DISCUSSIONS

Undergraduate Research Journal of CWRU

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Photo courtesy of John Twohig

Letter from the Editor

Dear readers,

Since 2006, Discussions has celebrated and promoted undergraduate research at Case Western Reserve University and other universities all over the world. As we enter our tenth year as a research publication, I want to take a look at the progress we have made and our goals for the future. Let me first say that it is truly my pleasure to introduce the newest issue of *Discussions* to you.

Discussions is the Undergraduate Research Journal of Case Western Reserve University. Although based at CWRU, we have become a global presence, partnering with numerous universities and research institutions around the country as well as receiving submissions from countries as far as China, Russia, Nigeria, and Turkey. As of this issue, *Discussions* has officially received submissions from every continent in the world (not including Antarctica). While *Discussions* was started by just a few dedicated students, we have grown to become a large organization dedicated to promoting undergraduate research from the best and the brightest students.

By taking the time to read this journal, you are promoting *Discussions* and, in turn, the long hours of research conducted by undergraduates, often without herald or pay. In choosing to read the papers included in this issue of *Discussions*, you are stepping into an oft-overlooked niche of research, learning about topics to which you may not have been exposed.

If you would like to see your research published in *Discussions*, visit our website at www.case.edu/discussions or our Facebook page for submission guidelines and more details.

As we continue to grow in size and prestige, I encourage anyone interested in research or the publication process to find ways to get involved with our publication. We accept submissions from around the world and distribute around the country; our organization's success has increased exponentially in recent years. Reach out to askdiscussions@case.edu to learn how to get involved.

As we continue to expand, our goal is to supply more students with an outlet through which they can share their passion for research and inquiry. It has taken nearly ten years to get to where we are now, and my hope is that in another ten years we will be able to look back, knowing our dedication to undergraduate research has only grown. Our primary goal, the same goal that encouraged students to form *Discussions* in 2006, is to become the premier undergraduate research journal in the country.

Finally, I would like to thank Sheila Pedigo, Bethany Pope, and the entire SOURCE office for their continued support.

Thank you for taking the time to read our journal, and I hope you enjoy the fantastic articles within.

Sincerely,

Benjamin Nudelman

Benjamin Nudelman

Editor-in-Chief, *Discussions* Research Journal

Faculty Spotlight

An Interview with

DR. RICHARD BOYATZIS

conducted by Sruthi Meka and Jacob Behrend

Photo courtesy of Case Western Reserve University

INTRODUCTION

In recent years, words like "dynamic," "evolving," "flexible," "inclusive," and "globalized" have risen within the business lexicon, reflecting a change in the entrepreneurial mindset. In many emerging businesses, employees are being seen more as intertwined, complex social-emotional individuals, rather than ubiquitous production cogs. With his widely used theory of intentional change, Dr. Richard Boyatzis, Distinguished University Professor in Organizational Behavior, is studying how and why groups and businesses change over time. Through the lense of leadership, emotional intelligence, and group cohesion, Professor Boyatzis is finding ways to predict such changes in order to guide businesses toward positive, sustainable change. Recently, I met with Dr. Boyatzis to discuss his research, his ideal business environment, and his advice for up-and-coming entrepreneurs.

1. What research topics interest you within your field?

Since 1967, the main thing I have studied is how individuals and social groups go through sustained, desired change. Over the years, I have developed the Intentional Change Theory, which describes how this process occurs. The complexity theory must be applied to this theory in order to understand it, since almost nothing with humans is linear or continuous. Right now, that is the grand design. Everything that I do fits into that in one way or another.

Within that, most of my research and the studies of the doctoral students I am currently supervising here in the two different doctoral programs and the Escuela Superior de Administración y Dirección de Empresas (ESADE) in Spain, fall into one area of study in particular.

Our research aims to continue expanding our understanding of the world of emotional intelligence, which is a set of behavioral capabilities that we all have to varying degrees. I specifically look at how that affects leadership effectiveness, management effectiveness, and professional effectiveness for doctors, nurses, engineers, etc.

Another important aspect of Intentional Change Theory is the power of vision, which I would argue is the main driving force behind any change process. A former doctoral student, a current doctoral student, and I are currently editing a special edition of "Frontiers in Psychology," in which we will have 10 to 12 articles that will come out in the special issue. Six of those articles discuss the power of vision for individuals, especially for women in STEM careers, to drive their career engagement and success.

2. Discuss the methodologies of your current research project:

In the fMRI, we are testing the part of my theory that essentially says: If you try to help people through what I call positive emotional attractors, where patients talk about their dreams rather than their problems, then you will activate physiological processes that make them more open to new ideas and people. We did the first fMRI study in 2009 with undergraduates at CWRU. We had students participate in 30 minutes of coaching around their vision of a fantastic life in 10 or 15 years. Another day, the subjects discussed their performance in their courses – whether they were doing the readings, the assignments, etc. In my theory, this is the difference between positive and negative emotional attractors. Talking about the obligations of each course is not necessarily negative in and of itself, but it does put subjects on the defensive. In this study, when different subjects went through the fMRI scan and were shown video segments of

> *"...our coaching studies can show people how to change their leadership methods and behaviors for the better."*

those who participated in the coaching sessions, specific portions of the subjects' brains were observed to light up. After further analysis, we recognized these parts of the brain as part of the default mode network, which is correlated to the human mind's openness to ideas, people, and moral concerns. Often, when students are asked about the courses they are taking and how they are performing in those courses, the task-positive network is activated, which causes them to be more analytic, defensive, and closed to new ideas.

We recently received funding to expand these findings by studying leadership effectiveness in a firefighting team. We hypothesize that effective leaders transition between both the default mode and task positive, which we studied previously. If we can show that, then we think our coaching studies can show people how to change their leadership methods and behaviors for the better.

3. What challenges do you face in your current line of research?

Time. I don't have enough time. The other challenge is the inevitable one of funding. It's a real struggle to get money. Fortunately here, Provost William A. "Bud" Baeslack III has provost initiative fund, which has been really helpful to us. We've even used some of my funds from my endowed chair. Now, we're starting to bring money in, but it's still slow.

4. What type of audience to you hope to address through your research?

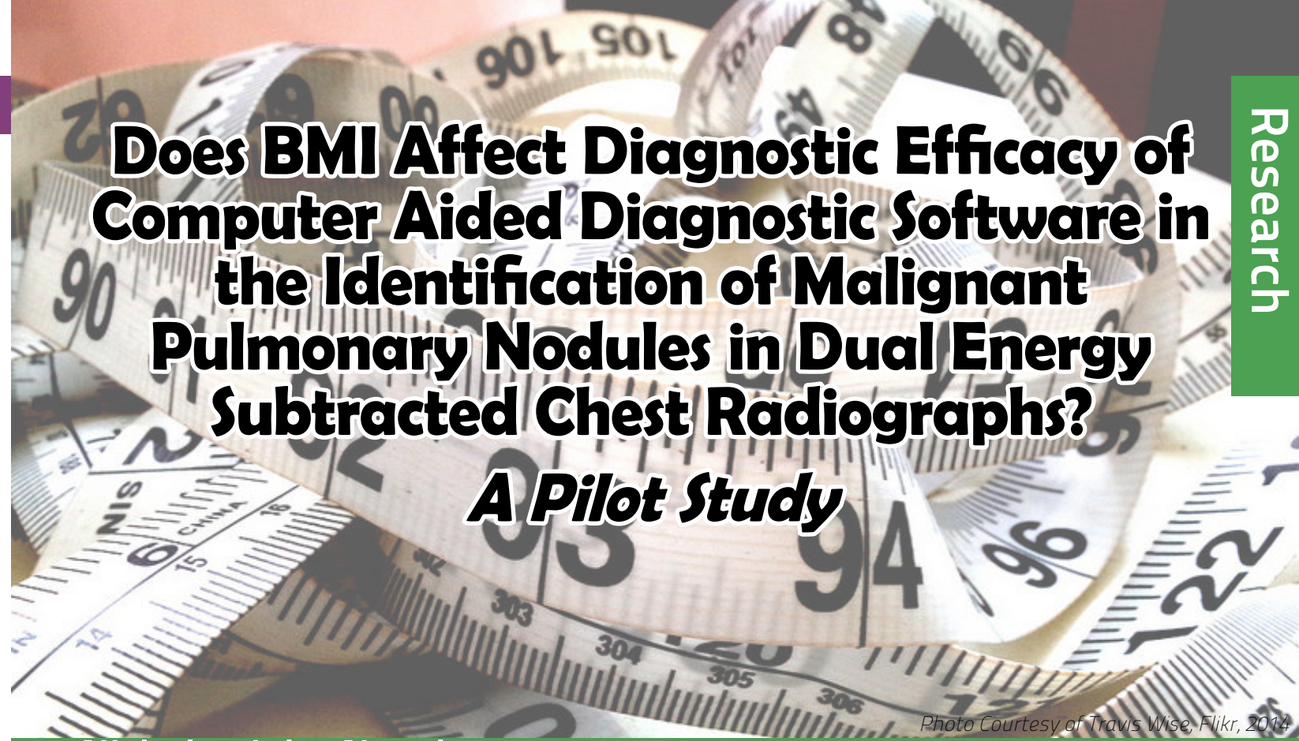
I'm trying to save the world, literally. My work really focuses on adults, so my work doesn't address individuals under 25. What I'm really focused on is trying to reach anyone and everyone to release the power of their dreams. There is something profound about people living lives with a sense of purpose. Now we know that when people feel driven by what you're doing in some way, you feel like it's of noble purpose, bigger than you and your activity, that one of the things that happens is your brain is more open. The second thing that happens is you activate parts of the body that allow your body to rebuild itself physiologically. I'm on the executive committee of the brain-health collaborative, which is in the process of becoming the brain-health institute at the medical school. Thus, we believe that our work will help individuals become more personally sustainable and more effective, and therefore lead better lives.

5. What advice would you offer young, emerging leaders?

Probably the first thing I would suggest a young leader is asking oneself, "What do you want out of life?" And I don't mean "What job do you want?"; it's really, "What kind of person do you want to be?" I think that sometimes, simply having goals can sometimes limit us. When the question is, "What is your dream?" people often think, "What would I like to do and how would I like to be doing it?" When you are 17 or 18, your answer to that question most likely is not fully yours; rather, it may simply be a reflection of what you think your parents or grandparents want for you. But that is what, in my model, is called "ought self." These are the things you feel you ought to do rather than what you dream about doing. You have to discern and work toward what you dream about. I think that is a constant process. You have to constantly ask, "Is this getting me closer to the person I want to be?"

A second major theme is recognizing that we do not go through life alone. You need friends, and you need really good friends. So, one of the things we've been trying to get money for is a program where we start teaching freshmen a set of techniques, including vision, meditation, yoga, and volunteering. As an important part of that process, we hope to have students gather into study groups of five to eight people, where they can learn specific techniques on how to help each other. The technical title is "peer coaching." The concept of finding and nurturing close friendships and task-based groups, regardless of how they come about, is extremely helpful and important to each of us.

> *" You have to discern and work toward what you can dream about. "*



Research

Does BMI Affect Diagnostic Efficacy of Computer Aided Diagnostic Software in the Identification of Malignant Pulmonary Nodules in Dual Energy Subtracted Chest Radiographs?

A Pilot Study

Photo Courtesy of Travis Wise, Flikr, 2014

Nicholas John Novak

ABSTRACT

The increasing level of obesity in the general population of industrialized nations is a major public health concern. While obesity increases morbidity and mortality, increasing body habitus also impacts the utilization and analysis of medical imaging. The purpose of this retrospective pilot study is to compare the diagnostic effectiveness of Computer Aided Diagnosis (CAD) software (OnGuard™ 5.2) in combination with a hardware based bone suppression tool, Dual Energy Subtraction (DES) radiography on thoracic radiographs of patients with varying levels of obesity. Chest radiographs from 30 patients with CT and pathology verified malignant pulmonary nodules (8-34 mm) and 23 CT negative patient controls with different levels of obesity as measured by Body Mass Index (BMI) were utilized for analysis. Twenty-six patients had a normal BMI (18.5-25) and twenty-seven patients were overweight, obese or morbidly obese (OOMO) with a larger BMI value (between 25-47). Test Sensitivity and Specificity, Analysis of Variance (ANOVA), Z-test for Equality of Proportions, Spearman's rank correlation coefficient and the independent sample Student's t-test were calculated. P-values less than 0.05 were considered significant. Age was not significantly different between the two BMI groups ($t=1.26$, $p=0.26$). CAD software Sensitivity =80.0% and Specificity=72.7% in normal BMI patients while for OOMO patients, Sensitivity=83.3% but Specificity was reduced to 44.4%. The difference in Specificity between the Normal and OOMO patients approached significance ($p=0.09$) using the one tailed equality of proportions test ($Z=-1.3$). Similarly, in normal patients, BMI and the number of Regions of Interest (ROI) were nearly significantly correlated ($\varrho=0.374$, $p=0.06$) while there was no significant correlation between BMI and ROI for OOMO patients, ($p>0.5$). There was no difference in Sensitivity between the Normal and OOMO groups; however there was likely a clinically significant difference in Specificity between the two groups, if not a statistically significant difference. Obesity appears to cloud the ability of CAD to identify the absence of malignant pulmonary nodules in OOMO patients. A study with a larger number of patients, particularly obese and morbidly obese patients, may provide a more accurate view of this discrepancy

INTRODUCTION

Chest radiography is one of the most commonly used forms of radiologic examination. One of the goals of chest radiography is the identification of malignant pulmonary nodules. Several authors have suggested that Computer Aided Detection (CAD) may help radiologists to detect cancerous pulmonary nodules (Li, Engelmann, Metz, Doi, &

MacMahon, 2008; Oda et al., 2009). CAD identifies Regions of Interest (ROI) on chest radiographs as areas suspected to be malignant. These ROI are identified as circles imposed on the radiograph. However, high numbers of false positives identified by early versions of CAD software on standard postero-anterior (PA) chest images limited the clinical utility of this technology (Bley et al., 2008; Kasai, Li, Shiraishi, & Doi, 2008; Kobayashi, Xu, MacMahon, Metz, & Doi, 1996). Meziane et al. (2011) indicated that CAD software improved recall rates and diagnostic accuracy, particularly in cases with small pulmonary nodules or inexperienced readers. Other researchers (Monnier-Cholley, Carrat, Cholley, Tubiana, & Arrive, 2004; Shah et al., 2003) determined that a large proportion of the false positives and missed lung cancer cases occurred because of bony structures in the chest, in particular clavicles and rib crossings. The literature on the subject suggests that the use of bone suppression improves the diagnostic accuracy of digital chest radiography. Many authors advocate the suppression of ribs and clavicles in digital chest images to improve malignant nodule detection by (CAD) software.

Currently, several methods exist to suppress bone and other calcified structures in digital chest radiographs. One method is hardware-based, Dual Energy Subtraction (DES) radiography (GE Healthcare). Oda et al. (2009) determined that the use of DES radiography significantly improved the radiologists' diagnostic performance in detecting ROI. Other studies found that hardware based bone suppression (DES) removed the presence of bony structures in digital chest radiographs and significantly improved the sensitivity of CAD, which reduced the false-positive diagnosis rate (Balkman, Mehandru, DuPont, Novak, & Gilkeson, 2010; Li et al., 2011; Szucs-Farkas, Patak, Yuksel-Hatz, Ruder, & Vock, 2010). Unfortunately, body fat attenuation of the x-ray beam used in thoracic radiography causes reduced image contrast and increased image noise that may lead to variance in the diagnostic performance of CAD software. These studies suggest that CAD together with bone-suppressed images improves the diagnostic accuracy of pulmonary nodule detection. Yet, the role of obesity in CAD efficacy has not been investigated despite the fact that the Centers for Disease Control notes that roughly two thirds of all Americans are overweight and over half of them are classified as obese (Buckley et al., 2009).

Obesity is measured by Body Mass Index (BMI) which is defined as mass in kilograms divided by the square of height in meters (kg/m2). Obesity has many serious short and long-term health effects that increase the risk of early morbidity and mortality including hypertension, diabetes, heart disease and cancer. Beyond these risks, obesity also creates difficulty with diagnostic imaging procedures and test interpretation. Several studies have concluded that obesity (BMI > 30) and particularly morbid obesity (BMI > 40) affects not only the quality of medical imaging (Reynolds, 2011; Uppot, 2007) but also the ability to use some imaging methods because patients are inhibited from using equipment designed for individuals of normal weight (Twaij, Sodergren, Pucher, Batrick, & Purkayastha, 2013; Uppot, 2007). Further, extreme obesity has been found to adversely affect the ability to interpret the results of radiological examinations (Larson, Franzblau, Lewin, Goodman, & Antao, 2014; Rajapakse & Chang, 2014). Increased levels of obesity make diagnostic determination of physiological landmarks more difficult (Ambardar et al., 2009), but also make the results of other imaging procedures less accurate (Carboni, Sedati, & De Marco, 2013; Twaij et al., 2013).

The purpose of this study was to compare the diagnostic effectiveness of CAD+DES bone suppression software combination to detect malignant pulmonary nodules when used on patients with differing levels of obesity as measured by BMI. As stated earlier, increased numbers of false positives (FP) and false negatives (FN) reduce the diagnostic efficacy and utility of CAD products in diagnostic radiology. The hypothesis of this study is that increasing levels of obesity alter the diagnostic efficacy and the number of ROI and FP marked by CAD.

METHODS

Institutional review board approval was obtained from University Hospitals Case Medical Center for this project. The approval for informed patient consent was waived and patient records were handled in compliance with Health Insurance Portability Accountability Act (HIPAA) regulations. All patient images and records were maintained on encrypted storage devices to maintain HIPAA compliance.

PATIENT SELECTION

Medical records and images from University Hospitals Case Medical Center were reviewed and sixty patients with either pulmonary nodules confirmed by 16 or 64 slice computed tomography (CT) and pathology verified lung carcinoma were selected. Individuals with single lung nodules 8-34 mm in size were selected as this size range is most likely to be missed by a radiologist. The nodules were located in a variety of locations in the lungs. This sample included 30 patients with CT and pathology proven malignant nodules and 23 patients without malignant nodules as determined by 16 or 64 slice CT. In individuals with malignant nodules, the size and location were measured and marked on standard Posteroanterior (PA) radiograph by an expert radiologist with 24 years of experience.

Patient records were reviewed for height and weight, gender and age. Patients were divided into obesity groups by BMI and classified as: Normal (BMI=18.5-25), Overweight (BMI= 25-30), Obese (BMI=30-40) and Morbidly Obese (BMI=40+). Underweight individuals (BMI < 18.5) were omitted from the analysis. Due to the small sample size, two groups were formed; normal individuals (18.5<BMI≤25) and overweight, obese and morbidly obese (OOMO, (25<BMI≤47)).

SOFT TISSUE IMAGE AND CAD SOFTWARE ANALYSIS

DES radiography , one form of bone suppressed image generation, was performed on a Revolution XRd/ Definium™ digital radiography unit (General Electric Medical Systems). This radiography unit consisted of a 41 x 41 cm2 amorphous silicon based flat panel detector. DES radiography is performed using the acquisition of a low energy 60 kVp PA chest radiograph taken after a 150 ms delay, and a high energy 120 kVp radiograph. Subtracted and bone-enhanced images are also presented after post-processing of the high and low energy radiographs as depicted in Figure 1.

BONE SUPPRESSION AND CAD SOFTWARE

OnGuard™ Computer Aided Diagnostic (CAD) software was used to identify potential ROI that may be identified as malignant nodules. The stand-alone version of CAD software known as OnGuard™ (Riverain Technologies, Miamisburg, Ohio, USA) was utilized for this study as OnGuard, Version 5.2. Unlike previous versions, OnGuard™ Ver. 5.2 incorporated an additional bone suppression algorithm to better identify areas of interest prior to applying the CAD markings to the radiograph. The CAD component of the OnGuard™ CAD system identified ROI by imposing circular markings on the radiographs.

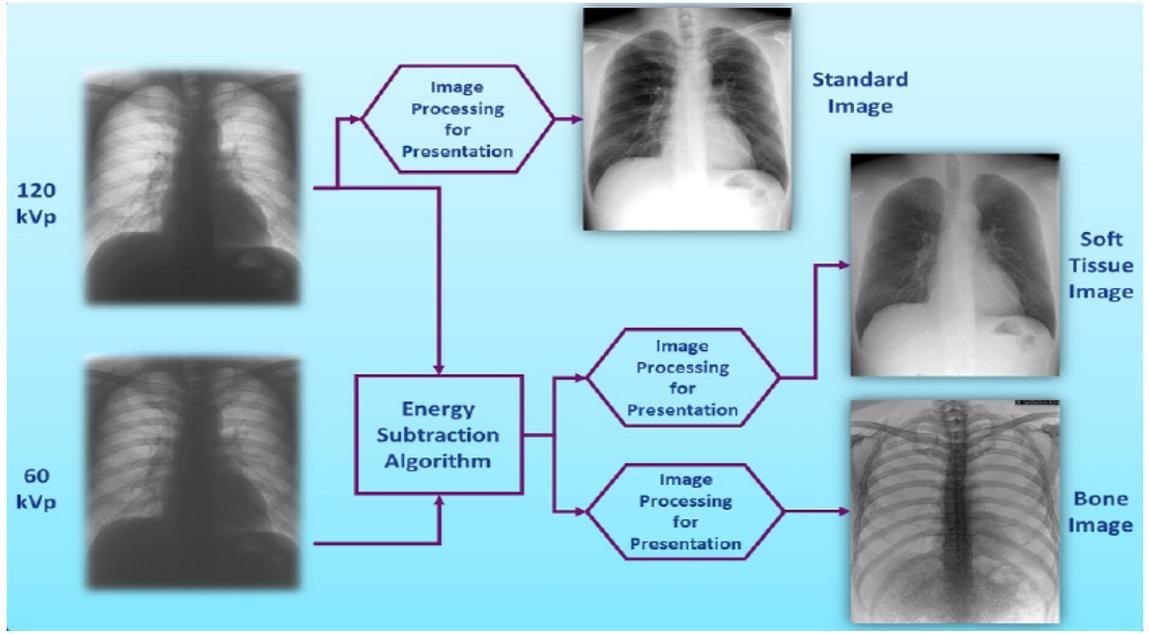


Figure 1: Dual Energy Subtraction Image Production Flowchart

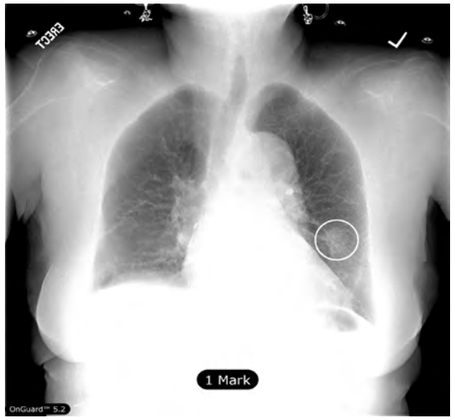


Figure 2: DES + OnGuard 5.2 CAD software Mark

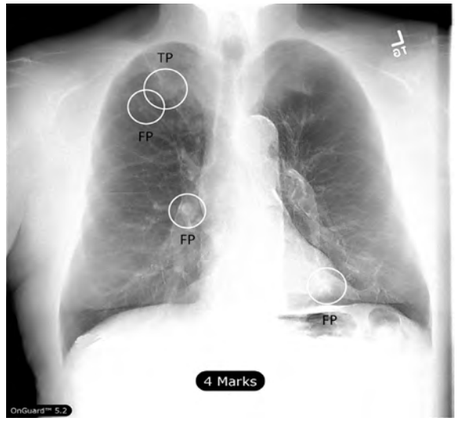


Figure 3: True Positive (TP) and False Positive (FP) CAD Markings

Examples of CAD markings on a bone suppressed DES image is shown in Figure 2. The circular marks were centered about a detection location that signified the identification of a probable malignant nodule. For identification of true positive detection and sensitivity, the known central point of the known nodule location must have been enclosed by the circular marking method of the CAD software and greater than 50% of the radiologist outlined nodule must have been also enclosed by the CAD mark. All other ROIs were determined to be false positives including those generated on CT proven negative cases. Figure 3 depicts true positive and false positive example markings on a bone suppressed DES image.

STATISTICAL ANALYSIS

SPSS Version 21 statistical software (IBM Corp, Armonk, NY) was used for statistical calculations. Correlation between several variables and ROI and FP frequency were evaluated by using the Spearman Rank Correlation test, chosen because of the non-normal distribution of ROI and FP. Test sensitivity and specificity were calculated using standard methods, Analysis of Variance (ANOVA), Chi-Square, the Z-test for Equality of Proportions and the independent sample Student's t-test were calculated to identify statistically significant differences between BMI groups. P-values less than 0.05 were considered significant.

RESULTS

Demographic and radiologic data was obtained on a total of 53 patients. Thirty patients had CT and pathology verified malignant pulmonary nodules and twenty-three were identified as malignant nodule free by CT. Overall, there were 24 females and 29 males. Sixteen females and seventeen males had malignant nodules and eight females and twelve males were in the non-malignant group. This distribution by gender was not significantly different by Chi-Square ($\chi 2=0.1$, $p=0.75$). The number of both ROI and FP did not differ by gender, ($0.24<p<0.27$). Patients with malignant nodules were significantly older than those that were malignancy free (69.1 yr. vs 48.8 yr.; $t=-20.4$, $p<0.001$). However, when divided into Normal ($n=26$) and OOMO ($n=27$) groups, there was no significant difference in age (59.3 yr. vs 63.5 yr.; $t=0.91$, $p=0.37$).

For all patients, the diagnostic efficacy of CAD in the detection of malignant pulmonary nodules was: Sensitivity=81.8%, Specificity=60.0%. For Normal patients: Sensitivity=80.0%, Specificity=72.7%. For OOMO patients: Sensitivity= 83.3% but Specificity was reduced to 44.4%. There was no significant difference between Normal and OOMO patients in the Sensitivity of CAD in detecting malignant nodules (80.0% vs 83.3%; $p=0.94$). The difference in Specificity between the Normal and OOMO patients approached significance ($p=0.09$) using the one tailed equality of proportions test ($Z=-1.3$). Similarly, in normal patients, BMI and the number of ROI were nearly significantly correlated ($\varrho=0.374$, $p=0.06$) while there was no significant correlation between BMI and ROI for OOMO patients, ($p=0.45$). For either the BMI or OOMO group, there was no significant correlation between BMI and the number of false positive markings.

CONCLUSIONS

This pilot study did not find a statistically significant difference in the Specificity of malignant pulmonary nodule detection between the Normal and OOMO BMI groups. However, while there was no statistically significant difference in Sensitivity between the Normal and OOMO groups, a reduction in Specificity from 72.7% in the Normal group to 44.4% in the OOMO group is likely a clinically significant difference. The presence of obesity in a patient appears to obscure the ability of CAD to correctly identify the absence of malignant pulmonary nodules in OOMO patients, possibly increasing the number of false positive markings in this group. While the use of CAD in the detection of malignant pulmonary nodules in digital chest radiographs was designed to supplement the diagnostic capability of thoracic radiologists, decreased diagnostic efficacy in obese patients may limit the utility of this technology. Further, both the statistical tests for differences in proportions for Specificity between the OOMO and Normal BMI groups and significant rank correlation between the ROI and BMI for the normal group approached significance, suggesting that obesity may cause a statistically valid decrease in the diagnostic efficacy of CAD in patients with increased levels of obesity. The results of this study were limited by the small number of patients available during the study timeframe, particularly in the obese and morbidly obese BMI groups. A study with a larger number of patients, particularly in these two BMI groups may provide more insight and a more accurate view of this apparent discrepancy. Since obesity has already been found to affect the ability of radiologists to accurately identify physiological landmarks, interpret test results and utilize specific procedures in diagnostic radiology, the determination of whether or not the diagnostic efficacy of CAD is altered by increased levels of obesity in evaluating digital chest radiographs for malignant pulmonary nodules seems to be a pertinent and timely question that should be investigated further.

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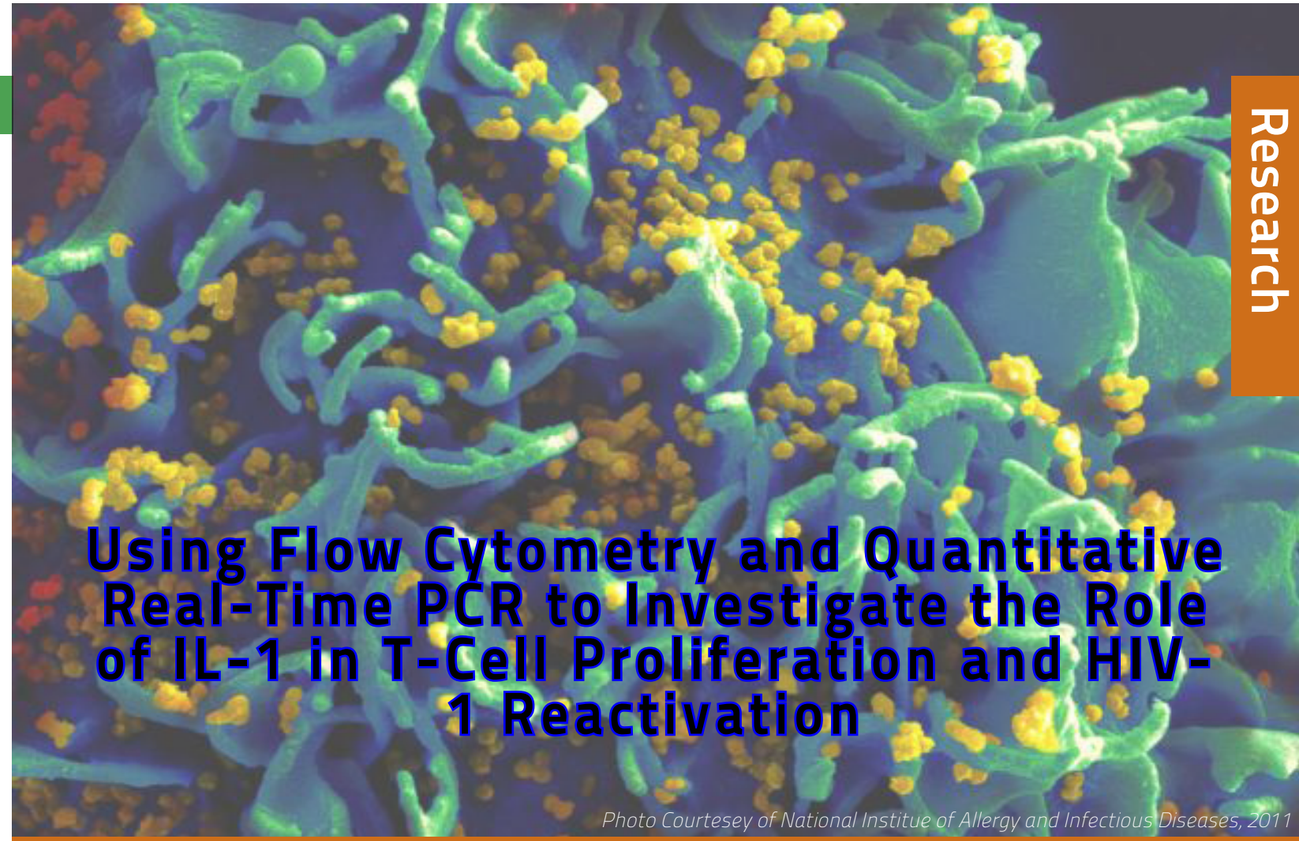

Using Flow Cytometry and Quantitative Real-Time PCR to Investigate the Role of IL-1 in T-Cell Proliferation and HIV-1 Reactivation

Photo Courtesey of National Institue of Allergy and Infectious Diseases, 2011

So Hee Moon

ABSTRACT

With the advent of antiretroviral therapy, suppressing the HIV-1 virus and stopping the progression of the disease are now possible. Even with long-term antiretroviral therapy, HIV reservoirs remain in individuals. These individuals experience an increase in inflammatory cytokines such as IL-6 and IL-1β that results in the proliferation of CD4 T-cells. In this study, we explored the relationship between inflammatory cytokine-induced proliferation and reactivation. To assess this relationship, we investigated the role of a specific inflammatory cytokine, IL-1β. It was concluded from our experiments that CD4 T-cells are able to proliferate in the presence of IL-1β. Although IL-1β may not induce overt reactivation of HIV-1, as shown in the flow cytometry data, further studies need to be conducted to see whether or not IL-1β propagates the reservoir.

INTRODUCTION

The single-stranded RNA virus human immunodeficiency irus-1 (HIV-1) presents a major public health crisis worldwide. Approximately 1.1 million people in the United States currently live with HIV, but only four out of five people realize that they are infected with the virus (Hall et al., 2008). The HIV-1 RNA and the reverse transcriptase enzyme , which synthesizes a complementary DNA strand is from this viral RNA, are contained within the nucleocapsid shell of the virus. This shell is encapsulated within a lipid bilayer that incorporates an integral membrane glycoprotein (gp41) and an associated glycoprotein (gp120). Associated glycoprotein molecules bind to CD4 molecules on the surface of helper T-cells. This interaction allows gp120 to insert its amino-terminal head into the host-cell membrane, causing the viral and T-cell membranes to fuse, which releases the viral core into the cytosol. Ultimately, the viral RNA can become DNA that is integrated into the host chromosome, which may kill the T-cell. The latent reservoir is a pool of cells that contain viral DNA but do not make active viruses (Berg et al., 2010). Therefore, HIV-1 results in decreased levels of the CD4 T-cell population. CD4 T-cells help individuals fight infections. Decrease in CD4 T-cells due to HIV is associated with chronic inflammation and acquired immunodeficiency syndrome (AIDS).

Due to its high mutation rate, the error-prone replication of HIV presents an ever-changing array of coat proteins, making it difficult to develop an effective vaccine. HIV-infected individuals are commonly treated with highly active antiretroviral therapy (ART) that utilizes a combination of several antiretroviral drugs. With ART, individuals typically experience an increase in CD4 T-cell-counts and an improvement in immune function. ART can reduce plasma HIV-1 RNA levels below the detection threshold of clinical assays, which is usually 50 copies/mL (Doyle et al., 2012). Due to this decrease in RNA level, previous studies have predicted that 2.3 to 3.1 years of ART could potentially cure HIV-1 infection (Perelson et al., 1997).

However, recent studies have shown that complete eradication of HIV is impossible even after two to three years of ART. For example, the "Mississippi baby," whose mother was HIV-1 positive, started ART within hours of birth. Although the child had undetectable levels of viral matter after a prolonged withdrawal from treatment and was thought to be cured of HIV, her virus still demonstrated reactivation after treatment was stopped (Stover et al., 2014). Similarly, although viral loads may appear low due to successful ART, patients experience an increase in inflammatory cytokines such as IL-6 and IL-1β. This, in turn, results in the proliferation of CD4 T-cells, a major site of latent virus reservoir (Shive et al., 2014).. When HIV reactivates upon removal of treatment, CD4 T-cells proliferate and produce more viral proteins. Therefore, HIV-1-infected individuals currently require lifelong ART to prevent a small but longstanding HIV latent reservoir from infecting more cells (Bosque et al., 2011).

The aim of this study is to assess whether proliferation induced by 1L-1β is associated with reactivation. 1L-1β, or catabolin, is a cytokine that induces various inflammatory and immune responses. Compared to uninfected subjects, untreated HIV-1-infected patients express increased levels of 1L-1β within all anatomical sites of the lymph nodes, especially in medullary cords, sinuses, and the T-cell zone (Shive et al., 2014). Patients who receive ART also express some level of 1L-1β (Shive et al., 2014). To assess the relationship between 1L-1β-induced proliferation and reactivation, we used flow cytometry and real-time polymerase chain reaction (PCR). Flow cytometry was used to measure the relative percentages of viral reactivation and proliferation, and to distinguish cells that divided after stimulation with 1L-1β from those that did not. A Carboxylfluorescein succinimidyl ester (CFSE) dilution was performed to assess the rate of proliferation of cells stimulated with IL-1β. In addition, real-time PCR was used to quantify viral RNA levels in the supernatant that was released from the cells.

MATERIALS AND METHODS

Ethics Statement

All participants in this study willingly provided written informed consent and fully understood the purpose and methods of this project. Patient studies were covered under the University Hospitals and Case Medical Center Institutional Review Board. Data analyses were conducted using an anonymous database.

Peripheral Blood Mononuclear Cell [PBMC] Purification from Whole Blood

Blood samples from three viremic patients were collected. Patient characteristics are shown in Table 1. Two samples of blood were rinsed with phosphate-buffered saline (PBS)., and the mixture was then added to the blood in 50mL Falcon tube. The mixture of blood and PBS was then slowly overlaid at a 45-degree angle onto a Ficoll-Hypaque solution (GE Healthcare) Once all the mixture was overlaid on the Ficoll-Hypaque, the Falcon tube was spun down in a centrifuge at 1800 rpm (680g) for 30 minutes. After separation on a Ficoll gradient, the middle buffy coat layer was collected. The volume of the cells was diluted with PBS, and then the cells were spun down once again in a centrifuge at 1200 rpm (300g) for 10 minutes. The supernatant was then decanted, and the cells were rewashed with PBS and spun down. 10mL PBS was added for counting by hemocytometer. The cells were then resuspended at the appropriate volume and concentration required for testing.

Table 1. Clinical characteristics of 3 Human Immunodeficiency Virus (HIV)-Infected Subjects Examined in this Study. ART indicates antiretroviral therapy. All three subjects are HIV-infected, viremic patients.

Patient Number	ART Status	Gender	Year Born	Plasma HIV RNA (Copies/mL)	CD4 T-Cell count (Cells/mm^3)
5210	Treated in the past	Male	1976	19691	574
5240	Untreated	Male	1991	11552	637
5224	Untreated	Male	1993	9618	770

Human Serum (HS), Fetal Bovine Serum (FBS) and Cytokine Treatment

After purifying cells by centrifugation over Ficoll, cells were labeled with CFSE dye (Molecular Probes Invitrogen), and quenched afterwards with FBS on ice. The cells were then cultured in a Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 1% penicillin/streptomycin (Gibco), 1% L-glutamine (Gibco), 1% sodium pyruvate (Gibco), and either 10% fetal bovine serum (FBS; Gemini Bio-products) or 10% human serum (HS; Gemini Bio-products). A sample of cells, plated in a well plate, was stimulated with either recombinant human IL-1β (R&D Systems) or phytohaemagglutinin (PHA, Sigma-Aldrich). The cells were incubated for 7 and 12 days at 37°C and 5% CO_2.

Harvest and Staining for Flow Cytometry

After 7 or 12 days, the plate was removed from the incubator. The bottom of each well was scratched to dislodge adherent cells and then placed into a flow tube. The flow tube was spun for 3 minutes, and supernatant was saved for later use (i.e. real-time PCR). After spinning, PBS and Live/Dead-Yellow (Molecular Probes) dye were added into each tube and the tubes were incubated in the dark for 20 minutes at room temperature. T-cell phenotypes were quantified using the following flourochrome-conjugated monoclonal antibodies: CD4-BV421 (Clone: RPA-T4; BD Biosciences), CD8-PerCP-Cy5.5 (Clone: RPA-T8; BD Biosciences), and CD3-allyphycocyanin (APC)-eFluor780 (Clone: SK7; eBioscience). Each sample was stained with antibody staining cocktail for 20 minutes at room temperature in the dark. Afterwards, the cells were spun, washed, and fixed in PBS-paraformaldehyde solution. To detect intracellular p24, the cells were washed and permeabilzed with a saponin-based buffer (BD Biosciences) and incubated with p24-PE antibody (Clone: KC57-RD1, Coulter Clone) for 40 minutes on ice. Cells were acquired on an LSRII flow cytometer (BD Biosciences) using BD FACSDiva software (version 6.2, BD Biosciences), and analyzed using FlowJo software (version 8.8.7, TreeStar).

RNA Isolation

A sample of supernatant from PBMCs stimulated with IL-1β or PHA was mixed with buffer AVL and a carrier RNA mix from RNeasy® Mini Kit (Qiagen). The mixture was incubated for at room temperature, and 100% ethanol was added. The mixture was then placed into a spin column from the mini kit, and spun down at 8000rpm (6800g) for one minute. The flow-through was discarded, and another sample of the mixture was spun down. Then buffer AW1 with ethanol was added, spun down at 8000rpm for one minute, and the flow-through was discarded. Buffer AW2 was added, the column was again spun down at 14000rpm (20800g) for 3 minutes, and the flow-through was discarded. The spin column was placed into an Eppendorf tube, followed by the addition of buffer AVE. The tube was incubated for at room temperature. Finally, the tube was spun down at 8000rpm for one minute and the elution was stored for complementary DNA (cDNA) synthesis.

cDNA Synthesis

cDNA was synthesized from reverse transcriptase with High-Capacity RNA-to-cDNA kit (Applied Biosystems, Grand Island, NY). A small sample of RNA from Qiagen kit RNA Isolation was added to HIV-1 reverse Gag primer (5'GTTCTTGCTATTGTCACTTCC-3'). The mixture was incubated in a thermal cycler. A mixture containing first strand buffer, 0.1M DTT, 10mM dNTPs, and RNase-free water was added. The new mixture was once again incubated in the thermal cycler. Then, a mixture containing Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT) and RNase-free water was added, followed by prolonged incubation in the thermal cycler.

RNA Measurement by Real-Time Quantitative Polymerase Chain Reaction (PCR)

A sample of cDNA was amplified using the StepOnePlus real-time quantitative PCR (Applied Biosystems). The cDNA sample was mixed with TaqMan Fast Universal PCR Master Mix, forward primer (5'-CCAGATCTGAGCCTGGGAGCTCTC-3'), reverse primer (5'-CTGTTCGGGCGCCACTGCAG-3'), and RNase-free water. The mixture was incubated in the thermal cycler.

Pre-amplified products were subjected to a nested real-time PCR. In a new PCR plate, a sample of the mixture from the previous step was added in duplicates to a mixture containing Taqman Fast Universal PCR Master Mix, forward primer(5' - T T A A G C C T C A A T A A A G C T T G C C -3'), reverse primer (5' - G T T C G G G C G C C A C T G C T A G A - 3'), and U5 probe (5' - 6FAM - C C A G A G T C A C A C A A C A G A C G G G C A C A -MGB-NFQ-3'). 6FAM indicates 6-carboxy fluorescein and Q indicates a 6-carboxytetramethylrhodamine group quencher conjugated though a linker arm nucleotide.

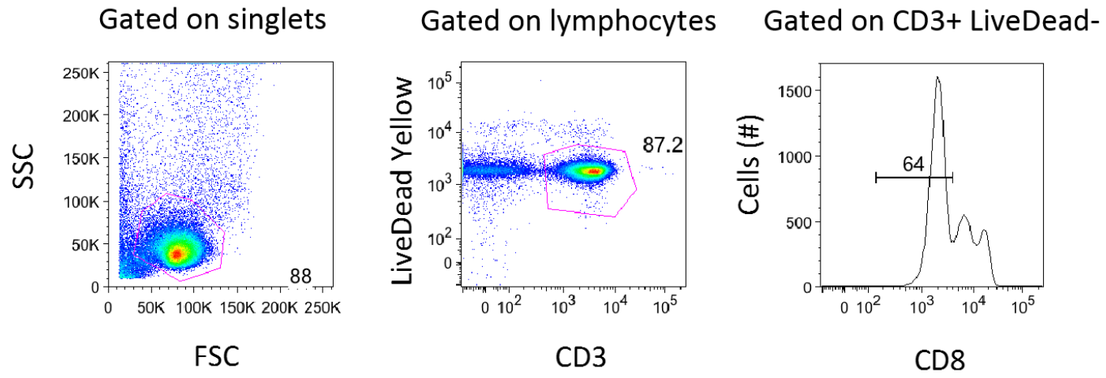

Figure 1. Gating Strategy for Flow Cytometry Analysis. Data for Media in FBS for Patient 5210 (Day 7) was analyzed using FlowJo Version 8.8.7. After gating on singlets, lymphocytes were gated using forward and side scatter. The forward scatter (FSC) measures the alterations of cell size whereas the side scatter (SSC) measures cell granularity. Live CD3+ T-cells were defined on a plot of CD3 vs. Live/Dead Yellow stain (b). CD8 negative T-cells were gated because reactivation causes downregulation of the CD4 molecule.

RESULTS

Gating Strategies for Flow Cytometry Analysis

As shown in Figure 1, cells were first gated for singlets to exclude cell aggregates (forward scatter area vs. forward scatter height, not shown) and then lymphocytes (side scatter vs. forward scatter shown in Figure 1a). The lymphocytes gate was further analyzed for its uptake of the Live/Dead-Yellow viability dye in order to determine live versus dead cells and their expression of CD3 (LiveDead Yellow vs. CD3 shown in Figure 1b). The live, healthy T -cell population (CD3 positive) was gated and dead cells were excluded. T-cells that lacked CD8 expression were used for further analysis (Figure 1).

Comparing HS and FBS

Previous studies suggested that in vitro studies about HIV-1 are usually performed in human cell cultures supplemented with FBS due to efficient proliferation (Perdomo et al., 2012), It was expected that 1L-1β would induce better proliferation in FBS than in HS. It was also expected that this would allow a second level of analysis to determine the relationship between proliferation and reactivation. When PBMCs treated with polyhydroxyalkanoates (PHA) were supplemented with HS, however, more cells reactivated, as shown in Figure 2. In addition, both of the cells that proliferated and those that did not reactivated The data suggests proliferation and reactivation are uncoupled from each other. It also suggests there is an additional factor with human serum that induces reactivation. The assay was not particularly sensitive because there was not a significant release of virus when PBMCs were left in the presence of 1L-1β, as measured by Gag protein expression.

Inflammatory cytokine IL-1β induces CD4 T-Cell proliferation

It was observed that cells stimulated by the inflammatory cytokine 1L-1β proliferated. Three PBMC samples were prepared from two viremic patients who received no ART and one viremic patient who had received ART in the past but was no longer on therapy (Table 1). Viremic patients were used for this study since the measurement of reactivating virus was expected to be more feasible givent their high viral loads. To track cell division, the cells were labeled with carboxyfluorescein succinimidyl ester (CFSE) dye and stimulated with 1L-1β or PHA for 7 days. CFSE dye allows for a direct measurement of cells undergoing division, since each cellular division dilutes the dye. As shown in Figure 2, 1L-1β induced cell division in both FBS and in HS. PHA induced the highest level of cell division, and media alone induced the lowest level of cell division. In all three cases, cells divided at a higher rate when they were subjected to FBS than HS.

Inflammatory cytokine IL-1β Downregulates CD4 T-Cells

As shown in Figure 1c, CD8-negative T-cells were gated because when cells are reactivated and express Gag protein, expressions of the CD4 molecules on the surface undergo downregulation. The expression of Gag protein was measured by its particular epitope, p24Gag. Examination of the cells that were p24Gag positive and CD4 negative, allowed for the conclusion that the virus was reactivated.

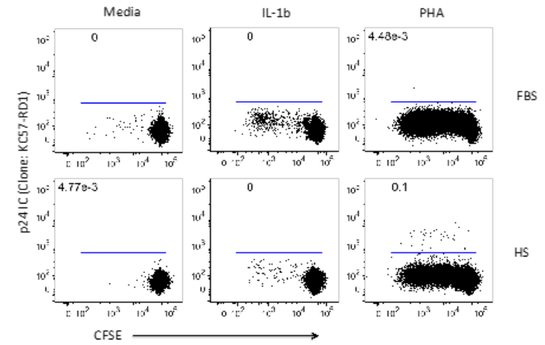

Figure 2. IL-1β Induces Cell Proliferation. Peripheral blood mononuclear cells (PBMCs) from representative patient 5210 (Viral load: 19691 copies/mL) were (1) left in the absence of cytokines (media) or in the presence of IL-1β or PHA, and (2) cultured in a medium supplemented with FBS or HS for 7 days. The cells were stained with 5,6-carboxyfluorescein-diacetate succinimidyl ester (CFSE) dye to track cell divisions. The cells were then assessed for intracellular p24Gag expression by flow cytometry. Numbers indicate percentages of the CD8-negative CD3-positive T-cell population.

IL-1β Does Not Cause Overt Reactivation of the Viral Reservoir

PBMCs were incubated for 7 days in the presence of IL-1β or PHA and the proliferated cells were examined for latent virus by assessing intracellular p24Gag by flow cytometry. Positive p24Gag indicates the presence of live virus that can make viral proteins. Each panel in Figure 2 and 3 shows the percentage of p24Gag-positive T-cells from the parent population. In representative patient 5210 (viral load: 19691 copies/mL), IL-1β caused proliferation without robust viral reactivation. In both FBS and HS treatment, zero percent of cells of the parent population were p24Gag-positive when stimulated with IL-1β. In media, zero percent of cells reactivated in FBS and 0.00477 percent of cells were reactivated in HS. PHA, a positive control, induced T-cell activation in a robust manner. When HS was present, 0.00448 percent of cells were reactivated, as shown in Figure 2, and 0.00672 percent of cells were reactivated, as shown in Figure 3. PHA supplemented with HS induced the highest level of reactivation. Under this condition, 0.1 percent of the parent population reactivated (Figure 2) and 0.092 percent demonstrated reactivation (Figure 3). This was due to the low number of infected cells.

Real-time PCR was used to quantify the amount of viral RNA that would be released in the culture supernatants. Representative standard curves and sample data are shown in Figures 4a and 4b, respectively. When the target sequence was amplified, IL-1β led to a very low but detectable level

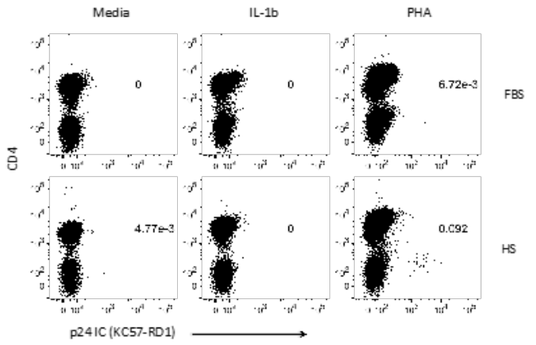

Figure 3. Reactivation causes downregulation of CD4. In representative patient 5210 (Viral load: 19691 copies/mL), virus in CD4 positive T-cells caused downregulation when it was reactivated. IL-1β did not induce reactivation when supplemented with FBS or HS.

of viral RNA in the supernatants. Although IL-1β did not induce viral reactivation as measured by flow cytometry, viral RNA levels increased in Real-time PCR to about five-fold in the presence of FBS and eight-fold in the presence of HS (Figure 4c). The results indicate that IL-1β can potentially lead to viral expression from latently infected cells, even in the absence of detectable Gag expression. As expected, PHA in the presence of HS resulted in the greatest amount of viral RNA.

Interestingly, when viral RNA levels were measured in copies/mL, a substantial amount of viral RNA was detected even in the negative control (Figure 4c). The presence of the viral RNA could be due to the amplification steps in which a small amount of RNA was amplified during the real-time PCR, or because viremic samples were used in this study. Samples from HIV-positive individuals who are not viremic may have lower baseline HIV RNA levels. In addition, the number of RNA copies was generally lower after 12 days of treatment, as shown in Figure 4c. Other cells may have stopped the virus, cells themselves may have died so that fewer cells were replicating the virus, or cells that reactivated may have become transient.

DISCUSSION

In this study, flow cytometry and quantitative real-time PCR were used to address how inflammatory cytokine IL-1β is associated with HIV latency. Measuring CFSE dilution by flow cytometry allowed direct measurement of cell proliferation stimulated with IL-1β. As the two acetate groups in CFSE fluorescence allowed the dye to readily cross the plasma membrane of the cell during division,

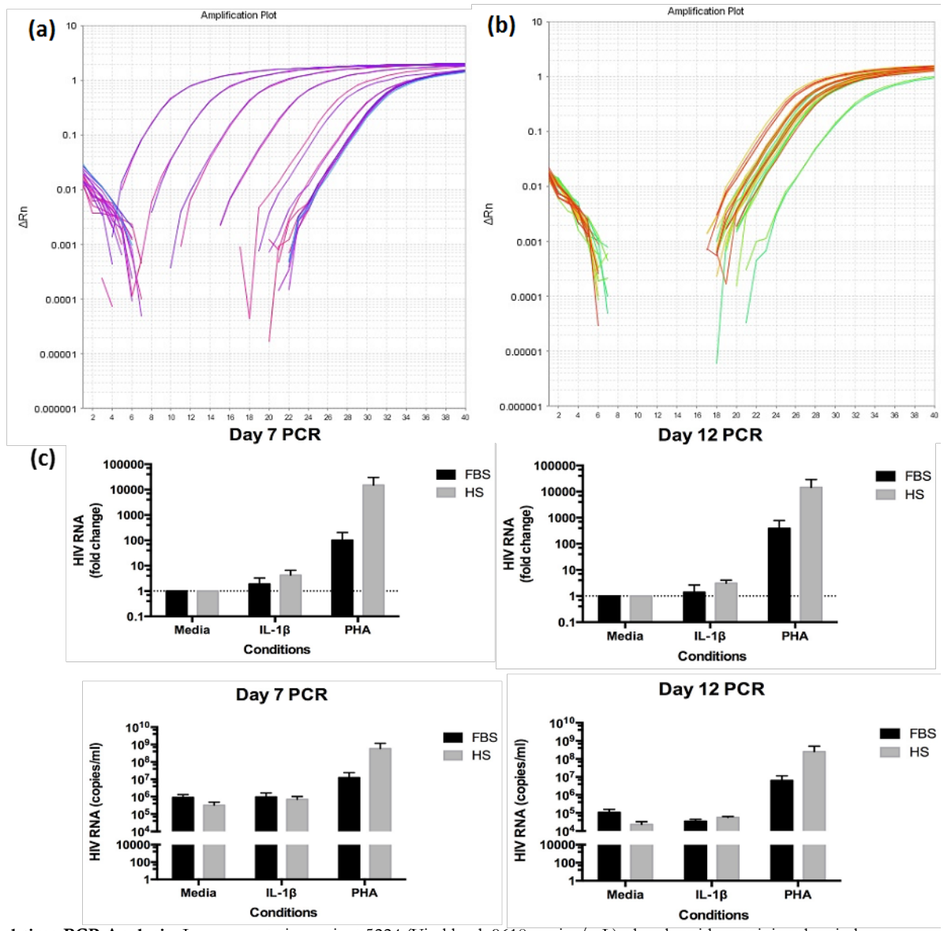

Figure 4. Real-time PCR Analysis. In representative patient 5224 (Viral load: 9618 copies/mL), the plasmid containing the viral sequence was amplified and analyzed by replicates using a real-time PCR. (a) Standard curves, made from plasmid containing the U5 sequence diluted in TE buffer and yeast RNA, were used to determine the quantity of RNA levels. (b) The graphs obtained from PCR made quantification of the level of residual viral matter possible. (c) After 7 and 12 days of treatment, IL-1β led to a low but detectable level of viral RNA. The viral RNA levels were shown as fold change (top) and actual copies per mL (bottom).

the succinimidyl group of CFSE retains the dye within the cell by covalently attaching to cellular proteins. Since CFSE fluorescence divided in half after each cell division, the intensity of fluorescence in the remaining cells indicated the extent of cell proliferation (Berg et al., 2010). It was observed that IL-1β induces cell proliferation (Figure 2 and Figure 3).

In addition, real-time PCR was used to detect viral RNA by amplifying the target sequence generated through denaturation, annealing, and extension. Since RNA is single-stranded, a complementary DNA strand is synthetized from the viral RNA using a reverse transcriptase. In the first step, the reaction temperature is raised to denature the DNA. When heat is applied, the hydrogen bonds within the DNA break to form two single strands. The temperature is lowered during the annealing stage. Specific primers and polymerases are attached to the sequences at each end of the target DNA. In the extension stage, the intervening DNA is synthesized by polymerase reaction in opposite directions in order to build a complementary strand.

PCR allows the virus to be detected in a small target sequence. A single-stranded DNA probe, which contains a fluorescent molecule and a quencher, is hybridized to the part of the DNA sequence synthesized between the two primers. By absorbing light energy emitted by the fluorescent molecule, the quencher allows the fluorescent molecule to emit detectable light when the fluorescent molecule is released from its neighboring quencher (Laird et al., 2013). Therefore, in each PCR cycle, the amount of emitted light doubles as the fluorescent molecule is released from the quencher. The amount of viral RNA is then determined by a reference to the rounds of PCR in which the amount of fluorescence first crosses the threshold of detection, calculated from the standard curves shown in Figure 4a.

The flow cytometry data indicates that IL-1β induces little viral reactivation, although it induces proliferation. However, in real-time PCR, viral RNA levels increased in the presence of IL-1β when the sequences were amplified. From these observations, it can be deduced that only a few T-cells produce virus make up a lot of virus to maintain the latent CD4 T-cell reservoir. Although IL-1β does not induce overt reactivation of HIV as shown in this study, this does not mean that IL-1β may not contribute to the maintenance of the reservoir. Over time, few cells may produce the virus after IL-1β treatment in the population that proliferates, or the population that does not. Therefore, further studies need to be conducted to assess the direct mechanism that addresses how the latent viral reservoir is maintained over time in HIV-1 infected patients.

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Research

Sodium Absorption in Summer and Winter Acclimated Freshwater Teleosts

Photo Courtesy of Francis Chung, Flickr, 2010

Katrina Thede

ABSTRACT

To maintain sodium (Na+) homeostasis in a hypotonic environment, freshwater teleosts must constantly absorb Na+ through their gills. Teleosts in temperate climates have the extra challenge of living in an environment in which ambient temperatures range from 0-30°C. It was hypothesized that 1. Na+ absorption through the gills occurs via a protein homologous to mammalian Na+/H+ exchanger (NHE), 2. Na+ is exchanged for NH4+—the nitrogenous waste product of the fish—rather than H+, and 3. gill proteins from 5°C winter-acclimated fish (WA) would be more active than those from 20°C Summer-acclimated fish (SA). To test this, the genome and transcriptome of SA fathead minnows (Pimephales promelas) were sequenced and assembled. RNA and DNA from SA were isolated using the Qiagen RNeasy Plus Mini-Kit, and the QIAamp DNA Mini-Kit, respectively. The tests found SAF expressed proteins homologous to four mammalian salt transport proteins: NHE, Na+/Cl- co-transporter (NCC), acid-sensing ion channels, and NH3 transporters (Rh factors). Transcriptomes of SA and WA are now being sequenced the activity of Na + transport proteins in the gills measured. Preliminary data showed that Na+ flux into gill vesicles saturated with a K1/2 of 7.5 mM indicating a carrier-mediated process. Subsequent tests will measure the activities of NHE, NCC, and acid-sensing ion channels in SA and WA under various ionic conditions.

INTRODUCTION

There are many models for how freshwater (FW) teleosts maintain Na+ homeostasis in hypotonic environments. August Krogh was the first to propose a model for Na uptake. He discovered that Na+ and Cl- absorption were independent leading him to propose that Na+ was exchanged for NH4+ and Cl- for HCO3- (Krogh, 1939). Later tests by Avella and Bornancin (1989) showed that Na+ was more likely exchanged for protons than ammonia.

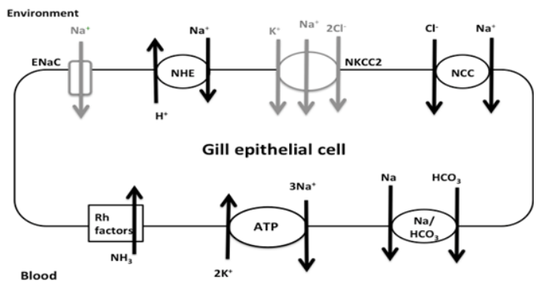

Figure 1. Na+ transporters of interest in warm acclimated fish. Transport proteins in black correspond to transcripts found in the gill transcriptome: NHE, NCC, Rh factors, NKA, and Na+/HCO3- co-transporter. ENaC and NKCC2 are in grey since they were not found in the transcriptome.

Recent research has shown there are three major models for Na+ uptake in FW fish: electrically coupled exchange using the epithelial Na+ channel (ENaC) linked to an H+-ATPase, uptake via Na+/H+ exchanger (NHE), and Na+ and Cl- co-transport (NCC) (Kumai & Perry, 2012). However, these models all have certain aspects that make them incomplete. For example, while the ENaC model works thermodynamically, there has been no evidence of ENaC, or its homologues, in sequenced FW fish genomes (Dymowska et. al, 2012). Genomic studies of the rainbow trout have shown the presence of NHE, but since it is driven by the concentration gradients of Na+ and H+, it should not be able to function in FW environments since extracellular Na+ is lower than intracellular Na+(Parks et al. 2008). One suggested way of overcoming the thermodynamics of NHE is through the use of ammonia transporters (Rh factors). Natawa and associates (2007) have proposed that Rh factor proteins transport NH3 out of the cell where it combines with H+ to form NH4+, causing an increase in local pH outside the cell making it favorable for Na+ transport via NHE. This model depends heavily on the creation of a microenvironment that is unstirred, but given the anatomy of the gills, and the high flow rate of water across the filaments, this unstirred layer is unlikely to exist. The last model, NCC, has only recently been identified in FW teleosts (Hiroi et al. 2008), but Na+ absorption via this transporter is also thermodynamically unfavorable given the extracellular and intracellular concentrations of Na+ and Cl- (Evans, 2011). It was proposed that Na+ absorption occurs by Krogh's original model in which extracellular Na+ is exchanged for intracellular NH4+ and that NHE is the protein that facilitates this exchange.

In addition Na+ absorption, FW teleosts in temperate climates must be able to maintain enzyme function from 0-30°C. Since enzyme function falls with decreasing temperatures, such a large range poses a challenge for epithelial transport proteins maintaining Na+ homeostasis. Research by Packer & Garvin (1998) has shown that Na+/K+ ATPase (NKA) activity is higher in cold acclimated teleosts compared to warm acclimated teleosts when assayed at the same temperature. NKA moves Na+ from the intracellular milieu to the blood, thus generating the concentration gradient needed for Na+ entry. Possible causes for the change in activity include changes in protein expression, membrane lipids, or transport protein subunits.

The Rosy Red fathead minnow (Pimephales promelas) was chosen for this study because of its use in ecotoxicology, commercial importance, and ability to tolerate environmental conditions under various temperatures, pH, and alkalinity. A member of the Cyprinidae family, the fathead minnow is broadly distributed across North America and is the model organism for aquatic toxicology studies and the Rosy Red strain is sold in pet stores as aquarium fish (Ankley & Villeneuve, 2006).

It was hypothesized that 1. Na absorption through the gills occurs via a protein homologous to mammalian Na/H exchanger (NHE), 2. Na is exchanged for NH4, the nitrogenous waste product of the fish, rather than H+, and 3. Gill proteins from 5°C winter-acclimated fish will have higher activity than those from 20°C summer-acclimated fish (WA). This hypothesis was tested by sequencing the transcriptome of a SA Rosy Reds and measuring the activity of transporters potentially involved in Na+ re-absorption using fluorescence tagging.

MATERIALS AND METHODS

RNA Preparation

Rosy Red Fathead minnows were acclimated to 22ºC prior to tissue extraction. Fish were injected with heparin then anesthetized in MS-222 Tricane solution and dissected on ice. The opercula were removed and the ventral abdomen opened from anal fins to gills. Tissue and bone were removed to expose the ventricle. A 30G needle was inserted into the conus arteriosus, then pushed into aorta and tied in with 8-0 suture to prevent back flow, and the gills were flushed with 6mL of physiological saline and heparin. Gill filament RNA was extracted using the Qiagen Rneasy® Plus Mini Kit by manufacturer's protocol (Cat. No. 74134, Qiagen, Hilden, Germany). Two samples were made, one from the right gill basket and the other from the left gill basket. RNA content and quality was determined using a Nanodrop Spectrophotometer, RNA samples were kept at -80ºC until sequenced.

RNA Sequencing, assembly, and annotation

RNA sequencing and library preparation was performed by the Case Western Reserve University Genome Sequencing Core using an Illumina HiSeq 2500 (Illumina, San Diego, CA, USA). Samples of 14.0 µg and 7.6 µg of RNA were used to construct two cDNA libraries using an Illumina TruSeq Stranded mRNA Sample Prep Kit (Illumina, San Diego, CA). Sequencing was done in two lanes, one for each

sample, producing 101 bp paired reads for each sample. Raw reads were stored as FastQ files.

George Washington University's Colonial One High Performance Computing Initiative was used to assemble and annotate the transcriptome. For quality control, the first and final thirteen base pairs of each scan were removed using the Fasts Toolkit (Hansen, et al. 2010). Both samples were then combined and de novo assembly performed with The Broad Institute's Trinity software package using default settings (Grabherr et al. and Haas et al.). The resulting transcripts were passed to the Broad Institute's TransDecoder software and transcribed into protein sequences.

The majority of functional gene annotation was done through the Broad Institute's Trinotate Software package and protocol. Other software, including WEGO gene ontology and Kyoto Encyclopedia of Genes and Genomes (KEGG), was used to create a more complete annotation. All references to default parameters refer to the parameters given in the Trinotate protocol, or when none are specified the defaults of the software itself. Statistical analysis of the output files was done using scripts provided by the Trinity software package.

Membrane Vesicle Preparation

Vesicle preparation was adapted from Flik and associates (1997). All vesicle preparation was performed at 4°C. After anesthetization in MS-222, the gills from two WA were homogenized in 1 mL of homogenizing solution which contained 50mM Sucrose, 10mM Tris/Hepes buffer 1:1, 1mM dithiothreitol, 1 mM ethylenediaminetetraacetic acid (EDTA). The resulting solution was centrifuged at 5,000xG for 15 minutes at 4°C. The supernatant was then spun at 50,000xG for 60 minutes. The pellet was resuspend pellet in 1mL sucrose solution which contained 300mM Sucrose, 10mM Tris/Hepes buffer 1:1, 1 mM EDTA and split into two 500uL samples. In one sample Na Green, a Na+-sensitive fluorescent dye, was added to make a 1µM final concentration and the other sample received the same volume of sucrose solution. Both were run through a 26.5G needle 20 times to allow the new solutions to be taken up by the vesicles. The solution was then spun at 50,000xG for 60 minutes to collect the vesicles. The pellets were finally suspended in 1.2 mL sucrose solution.

Table 1. The table below lists the wavelength settings on the fluorometer.

Excitation Wavelength	500nm
Emission Wavelength	530nm
Excitation Slit	5nm
Emission Slit	10nm
PMT Voltage	700V

Table 2. Summary of transcriptome analysis. Over 42,000 unique genes were found, and the high mean length indicates a transcriptome of high quality.

Total Reads	470,812,874
Length of Cleaned Reads	87 bp
Total Length of Assembly	40.9 Gbp
Number of Transcripts	152 Mbp
Mean Length	153,118
Transcripts Annotated	1,130
Unique Genes Represented	42,107

Fluorescence

Each sample was placed into a 3 mL cuvette with stirring. Over the course of 60 seconds, volumes of 150 mM NaCl were added corresponding to final Na+ concentrations of 2, 4, 6, 8, 16, and 32 mM and the change in fluorescence measured.

RESULTS & DISCUSSION

The results of sequencing the gill RNA of P. promelas using an Illumina HiSeq 2500 resulted in high quality sequencing and assembly, as determined by the large mean length of each transcript (Table 2).

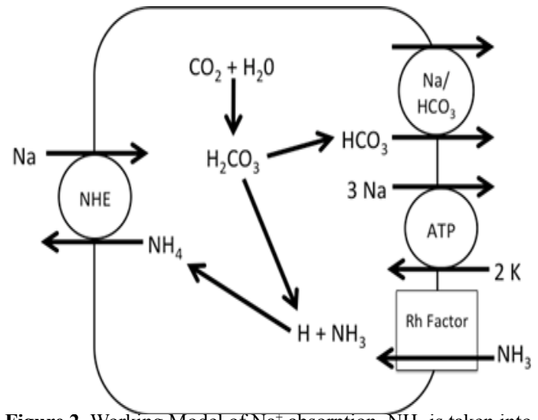

Figure 2. Working Model of Na^+ absorption. NH_3 is taken into the cell via Rh factor proteins where it combines with H^+ from carbonic acid in the cell making NH_4^+. The NH_4^+ is used to in place of H^+ in NHE allowing Na^+ uptake into the cell.

While the entire transcriptome was annotated, transporters related to Na+ uptake were detected: namely, transcripts corresponding to NHE, NCC, Na/HCO3 and Na/K ATPase (NKA). No transcripts related to ENaC or the Na/K/Cl cotransporter (NKCC) were detected. Initial query showed 14 different Rh factor proteins present in the transcriptome though due to false positives this number may be lower (Figure 1).

From the transporters found in the transcriptome, a working model was developed for sodium entry. In this model, NH3 is taken into the cell via Rh factor proteins where it

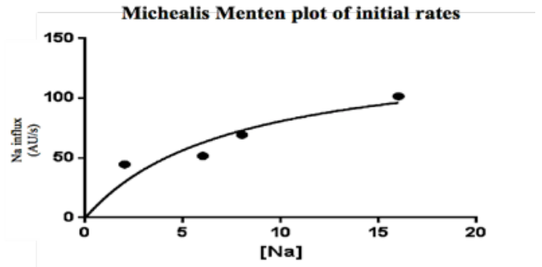

Figure 3. Calculated rate of Na influx as a function of extravesicular Na (in mM) in warm acclimated fish. The V_{max}=141 and the $K_{1/2}$= 7.5.

combines with H+ from carbonic acid in the cell making NH4+. The NH4+ is used in place of H+ in NHE thus allowing Na+ uptake into the cell. Na+ is then transported into the blood via NKA and the Enzyme activity of gill membrane vesicles containing the sodium-sensitive fluorescent dye, Sodium Green, was measured. Using the initial rates from each concentration, a Michealis-Menten plot was created where Vmax=141 and K1/2 = 7.5. These indicate that rises in fluorescence were due to carrier-mediated transport of Na+ into the vesicles (Figure 3).

These data suggest the existence of Na+/H+ exchanger, Na+/HCO3-, Na+/Cl- co-transport, Na+/K+ ATPase, and NH3 transporters as these were identified in the gill transcriptomes but neither the ENaC nor NKCC2 were present in the transcriptomes. Regardless, Na+ influx kinetics remained consistent with NHE as a mechanism of entry. With these data, it is possible to construct a novel working model for how Na+ is absorbed through the gill epithelium. Future studies should focus on differential expression between SA and WA gill transcriptomes to identify differences at the mRNA level. These differences can then be linked to changes in function using fluorescent tagging.

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Case Study

Interviews of College Students with Siblings who have Genetic Diseases

Photo Courtesy of eflon, Flickr, 2009

Melanie DeArdo

ABSTRACT

Purpose

There are more than 6,000 different genetic diseases manifested in 1/200 live births. These children are often part of a family with siblings. The purpose of the study was to learn through interviews the lived experience and needs among college students of siblings with genetic disease.

The aims of the study: 1) explore the lived experience of the college-aged student in a family living with a genetic disease process, 2) learn how universities may support these students, and 3) identify nursing interventions that would benefit these siblings. A literature review shows limited research has been done examining siblings of children with genetic diseases, especially college-aged siblings.

Study Design and Methods

This is a cross-sectional qualitative pilot study with a phenomenological perspective. Developmental theories of the college-aged student represent the conceptual framework. Students were recruited through university e-mail, and a single, one-hour interview was held on campus. Taped dialogue was transcribed and the "long table approach" for data analysis followed to identify reoccurring themes.

Results

Three female, Caucasian students participated. Three themes were identified: defining a new normal, caregiver role strain, and experiencing a crippling social life. Participants made suggestions for supportive nursing and university interventions.

Clinical Implications

It is acknowledged that sample size was low and results may be anecdotal. However, there were repeating comments and themes identified. Universities are in a position to nurture these students towards adulthood. Pediatric nurses need to recognize the needs of siblings of patients and deliver family-based holistic care. Future research calls for a larger, more diverse sample.

INTRODUCTION

It has been noted that there are more than 6,000 different genetic diseases, manifested in 1/200 live births (Stoppler, 2014). Birth defects often associated with genetic disease affect one in every 33 infants born in the United States every year (Centers for Disease Control and Prevention, 2014). A child with genetic disease often requires many planned and unplanned hospitalizations, and the family unit along with that sibling may experience unexpected changes in routines, priorities, and increased demands on parental resources.

The purpose of this qualitative study was to learn the lived experience and needs among college students with siblings who have a genetic disease such as Cerebral Palsy, Muscular Dystrophy, Sickle Cell Anemia, Cystic Fibrosis, Down syndrome, Trisomy 13, Schizophrenia and Bipolar Disorder that requires multiple hospitalizations. The aims of the study were to: 1) explore the lived experience of the college-aged student in a family unit that is living with a genetic disease process, 2) learn how the university may support these students, and 3) identify nursing interventions that would benefit all-aged siblings of patients with a genetic disease.

REVIEW OF THE RECENT LITERATURE

Orsmond and Seltzer (2009) have reported that examination of the status and needs of those who have siblings with a genetic disease has been limited. Those published largely focus on younger sibling children up to 18 years of age. There are numerous gaps and discrepancies in quality that currently plague sibling research, attributed to inconsistencies in methodology and lack of rigorous sampling (Macks & Reeve, 2007). Often seen in sibling research are survey instruments utilized to assess psychological functioning, family functioning, depression, family burden, child behavior, and quality of life (Gold, et al., 2008; Read, et al., 2008; Read, et al., 2011). Researchers also examined the healthy child's knowledge of their siblings' disease, such as sickle-cell disease, and findings indicated that such knowledge may be a critical factor related to their behavior (Gold, Treadwell, Weissman, & Vichinsky, 2011; Lobato & Kao, 2002).

Clinicians are not adequately aware that a healthy sibling in a family that has a child with a genetic disease may be at a greater risk for negative psychological outcomes, an effect that has been more recently addressed in the literature (Gaur, et al., 2008; Gold, et al., 2011). Health care providers should be encouraged to examine healthy siblings' quality of life as many experience hidden stress with the daily demands of the illness on themselves, their parents and their families (Havermans, et al., n.d., ahead of print).

Qualitative studies have also been conducted exploring feelings of loneliness, social support and psychosocial adjustment (Baumann, Dyches, & Braddick, 2005; Graff, et al., 2012; Kaminsky & Dewey, 2002; Rampton, et al., 2007). In a grade school focus group, feelings of embarrassment were shared. One subject stated, "…she bugs me so much. Sometimes I wish she wasn't even here," (Baumann, Dyches, & Braddick, 2005, p. 55). Another study reported similar themes of stress over the increasing role being the caregiver, feelings of guilt for being healthy, lack of necessitated parental attention, and withdrawal from social activities (Read, et al., 2011). This finding confirmed both studies by Orsmond & Seltzer, 2007 & 2009 where siblings "feel obligated under a sense of precocious responsibility for protecting the individual with (autism spectrum disorder and helping their parents, and relationships are negatively affected" (Orsmand, & Stelzer, 2009, p. 3). Not only have relationships been negatively affected, but it has also been found in one study that most siblings find their role in their family as the supporter of the ill sibling, as well as of their parents (Sin, et al., 2013).

In Australia, families living with a member who has a genetic metabolic disorders have expressed a need for support as the psychosocial and emotional impact are significant for both patients and families (Anderson, Elliott, & Zurynski, 2013). Findings also noted that families often experienced a lack of appropriate peer and community support services. In the survey, 70% of families indicated that they had become closer to their family because of their shared experiences, and 57% of them were interested in a peer or support group, but less than half could find one that pertained to them.

Few articles went beyond childhood into early adulthood and included 18-21 year olds in the sample and only one study focused on the parent/well-sibling relationship and the well- siblings' coping and stress (Graf, et al., 2010). One qualitative study interviewed 21 adolescent siblings of children with Down syndrome who needed extensive medical care and participants shared both positive and negative aspects of the experience (Graffe, et al., 2012). Skotko, Levine, and Goldstein (2011) found that 88% of older siblings of those with Down syndrome said they felt that they were better people because of their siblings with Down syndrome and that they planned to remain involved in their sibling's lives. In another study with young adults of siblings with cystic fibrosis, the healthy sibling felt that they were diplomatic, responsible, mature, important and loyal (Wennström et al., 2011). However, in this same study, participants remembered being angry, envious, and neglected when they were younger as parents had time constraints and they felt the burden of helping. No literature could be found that focused on college-aged students who have a sibling with a genetic disease.

METHODS

This is a cross-sectional qualitative pilot study with a phenomenological perspective. Phenomenology is a method of inquiry based on the premise that reality is what a person perceives or understands (Burns & Grove, 2009). Phenomenology is about the lived experience.
The conceptual framework included the developmental theories of Erikson and Sullivan. Erikson (1993) speaks of stages that need to be resolved in each age group to move successfully toward adulthood. The university student straddles two stages. Erik Erikson theorized that the university student would be leaving the stage of identity versus role confusion – where there is a questioning of self and where the student is going in life – and entering the stage of intimacy versus isolation – the first stage of adult development – where friendships and dating are important. Sullivan's Interpersonal Theory includes dynamisms of self-system that protects one from anxiety and maintains interpersonal security (Stern & Marchesani, 2004). For the college-aged student, it is the stage of forming lasting, intimate relationships that are crucial for the university student to develop. Relationships have the power to transform an immature preadolescent into a psychologically healthy adult individual (Evans, 1996).

Sample and setting

The setting was a university campus with an enrollment of approximately 3,700 students. The University is a liberal arts college that draws on the Lutheran principle of free inquiry. Students of the University were the target sample. Inclusion criteria included: age 18 to 25 years old, a University student having a living sibling with a genetic disease, they must have lived with that sibling for a least five years, been able to participate in a focus group conducted in English, and signed a consent form. Genetic diseases of siblings include but are not limited to: Tay Sach's disease, Sickle Cell Anemia, Cystic Fibrosis, Hemophilia, Down syndrome, and Schizophrenia or bipolar disorders and all genetic diseases or conditions that required multiple hospitalizations.

Procedure

This study was approved in expedited review by the university Institutional Review Board. Recruitment flyers approved by the Student and Community Engagement Office were posted on bulletin boards in academic buildings and resident halls recruiting candidates for the study. E-mails were also sent out to students and faculty with the flyer attached. Interested students were asked to contact an electronic mail account that was created exclusively for the study's purpose. In response to the e-mail inquiry of interest, a calendar was sent to establish a mutually convenient time for the interviews. Training was held prior to in conduct of the study. Initially, the study was designed to conduct a focus group, but limited student responses lead to the need for modification to conduct group and individual interviews.

Upon entrance into the study room the research team greeted the students. The study was explained and the potential participants were given time to ask questions. The consent form, the university Human Dignity Policy (that addresses respect for others and intolerance for bullying and harassment), and demographic form (student age, gender, race, ethnicity, birth order, and sibling's gender, age, diagnosis, number of hospitalizations, medications or therapies needed by the sibling, number of children in the family, and marital status of the parents) was e-mailed out in advance so that student participants had time to carefully read it over. The prospective study participants were given an opportunity to sign a consent form that acknowledges their voluntary participation and notes that the focus group will be audio-recorded. All identities were protected by pseudo-names taken by the study participants and the wearing of pseudo-name badges. Tent place cards with the fake name were made and placed in front of the participant for "identification." The only rule of the group interview was not to interrupt one another and to know that the forum was an open, trusting, non-threatening place where no judgments were made. A flip chart was used to visually record comments of the group, and reviewed with participants at the conclusion of the session.. The qualitative session was one hour in duration and a $10 gift card was given as compensation for participation time.

Questions were asked of participants that included inquiry into their perceptions of family life such as: Share with us what it was like at home when your sibling was hospitalized? And how did your family talk about the genetic disease of your sibling? Questions were presented that sought insight into their experiences in the hospital setting when their sibling was admitted for care. Tell us a story about when you visited your sibling in the hospital. What, especially the nursing staff, could they have done to make you feel better? The concluding questions sought information on student support needs the campus community may need to consider. How do think your sibling(s)' disease or syndrome has impacted your personal growth in regard to development/ maturation/independence/ strategies for facing adversity? What kind of support during your college years would you like to see on campus?

DATA ANALYSIS

Audio recording of dialogue was professionally transcribed verbatim, a person unknown to group members. The "long table approach" following the methodology of Morgan (1998) and Krueger (1998) and no computer software was used. In this process, the transcript was cut apart; common occurring phrases were put together and arranged into different categories found through the study. Themes were then identified from the categories. The use of a flip chart, field notes, debriefing, and data reconstruction also assisted in the identifications of themes, findings and conclusions.

RESULTS

Sample

All participants were Caucasian, non- Hispanic females. Two students were 22 and one was 20. There were two seniors and one freshman student. Their siblings with genetic diseases were: male with Arrhythmogenic Right Ventricular Dysplasia (a congenital heart disease), female with autoimmune Rheumatoid Arthritis, and male with Down's syndrome. All students stated that they participate in home therapies such as physical therapy, occupational therapy, speech therapy, and act as a home health aide when outside assistants are not available. All university students shared stories of their sibling having multiple and unexpected hospital admissions. Two students had divorced parents. The hospitals frequented by the siblings ranged from 25 minutes to two and one-half hours away. A focus group style methodology was used for two participants. The third participant was unable to attend the session due to a conflicting academic schedule and asked to be interviewed. The same questions were used and the study was conducted in the same manner as possible.

General Comments on Shared Experiences

College-aged participants were asked to tell a story when they visited their sibling in the hospital. "It was just scary not knowing whether he was going to live. It was really a traumatic experience, and being so young I don't think I fully realized what happened, what was going on with him and it was just very difficult." "He was in ICU [intensive care unit] and they thought I was too young to see what was going on." The second participant agreed that, "It was kind of scary when you see your sister in there [ICU]." Participant A recounted that, "I knew he was in a lot of pain, but it was scary at the same time, I didn't know what was going on." Participants shared the disruption in family life created for them during their siblings' hospitalization. Participant A's entry into school was delayed one year because of her brother's surgery. Participant B explained how a routine appointment is never routine. "You don't expect anything to go wrong at an eye doctor appointment, right? Just typical, you go in, get your eyes checked … 'Oh no. Something else is wrong with her." Participant A shared how she was often living with friends and family. "I just, I felt like I was just, you know, kind of living, just living at a place. I wasn't with my family. It was difficult."

From responses from the open-ended focus group questions, three themes were identified.

A New Normal

Participant A said, "...now it's like I feel like I don't have a normal life, but it is normal to me just because I'm used to it." Participant B expressed acceptance of her life's situation, "Everyone's normal is something different, going back would be weird." Participant C whose brother had Down's syndrome said, "Kids would ask me what was wrong with him [my brother]; I'd say nothing is wrong with him."

Caregiver Role Strain

Participants talked about the conflict of being an adolescent and young adult and the unique roles these siblings experienced and maintained in their families. One participant shared a story about participating in various therapy sessions as if he or she was the client. It was like I had to be his older sister [instead of the reality of the situation being the younger sister]." "We are partners in crime, my brother and me. I went through physical therapy and speech therapy too to make it fun and easier for him, otherwise he wouldn't do it." Another participant expressed resolution about her personal future. "What can I do? and I can't really do anything about it." She continued"…am I going to be the one to take care of her [when the sibling gets older] 'cause I don't know who else would be there for her?" In a matter-of-fact manner the third participant replied to a comment, "Yeah, I did [had to grow up early]." And the other two agreed.

Crippling university social life

The three participants all shared stories about the impact on their college personal life. The first student said, "I had exactly 2½ friends my freshman year at [university]. It was crippling socially. Like I mean gone every weekend. Gone social life." The second student talked about her returning home to assist in care giving to respite her parent. "I go home every single weekend. I haven't stayed one weekend this year." The third participant talked about sleep disturbance when her sibling telephoned. "Sometimes by brother calls me at 2 AM to say, 'just come home', and, 'why can't I be in college.'"

Participants' suggestions for nursing and university implications

To conclude the sessions participants were asked or suggestion on how nurses and universities could help them. They all agreed that acknowledgement of the well-siblings' presence was important. They felt that nurses ignored them when they were visiting. "It's like 'I exist too. I know that I'm here to support my sister, but I exist too, kind of thing." The second student said, "I don't remember any of the nursing staff ever explaining anything really to me. They kind of left that up to my parents." The third participant summed up the group's thoughts by saying, "It just doesn't affect the siblings [child with genetic disease], but everyone…"

The participants were then asked what suggestions they had for universities to help in the well-siblings' role in academic and university life. They offered three suggestions. The first was to establish a university support group. The purpose of the support group would enable them to "…just sit down and talk." They continued, "Yeah, [the support group] will understand." And "It would be nice to talk to someone who goes through the same thing, understands." Participant C whose brother had Down's syndrome suggested an idea for siblings' visitation weekend. She said, "Have a little siblings' weekend with a different theme [for siblings with genetic diseases]. Other siblings [with genetic diseases] could maybe hang out [together]." When asked how faculty could support these students Participant A suggested, "Sometimes flexibility with due dates. I've pulled lots of all-nighters trying to get things done and then [assignments have] poor quality because I'm trying to take care of something at home." When asked about sharing family information with university advisors they unanimously agreed when one said, "I don't feel like I want to be singled out, that's for sure."

Positive experiences

Only one of the three participants viewed having a sibling with a genetic disease as having a truly positive influence on her life and that was participant C whose brother had Down syndrome. "They [other peers in high school] acted like it was a negative thing and said, 'oh I feel so bad for you.' But it wasn't a negative thing." She continued that her experience at the university was much difference than what she had experienced in high school. "Maybe everyone grew up." This participant recounted that while her brother had proposed to every girl on her dormitory floor, he was well liked and warmly received when he visited. "There is so much better in being different. It taught me to like all different kinds of people, I feel I have learned a lot, I don't judge people."

Challenges of the study

It is acknowledged that the sample size was low and results may be anecdotal. However, even with a small sample size there was repeating comments and themes. While working with the Student and Community and Engagement Office in regards to distributing the flyers on campus, there was a break in communication and the flyers did not get posted until three weeks after the anticipated date, and another week was lost due to spring break. Attempting to recover lost time, we obtained permission to send a general email to the student body and faculty, and again posted flyers, but the end of the academic semester was approaching. The student researcher knew of other university students who met the inclusion criteria of the study, but they did not come forth. A second challenge was scheduling conflicts of the participants.

Box 1. Listed are the clinical nursing implications suggested by participants in the study

- Acknowledge siblings' presence in the hospital room
- Offer a blanket, pillow, cookies, items of comfort
- Ask parents what information on the patient's condition has been shared with the siblings. Be a conduit for communication.
- Incorporate Child Life Specialists whenever possible.
- Don't assume siblings want to be sent to the hospital playroom
- Present information to family and siblings on appropriate hospital and community-based support groups

CONCLUSION

College-students did not want to use their siblings genetic disease as an excuse, but would appreciate some flexibility in due dates of assignments when family crises arose. They did agree, however, that speaking with advisors to potentially make deadline extensions would decrease schoolwork-associated anxiety. Participants suggested a "sibs" weekend that would enable visits by their siblings with genetic diseases. Something that was noteworthy was that, by the engaging interactions of the participants it was shown that a support group would be therapeutic as they shared a common bond. They also expressed conflict regarding getting tested for the genetically linked disease of their sibling. In addition, at the close of the interviews participants agreed they felt more confident to discuss their family circumstances with faculty advisors.

Participants felt that holistic family-based care should include siblings in the delivery of nursing interventions. The two main opinions expressed by the participants were the nurses needed to increase communication and acknowledge the siblings' presence (Box 1). Nurses also need to remember that there are multiple resources to utilize, such as child life, pastoral care, social work, or psychology.

Early hospital experiences by siblings of patients who have a genetic disease appear to remain fixed in their memories and as college-students their role has evolved to be an active member of that siblings' caregiver team. These family demands surface to affect their college experience and may impact the stages of development that need to be achieved by this age group to reach healthy adulthood. It is hoped that the results of this pilot study will increase nursing awareness of the needs of siblings, as well as help the University learn how to support these students who have similar life experiences by developing new supportive programs. Through dissemination, these programming endeavors can then be shared with other university campuses. Future research could include conducting similar focus groups with larger, more diverse samples along with improved recruitment strategies.

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The Human Rights Impacts of VAWA 2013:

A True Victory for Native American Women?

Photo Courtesy of Catching.Light, Flickr, 2008

Lauren Rose Kelly

INTRODUCTION

The level of gender violence against native women in the United States has reached epidemic proportions. Furthermore, the vast majority of Native American gender violence victims are abused at the hands of non-native men. Native American tribes are considered to be "domestic dependents" of the United States, meaning that they have the inherent authority to govern themselves and also maintain U.S. citizenship rights. This system creates multiple overlapping governing systems on tribal reservations, as illustrated by determining jurisdiction over gender violence crimes. Tribal courts do not have the power to persecute non-native persons, and federal prosecutors, who do have jurisdiction, have declined approximately two-thirds of sexual abuse cases from Native Americans in recent years. Native women are essentially left legally defenseless against sexual abuse.

Consequently, Native Americans have reached out to the international human rights community for support in gaining more legal protection for the victims of gender violence. Independent international experts and human rights organizations have repeatedly called on the United States to take action and increase legal protection for Native American women. Amnesty International claims that "In failing to protect Indigenous women from sexual violence, the US is violating these women's human rights." The human rights organization is calling on the United States government to take necessary steps to end sexual violence against Native American women. James Anaya, the United Nations Special Rapporteur on the Rights of Indigenous Peoples, also expressed concern about the prevalence of gender violence on Native American reservations. Following his visit to the United States in 2012, he reported that numerous cases of violence against native women are committed by non-native individuals, many of whom are not subject to indigenous prosecutorial authority because of their non-native status. He asserts that "Congress should act promptly to pass key reforms to the Violence Against Women Act that bolster indigenous tribes' ability to prosecute cases involving violence against indigenous women."

Fortunately, in March of 2013 the Violence Against Women Act (VAWA) was reauthorized to include a provision,

which gave tribal governments criminal jurisdiction over some non-Indians who commit crimes on reservations. This recent legislation is hailed as a victory for indigenous women seeking justice and protection from the legal system, but there are also many critics and notable shortcomings of the new provision. This paper seeks to examine whether the reauthorized Act is truly a victory for Native American women and if it adequately protects their human rights. This study will focus on three key human rights established by the United Nation's Declaration of Human Rights: "the right to life, liberty and the security of person, the right to recognition and equality everywhere as a person before the law, and the right to an effective remedy by the competent national tribunals for acts violating the fundamental rights granted him by the constitution or by law".

HISTORY OF GENDER VIOLENCE AND TRIBAL JURISDICTION

Sexual abuse against Native American women is astonishingly prevalent and particularly violent. According to U.S. Department of Justice, Native American women are more than two and a half times more likely to be raped or sexually assaulted than are other women in the United States. Approximately one in three Native American women will be raped in their lifetimes. This rate is most likely even higher due to the underreporting of sexual violence crimes; according to the Department of Justice, seventy percent of sexual assaults of Native women are never reported. Many of the Native American women interviewed by Amnesty International said they did not know of any women in their community who had not experienced sexual violence. Furthermore, there is evidence that native women are more likely than other American women to suffer additional violence at the hands of their attackers. Rapes against native women are three times more likely to involve weapons than all other rapes in the United States. In a 2006 study, ninety-six percent of American Indian respondents who had been a victim of rape or sexual assault had experienced other physical abuse as well.

Lisa Brunner, the Director of the Sacred Spirits First Nations Coalition and advocate for the survivors of sexual violence in the Native American community, says that rates of rape are so high in her community that girls discuss rape in terms of what to do "when raped," and not "if [I am] raped." She recalls what one young girl told her: "My mom and I already talked about that. When I'm raped, we won't report it, because we know nothing will happen. We don't want to cause problems for our family." Sexual assaults are a constant threat and, as illustrated by Lisa Brunner's experience, a definite reality in native women's lives.

According to the US Department of Justice, in at least eighty-six percent of the reported cases of rape or sexual assault against Native American women, victims report that the perpetrators are non-Native men. This represents a substantially higher rate of interracial sexual violence than is experienced by Anglo or African American women within the United States. Reservation demographics are partially responsible for this. A significant portion of residents on most reservations are non-Indian, largely a result of the United States government's sale of tribal land to white settlers around the turn of the century. More than half of all married Native American women have non-native husbands and, arguably as a consequence, Native American women experience some of the highest domestic-violence victimization rates in the country. However, reports also state that sexual predators from outside tribal lands will often travel to reservations with the intent to rape. Accounts of violence against women living in tribal communities generally increase during hunting season.

Historically, Indian tribes have exercised full authority over its inhabitants. Early federal treaties specifically noted a tribe's power to punish non-Indians. However, towards the end of the nineteenth century, there was a push within the United States government to dismantle tribal government systems. Criminal law enforcement, especially in cases involving non-Indians, became federal or state matters, and tribal court powers became increasingly limited. In 1968, the Indian Civil Rights Act limited the criminal sentences that tribal courts could impose up to one year in jail and a $5,000 fine. To put this restriction into perspective, in cases of rape, state court sentences typically exceed eight years and federal sentences generally surpass twelve. Moreover, in the 1978 case Oliphant v. Suquamish Indian Tribe the Supreme Court ruled it unconstitutional for tribal courts to try non-natives without Congress' consent. This was an injustice to Indian victims of all crimes, but most exacerbated in regards to gender violence because sexual assaults on Native American women are overwhelmingly interracial. David Perez, a graduate of Yale Law School and legal commentator, states, "the biggest problem Native American women face isn't related to crimes committed by Native Americans—it's crimes committed by non-Indians on tribal land. But those who commit violence against women on tribal lands are roaming this legal maze with absolute impunity."

Native women who come forward today to report sexual violence are caught in a jurisdictional maze between three justice systems: tribal, state and federal. Whether the victim is a member of a federally recognized tribe, the accused is a member of a federally-recognized tribe, and whether the offence took place on tribal land, are the three most significant deciding factors in determining which justice system has the authority to prosecute the crime. David Perez explains that, "if neither the victim nor the perpetrator is Native American, then only State authorities can make the arrest and try the case. If the victim is Native American, but the perpetrator is not, then only federal agents can make the arrest. And if the victim is not Native American, but the perpetrator is? Then tribal authorities can make the arrest, but only federal courts would have jurisdiction to try the case." There are often significant delays before police, lawyers, and courts can eventually agree upon who has jurisdiction over a particular crime. There have even been cases where there was so much confusion over jurisdiction that the case was never tried.

The bureaucracy regarding jurisdiction has ultimately led to inefficient law enforcement on tribal lands. According to recent studies, law enforcement on reservations rarely leads to prosecution and conviction of non-Indian offenders. N. Bruce Duthu points out in the New York Times article "Broken Justice in Indian Country" that "the Department of Justice's own records show that in 2006, prosecutors filed only 606 criminal cases in all of Indian country. With more than 560 federally recognized tribes, that works out to a little more than one criminal prosecution for each tribe."

This situation is the result of not only jurisdictional confusion, but also the lack of law enforcement officers in tribal communities. There is less law enforcement per capita in tribal communities than there is in other rural areas nationwide. A 2001 survey of police departments on tribal reservations across the country found that many tribal police departments are underfunded and lack necessary resources like sufficient staffing (administrative and law enforcement personnel), technology, vehicles, and equipment. The lack of available resources means that law enforcement responses to crime scenes are extremely delayed. Furthermore, upon arrival the officer must first take the time to determine whether the crime is within tribal jurisdiction. If not, the state or federal law enforcement officers must be contacted, creating even more delay before the investigation may begin and the victim is actually tended to.

Moreover, federal and state prosecutors often lack the time and resources to pursue cases, and yet they are responsible for all cases involving non-native offenders. According to the Government Accountability Office, between 2005 and 2009, 67 percent of sexual abuse cases sent to the federal government for prosecution were declined. Although the Justice Department claims it has mandated extra training for prosecutors and has directed each field office to develop its own plan to help reduce violence against women, there have been no significant or quantitative improvements in recent years. Some advocates for Native American women say they no longer urge victims to report rapes. Even if a case is accepted by the federal government, relying on federal or state authorities often means having to travel hundreds of kilometers to the nearest forensic examiner or prosecutor.

Sarah Deer, an assistant professor of law at William Mitchell College and a citizen of the Muscogee Nation of Oklahoma, states: "There's never really been accountability for non-Indians coming into tribal communities and committing acts of rape or domestic violence; tribal governments can't prosecute them. I've heard tribal police say that the white men on the reservation basically flaunt their violence and taunt the police: 'You can't do anything, I know I'm outside the bounds of the law." The United States legal system essentially leaves Native American women helpless and vulnerable. According to Colorado State University expert Roe Bubar, an assistant professor in the school of Social Work and Ethnic Studies and Pamela Jumper Thurman, a senior researcher at the Tri-Ethnic Center for Preventative Research , "The message to Native women and their children is that they are expendable and there is no real help or assistance within the system."

VIOLENCE AGAINST WOMEN ACT

The Violence Against Women Act is a segment of the Violent Crime Control and Law Enforcement Act of 1994. VAWA has been widely credited with helping law enforcement and prosecutors crack down on domestic violence nationwide. The Act provides additional tools for protecting women on native reservations by defining a new federal habitual-offender crime penalty and authorizing warrantless arrests to federal law enforcement officers who determine probable cause during domestic violence cases. VAWA also created the Violence Against Women Office (VAWO), which currently administers 21 grant programs and subsequent legislation, four of which are specifically targeted to Native American populations.

On March 7, 2012, Congressman Dan Boren introduced the Stand Against Violence and Empower (SAVE) Native Women Act in addition to VAWA. The Act allows tribes to exercise sovereignty in investigating, prosecuting, convicting, and sentencing both Native Americans and non-natives who assault Native American partners in native lands. The reauthorized Act also clarifies tribes' sovereign power to issue and enforce civil protection orders against natives and non-natives. According to the Act, tribes' criminal jurisdiction over non-natives is limited to domestic violence crimes, dating violence, and criminal violations of protection orders. Crimes between two strangers (including sexual assaults), crimes committed by a person who lacks sufficient ties to the tribe, child and elder abuse that does not involve the violation of a protection order, and crimes between two non-natives are still not protected against. In addition to addressing issues of jurisdiction, the SAVE Act requires the Attorney General to submit an annual report of suggestions given by Indian tribes and actions taken to respond to recommendations from years prior. The purpose of these annual reports is to facilitate cooperation and consultation between tribes and law enforcement agencies.

The other major additions to VAWA that the 2013 reauthorization creates, aside from the sovereignty of tribal courts, is the protection against intimate partner violence of lesbian, gay, bisexual, and transgender people and extended access to United States' visas for immigrant victims. Ultimately, the reauthorized act extends the protections against gendered violence to minorities who did not receive the full benefit of the original Violence Against Women Act in 1994.

A strengthened version of the Violence Against Women Act was signed by President Obama on March 7, 2013. It is still hailed as a great victory for Native American women. When President Obama signed the reauthorization, he commented, "This is the day of the advocates, the day of the survivors. This is your victory. This victory shows that when the American people make their voices heard, Washington listens." Native Americans are commonly thought of as the biggest benefactors of the reauthorization. "It's a great victory for women everywhere but especially tribal women," said Rep. Ann Kirkpatrick. Secretary of the Interior Ken Salazar praised the new Act, stating, "This historic legislation, which recognizes and affirms inherent tribal jurisdiction over non-Indians in domestic violence cases, will provide much needed tools to tribal justice systems to effectively protect Indian women from abuse." This new law takes general effect on March 7, 2015, but it also authorizes a voluntary "Pilot Project" to allow certain tribes to begin exercising SDVCJ sooner. Today, there is barely any public discourse regarding the remaining limitations of the new Act.

ANALYSIS

It is dangerously easy to assume that since Native Americans won the 2013 VAWA reauthorization they have also achieved the proper human rights protection they deserve. However, political standards and human rights are far from the same. Upon closer reflection of how the VAWA reauthorization impacts Native American women's specific human rights, it becomes apparent that the new Act is not complete victory for the native victims of sexual violence.

The VAWA Reauthorization of 2013 only goes halfway to ensure the human rights of life, liberty and security of person for Native American women. While the new tribal provisions allow Native American communities much more sovereignty than previous versions of VAWA, there are still extreme jurisdictional limitations. For example, crimes between two strangers (including sexual assaults), crimes committed by a person who lacks sufficient ties to the tribe (e.g. living or working on its reservation), and child or elder abuse that does not involve the violation of a protection order are still out of tribal courts' jurisdiction. Sarah Deer, a citizen of Muscogee (Creek) Nation and assistant law professor at William Mitchell College of Law, explains that the law will only apply to non-Indians who are married or in an intimate relationship with a tribal member, and it is limited to cases of domestic violence. Bruce Duthu, an internationally recognized scholar of Native American law and professor at Dartmouth College, points out that "The reauthorized VAWA goes part of the way by affirming tribal sovereignty over all offenders for a very limited class of offenses."

Furthermore, while the VAWA reauthorization gives tribal courts more sovereignty, it does nothing to address the concerning inadequacies of these tribal courts. According to Amnesty International, "Tribal law enforcement agencies are also chronically under-funded and federal and state governments provide significantly fewer resources for law enforcement on tribal land than they provide for comparable non-Native communities. The lack of appropriate training in all police forces—federal, state and tribal—also undermines survivors' right to justice." Tribal courts' recently enlarged jurisdiction is currently regarded as a great victory, but it is important to remember that these tribal law enforcement systems do not presently have the necessary means to successfully pursue justice for victims. Furthermore, tribes yearning to take advantage of VAWA's jurisdictional provisions must first enact many institutional changes, including amending current tribal codes, hiring new judges, and devoting resources to pay for public defenders. Thus, the VAWA reauthorization does not ensure Native American women's right to recognition and equality everywhere as a person before the law. It is undeniable that Native American women still receive substandard legal protection and security compared to the protections other American women receive.

As decreed by the United Nations, it is a human right to "an effective remedy by the competent national tribunals for acts violating the fundamental rights granted him by the constitution or by law." The federal advisory bodies, equivalent to "national tribunals," suggested that federal agencies responsible for investigating and prosecuting sexual violence in Indian Country need to prioritize these cases and improve the transparency of their processes. The councils also recommended that tribal authorities have jurisdiction over non-Native offenders in Indian Country. According to the White House, most of the committees' suggestions are included in the reauthorized version of the Violence Against Women Act. However, the reauthorized Act does not dictate any necessary requirement for federal agencies to make sexual violence cases on tribal lands a priority. Furthermore, tribal authorities only have jurisdiction over some of the non-native offenders on tribal lands. Ultimately, Native Americans were not granted the full "effective remedy" that these interagency councils recommended.

Another major limitation of the 2013 VAWA reauthorization is that Alaskan Native women are left out. As Native Americans and supporters across the country celebrate the VAWA reauthorization, the media has largely ignored the controversial provision that excluded Alaskan Native tribes from the tribal jurisdiction provisions. This new provision prevents Alaskan Native women from gaining the same benefits that all other Native American women in the United States now receive from the VAWA reauthorization. The cause of this disparity between Alaska Natives and Native Americans is differences in land ownership. In Alaska, tribes do not have reservations, so they cannot base claims of jurisdiction on reservation boundaries. Tragically, for the same reason, Native Alaskan women are probably in the most need of tribal sovereignty. There are currently 140 Alaskan villages with no state law enforcement. Because of the vast distances, weather conditions, and lack of state trooper posts in Alaska, law enforcement response times can be very slow to help. The only place many women can turn to for protection against gender violence is their tribe. Thus, Native Alaskan women's human rights continue to be grossly violated.

RECOMMENDATIONS

In order to best protect Native American women's human rights, VAWA needs to extend tribal jurisdiction to all crimes of gender violence that occur on tribal lands. As sovereign entities closest in physical distance to the actual crime, tribal courts are likely to be the most effective means of law enforcement and may be able to engage the community in efforts to prevent gender violence in the first place. Tribal courts undeniably care more about the wellbeing of victims, and the argument that tribal courts are incapable of producing impartial juries is unfair and discriminatory. Moreover, Native American tribes are privy to the collective human right of governing themselves, as decreed by the United Nations as, "indigenous peoples, in exercising their right to self-determination, have the right to autonomy or self-government in matters relating to their internal or local affairs." In my opinion, a crime of gender violence which occurs on tribal land is an internal matter, no matter whether the offender is native or not. The loopholes in the justice system concerning the prosecution of non-natives are precisely what perpetuate the culture of rape on tribal reservations. In order to dismantle this cultural norm of sexual assault, it is critical that more federal funds from VAWA should go towards improving the tribal courts and law enforcement system so that they are actually effective.

I also recommend that tribal court systems create stronger statutory laws. Sarah Deer states that "during the last century, various criminal codes have been written and signed into law by tribal legislatures—some much better than others. Statutes are sometimes out of date. In addition, some of the earliest tribal criminal codes were taken from "boilerplate" codes which were drafted by non-Indians. Over the past several years, [she has] reviewed over 100 tribal sexual assault laws. What [she has] found is that many tribal codes have weaknesses—and most of the time, these weaknesses are inconsistent with tribal traditional laws which served to protect women and children." For example, some tribal codes require that a prosecutor prove that physical force was

used to commit sexual assault and some even have a marital exemption clause. These standards of gender violence are outdated and, frankly, insulting to women. The sovereignty of tribal courts can only benefit Native American women if tribal laws are updated to efficiently protect native women's human rights. If there is no sexual assault code in place at the tribal level, no justice can be found in tribal court.

Furthermore, in order to equalize the treatment of Native American women and non-native women before the law, it is imperative to create a system to collect, analyze and disseminate crime and victimization data on tribal lands. There is currently no systematic national data collection effort focused on crime on reservations, whereas there are extensive programs for this purpose throughout the rest of the United States. It is very rare that federal, state, and local crime data reports even distinguish between offenses committed in Indian Country from those committed elsewhere. According to the National Institute of Justice's Program of Research on Violence Against American Indian and Alaska Native Women, "The primary mechanisms for reporting crime data—the FBI's Uniform Crime Reporting (UCR) program and the National Incident Based Reporting System (NIBRS)—do not include offenses committed on reservations or criminal and delinquency arrests and subsequent processing by federal agents (e.g., FBI, BIA, The Drug Enforcement Administration [DEA], Bureau of Alcohol, Tobacco, Firearms and Explosives [ATF], U.S. Immigration and Customs Enforcement [ICE])." Collecting such information allows the government to anticipate, monitor, and prevent such criminal activity. The next VAWA reauthorization should create such a system so that Native American women can finally begin to receive the same protection against gender violence that all other American women receive.

Ultimately, it is important to remember that the condition of Native American women today can be explained through history. The high rate of gender violence experienced by native women today at the hands of white men is almost a tradition of American society. In order to truly change such an ingrained problem, the attitude of an entire nation must be reformed. The VAWA reauthorization of 2013 represents a step in the right direction, but there is still much more to be done to safeguard native women's human rights.

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About the Authors

NICHOLAS JOHN NOVAK

Nicholas Novak is a senior Integrated Graduate Studies student in Anthropology with a double major in Medical Anthropology and Evolutionary Biology at Case Western Reserve University. In his spare time, he is an officer in Mortar Board, an ELP Mentor and a volunteer at University Hospitals Seidman Cancer Center.

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SO HEE MOON

So Hee Moon is a sophomore from West Chester, PA majoring in Chemical Biology at Case Western Reserve University with a strong science, liberal arts, and experiential fund of knowledge. Her academic interests include genetics, disease, and organic chemistry. So Hee loves getting involved on campus through her activities as a peer tutor, a SELP mentor for Educational Service for Students (ESS), and a president of Carlton Road Community Council for Residence Hall Association (RHA). Currently, she works in Dr. Lederman's lab studying latent reservoirs of HIV.As a student in the 6-year Pre-Professional Scholars Program in Dentistry, she aspires to become an orthodontist in the future.

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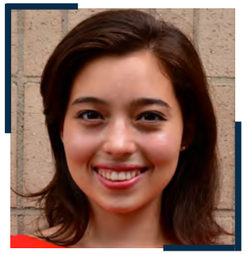
KATRINA THEDE



Katrina Thede is a senior Biology and Music major at Case Western Reserve University. Her future plans after graduation are to attend medical school and continue doing research in order to help create new technologies to better treat patients and cure diseases.

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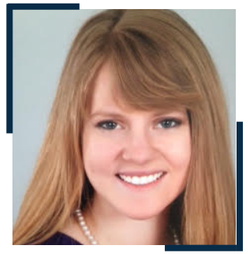
MELANIE JOY DEARDO



Melanie DeArdo, RN, BSN, graduated from Capital University in 2013. She currently is a staff RN at Texas Children's Hospital on the Progressive Care Unit.

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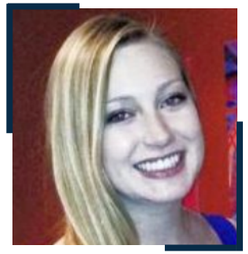
The writers thank the editors of the Discussions magazine for the opportunity to revise the manuscript and to resubmit. It has been shortened for better flow and momentum of the research story that is being told, but none of the research has been lost. Also, possible replication of information was removed to avoid redundancy. The authors of this project feel that it is very important to share, especially with those in the college community.

LAUREN ROSE KELLY



Lauren Kelly is a junior at Duke University. She is majoring in Public Policy and hopes to attend law school after graduation. She has interned at New York City's Office of Emergency Management and the Women's Legal Centre in Cape Town, South Africa. She is the Director of the student-run non-profit North Carolina Common Sense, co-chair of the Student Advisory Board to Duke's Human Rights Center and a member of the Duke Dance Marathon committee. She is a member of the Kappa Alpha Theta Sorority. This paper served as her final culminating project for the Duke University program "Duke Immerse: Rights and Identities in the Americas." She would like to thank her faculty advisors, Robin Kirk and Robert Korstad, for an incredibly transformative and enlightening Duke Immerse experience.

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