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Assessment of Virus-Induced Myocarditis in Human Heart Tissue Samples

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Key Word abbreviation meaning

Abbreviation	Word	Abbreviation	Word
MRI	Magnetic resonance imaging	NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
cTn1	Cardiac troponin 1	CRP	C-Reactive Protein
DAF	Decay accelerating factor	MAPK	Mitogen activated protein kinase
TNF-α	Tumor necrosis factor alpha		
TGF-β	Transforming growth factor beta		
IL-1	Interleukin-1		
IL-2	Interleukin-2		
IL-3	Interleukin-3		
IL-4	Interleukin-4		
IL-5	Interleukin-5		
IL-6	Interleukin-6		
IL-7	Interleukin-7		
IL-8	Interleukin-8		
IL-9	Interleukin-9		
IL-10	Interleukin-10		
HRSV	Human respiratory syncytial virus		
HSV1	Herpes Simplex Virus 1		
EBV	Epstein-Barr virus		
BZLF1	BamHI Z fragment leftward open frame 1		
CAR	Chimeric Antigen Receptor		
DNA	Deoxyribonucleic Acid		
RNA	Ribonucleic Acid		
ACE	Angiotensin-converting enzyme		
ECMO	Extracorporeal membrane oxygenation		
qPCR	Quantitative polymerase chain reaction		
RT-PCR	Reverse transcript polymerase chain reaction		
PCR	Polymerase chain reaction		
EMCV	Encephalomyocarditis Virus		
H&E	Hematoxylin and Eosin		
HCoV-229E	Human Coronavirus 229E		
HCoV-NL63	Human Coronavirus NL63		
HCoV-OC43	Human Coronavirus OC43		
HCoV-HKU	Human Coronavirus HKU		

Abstract

The heart is a major organ whose function is to transport nutrients and waste throughout the body. This organ can become infected by pathogens, such as viruses, bacteria, or parasites. Infection of the middle heart layer, or myocardium, is often caused by a viral agent. This disease has three stages: viral infiltration, adaptive immune system activation, and finally either viral clearance or cardiac cell remodeling. During this process the immune system will begin to secrete cytokines, which are signaling molecules that alert other members of the immune pathways, and also participate in cardiac remodeling. Evaluating the correlation between the cytokine expression levels with the viruses present in cardiomyopathy-positive heart tissue samples can lead to a better understanding of cardiac disease process, and aid in the development of new diagnostic tools.

In order to accomplish this study nucleic acid (DNA and RNA) from heart tissues obtained from cardiomyopathy-positive samples, as well as donor samples, was isolated. The DNA was then used in a PCR reaction with viral primers corresponding to DNA viruses. Next, RNA was used to synthesize cDNA. With cDNA made two types of analyses took place: a PCR with primers specific to RNA viruses was performed to identify viral genomes, and a qPCR assay was done to evaluate the expression levels of cytokines.

The most common viruses identified in the study were HRSV, Herpesviruses 5/7, and Hepatitis-C virus. The virus that was least prevalent was Coxsackievirus-B3. The analysis of cytokine expression profiles and genes involved in cardiac remodeling revealed that samples BIF28 and 48CAF had the highest expression levels of immune system, and cardiac, markers. The two markers with increased expression levels were Tnnt2 and TGF-β. When the cytokine expression levels were compared for each of the three heart layers (endocardium, epicardium, and

myocardium) it was seen the epicardium had the highest expression levels, suggesting highest levels of inflammation. In sum, the generated data revealed a correlation between viral infection and the degree of heart inflammation. Interestingly, based on samples analyzed in this study, I showed that distinct layers of the heart can have various inflammatory profiles, suggesting different levels of cardiac damage. Follow up studies will help delineate the association of viral infection of cardiac muscle and inflammation, which may help develop better diagnostic tools.

Introduction

The heart is a major organ within the human body, whose main function is to act like a pump (1). This pump is in charge of collecting deoxygenated blood from the body and transporting it through the veins to the lungs (1). In the lungs the blood becomes reoxygenated, which allows the heart to pump this blood to the tissues in the body (1). During this process, nutrients, hormones, and waste products are also transported throughout the body to the correct tissues (1).

The anatomical structure of the heart consists of 4 general regions: the right and left ventricle along with the left and right atrium (1). These regions are all connected through various valves preventing backflow of blood. There are also three main cellular levels within the heart, the epicardium, the outer most layer, the myocardium, the middle most layer, and the endocardium, the innermost layer (1). Cardiac muscle cells, otherwise known as cardiomyocytes, make up between 70 to 85 percent of the volume within the heart (1). The other cells known to make up the human heart are fibroblasts, endothelial cells, and peri-vascular cells (22). These other cell types ensure that the cardiomyocytes can contract properly and thus allow for the survival of the individual by building up the extracellular matrix and allowing intercellular communication (22). The endothelial cells outnumber cardiomyocytes; however, they are located very close to the cardiomyocytes, leading to the conclusion that the endothelial cells trigger the response to stress, and help regulate heart contraction (22). Fibroblasts are not very abundant in the human cells, and they aid heart remodeling and arrhythmogenesis (22). Like all other cells in the body, the cardiomyocytes are prone to infection. When these cells become infected by a pathogen, such as a virus, a bacterium or a parasite, heart disease may develop (2).

In the United States heart disease is the leading cause of death, with an average 1 out of every 4 deaths being attributed to a heart condition (2). Some of the more common heart diseases are

heart valve disease, in which at least one of the valves malfunctions, pericardial disease affecting the pericardium of the heart, and coronary heart disease affecting the arteries within the heart (2). The key factors that lead to heart disease are high blood pressure, high cholesterol levels, and smoking (3). These factors, and many more, lead to plaque built up in the arteries preventing proper blood flow throughout the body (3). When a virus infects the myocardium in the heart then this is known as myocarditis.

The proper function of the myocardium is to ensure heart contraction (4). This layer is able to generate force through cardiomyocytes communicating with each other via gap junctions (4). In a majority of cases myocarditis will be asymptomatic, not requiring any intervention, but in severe cases the heart is permanently damaged resulting in heart failure, stroke, or death (5). To diagnose myocarditis a doctor will order an MRI to detect any heart inflammation, use a blood test to determine the immune response, and an electrocardiogram to detect an abnormal heartbeat (6). In the blood test it is often that the levels of Troponin-I are evaluated given it is 89 percent specific in diagnosing myocarditis in patients (7). However, this biomarker is only 34 percent sensitive in the diagnosis of myocarditis displaying the need to further develop better diagnostic tools. Troponin is a regulatory protein that controls the interaction between actin and myosin in the myocardium. (18). It has been noted that nearly all patients have had elevated levels of cardiac troponin, cTn1, during a viral infection (18). On the electrocardiogram, a device measuring electrical signals in the heart, often the Q waves and St waves are evaluated (8). This is because in 27 percent of deaths these two waves are upregulated (8). In more severe cases, a heart biopsy sample will be obtained, but the American Heart Association and American College of Cardiology require that this approach is only taken after the onset of heart failure, and if the patient's left ventricle have become dilated (9).

The pathophysiology of myocarditis is broken down into three distinct parts. The first part of this process is known as acute myocarditis, which typically lasts for one week and involves viral entry into the heart (23). For this to happen a virus, such as a Coxsackievirus, binds to a receptor on a cardiomyocyte (23). The receptor in this case is DAF (23). This viral entry first triggers the innate immune response, which involves cells such as the natural killer cells and macrophages (23). This results in myocyte breakdown through protease apoptosis (23). The innate immune response also results in cytokine expression, especially Interleukins 1 and 2 (Il-1, Il-2), Tumor necrosis factor alpha (TNFα), and Interferon gamma (23).

The second phase of myocarditis is known as the subacute phase. In this phase the adaptive immune system is activated, which results in the upregulation of B cells and T cells, as well as in the production of cardiac autoantibodies (23). This leads to the heart tissue becoming inflamed causing more damage to the patient (23). The subacute phase lasts between 1 to 4 weeks. The final phase is known as chronic myocarditis, or viral clearance. In this phase the virus has either been properly cleared from the body resulting in normal heart function, or the heart has not been unable to clear the virus (23). This failure to clear the viruses results in chronic inflammation (23). When the heart is inflamed for an extended period of time the myocytes are degraded and the heart cells are remodeled (23). If this is not treated the patient's heart will fail, requiring a transplant to avoid death.

The immune system response in the heart is similar to that in the rest of the body, and it involves both an innate and an acquired pathway. The innate immune system is nonspecific (10), and it consists of barriers such as the skin and cilia, white blood cells, and other proteins (10). Many of the cells of the innate immune system, such as the natural killer cells, use enzymes in order to break down the virus infected cells (10).

The adaptive immune system is activated if the innate immune system is unable to properly clear the virus from the cells. The adaptive immune system is made up of the T-lymphocytes and B-lymphocytes. B-lymphocytes are produced and mature within the bone marrow (10). B-cells communicate with many nearby cells, as they lead to the secretion of cytokines, and are activated by T-cells (10). Once a virus has been properly dealt with, memory cells are created to ensure the body is prepared if the pathogen ever reinfects an individual (10). T-cells are also created in the bone marrow, but they mature in the thymus. There are three main types of T-cells in the body, the cytotoxic T-cells which break down the infected cells, the T-helper cells which recognize infected cells and activate B-cells, and the T-memory cells which are activated if a pathogen ever reinfects the individual (10).

During this immune response small specific proteins, known as cytokines, are activated (11). These cytokines are able to have an effect on the cells that secreted the nearby cells, and also cells from all over the body (11). In the event that the immune system is overactive, a proinflammatory response known as a cytokine storm can result (11). If the immune system continues to be upregulated, then this could result in the death of a cell (11). Some of the cytokines known to cause this inflamed response in the heart are TNF- α, TGF-β, IL-1, IL-4, IL-6, IL-8 and IL18 (12). Additionally, not all cytokines are detrimental, and it has been determined that the following cytokines: IL-2, IL-4, IL-6, IL-8, IL-10, are beneficial when it comes to clearing virus infected cells.

Some of the known viruses known to cause myocarditis are Herpesviruses (including Human Herpesvirus 6 and Epstein-Barr virus), Parvovirus, Hepatitis-C, Shingles, HRSV, Adenovirus, and more recently, Covid-19. Herpesvirus is of high importance because it is estimated that 95 percent of adults worldwide are infected with Herpesvirus-1 (HSV1) (13). More generally,

Herpesvirus is a latent virus meaning that viral DNA inserts itself in the hosts' nucleus and replicates along with the hosts cells (13). Herpesvirus has a genome size of around 250 kilobase pairs and four structural components (13). These are the capsid, envelope, tegument, and glycoprotein spike (13). Another important factor in dealing with Herpesviruses is that there is no known cure for this disease, and only treatments that result in the temporary prevention of symptoms are currently available (14).

Another type of Herpesviruses that is known to cause myocarditis is Epstein-Barr virus (EBV) (15). This virus targets the adaptive immune system within the body working to infect the B-lymphocytes (15). Over time EBV is able to infect T-cells and natural killer cells resulting in hemophagocytic lymphohistiocytosis (15). What makes this virus different from most types of Herpesviruses is that it is only ever active at low levels in the body, remaining inactivated a majority of the time (15). EBV encodes for the proteins BZLF1 and BRLF1, which are known to enable cell destruction assisting in the virus's negative effect on the patient (15).

A second virus known to cause myocarditis is Adenovirus (16). This virus is double stranded DNA-based with a total diameter of 100 nm (16). To date, there are 67 known strains of Adenoviruses with new sub-types often being found given the high mutation rate of this virus (16). The outside component of this virus contains a coat with 20 triangular faces (16). This virus family enters the cell through the CAR receptor and once inside cardiomyocytes, the infection results in cytoskeletal disruption creating an uncontrolled immune response even after the viruses has been cleared from the cell (24). Over time, the infection with these viruses, or viral persistence, may result in left ventricle dysfunction (24).

Parvovirus is another DNA virus that results in anemia due to the effect the virus has on red blood cells (17). Parvovirus is often referred to as the filth disease, which often affects young

children (17). This disease results in a mild rash for many individuals and is spread within the patient's albumin and immunoglobulins (17). Parvovirus has been seen to infect endothelial cells resulting in cardiomyocyte apoptosis (24). This particular disease is interesting as it can be found in autopsy samples from people who were not positive for myocarditis suggesting that the viral presence may not necessarily indicate a disease process (24).

Another group of viruses that has been known to historically cause myocarditis is the *Coronaviridae* family. The latest example of a virus from this family that has been able to do this is SARS-CoV-2. This virus is able to damage the heart through an imbalanced immune response (24). This excess of T-helper cells triggers a cytokine storm that leads to myocardial damage (24). SARS-CoV-2 has been able to enter the cell through a ACE2 receptor which in turn decreases the levels of angiotensin in the heart (24). In lab however it is unlikely that this strain will be found due to the fact the samples have been collected before the pandemic, but this virus displays the need to test the samples for other viruses in the *Coronaviridae* family. Some of the other known coronaviruses are HCoV-229E, HCoV-NL63, HCoV-OC43, and HCoV-HKU (25).

Interestingly, myocarditis can infect anyone regardless of age, but children often present different symptoms than adults. Some of the symptoms seen in children who are diagnosed with myocarditis are poor appetite, cyanosis, and anxiousness (19). Children are also more likely to have sudden death due to the disease and a more severe onset of symptoms (19). Also, in a majority of cases a heart transplant is required (19). It has also been noted that children often have a much higher cytokine expression patterns when exposed to the virus, and more viruses are often found in the heart tissue at one time (20).

In order to treat myocarditis, the first step is to use corticosteroids (21). These are medications that suppress the patient's immune system decreasing the inflammation seen in the heart (21).

This type of treatment is most effective in giant cell and eosinophilic myocarditis (21). Other medications that are often used are diuretics to help regulate blood osmolality, beta blockers to decrease the blood pressure, ACE inhibitors and angiotensin II blockers which are important to prevent angiotensin (a substance that narrows blood vessels) from being produced (21).

In more severe cases procedures will be used to help the heart pump blood more efficiently. One example of this is a ventricular assist device, which helps the ventricles pump blood throughout the body (21). Similarly, in the case of heart failure an ECMO extracorporeal membrane oxygenation machine mimics the lungs by removing carbon dioxide from the blood and adding oxygen (21). If none of the above procedures work, a heart transplant is required to keep the patient alive. Therefore, it is the goal of this study to create a diagnostic tool to screen patients for myocarditis through the identification of viruses present in human heart samples and the subsequent determination of the cytokine expression levels.

Materials and Methods

This study sought to use human heart tissues to identify the cytokine expression level pathways associated with viral infection. Then using this correlation, the aim was to define pathological features related to the damage seen in the heart samples. To accomplish this, genetic material from the human heart samples was isolated. These heart samples were isolated from patients who have been suffering from heart inflammation. The nucleic acid (DNA and RNA) from these samples was isolated and used in subsequent qPCR assay to determine the cytokine expression levels in each sample. This assay will be complemented with histological examination to clearly show the cardiac damage.

Nucleic Acid (DNA/RNA) Isolation from human heart tissue samples

To investigate how viral infection affects the cytokine expression patterns, I evaluated human heart biopsy samples obtained from the Campbell Muscle Lab at the University of Kentucky, and the Gill Heart & Vascular Institute in Lexington, Kentucky. All samples utilized in this analysis were excised from the hearts of patients diagnosed with either dilated cardiomyopathy or myocarditis. ZYMO DNA/RNA purification kit (Zymo Research) was used to derive DNA and RNA, with minor modifications. In order to accomplish the nucleic acid extraction, small pieces (>5 mg) of individual human heart tissue samples were isolated, and then homogenized in a solution containing appropriate ZYMO Tissue Kit buffers. This step typically yielded over 100 ng of DNA per sample, allowing for downstream assays to be conducted properly. RNA was than extracted using specific spin columns. This process began with an initial centrifugation that bound the RNA to the filter column, followed by the intermittent centrifugation and removal of flow through of the following buffers: DNA/RNA Prep Buffer and two rounds of DNA/RNA Wash Buffer. The amount of eluted RNA was usually over 500 ng per µl in each sample.

Estimation of DNA/RNA quality and quantity using a spectrometer and agarose gel electrophoresis

The extraction of the nucleic acid was then confirmed through gel electrophoresis with bands corresponding to the sizes of DNA and RNA extracted. The agarose gel was made with Tris acetate EDTA, TAE, and water were used to dissolve agarose at high temperatures. Then Safe DNA stain from the Thermo Fisher Scientific gel electrophoresis kit was added to the mixture and it was allowed to cool in a gel plate. Once cool the DNA/RNA samples were mixed with 6X DNA loading dye which was then added to the wells of the gel with a DNA ladder. A negative current was applied to the gel so that the samples would migrate through the gel. Once the markers have migrated the appropriate distance the gel was visualized. The DNA band was then compared to the ladder to evaluate if it has been properly isolated. For the RNA three bands were expected to be seen, corresponding to 28S, 18S, and tRNA.

While this was happening the DNA and RNA was the quantified using the nanodrop. For this procedure to work, 2µl of sample was added to the nanodrop plate along with 2µl of blank which was DNA/RNAse free water in this case. This procedure allows for the concentration to be known by measuring the amount of light that was not absorbed by the samples and quantifying that number.

Next, the purity of each of the extracted nucleic acids was also determined. This was done by seeing how much light of wavelength 260nm and 280nm has been absorbed by the samples. Then the ratio of 260nm wavelength absorbed was compared to the amount of 280nm light that has been absorbed. The expected ratio of 260/280 would be approximately 1.8 for DNA and 2.0 for RNA as these ratios correspond to “pure” nucleic acids, which was what was seen in a majority of the cases.

PCR analysis with virus specific primers

Following the nucleic acid extraction, I performed PCR analysis with primers specific to viruses. The reagent kit used was Thermo Scientific Phusion DNA Polymerase. For this reaction the sample was added into a tube with various reagents, primers, sample, and the DNA polymerase enzyme. The primers chosen for DNA samples were for known DNA viruses previously shown to be linked to heart disease (Adenovirus, Parvovirus-B19, Epstein-Barr virus, Varicella-Zoster virus, herpes simplex 1 virus and cytomegalovirus). Next, the samples were put in the thermocycler. For each virus slightly different conditions were used to optimize product yield and purity.

For the RNA an extra step was required. This was to synthesize cDNA from the total RNA samples. This began by using the ThermoFisher Scientific DNase I treatment to remove any excess DNA in the sample. Next, cDNA synthesis mix was made with reagents from the SuperScript III First-Strand Synthesis System for RT-PCR kit. This mixture was then added to the samples which contained RNA that has been previously incubated with random hexamers and dNTPs. The samples were then placed in the thermocycler where they were subjected to 25 degree Celsius for ten minutes, 50 degrees Celsius for 50 minutes, and 85 degrees Celsius for five minutes. With the cDNA synthesized primers for RNA viruses that are known to cause myocarditis, such as Coxsackievirus-B, EMCV, Influenza viruses or Scaffold viruses were used. This was done with the same procedure as seen above.

The products of both PCR's were then then run on an agarose gel, as described above. During the visualization of the gel, it was expected to see one band that corresponds to the viral genome being analyzed for. If no band was seen, then that sample likely was not positive for that particular virus.

Quantitative PCR (qPCR) analysis of immune system and cardiac gene expression in selected human heart tissue samples

Using the RNA samples, the cytokine expression levels were also determined. This was done by first converting the RNA into cDNA. The detailed procedure for this can be seen in the section above. With the cDNA synthesized this samples were then used to evaluate the cytokine expression levels, using the ThermoFisher Real-Time PCR Applied Biosystem SYBR kit. Using the reagents within this kit and primers for cytokines associated with myocarditis (NF-κB, TGF-β, IL-10, IL-1β, IL-6, TNF-α, CRP, IL-1RA, MAPK, IL-18, (MCP)-1/CCL2, IL-8/CXCL8). This mixture was then pipetted into the wells on the qPCR plate. Using the cycler, the samples were exposed to the manufacturer's conditions of 98 degrees Celsius for 30 seconds, then 37 cycles of 98 degrees Celsius for 10 seconds, 60 degrees Celsius for 30 seconds, and 72 degrees Celsius for 30 seconds. The cycle then ended with the samples being heated to 72 degrees Fahrenheit for 30 seconds.

After the cycle was completed, the data was exported from the computer for processing. All measurements were compared to actin levels allowing for normalization. Actin was used for normalization as it is a housekeeping gene expressed in all tissues normally. Using this data graphs were constructed to compare the expression levels of the various markers evaluated.

Results

Through the evaluation of cytokine expression levels, and viruses present in myocarditis-positive heart tissue samples it is theorized that myocarditis diagnostic tool can be developed. At the beginning of this experiment many of the samples were given an ID number to help with identification. These samples were then compiled in a table, where the cause of death for each individual and any additional comments from the doctors were added. This can be seen below.

Table 1: Isolated Heart Samples IDS and corresponding patient health information

Sample Number	Sample Database Identification Number	Heart disease, or cause of death	Additional comments
X	B23E3	Organ donor, passed away of stroke	Sinus tachycardia Non-specific T wave abnormality Abnormal ECG
Y	7ACC4	Heart transplant due to heart failure	Baseline artifact throughout, Normal sinus rhythm, Left atrial abnormality, Left anterior fascicular block, Incomplete right bundle branch block vs RVH, Abnormal ECG
Z	8F6OA	Heart transplant due to heart failure	Sinus tachycardia, Inferior infarct, Anterior infarct, T wave abnormality, consider lateral ischemia, Abnormal ECG
2	78BBB	Organ donor, death due to head trauma	Normal sinus rhythm
4	63513	Heart transplant due to heart failure	Atrial-sensed ventricular-paced rhythm, Biventricular pacemaker detected, Abnormal ECG
5	ED94C	Heart failure	
9	27E20	Heart failure	Dilated cardiomyopathy
11	4D931	Organ donor	Sinus tachycardia, Otherwise normal ECG
13	E98EE	LVAD removal and heart transplant due to heart failure	
16	BOBD1	Heart transplant due to heart failure	Electronic ventricular pacemaker, Abnormal ECG
20	7E551	LVAD removal and heart transplant due to heart failure	Normal sinus rhythm, Left anterior fascicular block, Anterolateral infarct, Abnormal ECG
23	063A3	Heart transplant due to heart failure	Supraventricular tachycardia with occasional premature ventricular complexes, Minimal voltage criteria for LVH, may be normal variant, Poor r-wave progression in precordial leads, Borderline ECG

Using this table, it has been seen that the leading cause of death was heart failure, furthering the evidence that there is an underlying immune system issue causing the patient's heart to malfunction.

Once the DNA was extracted, a PCR was conducted using viral primers. This was done for all of the samples. Next, the DNA was run on a band where a sample that was positive for the sample will see a band within the gel corresponding to the size of the viral genome. The viruses identified in each sample can be seen in the image below (table 2).

Table 2: Viruses identified in each human heart sample

Viral Genomes Found in Individual Samples	
Adenovirus	2, 4, 9, 11, 13, 16, 23
Human Respiratory Syncytial Virus (HRSV)	X, 2, 4, 5, 9, 11, 13, 16, 23
Parvovirus-B19	Y, Z, 2, 4, 5, 9, 11, 13
Hepatitis-C	X, 2, 4, 5, 9, 11, 13, 16, 23
Human Herpesvirus 5 (Cytomegalovirus)	X, 2, 4, 5, 9, 11, 13, 16, 23
Human Herpesvirus 7	X, 2, 4, 5, 9, 11, 13, 16, 23
Human Respiratory Syncytial Virus (HRSV)	X, 2, 4, 5, 9, 11, 13, 16, 23
Human Herpesvirus 4 (Epstein-Barr virus)	2, 4, 5, 9, 11, 13, 16, 23
Enterovirus	Z, 1, 5, 13, 23
Coxsackievirus-B3	Z

With the viruses identified, the cytokine expression levels were then determined to evaluate the molecular profiles of heart damage. To do this, RNA was isolated from each sample and converted to cDNA. This cDNA was used in the qPCR assay with cytokine primers so the inflammation levels of each heart sample could be evaluated. The first trials were done with 2 organ donor heart samples from patients who did not have myocarditis, and 8 myocarditis/ dilated cardiomyopathy-positive samples. The two donor samples were 4D931 and 2B487, while the six experimental samples were 063A3, 63513 7E551, E98EE, 48CAF, BOBD1, EOABF, and BIF28. The results from this analysis can be seen below (figure 1+2):

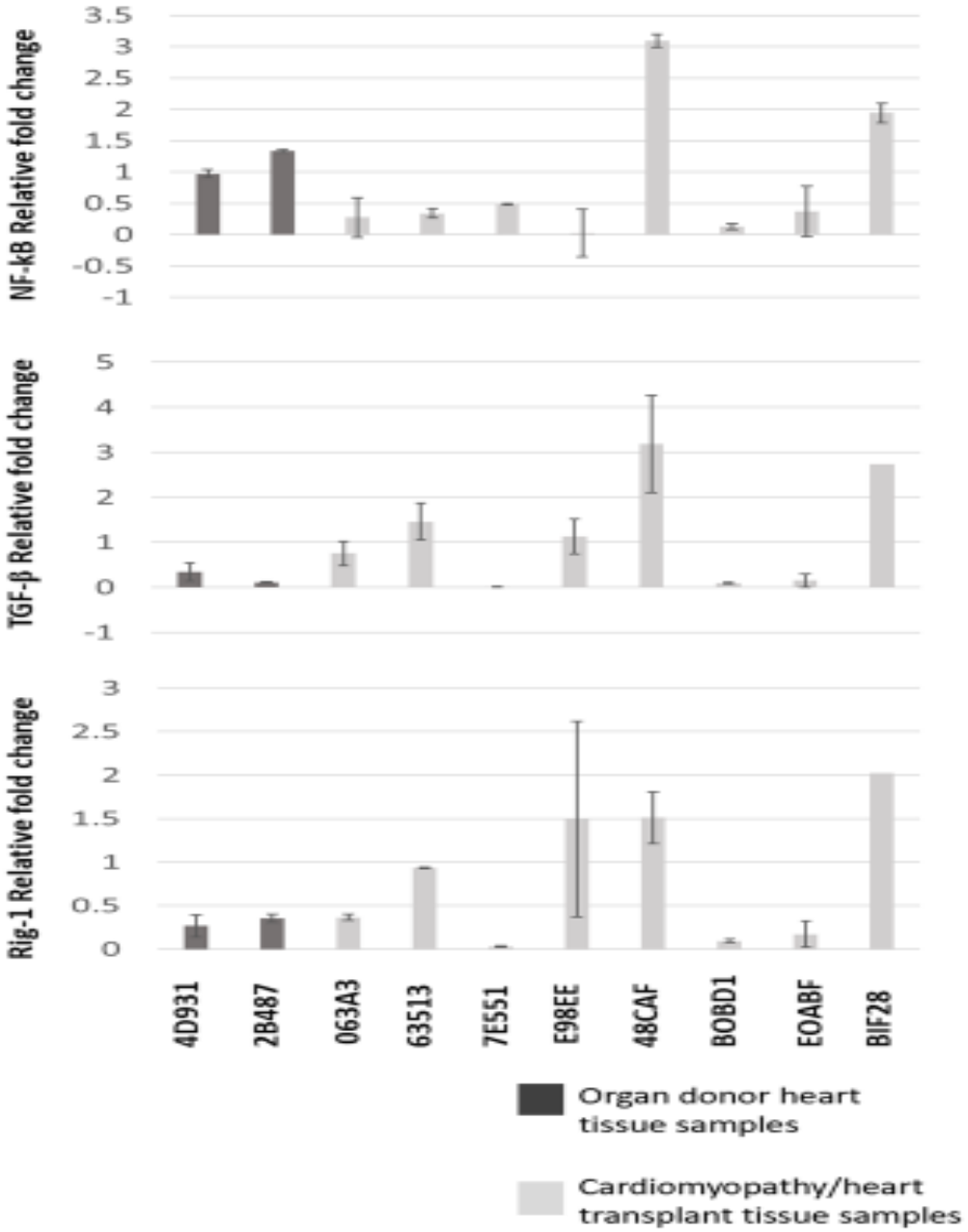

Figure 1: Relative fold expression profiles of NF-kβ, TGF-β and Rig-1 in organ donor samples and cardiomyopathy samples

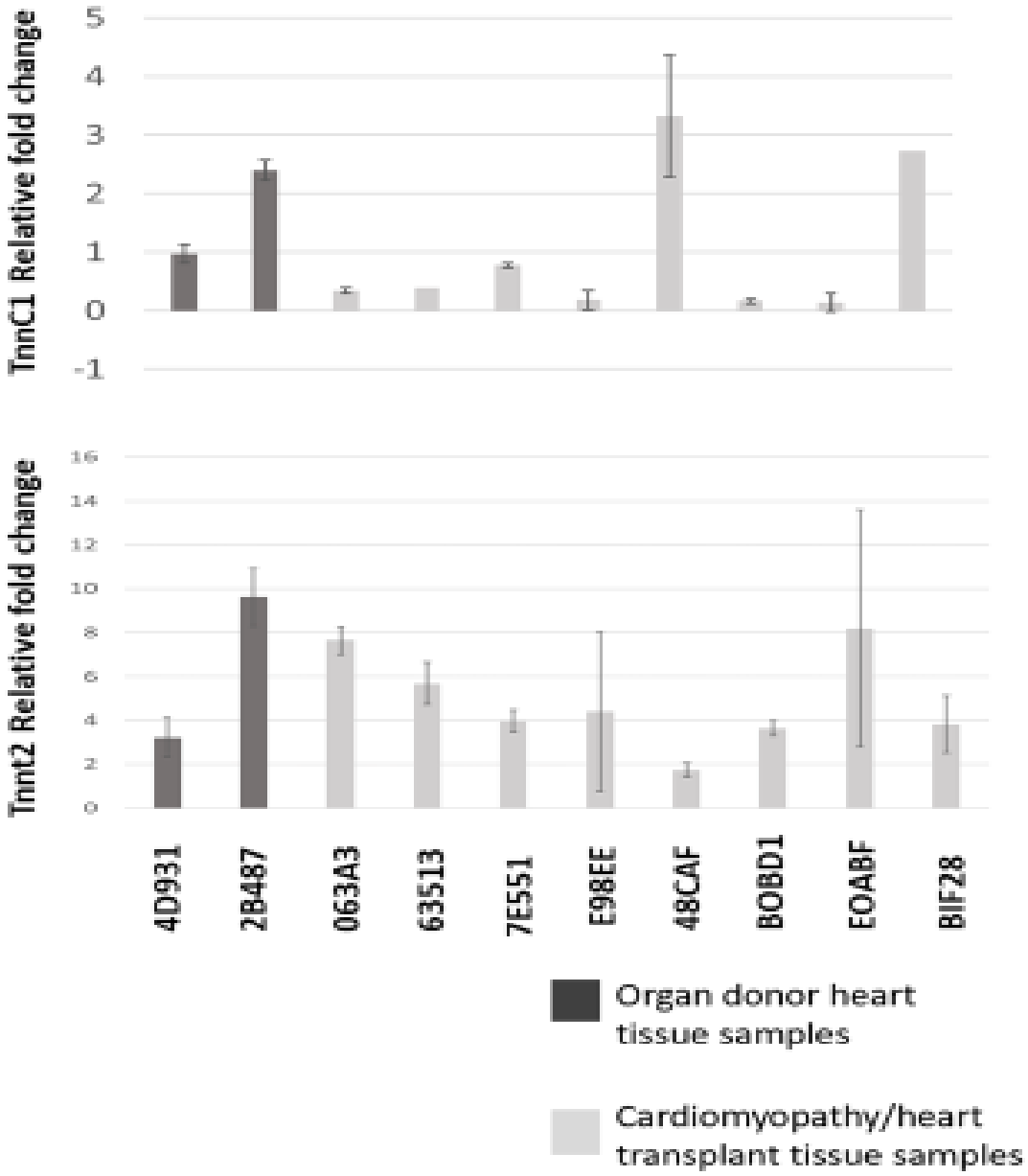

Figure 2: Relative fold expression profiles of Tnnc1 and Tnnt2 in organ donor samples and cardiomyopathy samples

Next, using RNA extracted from three samples, 632FD, 59386, and 48CAF, the inflammation of each of the heart layers was determined. This analysis can be seen below (figure 3+4):

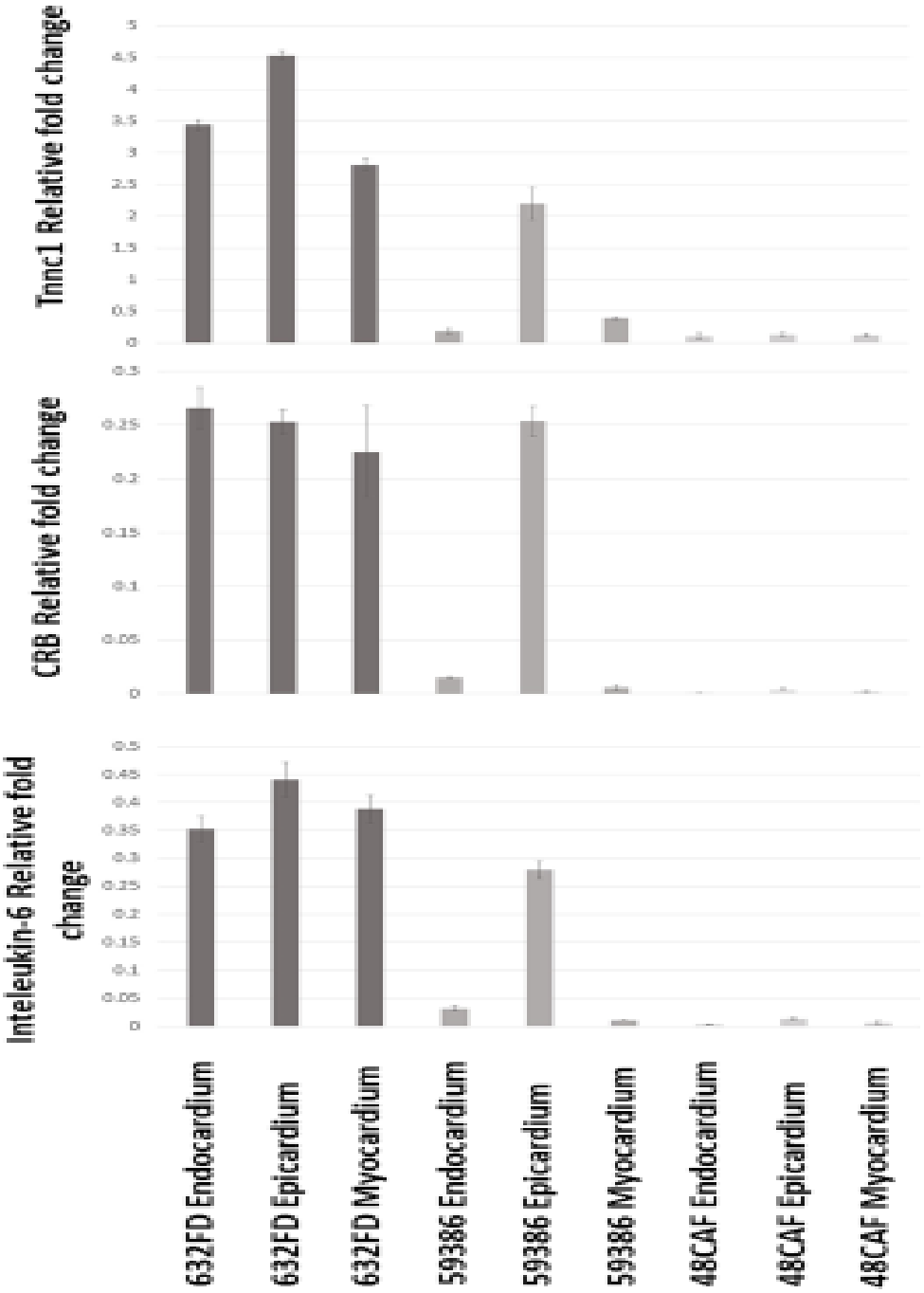

Figure 3 Relative fold expression profiles of Tnnc1, CRB, and Interleukin-6 in the three heart regions of cardiomyopathy samples

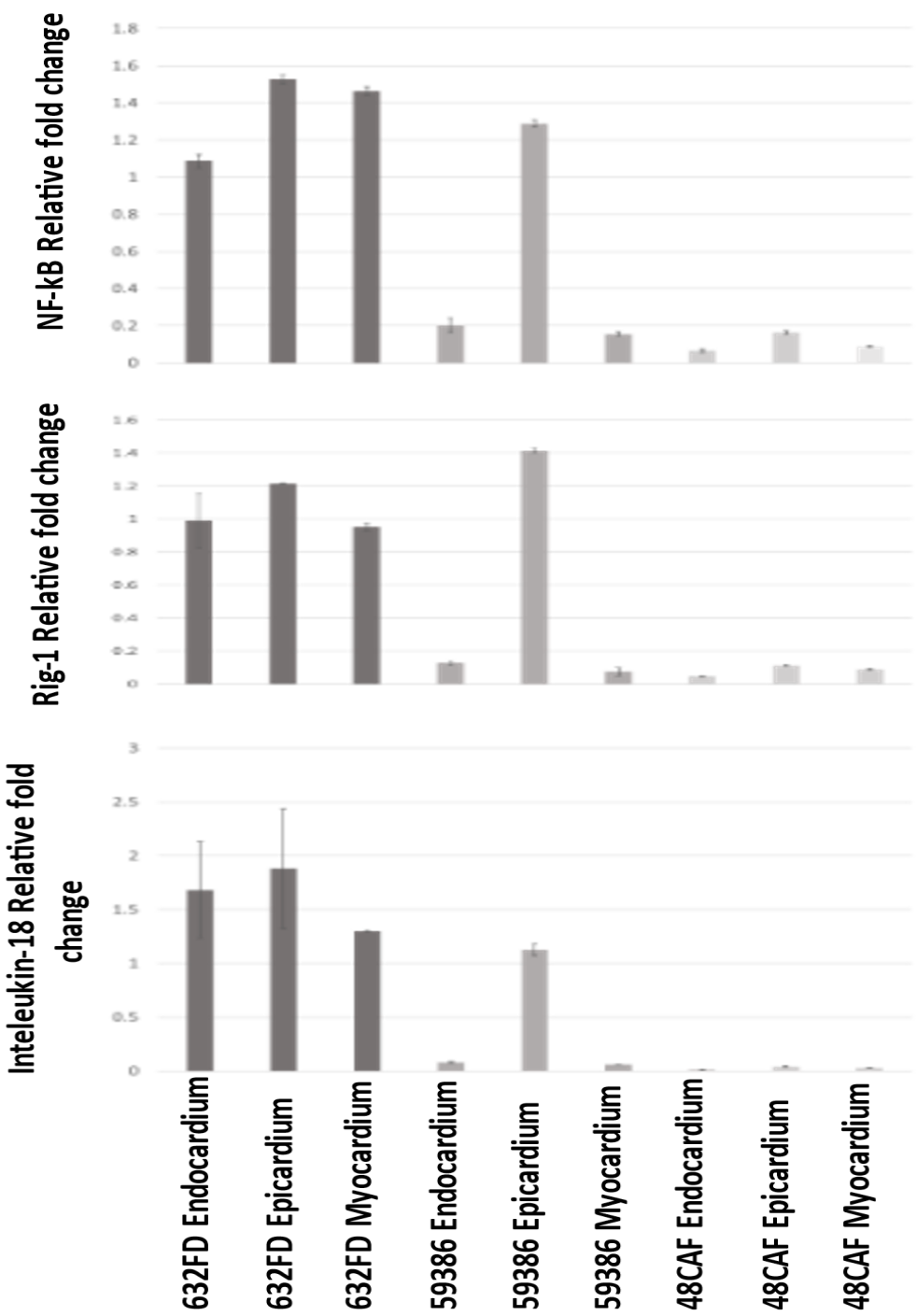

Figure 4 Relative fold expression profiles of NF-κB, Rig-1, and Interleukin-18 in the three heart regions of cardiomyopathy samples

Discussion

Each year in the United States, 1 in 4 causes of death can be attributed to heart disease, making it the leading cause of death. One cause of heart disease is myocarditis, which is the inflammation of the middle heart layer, myocardium. Viruses are thought to be responsible for most of the myocarditis cases. It is important to understand how these viruses contribute to myocarditis as the myocardium is essential for the heart to properly contract (4). In severe cases, myocarditis will lead to the heart being permanently damaged, causing the patient to have a stroke, heart failure, or at its worse die (5).

It has been seen that the progression of myocarditis can be broken into three distance phases. The first is acute myocarditis, when a virus can insert its genome into the heart, activating the innate immune system (23). During this phase, cytokines begin to be expressed, causing the heart to become inflamed (23). In the second phase, the subacute phase, the adaptive immune system becomes activated within the heart (23). This phase of myocarditis will last four weeks at its longest, and once again, the heart becomes further inflamed, which adversely affects the patient (23). In the third phase, the viruses are either cleared from the heart, and the patient returns to normal, or the viruses remain in the heart leading to chronic inflammation (23). During this time of increased inflammation, the myocytes begin to become remodeled, resulting in heart failure (23). It is important to understand the mechanism of each virus so that treatments or diagnostic tools can be created to help save the lives of many. For example, a doctor could better treat a myocarditis-positive patient if they could determine which virus was causing the condition and that virus's mechanism.

For many viruses evaluated in this study, the pathogenic mechanisms and site of cardiac infection are currently unknown. When it comes to enteroviruses (such as Coxsackievirus B3), it

has been seen that these viruses attack the heart as a secondary target organ (26). This happens because when one of these viruses infect the host through the gastrointestinal or respiratory tract and form an extracardiac reservoir (26). Then the viruses are recruited to the heart tissues, breaking down the cardiomyocytes (26).

Parvovirus, however, has a very different mechanism that starts with this virus infecting the vascular endothelial cells (26). Most people are exposed to these viruses when they are children, and then these viruses asymptomatically form reservoirs in the bone marrow (26). It has been seen in cases of myocarditis that this virus has been localized in the endothelial cells of venules, small arteries, and the arterioles (26). Parvovirus is released from the bone marrow by infected capillary cells and then induced damage of the endothelial cells by a direct cell virus interaction (26).

Herpesviruses have been found to affect several regions of the human heart, including the myocardium, left ventricle, and right ventricle. This virus has been able to infect both the mononuclear and endothelial cells (26). It further has been seen that cardiac myocytes and endothelial cells are reservoirs for latency and reactivation (26). Additionally, herpesviruses, specifically human herpesvirus-6 is reactivated by other infections (26). This leads to the theory that this virus enhances the pathogenicity of other viruses instead of being the main pathogen itself (26). It has been seen that human herpesvirus-6 is present in 50% of the progenies (26).

Epstein-Barr virus is another virus from the herpesvirus family. The mechanism of this particular virus is that it infects the cells of the adaptive immune system. It begins by infecting the B-cells and epithelial cells (15). What is unique to this virus is that it is usually active in the cells of the body at very low levels meaning it is likely also enhancing the pathogenicity of other viruses (15). Epstein-Barr viruses' main effect on patients is that the EBV genome encodes for two

proteins BZLF1 and BRLF1, which will lead to cell destruction, further affecting any myocarditis-positive patient (15).

Another virus that is often found in cases of myocarditis is Adenovirus. This virus is a very common source of infections in humans (common cold), and its high mutation rate makes it more difficult for the immune system to properly clear (16). The *Adenoviridae* family of viruses is able to enter cardiomyocytes through the CAR receptor on the outside of the cell (16). Once the virus has entered the cell, it causes cytoskeletal disruption (24). This disruption leads to an uncontrolled immune system response, causing dysfunction of a patient's heart's left ventricle.

This research project aimed to identify viruses in human heart tissue by examining samples obtained from patients suffering from myocarditis or dilated cardiomyopathy, and to establish an inflammatory gene expression profile in these samples. A series of donor samples were included in this analysis to further learn about the molecular characteristics of the immune system response to viral infection. It was seen that Tnnc1 and Tnnt2 had an over 2 relative fold change in a majority of samples, which is considered upregulated (Figure 2). When compared to the other cytokines, it was seen that CRB, and Interleukin-6 is had a low expression profile with under a 0.5 relative fold change (Figure 2+3). Interleukin-6 is of particular importance as it is a proinflammatory cytokine that, over time will lead to the breakdown of both the cytokine network and viral clearance pathways (27). Given it has a relatively low expression level, it is theorized that the virus might not be actively replicating in these samples, despite viral presence. The fact that this cytokine is active could be due to viruses, either active or dormant, being present in the heart. Over time the expression of this cytokine even at this low level can cause cardiac remodeling, resulting in complete heart failure if not diagnosed and treated properly.

The other findings can be seen when the organ donor heart samples, 4D931 and 2B487 are compared to the other samples, which were confirmed to have myocarditis or dilated cardiomyopathy. Tnnt2, which functions by making a cardiac troponin protein that helps in heart development, gave mixed expression profile results (28). The 2B487 sample had higher expression levels than many of the samples, while 4D931 had less expression or no statistical difference of expression when compared to each of the experimental trials (Figure 2). This higher expression levels could be due to sample 2B487 being infected with a virus that has yet to be tested or possibly having a higher level of viral infection when compared to the other samples. Sample 4D931 had many of the same viruses present as the other 8 samples (Table 2), suggesting that herpesviruses, Adenoviruses, and Epstein-Barr viruses can lead to an increase in expression levels Tnnt2 regardless of whether the patient has myocarditis or not.

Rig-1 is a cytokine that is active in the innate immune system and is needed to find cells that have become infected by a virus (29). It was observed that samples 63513, 48CAF, and BIF28 have this cytokine more upregulated than the two donor samples (Figure 1). This means it is likely that one virus that is found in these three samples is not found in the others, leading to these differences. In the future, it would be beneficial to screen these samples for even more viruses to see which combination can cause this increased expression levels of Rig-1.

The TGF-β cytokine becomes activated during cell repair, and it can regulate inflammation (30). It was seen that the cytokines fold change was near 0 for the two donor signals but higher in 5 of the 8 experimental trials (Figure 1). It can be speculated that in these two samples the immune system is actually attempting to decrease inflammation levels and repair the heart cells.

The final cytokine evaluated as NF-kB is a proinflammatory cytokine seen to trigger chronic inflammation (31). Only two samples were seen to have higher fold changes than the two donors,

48CAF and BIF28 (Figure 1). Once again, this supports the theory that the other experimental samples were actually attempting to clear the virus from the heart when they needed a transplant. Additionally, it was seen that BIF28 had higher cytokine expression levels meaning a full virus panel should be run to make more correlations between the virus presence and this increase in cytokines regardless of type.

In the next part of this study, the three heart layers were directly compared in three samples 632FD, 593836, and 48CAF, to evaluate the cytokine expression levels. For Interleukin-6, it was seen for samples 632FD that there was no statistical difference for cytokine expression level in the endocardium and epicardium, but both layers had a high expression when compared to the myocardium (Figure 3). This was interesting because it shows that the viruses present can impact multiple heart layers during myocarditis, a possible oversight in the current diagnostic methods. For 632FD this trend continued throughout each of the cytokines evaluated for, with all three heart levels being expressed at relatively high levels (Figure 3+4). The epicardium of this heart sample was seen to never have the lowest expression levels statistically in any of the samples, meaning in the future, this heart layer should be further evaluated to see if doctors are missing key data when diagnosing myocarditis in patients.

In the 59386 sample, the epicardium was seen to have much higher expression levels when compared to the other two layers, which were determined to be statistically similar in a majority of the cases (Figure 4). Once again this represents a potential oversight in the current way to diagnose myocarditis. It is currently thought that the myocardium is the main layer of the heart being affected in many cases, but for this sample, it can be suggested that the epicardium has even greater effects than the other two heart layers and should be further evaluated.

For 48CAF there were little levels of expression seen for any of the three regions (Figure 3+4). This means that it is likely that the immune system is not overly active in these samples regardless of the heart layer chosen. In the future, this sample could be used to see which viruses do not affect any of the heart layers in the sample.

Future Directions

Diagnosing heart inflammation relies on accurate identification of a pathogen behind cardiac infection. In the future, it would be beneficial for many of the results seen in this thesis to be optimized allowing for a more accurate identification. For example, during the viral identification portion of this study, it was expected there would be one clear band seen which would correspond to the viral genome present in the heart sample. In many of the images, there was one clear band than many bands surrounding the clear band. This was likely due to the fact that the PCR was not optimized. In order to overcome this, a new PCR can be done using varying annealing temperatures. Then the resulting product can be run on a gel electrophoresis to evaluate the proper temperature for that specific viral primer set. Doing this will allow for a clearer band to be seen.

Improving the qPCR results would allow for more accurate expression profiles. Some data points, especially those in the plate's bottom right, were calculated to have significant error bars often stretching into negative expression levels. In the future, it would be beneficial for these samples to be run in a different well on the plate so that more accurate data could be collected. Similarly, all of my data was done with duplicates but to increase the confidence seen in my data, it would be beneficial to run another trial so that the three data points can be compared amongst each other allowing for more accurate conclusions to be made.

Additionally, it would also be beneficial to send all of the samples out to be sequenced. Doing sequencing would allow for one to search for the viral genome. For example, if one of the samples was positive for Adenovirus, then it could have its genome searched for the known Adenovirus genome. This gives more confidence in the conclusions the samples were positive for the viruses evaluated for if it were found. Furthermore, the PCR sequences used can be aligned to the viral genome sequenced allowing for any mutations to be uncovered. This data would be beneficial in further understanding the mechanism of viral life cycles.

In the future, more qPCRs should be conducted using the three regions from each of the samples obtained. This would allow for the research team to better evaluate the inflammation levels in all three heart layers. Using this data will give more of an insight into how viruses affect the heart in its entirety and if the current medical system is overlooking important information in the diagnosis and treatment of myocarditis.

One other aspect that must be done in the future is the hematoxylin and eosin (H&E) staining on each of the heart samples. This assay will allow for the visualization of cardiac injury, such as infarcts, inflammatory cell infiltration and the rupture and necrosis of myocardial cells in each of the samples. The amount of scar tissue can also be seen in each of the samples after they are properly stained. This analysis is important as it would be tying together the identification of the viruses and qPCR to evaluate what is happening in the heart in its entirety. This analysis will give a definitive look into the heart, making conclusions with a higher degree of confidence.

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