorable results in the TNBC sub-population [22,23]. Neratinib, an inhibitor for HER2, HER4, and EGF receptors has shown efficacy in metastatic HER2-mutated, HER2-low BC [24]. Other anti-HER2 Tyrosine Kinase Inhibitors (TKIs), including poziotinib (NCT02544997) and pyrotinib (NCT03412383), are also being evaluated [25,26] The androgen receptor (AR) is part of the steroid receptor family and functions as a nuclear transcription factor [27]. AR-positive TNBC has a lower Ki-67 (a good marker of proliferation) index than AR-negative TNBC and could be less sensitive to chemotherapy, which is in accordance with the Luminal androgen receptor (LAR) subtype having a lower pathological complete response (pCR) rate relative to other subtypes [28,29]. The introduction of immune checkpoint inhibitors (PD-1 inhibitors and PD-L1 inhibitors) as a strategy to wake up the immune cells and reduce the tumor growth, is playing a critical role in improving treatment of TNBC [30]. The phosphatidylinositol 3-kinase (PI3K) pathway is another key regulator of survival, growth, proliferation, angiogenesis, metabolism, and migration [31]. Moreover, chromosomal translocations are well-known oncogenic drivers in malignancies, and targeting gene fusions have become a highly effective strategy to treat rearrangement-driven cancers [32, 33, 34]. Still, a critical need remains for the development of more modern next generation sequencing (NGS)-based biomarker to continue to improve outcomes in patients with TNBCs.

In the realm of cancer development, infectious agents rank as the third major risk factor. According to the American Cancer Society, approximately 20% of cancer cases are attributed to

infections [35]. For instance, lung cancer has been associated with *Mycobacterium tuberculosis* infection, while *Helicobacter. pylori* infection is linked to increased migration and invasion rates in gastric cancer, and *Campylobacter. jejuni* has been correlated with small intestine lymphomas [36]. Notably, the presence of the bacterium *Fusobacterium nucleatum* in breast cells has been linked to accelerated tumor growth [37]. One of the most recent findings involves tumor-resident microbiota, playing an important role in promoting cancer metastasis, intervention of which might therefore be worth exploring for advancing oncology care [38]. Also, another recent study implied that *F. nucleatum* orchestrates a molecular network of the Toll-like receptor, micro-RNAs (miRNAs), and autophagy to clinically, biologically, and mechanistically control colorectal cancer chemoresistance [39]. Overall, these studies underscore the importance of considering infectious agents as potential contributors to cancer development and progression and thereby emphasizing the need for further research in this area to better understand and target these complex interactions.

Identification of biomarkers in the infection origin of cancer is critical for improving cancer prevention, early detection, diagnosis, and treatment. It has the potential to significantly impact patient care, enhance treatment outcomes, and advance our understanding of these interactions between infectious agents and cancer development. Currently, the identification of specific markers for targeting pathogen-associated breast cancer is an area of ongoing research, and no widely accepted markers are available for clinical use. However, there are some potential markers that have been studied in the context of pathogen-association to several cancers such as HPV E6 and E7 Oncoproteins in cervical cancer [40], EBV-Encoded RNAs (EBERs) and CA-724 in gastric cancer [41,42], PI3/Akt pathway in lung cancer [43], LMP2A in nasopharyngeal

cancer [44], Carcinoembryonic antigen (CEA) in colorectal cancer [45] and Alpha-fetoprotein (AFP) in liver cancer [46].

A groundbreaking study from 2018 identified bacterial signatures in breast cancer, with *Borrelia burgdorferi* showing an association with poorer prognosis in HER2-positive breast cancer [36]. *Borrelia burgdorferi*, the causative agent of Lyme disease (LD), is known to cause a range of clinical manifestations, including musculoskeletal inflammation and neurological symptoms [47-50]. This pathogen enters the body through tick bites and can spread to different tissues and organs [47, 49, 50]. Past research has revealed that *B. burgdorferi* employs various strategies to evade the host immune response, such as altering its membrane proteins, actively suppressing the host immune system, and existing in different forms [50,51]. Furthermore, the bacterium can hide from the immune system by internalizing into host cells without harming their viability [51-53]. Our recent study reported that *B. burgdorferi* can invade both normal and cancerous mammary epithelial cells, impacting the invasion and matrix remodeling potential of cancer cells [54]. This capability, combined with cancer cells' uncontrolled growth, may ensure the bacteria's survival within the host. Notably, our research demonstrated that *B. burgdorferi* infection could potentially make TNBC cells more aggressive [54]. In addition, data from the prior thesis research study of our research group have demonstrated that *B. burgdorferi* can alter the expression of cancer-associated genes in MDA-MB-231 [55]. Also, one of the most recent published studies identified potential miRNAs (miR-206 and 214) which could be further evaluated as biomarkers for tumorigenesis caused by *B. burgdorferi* in breast cancer cells [56]. As a result, it becomes crucial to comprehend the physiological effects of *B. burgdorferi* on TNBC cells and identify the underlying molecular mechanisms through which it induces these aggressive cancer traits.

RNA-seq is a powerful technique used for quantifying gene expression, allele-specific expression, and splice variants in high-throughput. This method is particularly useful for studying the differential gene expression in cells infected with bacteria [57]. In previous research, RNA-seq was successfully employed to comprehensively map genes with altered expression in response to *B. burgdorferi* infection in human brain microvascular cells [58]. Interestingly, many of these differentially expressed genes (DEGs) were associated with dysregulated pathways in cancer, including breast cancer [59, 60]. Building upon this knowledge, we utilized RNA-Seq technology and KEGG, REACTOME pathway enrichment analysis to investigate potential biomarkers through which *B. burgdorferi* may impact triple-negative breast cancer and normal breast epithelial cell lines - MDA-MB-231 and MCF10A. By conducting two separate RNA-Seq runs for MDA-MB-231, we aimed to ensure the reliability and reproducibility of our results, thereby strengthening the confidence in the identified DEGs and the subsequent pathway analysis. This research offers crucial understanding of how *B. burgdorferi* infection impacts gene expression and pathways in TNBC cells. The potential biomarkers identified in this study hold great promise for further investigations to unravel the mechanisms underlying *B. burgdorferi*-associated TNBC. Moreover, these findings could lead to the development of innovative diagnostic panels and therapeutic strategies for combating this aggressive and complex form of breast cancer.

MATERIALS AND METHODS

Borrelia burgdorferi culture:

The B31 strain of *Borrelia burgdorferi* (ATCC 35210, Manassas, VA) was cultured in Barbour-Stoner-Kelly-H (BSK-H) medium (Sigma Aldrich, St Louis, MO), supplemented with 6% rabbit serum (GeminiBio, West Sacramento, CA) in sterile 15 mL glass tubes at 37 ºC and 3% carbon dioxide. Low passage numbers (<P6) were used for all experiments.

Mammalian cell culture and infection with *B. burgdorferi*:

Normal breast epithelial cells and triple-negative breast cancer cells (TNBC) were cultured using standard cell culture techniques at 37 ºC and 5% carbon dioxide. MDA-MB-231, was cultured in Dulbecco's Modified Eagle Medium (DMEM, Sigma Aldrich, Louis, MO) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin-glutamine (PSG, ThermoFisher Scientific, Waltham, MA), along with the 20 ug/mL gentamycin. MCF-10A was cultivated in Dulbecco's Modified Eagle Medium-F12 (DMEM/F-12), (Sigma Aldrich, St Louis, MO) supplemented with 5% Horse Serum (Gibco^{TM}; Waltham, MA, USA), 10 ug/mL bovine insulin (Sigma Aldrich, St Louis, MO), 100 ng/mL cholera toxin (Sigma Aldrich, St Louis, MO), 20 ng/mL epidermal growth factor (EGF, ThermoFisher, Waltham, MA), 0.5 mg/mL hydrocortisone (Sigma Aldrich, St Louis, MO), and 1% Penicillin-Streptomycin (PS, Gibco^{TM}; Waltham, MA, USA). The cells were serum deprived overnight in serum-free basal growth media supplemented with 1% of required antibiotic (PSG for MDA-MB-231 and PS for MCF10A) and infected with *B. burgdorferi* at an MOI of 60 for 48 hours in co-culture media composed of 1/3rd serum- and antibiotic-free growth media and 2/3rd of BSK-H supplemented with 6% rabbit serum.

RNA extraction:

Infected cells along with co-culture controls were trypsinized and pelleted by centrifuging at 2200 rpm for 10 minutes at room temperature (RT). The medium was aspirated, and the pellet was resuspended in phosphate-buffered saline (PBS, Sigma Aldrich, St Louis, MO) followed by centrifugation at 2200 rpm for 10 minutes at RT. RNA was extracted using RNeasy mini kit (Qiagen, Hilden, Germany) following the manufacturer's instruction and quantitated using the Nanodrop Lite spectrophotometer (ThermoFisher Scientific, Waltham, MA). The total RNA extracted from three independent sets of infected and uninfected samples for each cell line was sent for sequencing at the Yale Center for Genome Analysis (Yale West Campus, Orange, CT).

cDNA library prep and RNA sequencing:

The integrity of the RNA was determined by running an Agilent Bioanalyzer gel (Agilent, Santa Clara, CA), to measure the ratios of the ribosomal peaks and to assign an RNA integrity/RNA quality number (RIN/RQN) to the samples. Total RNA was subjected to either polyA selection (MDA-MB-231) using Roche Kapa mRNA Hyper Prep Kit (Roche KAPA Biosystems, Wilmington, MA) or ribosomal depletion using the Roche KAPA RNA Hyper Prep Kit with RiboErase (Roche KAPA Biosystems, Wilmington, MA). Samples with a yield of ≥ 0.5 ng/uL and a size distribution of 150-300 bp were used for sequencing. Samples were sequenced on Illumina NovaSeq6000 according to Illumina protocols.

Analysis of differentially expressed genes (DEGs) and pathway enrichment analysis:

Volcano plots were generated using R (EnhancedVolcano library) to illustrate significantly DEGs from the RNA-seq data. Genes were considered significantly differentially expressed if the $\log_2$ fold change was > 0.585 (fold change 1.5) and the adjusted p-value (false discovery rate (FDR)) was < 0.1. The DEGs were run through the Database for Annotation, Visualization,

and Integrated Discovery (DAVID) [60] to determine cellular processes that the DEGs were enriched in. Within DAVID, two different servers – KEGG and REACTOME were used for the pathway enrichment analysis. KEGG is a comprehensive database that integrates genomic, chemical, and systemic functional information. It contains various types of biological pathways, including metabolic pathways, signaling pathways, and disease-related pathways. KEGG pathways are represented as networks of interacting genes and gene products that participate in specific biological processes. The KEGG pathway analysis involves mapping DEGs from experimental data onto the known pathways in the KEGG database. REACTOME is another widely used pathway database that provides curated and peer-reviewed information about biological pathways and processes. It covers a broad range of biological areas, including cell signaling, metabolism, immune response, and DNA repair, among others. REACTOME pathway analysis involves inputting gene lists from experimental data and identifying overrepresented pathways. The analysis assesses the statistical significance of the overlap between the input gene list and the genes associated with specific REACTOME pathways. For this study, pathways that highlighted the top 12 genes (FC ≤ 8) for MDA-MB-231 both runs (1+2) and the top 12 genes (FC ≤ 5) for MCF10A have been discussed. Each pathway was then mapped to the significantly expressed genes for both cell lines in EXCEL according to the following rules: (1) genes with adjusted p values < 0.0001 and base Mean greater than 400; (2) pathways with the Benjamini-Hochberg adjusted p-value ≤ 0.10.

RESULTS

In this study, RNA sequencing was performed on TNBC cell line MDA-MB-231 and a normal mammary epithelial cell line, MCF10A. The reliability and reproducibility of our findings were confirmed by performing two distinct, independent RNA sequencing runs for MDA-MB-231 cells

(Run 1 and 2). For each cell line run, 1 x10^6 cells were plated in biological triplicates for 6 hours after which they were serum starved for 10 hours and then infected with *B. burgdorferi* at a multiplicity of infection (MOI) of 60 alongside uninfected controls. The RNAs extracted from these biological triplicates of uninfected and *B. burgdorferi*-infected cells were determined to be good quality as assessed by an RNA Quality Number (RQN) value >7.

RNA-seq on triple-negative breast cancer and normal-like mammary epithelial cells

To investigate the changes in gene expression after *B. burgdorferi* infection, computational analysis of the DEGs between the infected and uninfected group was performed. Overall, 142 DEGs between uninfected and infected samples were identified in MDA-MB-231 cells (adjusted p-value < 0.01) for Run 1 out of which 122 significant genes were differentially expressed by over 1.5-fold.

The volcano plots display the statistical significance of the difference relative to the magnitude of difference for every single gene in the comparison, through the negative base-10 log of the adjusted p-value (FDR) and $\log_2$ fold-change, respectively. Since the p-values have a negative transformation, the higher along the y-axis a data point falls, the smaller the p-value. Data points closer to 0 represent genes that have similar or identical mean expression levels. Extreme data points along the x-axis depicts upregulated genes on the right and downregulated genes towards the left of the origin. From Figure 1, we observed a total of 122 genes upregulated for MDA-MB-231 Run 1 as the data points skews towards the right, out of which 13 of these significant genes showed a ≥3-fold change. These included the CXCL family (CXCL-1,3,8,10 & 11), CCL20,

interleukin-6 and SAA1 to name a few. A total of 20 genes were downregulated out of which the most significant ones are shown as data points to the left of the origin.

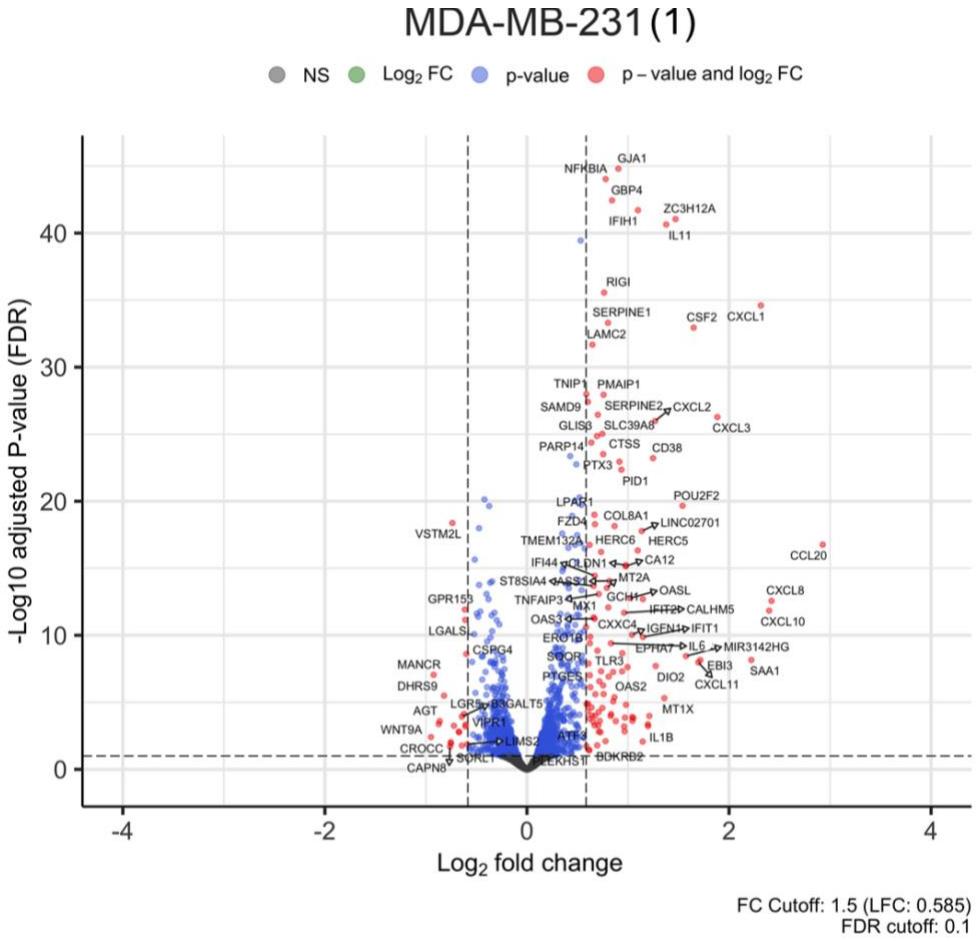


Figure 1: Volcano plot of DEGs in MDA-MB 231 cell line Run 1. The x-axis represents the $\log_2$ fold change and the y-axis represents the negative log10 of the adjusted P-value. The cutoff criteria were adjusted P-value ≤0.10 and $\log_2$ fold change greater than 0.585. Points in red represent genes that exceed both cutoff criteria.

By conducting two independent RNA sequencing runs for MDA-MB-23 (6 months apart), we aimed to ensure the reliability and reproducibility of our results. A total of 113 DEGs between uninfected and infected samples were identified in MDA-MB-231 cells (adjusted p-value < 0.01) for Run 2 out of which 60 significant genes were differentially expressed by over 1.5-fold. From Figure 2, we observed those 60 upregulated genes for MDA-MB-231 Run 2 as the data points

skews towards the right, out of which 8 of the significant genes showed a ≥3-fold change. These included genes from the CXCL family (CXCL-1, 8, 10), C3, PTGS2 (COX2), C15orf48 (chromosome 15 open reading frame 48), PI3 and SAA1. A total of 60 genes were downregulated out of which the most significant ones are shown as data points to the left of the origin.

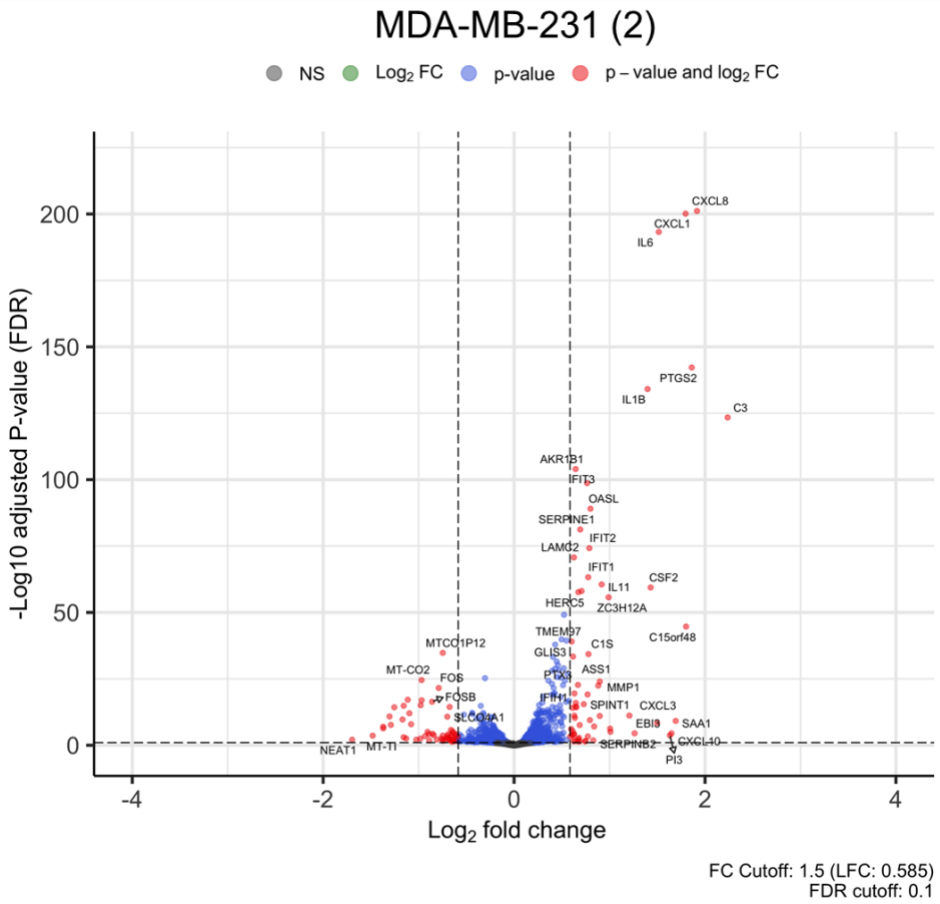


Figure 2: Volcano plot of DEGs in MDA-MB 231 cell line Run 2. The x-axis represents the $\log_2$ fold change and the y-axis represents the negative log10 of the adjusted P-value. The cutoff criteria were adjusted P-value ≤0.10 and $\log_2$ fold change greater than 1.5. Points in red represent genes that exceed both cutoff criteria.

In order to have a reference point for comparison, normal epithelial cell line, MCF10A was used for which 95 DEGs between uninfected and infected samples were identified (adjusted p-value < 0.01). Within these DEGs, a subset of 38 genes were differentially expressed over 1.5-fold (p <

0.01, Table 1). From Figure 4, a total of 38 genes upregulated for MCF10A can be seen as the data points skews towards the right, out of which 3 of the significant genes showed a ≥3-fold change. These included CXCL10, RSAD2 and TNF alpha induced protein 6 (TNFAIP6). A total of 57 genes were downregulated out of which the most significant ones are shown as data points to the left of the origin.

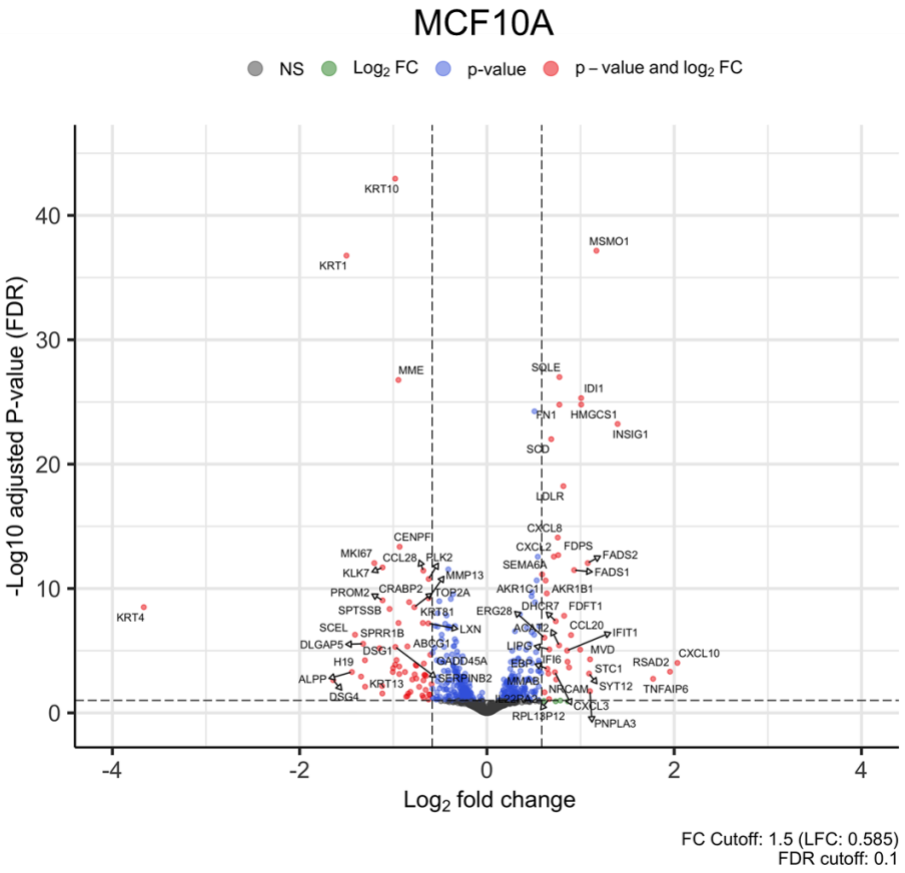


Figure 3: Volcano plot of DEGs in MCF10A cell line. The x-axis represents the $\log_2$ fold change and the y-axis represents the negative log10 of the adjusted P-value. The cutoff criteria were adjusted P-value ≤0.10 and $\log_2$ fold change greater than 1.5. Points in red represent genes that exceed both cutoff criteria.

Screening of DEGs in triple-negative breast cancer and normal-like mammary epithelial cells

In this study, following the data analysis for the independent RNA sequencing runs (Run 1 and Run 2) for the MDA-MB-231 cell line and MCF10A, Venn diagrams were generated to identify the overlapping genes between the multiple cell lines illustrating the common DEGs that were consistently identified in the multiple cell line RNA sequencing runs after *B. burgdorferi* infection. A total of 122 and 60 significantly upregulated genes were observed for MDA-MB-231 Run 1 and 2 respectively. MCF10A on the other hand showed 38 significantly upregulated genes.

From Figure 5, the intersection of the Venn diagram illustrates the genes that were commonly upregulated in response to *B. burgdorferi* infection in MDA-MB-231 Run 1 and Run 2. About 49 genes were observed that were consistently upregulated in both runs with a cut off ≥1.5 FC. As the fold change cut off was increased to ≥2, about 15 genes were observed that were consistently upregulated in both runs (Figure 6). As a more stringent fold change cut off ≥3 was applied, a subset of genes that remained consistently upregulated was observed among MDA-MB-231 Run 1 and Run 2 in response to *B. burgdorferi* infection. Specifically, about 6 genes were identified as consistently upregulated with a fold change of ≥3 in both runs, as observed in Figure 7. The genes include genes from the CXCL family (CXCL-1,8 & 10), C3, PTGS2 (COX2) and SAA1.

These genes may represent the most significantly regulated targets in the cellular response to *B. burgdorferi* in the MDA-MB-231 cell line, suggesting their potential importance in *B. burgdorferi* -associated in the physiology of MDA-MB-231 cells.

Figure 4: Venn diagram showing the common up-regulated DEGs in the RNA Sequencing data of MDA-MB-231 Run 1 and 2. Genes with a differential expression ≥ 1.5-fold and p-value < 0.01 in MDA-MB-231 cells for both runs were considered.

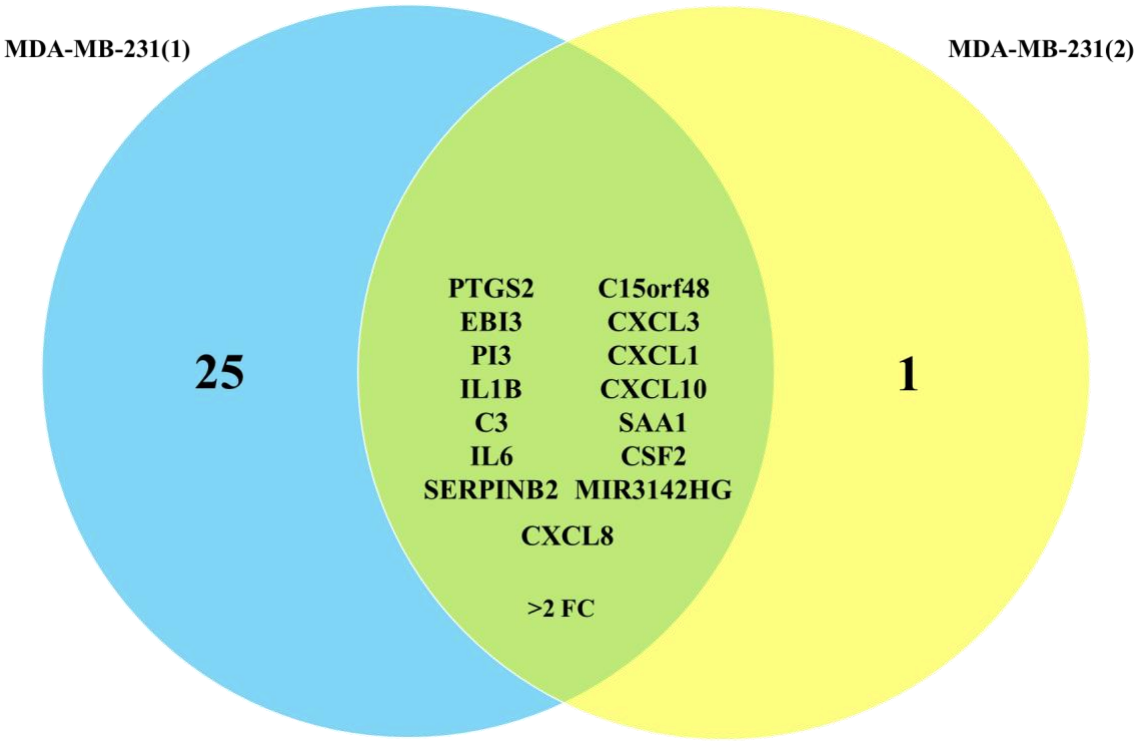


Figure 5: Venn diagram showing the common up-regulated DEGs in the RNA Sequencing data of MDA-MB-231 Run 1 and 2. Genes with a differential expression ≥ 2-fold and p-value < 0.01 in MDA-MB-231 cells for both the runs were considered.

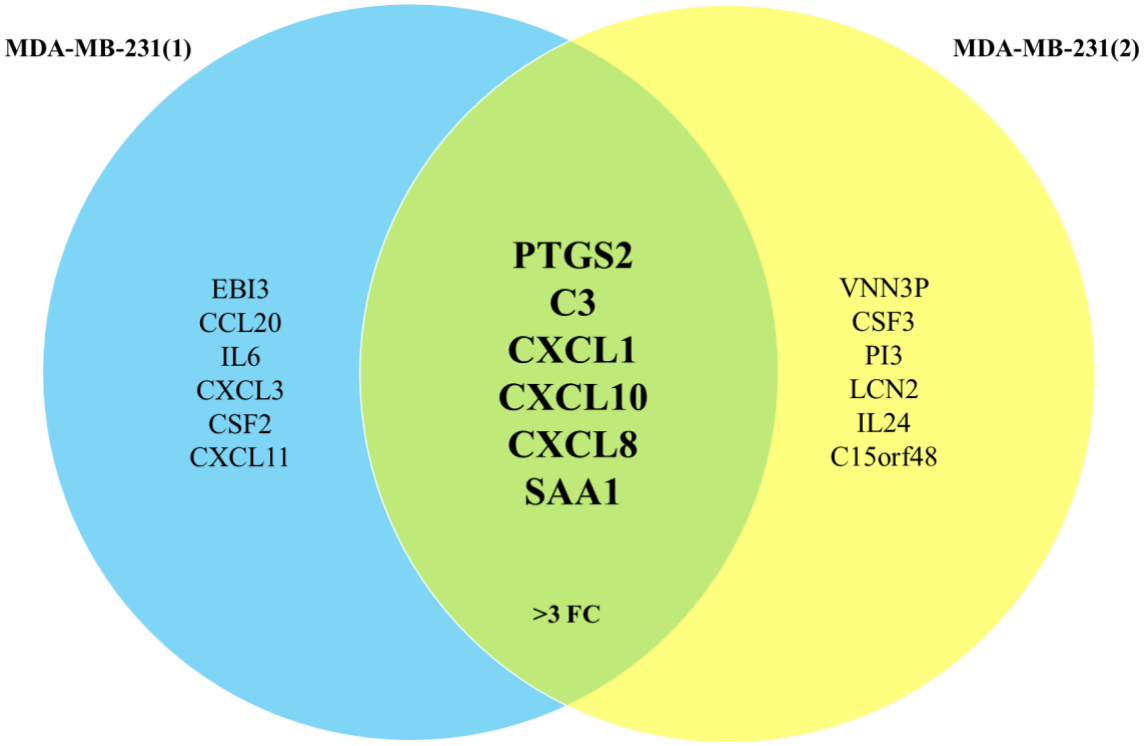


Figure 6: Venn diagram showing the common up-regulated DEGs in the RNA Sequencing data of MDA-MB-231 Run 1 and 2. Genes with a differential expression ≥ 3-fold and p-value < 0.01 in MDA-MB-231 cells for both the runs were considered.

For further screening, MCF10A, the normal epithelial cell line was included which serves as a reference point for understanding the gene expression changes specific to *B. burgdorferi*-infected TNBC cells. Investigating that, a fold change cut off ≥1.5 was considered to identify genes that were consistently upregulated commonly for the MDA-MB-231 Run (1+2) (Refer to Figure 5) and MCF10A cell line. Figure 9, illustrates the common genes that were upregulated in response to *B. burgdorferi* infection in both MDA-MB-231 and MCF10A cell lines. A total of 6 genes that were consistently upregulated in both cell lines under these conditions were observed. These included

the CXCL family genes- CXCL10, 8, 3 & 2, CCL20 and IFIT1. The identified common upregulated genes between MDA-MB-231 and MCF10 cells may indicate 6 critical markers of the host response to *B. burgdorferi* infection, irrespective of the cell's malignancy status.

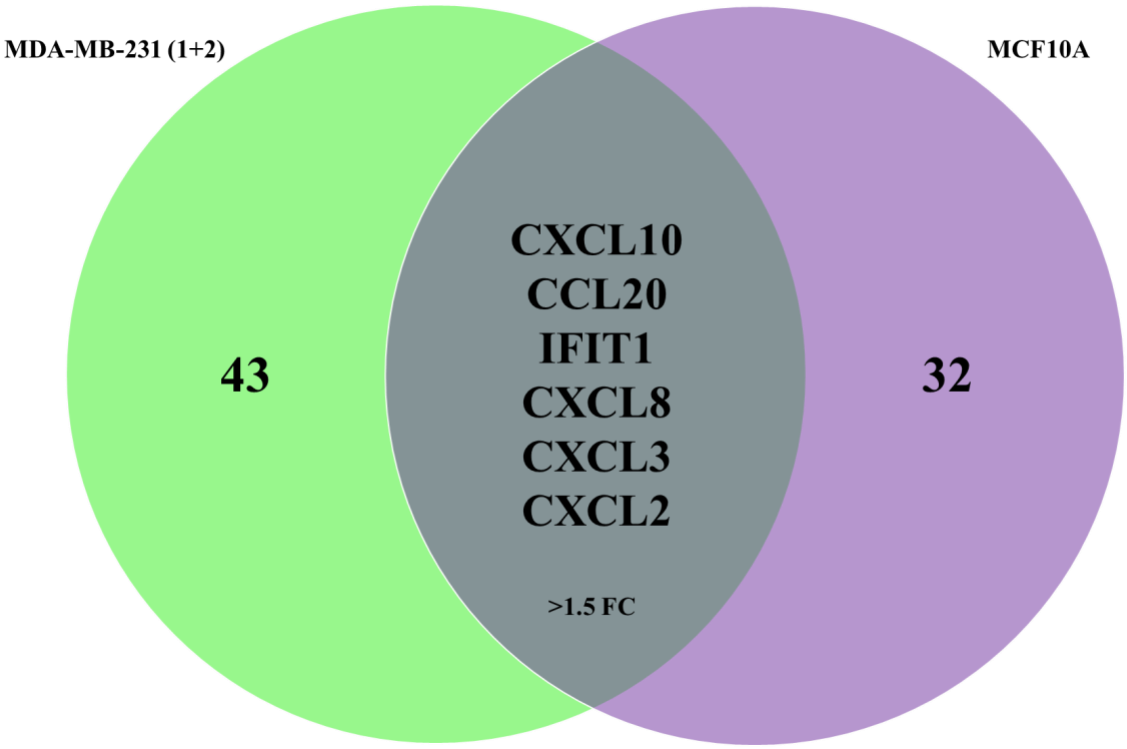


Figure 7: Venn diagram showing the common up-regulated DEGs in the RNA Sequencing data of MDA-MB-231 Run 1+2 and MCF10A. Genes with a differential expression ≥ 1.5-fold and p-value < 0.01 in MDA-MB-231 cells for both the runs together and MCF10A were considered.

KEGG and REACTOME pathway enrichment analyses of DEGs in triple-negative breast cancer and normal-like mammary epithelial cells

To further explore the functional significance of these common identified genes, pathway analysis was performed using the KEGG and REACTOME pathway enrichment analyses on the DAVID Bioinformatics site (https://david.ncifcrf.gov). The enrichment analysis using the screened DEGs from the above analysis revealed pathways related to inflammation, infection,

metabolism, and cancer pathways, supporting the notion that these genes may be involved in critical biological processes implicated in *B. burgdorferi* -associated TNBC cells.

In order to gain insights into the functional implications of the identified upregulated genes in MDA-MB-231 cells, pathway analysis was performed using the top 12 genes consistently upregulated in both MDA-MB-231 Run 1 and Run 2 (Figure 5) upon *B. burgdorferi* infection. These 12 genes were selected based on their highest average fold change values and their consistent upregulation across independent RNA sequencing runs. The heatmap (Figure 11) represents the expression patterns of these 12 upregulated genes and their corresponding fold changes in response to *B. burgdorferi* infection. This expression pattern was studied using the KEGG pathway analysis. The color intensity in the heatmap reflects the magnitude of gene expression changes, with red indicating higher expression levels and light yellow indicating lower expression levels. Pathway analysis of these top 12 upregulated genes revealed their involvement in approximately 15 KEGG pathways associated with diverse inflammation processes. Notably, several signaling pathways including the IL-17, TNF, NOD like receptor, TLR, NF-kB and Chemokine-signaling pathways were significantly enriched. Moreover, receptor interactions such as cytokine-cytokine receptor interaction and viral protein interaction with cytokine receptor was significantly enriched. Inflammatory disease pathways such as for Rheumatoid Arthritis, Alzheimer's, Alcoholic liver disease and Atherosclerosis were enriched too in the KEGG pathway analysis. Additionally, several of the DEGs were also enriched in pathways implicated in cancer. From the top 12 genes common to both the RNA Sequencing runs for MDA-MB-231(1+2), the CXCL-family genes (CXCL8,3 & 1) and IL6 were the most common in all the KEGG inflammatory pathways enriched. Also, IL1B was one of the most common genes with a lower fold-change however making a very significant difference by being

involved in very important pathways as depicted in Figure 11. These findings align with the emerging evidence of *B. burgdorferi 's* influence on inflammation in MDA-MB-231 cells.

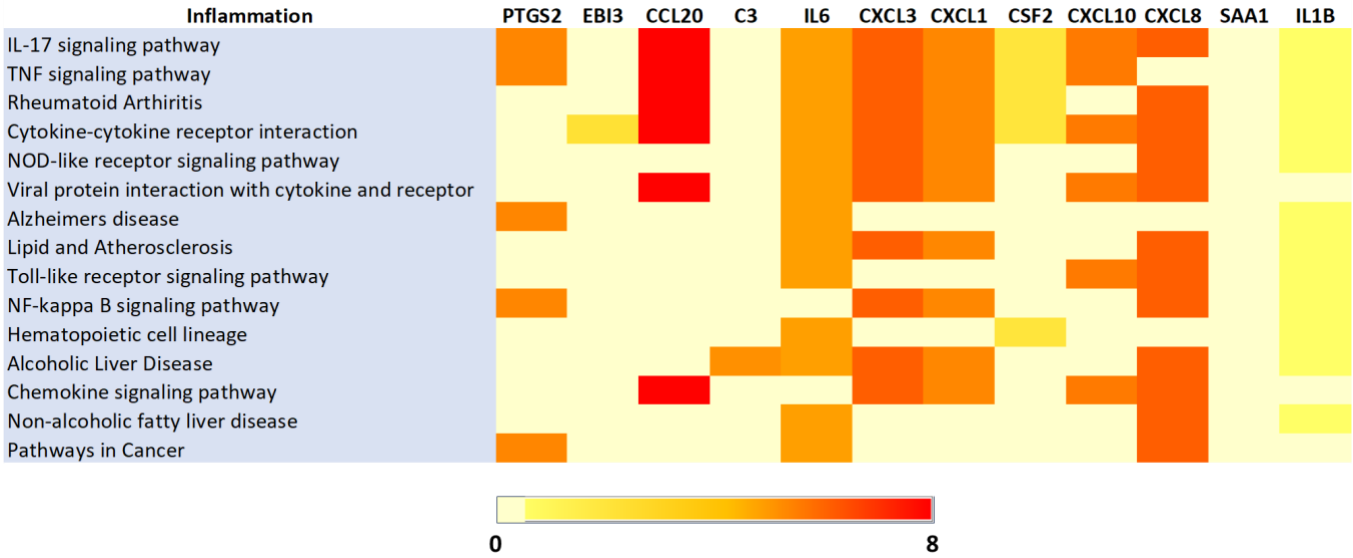


Figure 8: Heatmap depicting the expression of top 12 upregulated genes and their fold changes associated with KEGG inflammation pathways from *B. burgdorferi* -infected MDA-MB-231 (1+2) Runs. Pathways with the Benjamini-Hochberg adjusted p-value ≤ 0.10 were considered. Red indicates higher expression levels and light yellow indicates lower expression levels.

Figure 12 represents the expression patterns of the same 12 upregulated genes and their corresponding fold changes in response to *B. burgdorferi* infection. Pathway analysis of these top 12 upregulated genes revealed their involvement in approximately 15 KEGG pathways associated with diverse infectious processes. The KEGG server highlighted the involvement of the 12 DEGs in pathways activated during various infectious diseases like COVID-19, Legionellosis, Prion disease, Influenza-A, Pertussis, Measles, Kaposi-sarcoma-associated herpesvirus and Chagas disease, among others. Notably, only one signaling pathway that is the RIG-I like receptor-signaling pathways was significantly enriched. Additionally, pathways involved in pathogenic *E. coli* infection and epithelial cell signaling in *H. Pylori* infection was also observed which shows potential connection to other infectious origin pathways in TNBC

cells- MDA-MB-231. From the top 12 genes common to both the RNA Sequencing runs for MDA-MB-231(1+2), the CXCL-family genes (CXCL8,3 & 1), IL6 and IL1B were also common in majority of the KEGG infection pathways enriched. Although, complement 3, which showed regulation for only one of the inflammatory pathways (Figure 10), was enriched for a total of seven out of the 15 pathways depicting a key role in body's infection related immune response. These findings align with the emerging evidence of *B. burgdorferi 's* influence on infection in MDA-MB-231 cells.

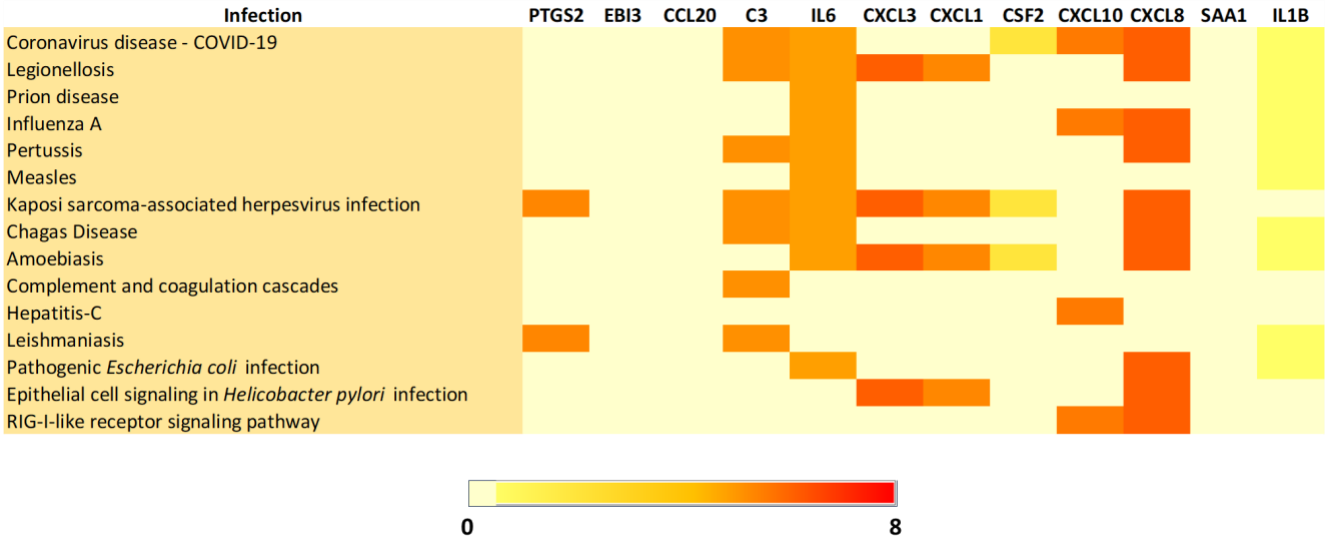


Figure 9: Heatmap depicting the expression of top 12 upregulated genes and their fold changes associated with KEGG infection pathways from *B. burgdorferi* -infected MDA-MB-231 (1+2) Runs. Pathways with the Benjamini-Hochberg adjusted p-value ≤ 0.10 were considered. Red indicates higher expression levels and light yellow indicates lower expression levels.

To further explore the functional significance of the top 12 upregulated genes consistently observed in both MDA-MB-231 Run 1 and Run 2 upon *B. burgdorferi* infection, REACTOME pathway enrichment analysis was conducted which showed 9 inflammation, 2 infection and 1 metabolic pathway enriched. From Figure 13A, out of the nine pathways enriched for inflammation, six of them were involved in important signalling pathways such as cytokine, interleukin (IL-10, 4 & 13), G alpha (I) and GPCR downstream signalling pathways. The other

important pathways indicate the receptors functioning such as the chemokine receptors, peptide ligand binding receptors and rhodopsin-like receptors. Among the 12 top regulated genes, CXCL8 was involved in all the pathways followed by CXCL10 & 1 and CCL20 being the most common genes expressed in majority of the inflammatory pathways. Surprisingly the expression of SAA1, which was not in the KEGG pathway analysis, was in 7 out of the 9 pathways enriched. Looking further onto the infection pathways enriched in the REACTOME server for MDA-MB-231 (Figure 13B), only two significant pathways were observed which includes the immune system pathway depicting the upregulation of all twelve genes except CXCL3. Also, for the IFIH1 mediated induction of interferon-alpha/beta pathway, only SAA1 was involved. Figure 13C shows the only involvement of PTGS2 in the Nicotinate metabolism pathway as per the REACTOME pathway analysis.

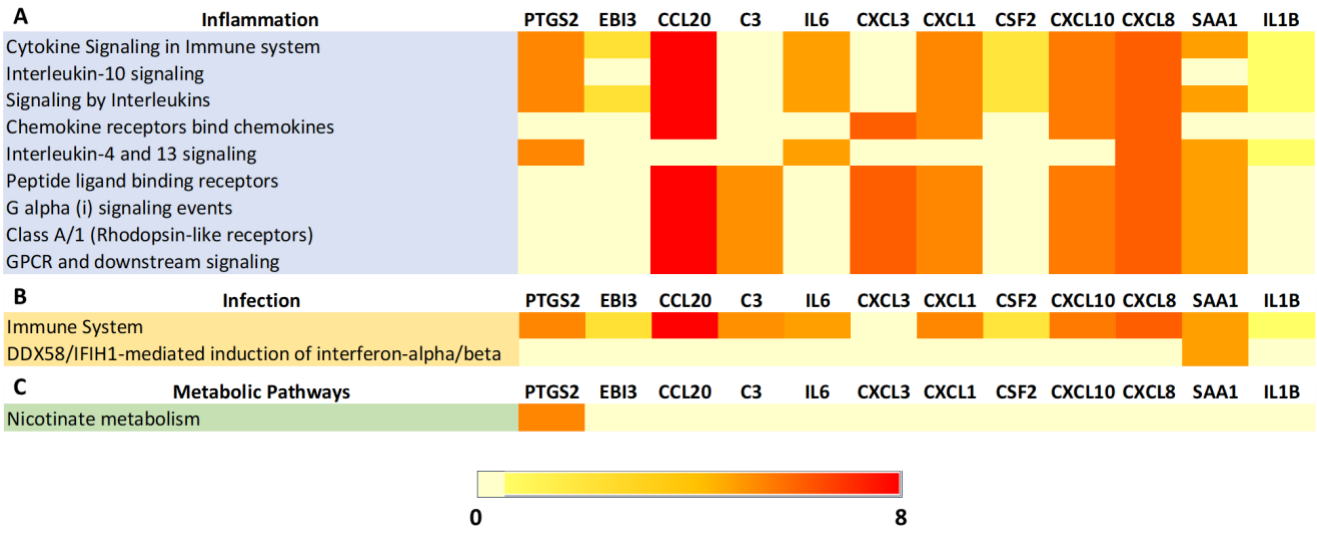


Figure 10: Heatmap depicting the expression of top 12 upregulated genes and their fold changes associated with (A) REACTOME inflammation pathways, (B) REACTOME infection pathways and (C) REACTOME metabolic pathways from *B. burgdorferi* -infected MDA-MB-231 (1+2) Runs. Pathways with the Benjamini-Hochberg adjusted p-value ≤ 0.10 were considered. Red indicates higher expression levels and light yellow indicates lower expression levels.

The comparative analysis of the effects of *B. burgdorferi* infection in both normal and cancerous cellular contexts was perpetuated by using MCF10A cells, which serve as a relevant control to discern pathway alterations that are specific to *B. burgdorferi* -infected TNBC cells, as opposed to general cellular responses to *B. burgdorferi*.

KEGG pathway analysis was performed using the top 12 genes consistently upregulated in MCF10A RNA sequencing data upon *B. burgdorferi* infection. Pathway analysis of these upregulated genes revealed their involvement in approximately 7 KEGG pathways associated with diverse inflammation processes. The heatmap (Figure 15A) depicts some already indicated IL-17, chemokine & TNF signaling pathways significantly enriched. Inflammatory disease pathways such as for Rheumatoid Arthritis and Alcoholic liver disease were also enriched in the KEGG pathway analysis for MCF10A. Additionally, several DEGs were enriched in pathways implicated in cytokine-cytokine receptor signaling as well as viral protein interaction with cytokine-cytokine receptors. From the top 12 genes, the CXCL-family genes (CXCL8,3, 2 & 10) and CCL20 were the most common in majority of the KEGG inflammatory pathways enriched. Also, the endoplasmic reticulum (ER) enzyme stearoyl-CoA desaturase (SCD) was involved in only Alcoholic liver disease pathway with a lower fold-change as depicted in Figure 15A. Figure 15B represents the expression patterns of the same 12 upregulated genes and their involvement in approximately 4 KEGG pathways associated with diverse infectious processes. The KEGG server highlighted the involvement of the DEGs in previously discussed pathways activated during various infectious diseases like amoebiasis, hepatitis-C, legionellosis and influenza-A. From the top 12 genes of the RNA Sequencing run for MCF10A, the CXCL-family genes (CXCL8,3,2 & 10) and RSAD2 were common in majority of the KEGG infection pathways enriched. Also, farnesyl diphosphate synthase (FDPS) showed regulation for only one of the

infectious disease pathways i.e., Influenza-A. Additionally, the KEGG server in DAVID, highlighted fatty acid metabolism and metabolic pathways in general, for MCF10A as shown in Figure 15C. Interestingly, several DEGs were enriched in pathways related to biosynthesis of steroids, terpenoid backbone and unsaturated fatty acids, which could be indicative of a cellular transformation in MCF10A. The most common genes involved in at least two of these 5 pathways related to metabolism and biosynthesis are Methyl sterol monooxygenase 1 (MSMO1), member of the fatty acid desaturase gene family-FADS1 & 2, enzyme involved in lipid metabolism- ACAT2, FDPS and SCD.

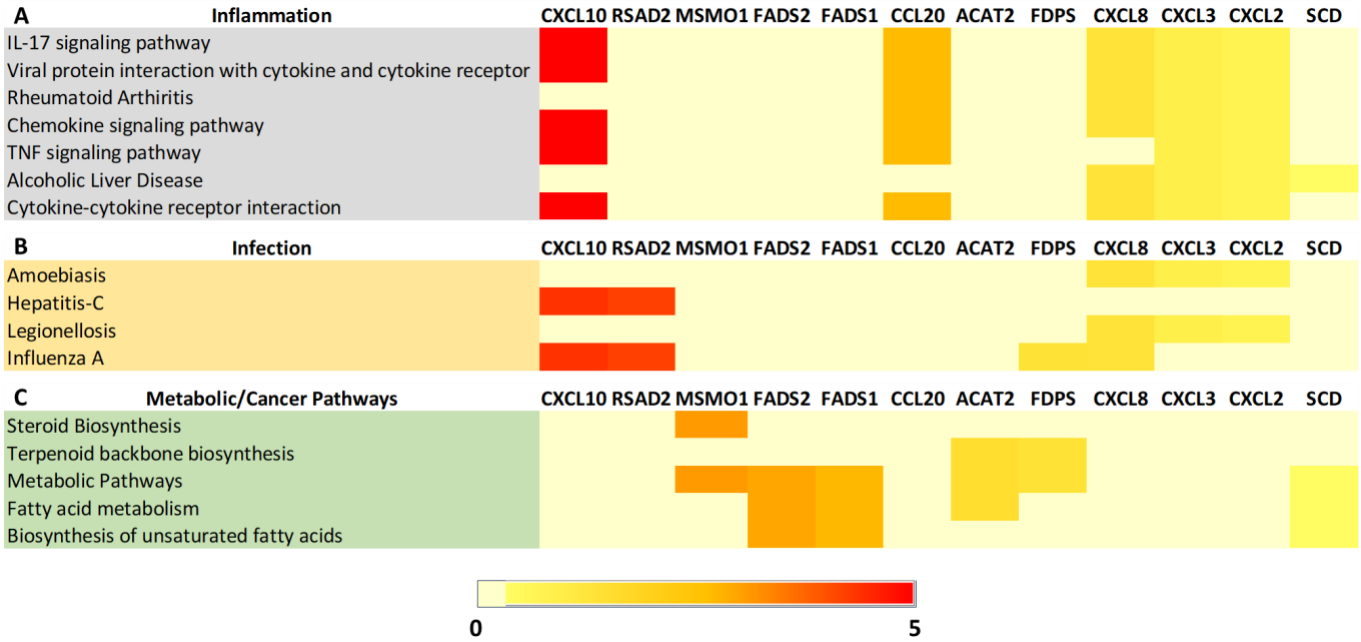


Figure 11: Heatmap depicting the expression of top 12 upregulated genes and their fold changes associated with (A) KEGG inflammation pathways, (B) KEGG infection pathways and (C) KEGG metabolic pathways from *B. burgdorferi* -infected MCF10A cells. Pathways with the Benjamini-Hochberg adjusted p-value ≤ 0.10 were considered. Red indicates higher expression levels and light yellow indicates lower expression levels.

To further explore the functional significance of the top 12 upregulated genes consistently observed in MCF10A upon *B. burgdorferi* infection, REACTOME pathway enrichment analysis was conducted which surprisingly showed 4 inflammation and 4 metabolic pathways enriched.

From Figure 16A, out of the four pathways enriched for inflammation, 1 of them is involved in the interleukin-10 signalling pathway. The other important pathways indicate the receptors functioning such as the chemokine receptors and the peptide ligand binding receptors. Cytokine signalling in the immune system is also one important pathway enriched in this analysis. Among the 12 top regulated genes, CXCL8, 2 & 10 and CCL20 was involved in all the pathways. CXCL3 was involved in binding of the chemokine receptors pathway and peptide ligand binding receptors pathway. Also, RSAD2 is involved in cytokine signalling in the immune system. Surprisingly the expression of SAA1, which was nil in the KEGG pathway analysis, was expressed in 7 out of the 9 pathways enriched. Looking further onto the metabolic pathways in the REACTOME server for MCF10A (Figure 16B), metabolism of steroids, lipids and metabolism in general was significantly enriched. Interestingly, several DEGs were enriched in pathways related to biosynthesis cholesterol which further confirms the chances of cellular transformation in MCF10A. The most common genes include MSMO1, ACAT2 and FDPS in all the metabolic and biosynthesis pathways.

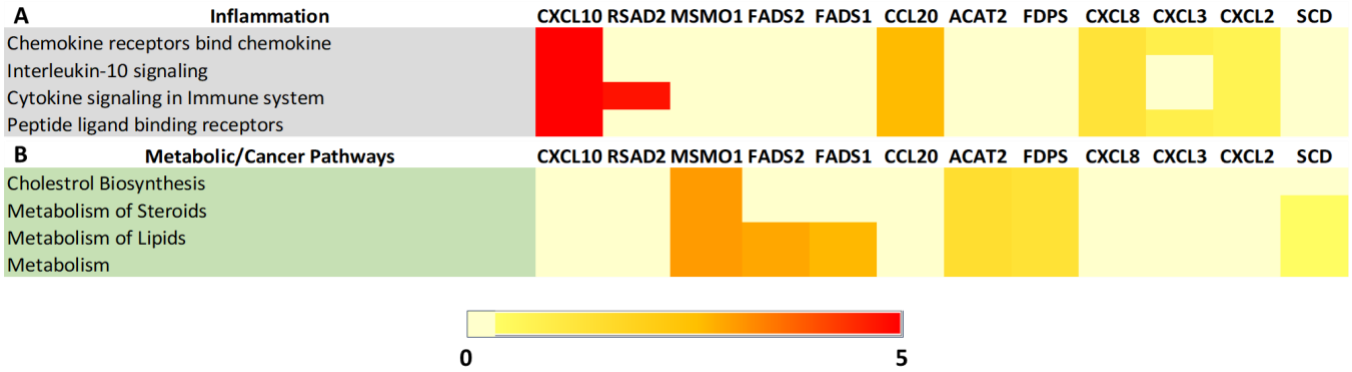


Figure 12: Heatmap depicting the expression of top 12 upregulated genes and their fold changes associated with (A) REACTOME inflammation pathways, (B) REACTOME metabolic pathways from *B. burgdorferi* -infected MCF10A cells. Pathways with the Benjamini-Hochberg adjusted p-value ≤ 0.10 were considered. Red indicates higher expression levels and light yellow indicates lower expression levels.

DISCUSSION

Although great efforts have been invested in the management of TNBC, the prognosis of infection associated TNBC patients is still poor. Thus, it is necessary to elucidate detailed mechanisms for early diagnosis, treatment, and prognosis of infection associated TNBC. Our previous studies demonstrated that by 48 hours, 96% of *B. burgdorferi* spirochetes were able to internalize within MDA-MB-231 cells, while 52% spirochetes were able to invade MCF10A cells [54]. Additionally, Matrigel based invasion assays have demonstrated a 4-fold increase in invasion by MDA-MB-231 cells upon infection with *B. burgdorferi* accompanied with a 48% increase in their migration rate in corresponding wound-healing assays [54]. Thus, tackling this complex form of infection associated cancer requires developing targeted therapies, enhancing treatment efficacy while reducing side effects.

With the progress in bioinformatics analysis, analysis of sequencing data has been regarded as a useful strategy for investigating general genetic alterations and deciphering the pathophysiology of TNBC. In this study, we performed RNA sequencing on two different cell lines: MDA-MB-231-twice and MCF10A. Through this multiple run approach, we could identify potential biomarkers that are consistently differentially expressed in response to *B. burgdorferi* infection across TNBC cell lines while also distinguishing TNBC-specific responses from general cellular responses to infection. From the intersection of the Venn diagram (Figure 5), 49 genes were identified that were commonly upregulated in both MDA-MB-231 Run 1 and Run 2 in response to *B. burgdorferi* infection. These 49 genes were consistently upregulated with a fold change cutoff of ≥1.5. Upon increasing the fold change cutoff to ≥3 (Figure 7), a subset of 6 genes were identified that were upregulated in both runs. These genes included members of the CXCL family (CXCL-1, CXCL-8, and CXCL-10), C3, PTGS2 (COX2), and SAA1. The CXCL family of chemokines

plays a crucial role in inflammation and immune response regulation. Previous studies have linked CXCL-1, CXCL-8, and CXCL-10 to breast cancer development and progression, and they have been associated with tumour growth, metastasis, and angiogenesis [61-63]. In breast cancer cells, CXCL1 expression is lineage-dependent, being highest in triple-negative MDA-MB-231 cells [64,65]. CXCL1 induces cell proliferation, migration, and epithelial-to-mesenchymal transition (EMT) [66,67]. It also contributes to the formation of metastasis, particularly in the bone, lung, brain, and lymph nodes [65]. CXCL1 is also said to be associated with chemoresistance and radio resistance, and its increased expression is linked to a worse prognosis in obese breast cancer patients [68-71]. In humans, Interleukin-8 (IL-8 or CXCL8) is a granulocytic chemokine with multiple roles within the tumor microenvironment (TME), such as recruiting immunosuppressive cells to the tumor, increasing tumor angiogenesis, and promoting EMT [72]. All these effects of CXCL8 on individual cell types can result in cascading alterations to the TME [73]. It has also been reported that CXCL10 also might be related to the prognosis in pancreatic adenocarcinoma [74] and TNBC [75]. For TNBC, a recent study demonstrated that CXCR4 and CXCL10 were the chemokines related to prognosis. Therefore, CXCL10 might be an effective prognostic factor and obviously potential therapeutic target for TNBC treatment [76]. The complement component C3 plays a significant role in triple-negative breast cancer (TNBC) and infection, exerting diverse effects in both contexts. In TNBC, C3 has been implicated in promoting tumour growth, invasion, and metastasis. Its expression is often elevated in TNBC tissues, and studies have shown that C3 contributes to tumour progression by interacting with complement receptors on cancer cells, leading to activation of downstream signalling pathways that promote proliferation and survival [77,78]. Moreover, C3 can interact with tumour-associated macrophages and immune cells, fostering an immunosuppressive microenvironment that hinders anti-tumour immune responses.

In the context of infection, C3 is a key component of the innate immune response. When pathogens invade the body, the complement system, including C3, is activated to opsonize and eliminate the invaders [79]. PTGS2 or COX2 a downstream target of TGFβ plays an important role in regulating cancer stem cells in TNBC. COX-2 catalyzes a key step in the formation of prostaglandins (PGs) and is highly induced at inflammatory sites and during tumor progression [80]. SAA1 on the other hand, an important marker for LD, induces suppressive neutrophils through the Toll-like receptor 2–mediated signalling pathway to promote progression of breast cancer [81]. Serum amyloid A levels are significantly elevated in several malignancies and correlate with poor prognosis [82]. Previous studies have reported that SAA is a critical mediator of pro-inflammatory cytokine production and recruitment of immune cells, and thereby promotes tumor development by facilitating angiogenesis, tumor invasion, and immunosuppression [83,84]. These six important genes identified, thereby hold promise as potential diagnostic or therapeutic targets for *B. burgdorferi* -associated TNBC cells.

To further analyse the common markers for *B. burgdorferi* infection in normal breast cells, a comparative analysis including MCF10A was carried out which identified genes in common to both the MDA-MB-231 runs (1+2) together. These genes included members of the CXCL family (CXCL10, CXCL8, CXCL3, and CXCL2), CCL20, and IFIT1. CCL20, is implicated in the formation and function of mucosal lymphoid tissues via chemoattraction of lymphocytes and dendritic cells towards the epithelial cells surrounding these tissues [86]. Additionally, the expression of CCL20 in triple-negative breast cancer cell lines (MDA-MB-231 and BT549) facilitated tumor migration by upregulating MMP1, MMP-2 and MMP-9 [87]. IFIT1 modulates the proliferation, migration, and invasion of pancreatic cancer cells via Wnt/β-catenin signalling [88]. In breast cancer, IRDS genes are regulated by exosome transfer from stroma cells, mediate

resistance to radiation- and chemo-therapy, predict poor prognosis and are enriched in cells exerting cancer stem cell like features [89]. In one of the previous studies, overexpression of GIRK1 in the benign MCF10A triggered enhanced expression of several IRDS genes such as IFIT1, IFIT3, IRF7 and ISG15 [90]. These results altogether suggest that CXCL8 and CXCL10 are promising therapeutic targets, having consistent expressions in all the three runs, for *B. burgdorferi* infected cells.

Infection-associated triple-negative breast cancer (TNBC) cells have been shown to exhibit dysregulation in various signaling pathways involved in immune responses and pathogen recognition. One of the key pathways implicated in infection-associated TNBC is the toll-like receptor (TLR) pathway. TLRs play a crucial role in recognizing pathogen-associated molecular patterns (PAMPs) and initiating innate immune responses against invading pathogens, including bacteria like *B. burgdorferi* [91]. Additionally, the nuclear factor-kappa B (NF-κB) pathway is often upregulated in infection-associated TNBC, as NF-κB plays a central role in regulating the expression of genes involved in inflammation and cell survival [92]. Moreover, the Janus kinase-signal transducer and activator of transcription (JAK-STAT) pathway have been implicated in infection-associated breast cancer, as it contributes to the modulation of immune responses and cell proliferation [93]. These pathways may collectively contribute to the host's response to *B. burgdorferi* infection in TNBC cells and potentially influence the tumor microenvironment, influencing cancer progression and immune evasion. In order to confirm the same, our pathway analysis identified several common pathways involved in inflammation, infection. From the KEGG server, the most common inflammatory pathways in TNBC cells included the IL-17 signaling, TNF signaling, NF-kB signaling and chemokine signaling pathways with several of the above identified genes in function. The other signaling pathways enriched by the REACTOME

server include cytokine signaling, IL-4, 10 & 13 signaling, G alpha (i) and GPCR downstream signaling pathways. From the KEGG server, the most common inflammatory pathways in MCF10A cells included the IL-17 signaling, TNF signaling and chemokine signaling pathways with several of the above identified genes in function. The other signaling pathways enriched by the REACTOME server include IL-10 signaling, and cytokine signaling pathways. Several pathways associated to inflammatory diseases were also identified for all the three runs, the most common being Rheumatoid Arthritis and Alcoholic Liver disease. From the KEGG server, the most common infectious disease associated pathways in TNBC cells included COVID-19, Legionellosis, Prion disease, Influenza-A, Pertussis, Measles, Kaposi-sarcoma-associated herpesvirus, and Chagas disease, among others. Additionally, pathways involved in pathogenic E. coli infection and epithelial cell signaling in H. Pylori infection was also observed which shows potential connection to other infectious origin pathways in TNBC cells- MDA-MB-231.The other infection associated pathways also common to MCF10A include Hepatitis-C and Amoebiasis. The most common enriched metabolic pathways for MCF10A from the KEGG and REACTOME servers include fatty acid metabolism, glycolipid metabolism and steroid metabolism. Biosynthesis pathways such as steroid and terpenoid backbone biosynthesis pathways were also observed common from the KEGG and REACTOME server for MCF10A. Further studies are needed to unravel the precise molecular mechanisms underlying these pathways in the context of infection-associated TNBC and their potential as therapeutic targets. From the REACTOME server, the most common signaling pathways common to MCF10A include the IL-10 signaling and cytokine signaling pathways. Several pathways associated to receptor binding were also identified for MDA-MB-231(1+2) and MCF10A, the most common being chemokine receptors and peptide ligand binding receptors. There were no common pathways for metabolism and biosynthesis in

any of the TNBC cell lines runs and MCF10A suggesting that cancer cells exhibit distinct metabolic characteristics compared to normal cells, a phenomenon known as "metabolic reprogramming." The altered metabolism in cancer cells is driven by various factors, including oncogenic mutations and the tumor microenvironment.

Altogether, the biomarkers identified show significant correlations to cancer and its progression. It is important to thereby understand what these pathways implicate. IL-17 signaling directly activates the early stages of cancer cell proliferation via STAT3 signaling [94]. IL-17 also promoted tumor graft development and directly inhibited apoptosis in several breast cancer cell-lines in a TGF-β dependent manner [95]. IL-17 secretion promotes the development of chemoresistance in breast cancer by suppressing the expression of pro-apoptotic signals [96]. This association to cancer progression suggests a similar phenomenon induced by *B. burgdorferi* infection. Similarly, the IL-10 signaling pathway is said to be involved in signaling via the JAK/STAT pathway. IL-10 upon binding to its receptor causes phosphorylation of JAK1 which inturn activates STAT3 [97]. But its implications on cancer have been paradoxical as both the pro- and anti-tumor effects of IL-10 have been well characterized [98]. IL-10 can promote CD8+ T cell activation and proliferation, which has a direct or indirect cytotoxic effect on the cancer cells. Second, IL-10 inhibits T cell-stimulated tumor-killing immunity by suppressing antigen presentation by APCs [99]. Other important signaling pathways implicated towards cancer include the other type 2 cytokine signaling pathways IL4 and IL13, which are said to be overexpressed in cancers including pancreatic cancer [100]. IL-4 was also considered to be closely related to the poor outcome of breast cancer thereby emphasizing the role of these important signaling pathways that have been enriched by *B. burgdorferi* infection in TNBC cells [101]. Receptor interaction pathways were also enriched significantly some of which included the cytokine-cytokine receptor

interactions depicting the various cytokines in play and the toll-like receptor pathway in response to the *B. burgdorferi* infection. TLR stimulation is said to have both pro-tumor role and anti-tumor roles depending on the cell type [102]. Stimulation of TLR 2, 4, and 7/8 can lead to tumor progression via production of immunosuppressive cytokines, increased cell proliferation and resistance to apoptosis [103]. Another significant receptor interaction is the NOD like receptors which are major players (and targets) in the interface between innate immunity and cancer. Overexpression of either NOD1 or NOD2, in the TNBC cells, can reduce cell proliferation but increase clonogenic potential in vitro [104]. The proteomic profile of these overexpressing cells suggests the involvement of several inflammation- and stress-related pathways (intersecting NF-κB, PI3K and MAPK cascades) in the modulation of protein degradation processes, cell cycle and cellular adhesion [105]. The disruption of these critical systems suggests a functional link between NOD1/NOD2 and the proliferation and migration of triple negative breast cancer cells [105]. These pathways induced by Borrelia infection could thereby have significant role in driving the progression of this complex breast cancer type. Also, importantly, these pathways implicate, irrespective of the fold change, it is important to understand the genes are involved in a number of these crucial pathways leading towards cancer development.

One of the recent studies presents a complete picture of the signaling events evoked in human brain microvascular endothelial cells (hBMECs) by *Borrelia bavariensis* infection. This study revealed several of the genes related to cytokines, chemokines, interferon, and TNF that may cause alteration in the blood-brain barrier (BBB) integrity [58]. While our study focused on investigating the gene expression changes in triple-negative breast cancer (TNBC) cells upon *Borrelia burgdorferi* infection, the endothelial cells of the brain microvasculature represent a distinct cellular context. Some of the most common upregulated genes between hBMECs and MDA-MB-

231 (Figure 5) include the CXCL family genes- CXCL8, CXC3CL1, CXCL1, CXCL10, CXCL3 and CXCL2 playing significant roles in signal transduction, innate immune response and in the Induction of the Pattern Recognition Receptors and Downstream Signalling Cascades. CCL20 was also identified common which plays a significant role in the latter of the two mentioned above. The interleukin family- IL1B and IL6 were common which were discussed to be responsible for remodulation of cell cytoskeleton and recruiting leukocytes during the *B. burgdorferi* l invasion. The binding and uptake of ligands by scavenger receptors was functioned by SAA1. Also, C3 was identified to be recurrent in their RNA Sequencing data. The other common genes include C15orf48, GoS2, POU2F2, LAMC2, CTSS, PTX3 and ZC3H12A involved in several of the Gene ontology biological processes. A previous study by our research group identified miR-206 and 214-3p as the most statistically significant miRNA upregulations in *B. burgdorferi*-infected breast cell lines compared to the uninfected breast cells [56]. The down streaming gene targets of miR-206 was identified to be common to two of the most significant genes expressed in our comparative analysis of the two MDA-MB-231 runs (Figure 6)- PTGS2 and IL-6. Inhibition of MALAT1 leads to the restoration of miR-206 levels, subsequently reducing the expression of PTGS2 [106]. Moreover, IL-6 induced miR-206 down-regulation by reducing the cropping process of primary miR-206 in EGFR-mutant lung cancer cells [107]. This further helps us target these significant genes with miRNAs to have a more comprehensive and detailed understanding of the molecular landscape underlying *B. burgdorferi* -infected triple-negative breast cancer.

CONCLUSION

B. burgdorferi infection has a significant impact on triple-negative breast cancer (TNBC) cells, leading to dysregulation of various genes and pathways involved in inflammation, immune

response, and cancer progression. The study utilized RNA sequencing to identify differentially expressed genes (DEGs) in response to *B. burgdorferi* infection in two cell lines: MDA-MB-231, and MCF10A (normal breast cells). By comparing the gene expression profiles of the two MDA-MB-231 runs, we identified common biomarkers of the CXCL family CXCL-1, CXCL-10 & CXCL-8, C3, COX2 and SAA1 in *B. burgdorferi* -infected TNBC cells. Pathway analysis revealed the IL-4, 10, 13 & 17 signaling, TNF, NOD like receptor, TLR, NF-kB and Chemokine-signaling pathways. The most enriched signaling pathways in MCF10A included the IL-10 & 17, chemokine, cytokine and TNF signaling pathways. The most common infectious disease associated pathways in TNBC cells included COVID-19, Legionellosis, Prion disease, Influenza-A, Pertussis, Measles, Kaposi-sarcoma-associated herpesvirus, and Chagas disease, among others with several of the above identified genes in function. The most common infectious disease associated pathways in MCF10A cells included Amoebiasis, Legionellosis, Hepatitis-C and Influenza-A. The most common enriched metabolic pathways for MCF10A from the KEGG and REACTOME servers include fatty acid metabolism, glycolipid metabolism and steroid metabolism. Biosynthesis pathways such as steroid and terpenoid backbone biosynthesis pathways were also observed common from the KEGG and REACTOME servers for MCF10A.Triple-negative breast cancer is one of the most aggressive types of breast cancer, which has a an extremely high treatment burden and no definitive cause and potentially a wide array of mechanisms that have gone awry. The prevalence of Lyme disease combined with the ability of the Lyme disease pathogen *B. burgdorferi* to infect breast cancer cells prompts to a potential cause-effect relationship which demands to be explored. High throughput transcriptomic studies such this, can prove to be extremely effective for preliminary screening to identify molecular markers that the bacteria may be implementing to orchestrate more severe breast cancer phenotypes,

particularly TNBC. In conclusion, our study provides valuable insights into the gene expression changes and pathways associated with *B. burgdorferi* infection in TNBC cells. The identified potential biomarkers represent a promising starting point for future research aiming to elucidate the underlying mechanisms of *B. burgdorferi* -associated TNBC and identify novel targets for diagnostic and therapeutic interventions.

REFERENCES

1. Breast Cancer Facts & Figures 2022-2024 American Cancer Society. https://www.cancer.org/content/dam/cancer-org/research/cancer-facts-and-statistics/breast-cancer-facts-and-figures/2022-2024-breast-cancer-fact-figures-acs.pdf Accessed June 26, 2023.
2. Yin L, Duan JJ, Bian XW, Yu SC. Triple-negative breast cancer molecular subtyping and treatment progress. *Breast Cancer Res*. 2020;22(1):61. Published 2020 Jun 9. doi:10.1186/s13058-020-01296-5
3. André F, Zielinski CC. Optimal strategies for the treatment of metastatic triple-negative breast cancer with currently approved agents. Ann Oncol. 2012;23 Suppl 6:vi46-vi51. doi:10.1093/annonc/mds195
4. Zhuang J, Dai S, Zhang L, et al. Identifying Breast Cancer-induced Gene Perturbations and its Application in Guiding Drug Repurposing. Current Bioinformatics. 2020;15(9):1075-1089. Accessed July 28, 2023. https://www.eurekaselect.com/article/104155
5. Wapnir IL, Price KN, Anderson SJ, et al. Efficacy of Chemotherapy for ER-Negative and ER-Positive Isolated Locoregional Recurrence of Breast Cancer: Final Analysis of the CALOR Trial. J Clin Oncol. 2018;36(11):1073-1079. doi:10.1200/JCO.2017.76.5719
6. Xu J, Liao K, Fu Z, Xiong Z. Screening differentially expressed genes of pancreatic cancer between Mongolian and Han people using bioinformatics technology. BMC Cancer. 2020;20(1):298. Published 2020 Apr 9. doi:10.1186/s12885-020-06722-7
7. Cheng L, Qi C, Zhuang H, Fu T, Zhang X. gutMDisorder: a comprehensive database for dysbiosis of the gut microbiota in disorders and interventions [published correction appears in Nucleic Acids Res. 2020 Jul 27;48(13):7603]. Nucleic Acids Res. 2020;48(D1):D554-D560. doi:10.1093/nar/gkz843
8. Gu Y, Gao Y, Tang X, Xia H, Shi K. Bioinformatics Analysis Identifies CPZ as a Tumor Immunology Biomarker for Gastric Cancer. Current Bioinformatics. 2020;16(1):98-105. Accessed July 28, 2023. https://www.eurekaselect.com/article/107967
9. Yuan G, Ding W, Sun B, Zhu L, Gao Y, Chen L. Upregulated circRNA_102231 promotes gastric cancer progression and its clinical significance. Bioengineered. 2021;12(1):4936-4945. doi:10.1080/21655979.2021.1960769

10. Cheng L, Qi C, Yang H, et al. gutMGene: a comprehensive database for target genes of gut microbes and microbial metabolites. Nucleic Acids Research. 2021;50(D1):D795-D800. doi:https://doi.org/10.1093/nar/gkab786

11. Zhang Z, Zhang X. Identification of m6A-Related Biomarkers Associated with Prognosis of Colorectal Cancer. Med Sci Monit. 2021;27:e932370. Published 2021 Aug 10. doi:10.12659/MSM.932370

12. Rode MP, Silva AH, Cisilotto J, Rosolen D, Creczynski-Pasa TB. miR-425-5p as an exosomal biomarker for metastatic prostate cancer. Cell Signal. 2021;87:110113. doi:10.1016/j.cellsig.2021.110113

13. Ao C, Yu L, Zou Q. Prediction of bio-sequence modifications and the associations with diseases. Brief Funct Genomics. 2021;20(1):1-18. doi:10.1093/bfgp/elaa023

14. Yu L, Wang M, Yang Y, et al. Predicting therapeutic drugs for hepatocellular carcinoma based on tissue-specific pathways. PLoS Comput Biol. 2021;17(2):e1008696. Published 2021 Feb 9. doi:10.1371/journal.pcbi.1008696

15. Federica Laudisi, Stolfi C, Bevivino G, et al. GATA6 Deficiency Leads to Epithelial Barrier Dysfunction and Enhances Susceptibility to Gut Inflammation. Journal of Crohn's and Colitis. 2021;16(2):301-311. doi:https://doi.org/10.1093/ecco-jcc/jjab145

16. Zhang T, Liu N, Wei W, Zhang Z, Li H. Integrated Analysis of Weighted Gene Coexpression Network Analysis Identifying Six Genes as Novel Biomarkers for Alzheimer's Disease. Li Q, ed. Oxidative Medicine and Cellular Longevity. 2021;2021(Article ID 9918498):1-16. doi:https://doi.org/10.1155/2021/9918498

17. Zhao H, Zhang Q, Chen H, Rahman MR, Faruquee HM. Integrated multi-omics approach identified molecular mechanism and pathogenetic processes of COVID-19 that affect patient with Parkinson's disorder. Saudi Journal of Biological Sciences. 2021;28(12):6939-6945. doi:https://doi.org/10.1016/j.sjbs.2021.07.074

18. Kretschmer C, Sterner-Kock A, Siedentopf F, Schoenegg W, Schlag PM, Kemmner W. Identification of early molecular markers for breast cancer. Mol Cancer. 2011;10(1):15. Published 2011 Feb 11. doi:10.1186/1476-4598-10-15

19. Manjili MH, Najarian K, Wang XY. Signatures of tumor-immune interactions as biomarkers for breast cancer prognosis. Future Oncol. 2012;8(6):703-711. doi:10.2217/fon.12.57

20. Sukumar J, Gast K, Quiroga D, Lustberg M, Williams N. Triple-negative breast cancer: promising prognostic biomarkers currently in development. Expert Rev Anticancer Ther. 2021;21(2):135-148. doi:10.1080/14737140.2021.1840984

21. Lord CJ, Tutt AN, Ashworth A. Synthetic lethality and cancer therapy: lessons learned from the development of PARP inhibitors. Annu Rev Med. 2015;66:455-470. doi:10.1146/annurev-med-050913-022545

22. Tarantino P, Hamilton E, Tolaney SM, et al. HER2-Low Breast Cancer: Pathological and Clinical Landscape. J Clin Oncol. 2020;38(17):1951-1962. doi:10.1200/JCO.19.02488

23. Mittendorf EA, Ardavanis A, Litton JK, et al. Primary analysis of a prospective, randomized, single-blinded phase II trial evaluating the HER2 peptide GP2 vaccine in breast cancer

patients to prevent recurrence. Oncotarget. 2016;7(40):66192-66201. doi:10.18632/oncotarget.11751

24. Hyman DM, Piha-Paul SA, Won H, et al. HER kinase inhibition in patients with HER2- and HER3-mutant cancers [published correction appears in Nature. 2019 Feb;566(7745):E11-E12]. Nature. 2018;554(7691):189-194. doi:10.1038/nature25475
25. A Phase II, Single-Arm Trial of Poziotinib as Salvage Treatment in Patients With Metastatic Breast Cancer Who Has HER2 or EGFR Mutation or Activated AR or EGFR Pathway - Full Text View - ClinicalTrials.gov. clinicaltrials.gov. Published July 7, 2020. Accessed August 1, 2023. https://classic.clinicaltrials.gov/ct2/show/NCT02544997
26. Pyrotinib in Metastatic HER2 Non-amplified But HER2 Mutant Breast Cancer - Full Text View - ClinicalTrials.gov. clinicaltrials.gov. Published January 30, 2018. Accessed August 1, 2023. https://classic.clinicaltrials.gov/ct2/show/NCT03412383
27. Davey RA, Grossmann M. Androgen Receptor Structure, Function and Biology: From Bench to Bedside. Clin Biochem Rev. 2016;37(1):3-15.
28. Santonja A, Sánchez-Muñoz A, Lluch A, et al. Triple negative breast cancer subtypes and pathologic complete response rate to neoadjuvant chemotherapy. Oncotarget. 2018;9(41):26406-26416. Published 2018 May 29. doi:10.18632/oncotarget.25413
29. Barton VN, D'Amato NC, Gordon MA, Christenson JL, Elias A, Richer JK. Androgen Receptor Biology in Triple Negative Breast Cancer: a Case for Classification as AR+ or Quadruple Negative Disease. Horm Cancer. 2015;6(5-6):206-213. doi:10.1007/s12672-015-0232-3
30. Schmid P, Adams S, Rugo HS, et al. Atezolizumab and Nab-Paclitaxel in Advanced Triple-Negative Breast Cancer. N Engl J Med. 2018;379(22):2108-2121. doi:10.1056/NEJMoa1809615
31. Fruman DA, Chiu H, Hopkins BD, Bagrodia S, Cantley LC, Abraham RT. The PI3K Pathway in Human Disease. Cell. 2017;170(4):605-635. doi:10.1016/j.cell.2017.07.029
32. Drilon A, Laetsch TW, Kummar S, et al. Efficacy of Larotrectinib in TRK Fusion-Positive Cancers in Adults and Children. N Engl J Med. 2018;378(8):731-739. doi:10.1056/NEJMoa1714448
33. Doebele RC, Drilon A, Paz-Ares L, et al. Entrectinib in patients with advanced or metastatic NTRK fusion-positive solid tumours: integrated analysis of three phase 1-2 trials [published correction appears in Lancet Oncol. 2020 Feb;21(2):e70] [published correction appears in Lancet Oncol. 2020 Jul;21(7):e341] [published correction appears in Lancet Oncol. 2020 Aug;21(8):e372] [published correction appears in Lancet Oncol. 2021 Oct;22(10):e428]. Lancet Oncol. 2020;21(2):271-282. doi:10.1016/S1470-2045(19)30691-6
34. Ross J, Chung J, Elvin J, et al. Abstract P2-09-15: NTRK fusions in breast cancer: Clinical, pathologic and genomic findings. Cancer Research. 2018;78(4_Supplement):P2-0915-P209-15. doi:https://doi.org/10.1158/1538-7445.sabcs17-p2-09-15
35. Infections that Can Lead to Cancer. Cancer.org. https://www.cancer.org/cancer/cancer-causes/infectious-agents/infections-that-can-lead-to-cancer.html. Published 2022. Accessed May 12, 2022.

36. Banerjee S, Tian T, Wei Z, et al. Distinct Microbial Signatures Associated With Different Breast Cancer Types. Front Microbiol. 2018;9:951. Published 2018 May 15. doi:10.3389/fmicb.2018.00951

37. Parhi L, Alon-Maimon T, Sol A, et al. Breast cancer colonization by Fusobacterium nucleatum accelerates tumor growth and metastatic progression. *Nature Communications*. 2020;11(1). doi:10.1038/s41467-020-16967-2

38. Fu A, Yao B, Dong T, et al. Tumor-resident intracellular microbiota promotes metastatic colonization in breast cancer. Cell. 2022;185(8):1356-1372.e26. doi:10.1016/j.cell.2022.02.027

39. Yu T, Guo F, Yu Y, et al. Fusobacterium nucleatum Promotes Chemoresistance to Colorectal Cancer by Modulating Autophagy. Cell. 2017;170(3):548-563.e16. doi:10.1016/j.cell.2017.07.008

40. Yim EK, Park JS. The role of HPV E6 and E7 oncoproteins in HPV-associated cervical carcinogenesis. Cancer Res Treat. 2005;37(6):319-324. doi:10.4143/crt.2005.37.6.319

41. Banerjee, A. S., Pal, A. D., & Banerjee, S. (2013). Epstein-Barr virus-encoded small non-coding RNAs induce cancer cell chemoresistance and migration. Virology, 443(2), 294–305. https://doi.org/10.1016/j.virol.2013.05.020

42. Li M, Xue F, Yang J, Pan X. Correlation between tumor marker CA72-4 and prognosis of patients with gastric cancer: A protocol for systematic review and meta-analysis. Medicine (Baltimore). 2020;99(52):e23723. doi:10.1097/MD.0000000000023723

43. Wu HH, Wu JY, Cheng YW, et al. cIAP2 upregulated by E6 oncoprotein via epidermal growth factor receptor/phosphatidylinositol 3-kinase/AKT pathway confers resistance to cisplatin in human papillomavirus 16/18-infected lung cancer. Clin Cancer Res. 2010;16(21):5200-5210. doi:10.1158/1078-0432.CCR-10-0020

44. Kong QL, Hu LJ, Cao JY, et al. Epstein-Barr virus-encoded LMP2A induces an epithelial-mesenchymal transition and increases the number of side population stem-like cancer cells in nasopharyngeal carcinoma. PLoS Pathog. 2010;6(6):e1000940. Published 2010 Jun 3. doi:10.1371/journal.ppat.1000940

45. Zhao S, Mei Y, Wang Y, Zhu J, Zheng G, Ma R. Levels of CEA, CA153, CA199, CA724 and AFP in nipple discharge of breast cancer patients. Int J Clin Exp Med. 2015;8(11):20837-20844. Published 2015 Nov 15.

46. Wang X, Wang Q. Alpha-Fetoprotein and Hepatocellular Carcinoma Immunity. Can J Gastroenterol Hepatol. 2018;2018:9049252. Published 2018 Apr 1. doi:10.1155/2018/9049252

47. Tilly K, Rosa PA, Stewart PE. Biology of infection with Borrelia burgdorferi. Infect Dis Clin North Am. 2008;22(2):217-v. doi:10.1016/j.idc.2007.12.013

48. Timmaraju VA, Theophilus PA, Balasubramanian K, Shakih S, Luecke DF, Sapi E. Biofilm formation by Borrelia burgdorferi sensu lato. *FEMS Microbiol Lett*. 2015;362(15):fnv120. doi:10.1093/femsle/fnv120

49. Miklossy J. Chronic or late Lyme neuroborreliosis: analysis of evidence compared to chronic or late neurosyphilis. Open Neurol J. 2012;6:146-157. doi:10.2174/1874205X01206010146

50. Dattwyler RJ, Luft BJ. Overview of the clinical manifestations of Borrelia burgdorferi infection. Can J Infect Dis. 1991;2(2):61-63. doi:10.1155/1991/902928
51. Dulipati V, Meri S, Panelius J. Complement evasion strategies of Borrelia burgdorferi sensu lato. FEBS Lett. 2020;594(16):2645-2656. doi:10.1002/1873-3468.13894
52. Vechtova P, Sterbova J, Sterba J, et al. A bite so sweet: the glycobiology interface of tick-host-pathogen interactions. Parasit Vectors. 2018;11(1):594. Published 2018 Nov 14. doi:10.1186/s13071-018-3062-7.
53. Karvonen K, Nykky J, Marjomäki V, Gilbert L. Distinctive Evasion Mechanisms to Allow Persistence of Borrelia burgdorferi in Different Human Cell Lines. *Front Microbiol*. 2021;12:711291. Published 2021 Oct 12. doi:10.3389/fmicb.2021.711291.
54. Gaur G, Sawant JY, Chavan AS, et al. Effect of invasion of Borrelia burgdorferi in normal and neoplastic mammary epithelial cells. *Antibiotics*. 2021;10(11):1295. doi:10.3390/antibiotics10111295
55. Unpublished: Vishwa K.: Global Transcriptomic Analysis of Breast Cancer Cells Infected with Borrelia burgdorferi [Master's thesis]. West Haven, CT: University of New Haven; 2022-23.
56. Debbarma A, Mansolf M, Khatri VA, Valentino JA, Sapi E. Effect of Borrelia burgdorferi on the Expression of miRNAs in Breast Cancer and Normal Mammary Epithelial Cells. Microorganisms. 2023;11(6):1475. Published 2023 Jun 1. doi:10.3390/microorganisms11061475
57. Kukurba KR, Montgomery SB. RNA Sequencing and Analysis. *Cold Spring Harb Protoc*. 2015;2015(11):951-969. Published 2015 Apr 13. doi:10.1101/pdb.top084970
58. Tkáčová Z, Bhide K, Mochnáčová E, et al. Comprehensive Mapping of the Cell Response to Borrelia bavariensis in the Brain Microvascular Endothelial Cells *in vitro* Using RNA-Seq. *Front Microbiol*. 2021;12:760627. Published 2021 Nov 8. doi:10.3389/fmicb.2021.760627
59. Robinot R, Bachy E, Chaubard S, et al. Chronic Borrelia burgdorferi infection triggers NKT lymphomagenesis. *Blood*. 2018;132(25):2691-2695. doi:10.1182/blood-2018-07-863381
60. Functional annotation tools. DAVID. https://david.ncifcrf.gov/tools.jsp. Accessed January 20, 2023.
61. Korbecki J, Bosiacki M, Barczak K, et al. Involvement in Tumorigenesis and Clinical Significance of CXCL1 in Reproductive Cancers: Breast Cancer, Cervical Cancer, Endometrial Cancer, Ovarian Cancer and Prostate Cancer. Int J Mol Sci. 2023;24(8):7262. Published 2023 Apr 14. doi:10.3390/ijms24087262
62. Waugh DJ, Wilson C. The interleukin-8 pathway in cancer. Clin Cancer Res. 2008;14(21):6735-6741. doi:10.1158/1078-0432.CCR-07-4843
63. Ejaeidi AA, Craft BS, Puneky LV, Lewis RE, Cruse JM. Hormone receptor-independent CXCL10 production is associated with the regulation of cellular factors linked to breast cancer progression and metastasis. Exp Mol Pathol. 2015;99(1):163-172. doi:10.1016/j.yexmp.2015.06.002
64. Fujisawa N, Sakao Y, Hayashi S, Hadden WA 3rd, Harmon CL, Miller EJ. alpha-Chemokine growth factors for adenocarcinomas; a synthetic peptide inhibitor for alpha-chemokines

inhibits the growth of adenocarcinoma cell lines. J Cancer Res Clin Oncol. 2000;126(1):19-26. doi:10.1007/pl00008460
65. Bièche I, Chavey C, Andrieu C, et al. CXC chemokines located in the 4q21 region are up-regulated in breast cancer. Endocr Relat Cancer. 2007;14(4):1039-1052. doi:10.1677/erc.1.01301
66. Wang N, Zheng Y, Gu J, et al. Network-pharmacology-based validation of TAMS/CXCL-1 as key mediator of XIAOPI formula preventing breast cancer development and metastasis. Sci Rep. 2017;7(1):14513. Published 2017 Nov 6. doi:10.1038/s41598-017-15030-3
67. Bhat K, Sarkissyan M, Wu Y, Vadgama JV. GROα overexpression drives cell migration and invasion in triple negative breast cancer cells. Oncol Rep. 2017;38(1):21-30. doi:10.3892/or.2017.5668
68. Sprung CN, Yang Y, Forrester HB, et al. Genome-wide transcription responses to synchrotron microbeam radiotherapy. Radiat Res. 2012;178(4):249-259. doi:10.1667/RR2885.1
69. Wang P, Song D, Wan D, et al. Ginsenoside panaxatriol reverses TNBC paclitaxel resistance by inhibiting the IRAK1/NF-κB and ERK pathways. PeerJ. 2020;8:e9281. Published 2020 Jun 3. doi:10.7717/peerj.9281
70. Dalmases A, González I, Menendez S, et al. Deficiency in p53 is required for doxorubicin induced transcriptional activation of NF-κB target genes in human breast cancer. Oncotarget. 2014;5(1):196-210. doi:10.18632/oncotarget.1556
71. Gibson JT, Orlandella RM, Turbitt WJ, et al. Obesity-Associated Myeloid-Derived Suppressor Cells Promote Apoptosis of Tumor-Infiltrating CD8 T Cells and Immunotherapy Resistance in Breast Cancer. Front Immunol. 2020;11:590794. Published 2020 Oct 6. doi:10.3389/fimmu.2020.590794
72. Ha H, Debnath B, Neamati N. Role of the CXCL8-CXCR1/2 Axis in Cancer and Inflammatory Diseases. Theranostics. 2017;7(6):1543-1588. Published 2017 Apr 7. doi:10.7150/thno.15625
73. Han ZJ, Li YB, Yang LX, Cheng HJ, Liu X, Chen H. Roles of the CXCL8-CXCR1/2 Axis in the Tumor Microenvironment and Immunotherapy. Molecules. 2021;27(1):137. Published 2021 Dec 27. doi:10.3390/molecules27010137
74. Qian L, Yu S, Yin C, et al. Plasma IFN-γ-inducible chemokines CXCL9 and CXCL10 correlate with survival and chemotherapeutic efficacy in advanced pancreatic ductal adenocarcinoma. Pancreatology. 2019;19(2):340-345. doi:10.1016/j.pan.2019.01.015
75. Adams S, Diamond JR, Hamilton E, et al. Atezolizumab Plus nab-Paclitaxel in the Treatment of Metastatic Triple-Negative Breast Cancer With 2-Year Survival Follow-up: A Phase 1b Clinical Trial. JAMA Oncol. 2019;5(3):334-342. doi:10.1001/jamaoncol.2018.5152
76. Chuan T, Li T, Yi C. Identification of CXCR4 and CXCL10 as Potential Predictive Biomarkers in Triple Negative Breast Cancer (TNBC). Med Sci Monit. 2020;26:e918281. Published 2020 Jan 11. doi:10.12659/MSM.918281
77. Jézéquel P, Kerdraon O, Hondermarck H, et al. Identification of three subtypes of triple-negative breast cancer with potential therapeutic implications. Breast Cancer Res. 2019;21(1):65. Published 2019 May 17. doi:10.1186/s13058-019-1148-6
78. Di Caro G, Castino GF, Bergomas F, et al. Tertiary lymphoid tissue in the tumor microenvironment: from its occurrence to immunotherapeutic implications. Int Rev Immunol. 2015;34(2):123-133. doi:10.3109/08830185.2015.1018416

79. Wu M, Jia BB, Li MF. Complement C3 and Activated Fragment C3a Are Involved in Complement Activation and Anti-Bacterial Immunity. Front Immunol. 2022;13:813173. Published 2022 Feb 25. doi:10.3389/fimmu.2022.813173
80. Tian J, Hachim MY, Hachim IY, et al. Cyclooxygenase-2 regulates TGFβ-induced cancer stemness in triple-negative breast cancer. Sci Rep. 2017;7:40258. Published 2017 Jan 5. doi:10.1038/srep40258
81. Niu X, Yin L, Yang X, et al. Serum amyloid A 1 induces suppressive neutrophils through the Toll-like receptor 2-mediated signaling pathway to promote progression of breast cancer. Cancer Sci. 2022;113(4):1140-1153. doi:10.1111/cas.15287
82. De Santo C, Arscott R, Booth S, et al. Invariant NKT cells modulate the suppressive activity of IL-10-secreting neutrophils differentiated with serum amyloid A. Nat Immunol. 2010;11(11):1039-1046. doi:10.1038/ni.1942
83. Cho WC, Yip TT, Yip C, et al. Identification of serum amyloid a protein as a potentially useful biomarker to monitor relapse of nasopharyngeal cancer by serum proteomic profiling. Clin Cancer Res. 2004;10(1 Pt 1):43-52. doi:10.1158/1078-0432.ccr-0413-3
84. Zhang Y, Wei Y, Jiang B, et al. Scavenger Receptor A1 Prevents Metastasis of Non-Small Cell Lung Cancer via Suppression of Macrophage Serum Amyloid A1. Cancer Res. 2017;77(7):1586-1598. doi:10.1158/0008-5472.CAN-16-1569
85. Wang WW, Lebrun (Supervisor) JJ. Characterizing COX-2-associated gene network in triple negative breast cancer: its role in distant metastasis and COX-2 inhibitor resistance. escholarship.mcgill.ca. Published December 14, 2021. Accessed July 28, 2023. https://escholarship.mcgill.ca/concern/theses/h989r812f
86. Baba M, Imai T, Nishimura M, et al. Identification of CCR6, the specific receptor for a novel lymphocyte-directed CC chemokine LARC. J Biol Chem. 1997;272(23):14893-14898. doi:10.1074/jbc.272.23.14893
87. Lee SK, Park KK, Kim HJ, et al. Human antigen R-regulated CCL20 contributes to osteolytic breast cancer bone metastasis. Sci Rep. 2017;7(1):9610. Published 2017 Aug 29. doi:10.1038/s41598-017-09040-4
88. Li TH, Zhao BB, Qin C, et al. IFIT1 modulates the proliferation, migration and invasion of pancreatic cancer cells via Wnt/β-catenin signaling. Cell Oncol (Dordr). 2021;44(6):1425-1437. doi:10.1007/s13402-021-00651-8
89. Boelens MC, Wu TJ, Nabet BY, et al. Exosome transfer from stromal to breast cancer cells regulates therapy resistance pathways. Cell. 2014;159(3):499-513. doi:10.1016/j.cell.2014.09.051
90. Schratter G, Scheruebel S, Langthaler S, et al. GIRK1 triggers multiple cancer-related pathways in the benign mammary epithelial cell line MCF10A. Sci Rep. 2019;9(1):19277. Published 2019 Dec 17. doi:10.1038/s41598-019-55683-w
91. Cervantes JL, Hawley KL, Benjamin SJ, Weinerman B, Luu SM, Salazar JC. Phagosomal TLR signaling upon Borrelia burgdorferi infection. Front Cell Infect Microbiol. 2014;4:55. Published 2014 May 20. doi:10.3389/fcimb.2014.00055
92. Van Laere S, Limame R, Van Marck EA, Vermeulen PB, Dirix LY. Is there a role for mammary stem cells in inflammatory breast carcinoma?: a review of evidence from cell line, animal model, and human tissue sample experiments. Cancer. 2010;116(11 Suppl):2794-2805. doi:10.1002/cncr.25180

93. Stevens LE, Peluffo G, Qiu X, et al. JAK-STAT Signaling in Inflammatory Breast Cancer Enables Chemotherapy-Resistant Cell States. Cancer Res. 2023;83(2):264-284. doi:10.1158/0008-5472.CAN-22-0423
94. Yu H, Kortylewski M, Pardoll D. Crosstalk between cancer and immune cells: role of STAT3 in the tumour microenvironment. Nat Rev Immunol. 2007;7(1):41-51. doi:10.1038/nri1995
95. Du JW, Xu KY, Fang LY, Qi XL. Interleukin-17, produced by lymphocytes, promotes tumor growth and angiogenesis in a mouse model of breast cancer. Mol Med Rep. 2012;6(5):1099-1102. doi:10.3892/mmr.2012.1036
96. Cochaud S, Giustiniani J, Thomas C, et al. IL-17A is produced by breast cancer TILs and promotes chemoresistance and proliferation through ERK1/2. Sci Rep. 2013;3:3456. Published 2013 Dec 9. doi:10.1038/srep03456
97. Acuner-Ozbabacan ES, Engin BH, Guven-Maiorov E, et al. The structural network of Interleukin-10 and its implications in inflammation and cancer. BMC Genomics. 2014;15 Suppl 4(Suppl 4):S2. doi:10.1186/1471-2164-15-S4-S2
98. Zhao S, Wu D, Wu P, Wang Z, Huang J. Serum IL-10 Predicts Worse Outcome in Cancer Patients: A Meta-Analysis. PLoS One. 2015;10(10):e0139598. Published 2015 Oct 6. doi:10.1371/journal.pone.0139598
99. Ouyang W, O'Garra A. IL-10 Family Cytokines IL-10 and IL-22: from Basic Science to Clinical Translation. Immunity. 2019;50(4):871-891. doi:10.1016/j.immuni.2019.03.020
100. Shi J, Song X, Traub B, Luxenhofer M, Kornmann M. Involvement of IL-4, IL-13 and Their Receptors in Pancreatic Cancer. Int J Mol Sci. 2021;22(6):2998. Published 2021 Mar 15. doi:10.3390/ijms22062998
101. König A, Vilsmaier T, Rack B, et al. Determination of Interleukin-4, -5, -6, -8 and -13 in Serum of Patients with Breast Cancer Before Treatment and its Correlation to Circulating Tumor Cells. Anticancer Research. 2016;36(6):3123-3130. https://ar.iiarjournals.org/content/36/6/3123.short
102. Manome Y, Suzuki D, Nishida R, et al. Immunotherapy for malignant tumors with focus on toll-like receptors. Oral Science International. 2019;16(1):3-7. doi:https://doi.org/10.1002/osi2.1011
103. Urban-Wojciuk Z, Khan MM, Oyler BL, et al. The Role of TLRs in Anti-cancer Immunity and Tumor Rejection. Front Immunol. 2019;10:2388. Published 2019 Oct 22. doi:10.3389/fimmu.2019.02388
104. Velloso FJ, Sogayar MC, Correa RG. Expression and in vitro assessment of tumorigenicity for NOD1 and NOD2 receptors in breast cancer cell lines. BMC Res Notes. 2018;11(1):222. Published 2018 Apr 3. doi:10.1186/s13104-018-3335-4
105. Velloso FJ, Campos AR, Sogayar MC, Correa RG. Proteome profiling of triple negative breast cancer cells overexpressing NOD1 and NOD2 receptors unveils molecular signatures of malignant cell proliferation. BMC Genomics. 2019;20(1):152. Published 2019 Feb 21. doi:10.1186/s12864-019-5523-6
106. Chen ZL, Liu JY, Wang F, Jing X. Suppression of MALAT1 ameliorates chronic constriction injury-induced neuropathic pain in rats via modulating miR-206 and ZEB2. J Cell Physiol. 2019;234(9):15647-15653. doi:10.1002/jcp.28213
107. Yang Y, Wang W, Chang H, Han Z, Yu X, Zhang T. Reciprocal regulation of miR-206 and IL-6/STAT3 pathway mediates IL6-induced gefitinib resistance in EGFR-mutant lung cancer cells. J Cell Mol Med. 2019;23(11):7331-7341. doi:10.1111/jcmm.14592