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Interaction of Disease, Drugs, and Disposition in Ewing's Sarcoma Patients

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SENIOR THESIS APPROVAL

This Honors thesis entitled

“Interaction of Disease, Drugs, and Disposition in
Ewing’s Sarcoma Patients”

written by

Madeline Martin

and submitted in partial fulfillment of
the requirements for completion of
the Carl Goodson Honors Program
meets the criteria for acceptance
and has been approved by the undersigned readers.

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Abstract

Ewing's Sarcoma is a pediatric bone cancer that is highly aggressive, leading to a five year survival rate of only 30% even with multi-modal treatment protocols. Improved therapeutic options are desperately needed. Our research has focused on the ability of the non-psychoactive cannabinoid, ajulemic acid, to induce apoptosis and decrease metastatic potential in cells from members of the Ewing Sarcoma family of tumors. Recently, we explored the effects of the naturally-occurring cannabinoid, cannabidiol, on three-dimensional spheroids that mimic the cellular components and microenvironment of Ewing's tumors. We looked at how this treatment affects VEGF, a mediator of angiogenesis, to determine if these cannabinoids work through similar cellular pathways. Data with cannabidiol can then be compared to data collected from ajulemic acid studies to determine if these cannabinoids work through similar cellular pathways.

Ewing's Sarcoma

“Ewing's Bone Sarcoma is a malignant childhood bone and soft tissue tumor known to be highly aggressive and invasive” (Sanceau, Truchet, and Bauvois, 2003). This cancer starts in the bones and spreads quickly to the soft tissues and often the lungs, making it extremely dangerous. People with Ewing's may feel pain, sometimes notice a mass, or a bone may fracture, but these symptoms usually go unnoticed until the cancer has already spread, making the disease even more dangerous. Because of the nature of the disease to spread before being noticed, the survival rates for Ewing's are considerably low. Children who are diagnosed after the disease has spread have less than a 30% survival rate. Teens typically have a survival rate of about 56%. Ewing's is commonly treated with chemotherapy,

radiation, and many times surgery to remove the mass. In many cases, amputation may be necessary. Alternative treatment plans are clearly needed considering the low survival rate even with treatment.

Cannabinoids are a class of compounds found in the *Cannabis*, or marijuana, plant that has been shown to have medicinal value. Over the past six years, students on our research team have been able to perform research using ajulemic acid, which is a synthetic, non-psychoactive compound structurally similar to those found in the *Cannabis* plant. ajulemic acid is shown to kill tumor cells, block angiogenesis, and decrease tumor cell invasiveness (Recht *et al.*, 2001). These factors all contribute to metastasis, which is the ability for cancer to spread from its primary site to other parts of the body. Because the debate over the legalization of using marijuana for medicinal purposes is growing, we wanted to compare the effects of ajulemic acid with the effects of the naturally occurring compound, cannabidiol (CBD).

Cannabidiol is a naturally-occurring compound derived from the *Cannabis* plant. Many people may be concerned with using a substance coming directly from the marijuana plant. However, CBD is a non-psychoactive constituent of marijuana extract with a low-toxicity profile. Studies have shown that cannabinoids can have a wide array of effects on the immune, digestive, reproductive, ocular, cardiovascular, and central nervous system (Kogan, 2005). It has also been shown that CBD can inhibit breast cancer cell proliferation and invasion, as well as Lewis lung carcinoma growth (McAllister *et al.*, 2010). The mechanisms by which it effects the various body systems are beginning to be researched, and the mechanisms vary. Studies suggest cannabinoids' anti-angiogenic effect may be linked to their

ability to modulate the response of cells to growth factors, such as VEGF. Cannabinoids can interfere with these growth factors, blocking growth factor-dependent cancers from spreading (Kogan, 2005). VEGF, vascular endothelial growth factor, is central in angiogenesis of cancer cells as it aids in the recruitment of a blood supply to tumors. Blocking angiogenesis is necessary to inhibit metastasis because it is the means by which a tumor receives blood supply to feed the tumor. If blood vessels are not within close proximity to the tumor, the tumor cannot survive (Huang *et al.*, 2003). Inhibition of VEGF production has been reported in tumor cells treated with CBD. For these reasons, our research is focusing on how CBD effects VEGF in spheroid models of Ewing's Sarcoma. We hypothesized that VEGF levels would decrease when exposed to CBD.

Two-dimensional cell cultures have led to many discoveries in understanding human tissues and diseases, but this type of cell culture does have limitations. In 2D cell cultures, there is limited cell-cell and cell-matrix contacts and a lack of realistic mass transfer gradients (Katz, 2013). Recently, systems have been developed to grow three-dimensional cultures *in vitro* that would mimic the microenvironment of the cancer *in vivo*. It is hypothesized that tumor cell biology and treatment response would be more accurate and informative if done in a setting that is similar to the tumor's microenvironment in order to observe the intra/intercellular interactions (Upreti *et al.*, 2011). Because of the greater accuracy, our research has turned to three-dimensional configurations. Spheroids were made containing components that would be found in a tumor setting in the body in order to observe the interactions. These spheroids were made in round-bottom well plates that encourage the cells to cling to one another but not adhere to the bottom of the plate. This model reflects the

suspended nature of a clump of tumor cells in the body. The spheroids contained three cell lines: endothelial cells, which aid in the formation of new blood vessels so the tumor can survive, fibroblasts, which serve as the structural framework holding the tumor cells together, and the cancer cell line, which was taken from a Ewing's sarcoma tumor that had metastasized to the lung. These cell lines together best resemble tumor conditions in the body that we can treat and analyze *in vitro*.

Materials and Methods

Cell Lines and 3D Tumor Spheroid Models

A standard spheroids protocol was utilized to create the spheroid models that would be used to grow and test the Ewing's Sarcoma tumors. In an ultra low-binding, round bottom well plate, three cell lines were plated: EPC endothelial cells, WI-38 fibroblasts, and TC71-PM4-GR, the Ewing's Sarcoma cancer cell line. Twenty-four of the 96 wells were plated with one-thousand cells per well. The cells in each cell line were counted, and the volume needed was then calculated. The cells were resuspended in 50 mL of mixed media and gently mixed. 50 μ L were added to each well, then the well plates were placed in a 37° incubator. After being in the incubator for three days, 50 μ L of fresh mixed media was added to encourage the cells to continue to grow. On day six, 100 μ L of Opti-MEM (low serum) was added. On day eight, treatment of cannabidiol was administered. Six well plates were left untreated, six were treated with 10 μ M, six were treated with 20 μ M, and six were treated with 32 μ M of cannabidiol. Samples were taken for an enzyme-linked immunosorbent assay (ELISA) at 18 hours, because it is shown that at 24-48 hours, CBD induces the cancer cells to die. We took samples before the 24-48 hour time period to see only the drugs effects on the VEGF levels.

ELISA

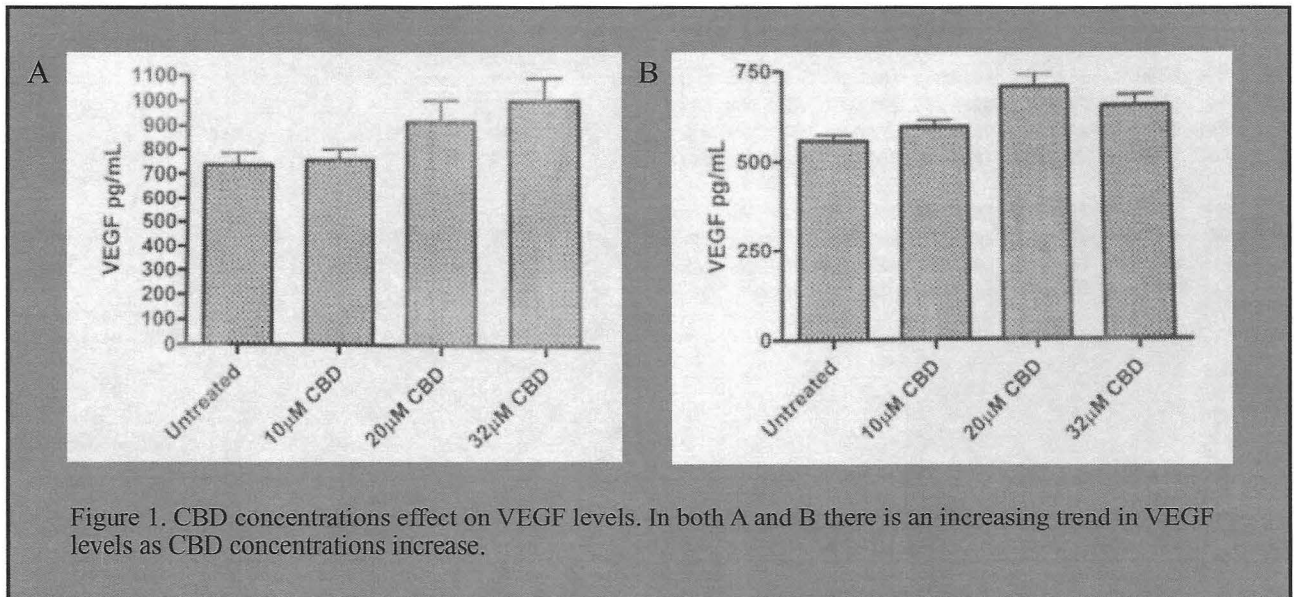
The DuoSet ELISA Development System kit and protocol were used on the cell lines. The samples were taken 18 hours after being treated with cannabidiol. The ELISA test measured the amount of VEGF present in the growth media of the spheroids. After performing the protocol for the ELISA test, the optical density of each well plate was determined using a microplate reader. Absorbance values were used to determine the VEGF levels in each well plate and to analyze the effects of various concentrations of CBD on the VEGF levels.

Results

Through this research phase, we wanted to determine how CBD affected the metastatic potential of Ewing's Sarcoma tumor cells, specifically through the VEGF mechanism. Studies with other cancer cell lines involved demonstrated the ability of CBD to inhibit VEGF production. For this reason, we expected to see VEGF levels decrease as the concentration of CBD increased. The study looked at untreated cell lines and cells treated with 10 μ M, 20 μ M, and 32 μ M of cannabidiol. The following graphs show the results from the ELISA tests, with the y-axis showing the VEGF level present after treatment, and the x-axis showing the concentration of CBD administered.

Referring to Figure 1, the first trial produced unexpected results. In Figure 1A, we found a dependence on VEGF levels to CBD concentrations, but in the opposite way expected: VEGF levels *increased* as CBD concentration *increased*. We expected inhibition of VEGF production, but instead saw more VEGF. One can also see that there was less than 100 pg/mL difference in VEGF levels for the untreated and 10 μ M concentrations of CBD. In

Figure 1B, the same trend was found, with the difference being in the drop of VEGF levels at 32 μ M.



After obtaining unexpected results, we ran the test again using the same cell lines to ensure that we did not make a systematic or random error. The results from Trial 2 are shown in Figure 2.

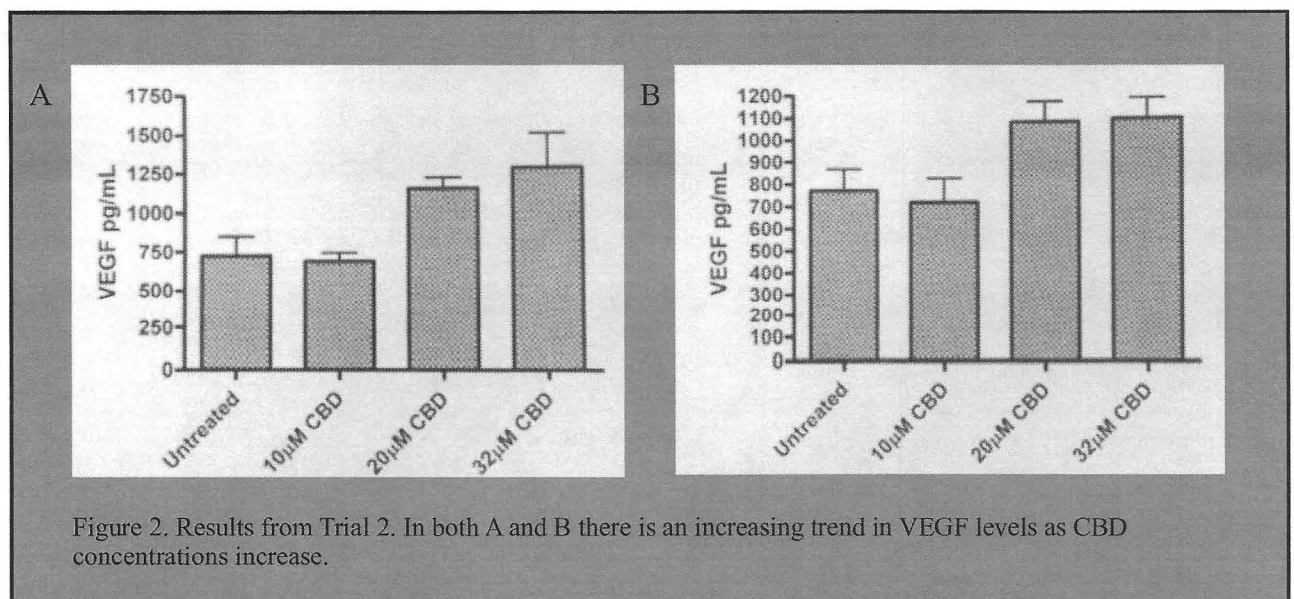


Figure 2. Results from Trial 2. In both A and B there is an increasing trend in VEGF levels as CBD concentrations increase.

With the second set of data, we again found an increase in VEGF levels as CBD concentrations increased. There was again less than 100 pg/mL difference in the VEGF level between the untreated and 10 μ M concentration. In both Figures 2A and 2B, the most significant increase in VEGF levels was between the 10 μ M and 20 μ M concentrations of CBD. There was less than 100 pg/mL difference in the VEGF levels between the 20 μ M and 32 μ M concentrations of CBD.

Again not getting the results that were expected, and after seeing inconsistencies, we decided to run the test on entirely new aliquots of cell lines. We ran the test on the old and new cell lines again for comparison sake. Figure 3 represents the old cell lines in Trial 3, and Figure 4 represents the new cell lines in Trial 3.

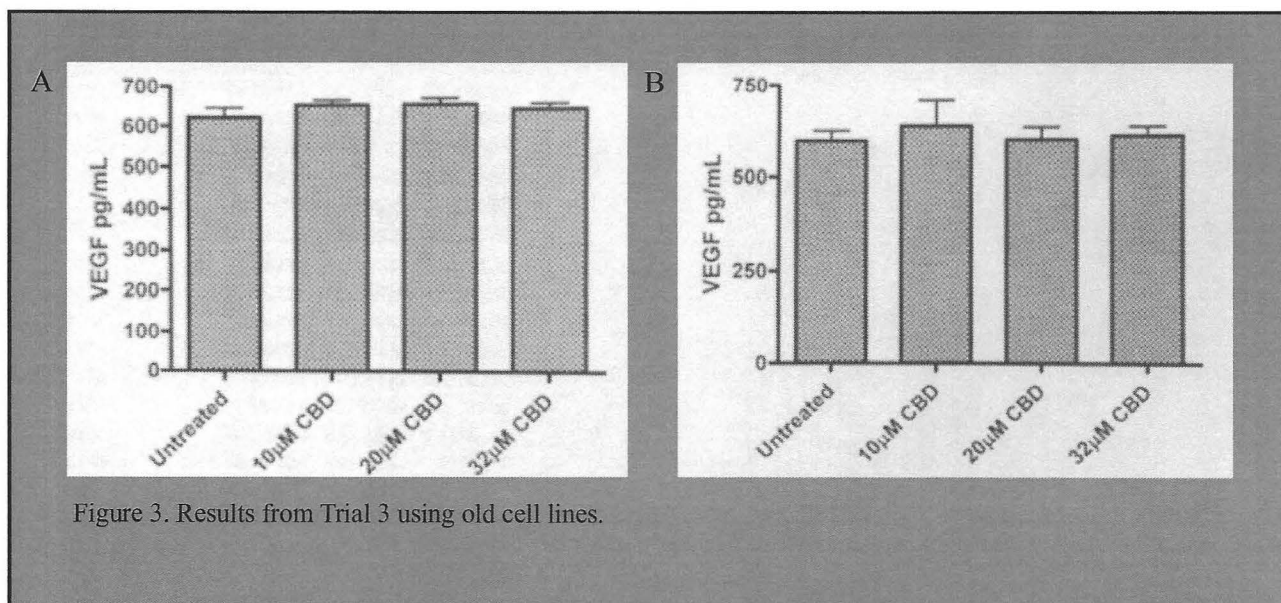
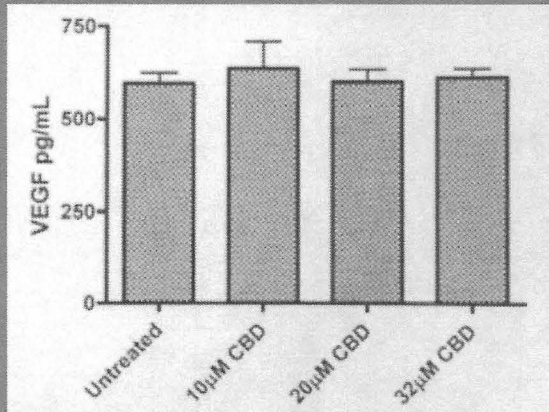


Figure 3. Results from Trial 3 using old cell lines.

With the old cell lines run once more in Trial 3, there was a leveling off of VEGF levels as concentrations increased. CBD did not appear to have an effect on VEGF levels.

A



B

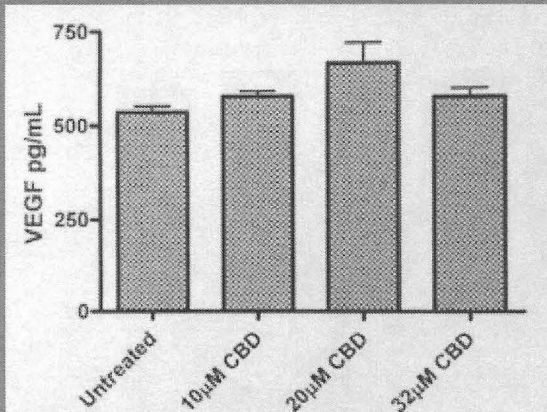


Figure 4. Results from Trial 3 using new aliquots of cell lines.

We hypothesized that completely starting new cell lines would result in an inhibition of VEGF production as CBD levels increased, but the result in Trial 3 with new cell lines did not produce support that hypothesis. In Figure 4A, results were different. There was a decrease instead of increase in VEGF levels at 20µM of CBD. In Figure 4B, we saw much the same as before: little change from untreated to 10µM and a spike in VEGF levels at 20µM. We again saw a decrease in VEGF levels at 32µM like we did in Figure 1B.

The data from all three trials were not incredibly consistent. In some trials, we saw an increase in VEGF levels as the concentration of Cannabidiol increased, and in others there was little change at all. These results suggest that Cannabidiol does not effect VEGF levels in a consistent manner in Ewing's Sarcoma tumor cells.

Significance

Our results showed that cannabidiol does not have an effect on VEGF levels. There are multiple pathways and mechanisms through which metastasis of cancer cells is achieved, and metastasis is a highly complicated process depending on multiple interactions (Sanceau, Truchet, and Bauvois, 2003). This study only looked at the VEGF mechanism. Cannabidiol has been shown in other studies to inhibit metastasis. Since each type of cancer is different, it is probable that CBD can have an inhibitory effect on Ewing's Sarcoma cells, just through a different mechanism than the one we studied. Because of this, further research is recommended on other possible mechanisms effecting metastasis. MMP-2 mediates another mechanism that induces invasion and migration, much like MMP-9 (a mechanism concurrently tested with similar results to VEGF and CBD). Our research team plans to test CBD's effect on MMP-2 levels through the same procedures discussed earlier. It is important that we not only demonstrate that CBD can inhibit Ewing's Sarcoma's spread, but also the mechanism that it effects. Other future research plans include working with a computer program called Oncomine. This program gathers clinical data from a wide variety of sources. Through these additional measures, we will seek to identify which proteins are causing the rapid spread of Ewing's Sarcoma and work backwards. Similar tests could then be run on these proteins to understand cannabidiol's effect on it.

While researching the effects of cannabidiol on Ewing's Sarcoma, we have also been studying the effects of the synthetic compound AJA. We hypothesize that AJA and CBD will inhibit Ewing's Sarcoma through the same mechanisms. Research will continue to determine the similarities and differences between the naturally occurring and synthetic form. A better

understanding of how these drugs compare can provide the public with a better background of knowledge when debating and voting on the issue of the legalization of medicinal marijuana.

Psycho-Oncology

Ewing's Sarcoma, and pediatric cancers in general, can put a great strain on families- particularly the cancer patients, parents, and their siblings. From diagnosis to treatment, each step in the life of cancer patients and their families can be extremely stressful and emotionally devastating. Because of these negative psychological effects, specialized intervention plans should be implemented for the patient and each family member. To understand how to best manage these psychological issues, we will look at the main problems concerning patients, parents, and families, and the coping methods that are shown to be most effective.

Patients, parents, and siblings of pediatric cancers all face different psychological issues because of their role and responsibilities within the family unit. It has been shown in studies that the diagnosis and treatment of pediatric cancer is a stressful and emotionally taxing event for the patient. Survival rates are improving for some types of pediatric cancers; however the "course of treatment for childhood cancer, such as chemotherapy, surgery or radiotherapy, is still a very stressful experience in the life of a child" (Li *et al.*, 2010). In a study examining physical, emotional, and psycho-social well-being in cancer patients from Hong Kong, a higher than normal level of anxiety, sadness, and worry was shown in the patients. Over half of these patients were also at risk for depression (Li *et al.*, 2010). Even the patients who survive are at risk for post-traumatic stress disorder (PTSD). "Nearly 20% to 35% of childhood cancer survivors meet diagnostic criteria for PTSD," which is more than double the rate for the general population (Stoppelbein, Greening, and Wells, 2013). More than 50% of pediatric cancer survivors show subclinical post-traumatic stress symptoms

(PTSS). Risk factors for developing PTSD or PTSS “include older age, being female, being on treatment, shorter length of time since treatment ended, anxiety, parents’ higher level of PTSS, and parents’ perceiving treatment as intense” (Stoppelbein, Greening, and Wells, 2013). With the increased stress level, emotional instability, risk for depression and PTSD, interventions need to be made to improve patients’ quality of life.

In order to create the most effective intervention plan for pediatric cancer patients, health-care professionals must understand what coping is and what coping strategies are used by these children. “Coping is a process involving cognitive and behavioral attempts to meet the demands of, and to control the emotions generated by, the situation” (Li *et al.*, 2010). There are two types of coping strategies that children may use either on their own or in conjunction during a stressful event: problem-focused and emotion-focused. According to Lazarus and Folkman, problem-focused coping includes “seeking social support, planful problem solving, and confronting. Emotion-focused coping includes “positive reappraisal, self-control, escape-avoidance, accepting responsibility, and distancing” (Li *et al.*, 2010). Based on many studies, it is often culturally based as to which coping strategy the child uses more frequently. In Western children, problem-focused coping was found most commonly, specifically seeking social support. In Eastern children, emotion-focused was most common, specifically through self-control. It seems that emotion-focused coping is used most often when it seems like nothing can be done to change the situation. There is also variance in coping strategies used by different age groups. Younger children rarely use confronting, positive reappraisal and accepting of responsibility as a coping method. Evidence suggests that as “children become older their locus of control shifts from an external to an internal

control” (Li *et al.*, 2010). Results from this study on coping mechanisms demonstrate the importance of targeting interventions based on culture, age, developmental stage, and coping strategy to reduce emotional stress. For example, those patients who use problem-focused coping in efforts to reduce the impact of the problem encountered, it would be best to use interventions that include providing detailed information about procedures that will be used along with instructions that enhance problem-solving activities. For patients that use emotion-focused coping, interventions that focus on regulating emotions by relaxation, distraction, or denial, would be most effective. By understanding how the child is approaching their diagnosis and treatment, more effective interventions can be created.

Another promising approach to supportive care for pediatric cancer patients, is massage therapy. “Massage Therapy as a Supportive Care Intervention for Children with Cancer” reviews literature on massage therapy “to inform healthcare providers about the potential uses of massage for children with cancer” (Hughes *et al.*, 2008). Massage therapy has been used to “alleviate stress and muscle cramping, induce relaxation, improve circulation and lymph flow, promote muscle tone, increase range of motion, and encourage recovery from injuries and medical procedures” (Hughes *et al.*, 2008). There are a number of physiological benefits from massage therapy. Parents of patients have reported the use of massage to help counteract toxicities from chemotherapy and radiation therapy, including fatigue and nausea. This article cites studies that have shown that massage therapy can lead to decreased heart rate, blood pressure, and respiratory rate. Massage therapy also appears to manage symptoms associated with cancer treatment such as nausea, mood, appetite, insomnia, pain, mobility, fatigue, bowel movement patterns, concentration, and appearance.

Massage therapy can also provide psychological benefits. “Overall quality of life measures improved significantly” for patients receiving massage therapy compared to a control group. There was a decrease in anxiety and depression. With the reduction of stress provided by the massage therapy, immune function benefits are also seen. Boosting the immune system can be highly beneficial during periods of profound immune suppression. “Massage therapy may suppress the activity of the hypothalamic-pituitary-adrenal axis, causing decreases in cortisol and neuropeptide levels” (Hughes *et al.*, 2008). This may improve immune function by increasing natural killer cell number and activity. For pediatric patients, massage therapy can be extremely beneficial to improve both physiological and psychological symptoms associated with cancer treatment.

Although the patient is suffering great physical and emotional pain, the parents are dealing with emotional instability and a number of new responsibilities and stressors. It is important to understand the parents’ perspective and ways to manage their psychological issues, because the parents emotional state can impact the rest of the family and the patient both mentally and physically. When a parents’ child is diagnosed with cancer, parents have been shown to exhibit “higher emotional distress levels and more psychosomatic complaints than norm populations” (Hoekstra-Weebers *et al.*, 2012). They are suddenly bombarded with frequent hospital visits and the responsibility of managing their child’s healthcare. Not only are the time demands and responsibilities difficult for parents to adjust to, they are also having to see their child suffer through intense treatment regimens and constant hospitalization (Gage, 2013). Because of these issues associated with parents of pediatric cancer patients, interventions are needed to help parents cope.

There are a number of ways for parents to cope with the diagnosis and treatment of their child's cancer such as social support groups and psycho-education programs. A study was conducted by the Department of Community Health and Behavior at the University of Buffalo to examine the mechanics of effective social support. They specifically looked at how people ask for, manage, and perceive offers of social support. To better understand the dynamics of social support, researchers interviewed parents about their child's diagnosis, their experience with social support, and how they managed their various responsibilities. The following are the responses from parents. They often felt like they received many offers of help immediately after diagnosis, however these quickly declined and they did not seem genuine. Parents felt unsure of how to manage all of these offers, because they felt awkward asking for help in what they really needed such as specific daily chores. For many families, they were able to ask close family friends and family to help with these everyday tasks. It was important for there to be a close relationship before the diagnosis, in order for the parents to feel as if they could ask for help. These close relationships were able to help in many daily tasks and help rally support from those that were not as close to the family. The study found that less personal support, such as financial support, was easiest for parents to accept from socially distant network members. This study provided important information about how the parents view social support and the issues associated with it. Because of the results from these studies, "it may be fruitful to design programs that counsel parents to identify their needs and identify potential support providers with these distinctions in mind" (Gage, 2013). With programs in place, parents can better receive support throughout the treatment process and to fit their actual needs.

Studies have also been done to examine the effectiveness of structured psycho-educational programs (PeP) for parents of children with cancer. Their main goals in the PeP was to “improve parents’ knowledge on childhood cancer, increase parents’ psychological awareness and acceptance to diagnosis, recommend effective coping strategies, and decrease their levels of anxiety and stress symptoms” (Othman *et al.*, 2010). Results showed that this type of program enhanced parents’ knowledge on childhood cancer and there was a trend towards reduced anxiety. However, no statistical difference was found on measures of the other goals in the study. Based on these results, the PeP can be used to increase knowledge and reduce anxiety, but other coping strategies should be implemented to address the other issues.

Finally it should be noted that coping strategies vary amongst genders. For example, mothers used social support more than fathers. Therefore, “when designing interventions for parents, differences between the effects coping has on fathers’ and mothers’ psychological functioning should be taken into account” (Hoekstra-Weebers *et al.*, 2012). It is important that parents find ways to cope and manage the stresses associated with pediatric cancer in order to increase their quality of life and their families. Studies have shown that parental stress, overprotection, and perceived child vulnerability, can cause negative effects on patient adjustment and quality of life (Hullmann *et al.*, 2010).

There is one more important component of the immediate family that can be greatly impacted by a pediatric cancer diagnosis and treatment. Those are the siblings of patients. They have been referred to as “the forgotten ones,” because they are often thought about the least during their brother or sister’s illness (Madan-Swain *et al.*, 1993). During this time,

there are many added stressors to the sibling's daily life that can impact them in a negative way and cause behavioral problems. These stressors include: "loss of parental attention..., changes in family structure, roles, and expectations; concerns about the cause of their sibling's illness; feelings of guilt, anxiety and shame; social avoidance; and avoidance of affectual disclosure within the family," (Madan-Swain *et al.*, 1993). These stressors have led to internal and external psychological problems in many children, such as anxiety and depression, problems in school, lower quality of life, and post-traumatic stress (Prchal and Landolt, 2009). These results show that interventions need to be accessible for siblings of pediatric cancer patients.

Studies have been conducted to evaluate the effectiveness of group therapy intervention programs. In a study conducted by Barrera and colleagues at the Hospital for Sick Children in Toronto, siblings of pediatric cancer patients participated in an 8 week group program. The group met weekly for 2 hours in a hospital and was led by two therapists. Each session focused on a different goal or theme, such as group rules, medical information, family, siblings' feelings, relationships, schools, hope for the future, and finally closing thoughts. The sessions were structured beginning with a fun activity or review of last weeks theme, and then continuing with the main theme of that week. Parents were kept updated on the status and progress of their child in the class. Group interaction improved each week, with the participants becoming more involved in discussion and the activities. At the end of the study, the participants were evaluated through self-reports and satisfaction questionnaires. Levels of anxiety and depression were significantly lower after the program's completion. Self-reports demonstrated a reduction in fear of cancer and improved thoughts and feelings

toward cancer. Participants said that they felt that the program was useful in reducing their worries. They felt like they learned more about pediatric cancer, could express their feelings better, and communicate with their parents and others more effectively. This study shows that programs can be effective at minimizing the negative effects cancer can have on patients' siblings (Barrera *et al.*, 2002).

Pediatric cancer can have a dramatic effect on the entire family. The need for intervention programs for each member of the immediate family is imperative for the positive adjustment of all members. Coping strategies, social support, and therapy programs are all effective ways for families to manage this difficult time. It is important to remember that each family member is facing different stressors from the diagnosis and treatment of the cancer. A unique plan should be established for each person. Family-centered care should become a vital part in the management of pediatric cancer.

Reasons for Research

As a senior in college, I have been exposed to a number of significant learning experiences that will impact me in my future endeavors. However, nothing in my undergraduate work will have such an impact as the research that I conducted under the direction of Dr. Lori Hensley, and the subsequent research that I have done to write this Honors Thesis. Why is this so important to me? After graduation, I plan on pursuing a career as a Physician Assistant. Although I do not know what speciality I will choose, I will be working with patients on a daily basis. This research has given me a glimpse into an emotional subject that many children and parents must deal with. With this background in cancer research and in psychological research, I will be able to relate to my patients more effectively and provide or

refer them to the resources they need to get through a difficult time. I can relate this research to other illnesses other than pediatric cancer. Any health issue that changes the patient or families' lives will have the potential to cause stress, anxiety, depression, and a number of other psychological issues. I will apply what I have learned through this research and will continue to learn in Physician Assistant school how to care for my patients fully and to the best of my ability.

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