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Research Exemplars from an Interdisciplinary Healthcare Genetics Program

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RESEARCH EXEMPLARS FROM AN INTERDISCIPLINARY HEALTHCARE GENETICS PROGRAM

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ABSTRACT

The first chapter of this dissertation provides an overview of the interdisciplinary Healthcare Genetics model, the framework for the studies described in Chapters Two, Three and Four. The model is comprised of five core constructs: Research, Environment, Clinical Practice, Ethical/Legal/Social Implications (ELSI) and Education. Each construct is defined. Chapter One also presents a brief overview of subsequent chapters.

Chapter Two details a biophysical approach to the phenotypic classification of mutations in the 7-dehydroxycholesterol reductase (*DHCR7)* gene, involved in Smith-Lemli-Opitz syndrome. This study utilized computer modeling to determine the biophysical effects of each mutation on structure and function of the DHCR7 protein.

The third chapter is a narrative review of the role of the cytokine interleukin 17 (IL-17) in the gynecological condition endometriosis. This review discusses the incidence, etiology and pathogenesis of endometriosis and outlines general guidelines for diagnosis and treatment are outlined. Finally, potential IL-17-targeted therapies are explored.

Chapter Four presents original bench research that evaluated the effects of the phyto-cannabinoid beta-caryophyllene (BCP) on a cell culture model of endometriosis. The effects of BCP on markers of inflammation such as cell proliferation and vascular endothelial growth factor (VEGF) production were assessed.

The final chapter, Chapter Five, summarizes the findings from Chapters Two, Three and Four. The significance and limitations of these studies are discussed. Finally, Chapter Five provides recommendations for future research.

DEDICATION

I would like to dedicate this dissertation to my family and friends for their continual support and encouragement throughout my years in the doctoral program. Thank you all so much for your love and prayers. I could not have reached this point without you!

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I'd like to thank Yunhui Peng and Wenxing Zhang, my co-authors on Chapter Two of this dissertation. Also, thank you to Dr. Patilee Tate, Dr. Bruce Lessey and Angela Houwing for their assistance and support during the research detailed in Chapter Four.

Finally, I would like to thank the faculty and staff of the Clemson University School of Nursing and my fellow Healthcare Genetics doctoral students.

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CHAPTER ONE

INTRODUCTION

In 2003, the Human Genome Project was completed, providing the world with access to the sequence of most of the human genome and launching the field of genetics into the forefront of medicine (Human Genome Sequencing Consortium, 2004). Continuing from that time, genetic research has proliferated exponentially, resulting in ever-increasing understanding of the molecular basis of disease and the development of more cost-effective technologies with rapid availability of detailed results (Wilson & Nicholls, 2015). The impact of genetics on healthcare is far-reaching, offering the promise of disease prevention, early disease detection, and personalized therapies (Rabbani, Tekin, & Mahdieh, 2014). Healthcare genetics is an emerging discipline seeking to meet the challenges associated with the rapidly-expanding and evolving genetics landscape.

This dissertation is comprised of five chapters with original research which illustrates and applies the Clemson University School of Nursing Healthcare Genetics (HCG) program model of interdisciplinary scholarship. In addition, the direct research topics in this dissertation are led by the HCG model constructs (see Figure 1.1). Chapter One introduces the HCG model and provides an overview of the dissertation chapters. Two important definitions, which serve as the foundation for this dissertation, are "exemplar" and "interdisciplinary research." An exemplar is defined as "a person or thing serving as a typical example or excellent model" (Merriam-Webster.com, 2018). The

National Academy of Sciences, National Academy of Engineering, and Institute of Medicine (2005) defines interdisciplinary research as:

> "A mode of research by teams or individuals that integrates information, data, techniques, tools, perspectives, concepts and/or theories from two or more disciplines or bodies of specialized knowledge to advance fundamental understanding or to solve problems whose solutions are beyond the scope of a single discipline or field of research practice" (p. 26).

These studies, which comprise this dissertation, serve as models of interdisciplinary research, utilizing a variety of techniques from different disciplines.

The theoretical foundation for this dissertation research is the HCG model (Figure 1.1). The HCG model was developed collaboratively by Healthcare Genetics PhD students and faculty in the theoretical foundations course (2014). The goal of the HCG program is to, "prepare interdisciplinary scientists to: collaborate with multiple disciplines to generate knowledge and develop theories that focus on the genomic aspects of actual and potential health problems, formulate health promotion, disease prevention and treatment strategies that translate and integrate genomic knowledge from a variety of disciplines and demonstrate leadership that facilitates interdisciplinary development and application of ethical guidelines and health policy in genetics." (Healthcare Genetics Program Goals, 2018). New scientific and technological breakthroughs have been occurring at a rapid rate in the past decades, resulting in significant impacts in multiple areas of genetics. These genetic breakthroughs are constructs of the HCG model, including Clinical Practice, Education, Environment, Ethical, Legal and Social Implications (ELSI), and Research. While each construct has unique elements, the

constructs also contain shared elements, thus enabling identification of new areas of genetic information and/or application in areas of overlap.

Figure 1.1. Interdisciplinary Healthcare Genetics Model

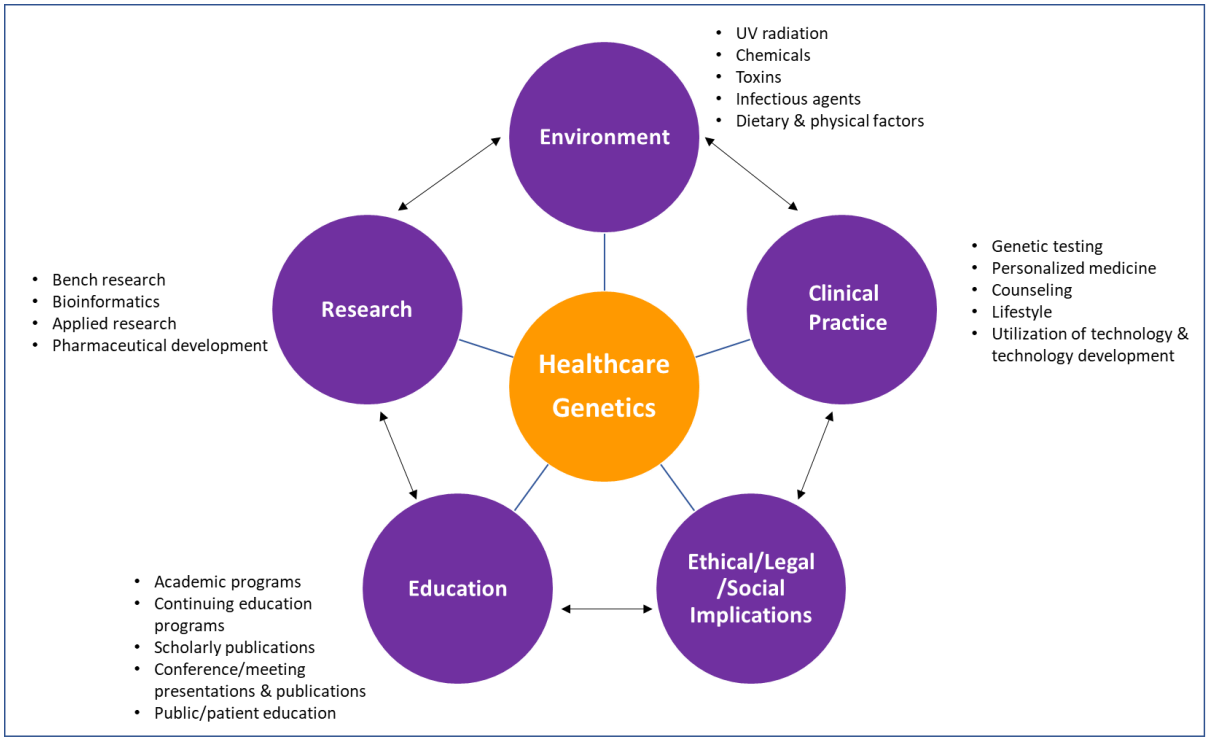

Figure 1.1. The model is built using constructs of Research, Environment, Clinical Practice, Ethical/Legal/Social Implications (ELSI), and Education, (HCG, 2014).

The *Research* construct, within the HCG model, is grounded in different research methods which include, but are not limited to, laboratory (bench) research, bioinformatics, biophysical research and applied research. Results from laboratory-based research enhance understanding of the mechanisms of disease at the molecular level. Bioinformatic and biophysical research employs computational techniques to analyze complex biological data such as DNA, ribonucleic acid (RNA) and protein sequences obtained from databases (Kucukkal et al., 2014). Applied research studies potentially

provide answers to issues associated with genetic diseases. e.g. developing safe and effective medications and medical devices to treat and/or manage a variety of health conditions (Coccia, 2018).

The *Environment* construct includes the interaction of external factors with the genome or the epigenome. Factors that affect the genome may potentially directly change the DNA sequence, resulting in permanent changes. These factors may include exposure to ultraviolet (UV) radiation, chemicals, diet, exercise or encountering genome-altering viruses (Boskovic & Rando, 2018). These factors affect the epigenome by altering gene expression, but not the actual DNA sequence. These factors may result in a form of epigenetic modification, the methylation or demethylation of cytosine-guanine (CpG) dinucleotides near gene promoters. The addition of methyl groups typically silences gene expression while their removal increases gene expression (Koukoura, Sifakis, & Spandidos, 2016). Epigenetic changes have also been shown to have transgenerational effects (Boskovic & Rando, 2018). For example, the chronic inflammatory disease endometriosis is driven by the effects of estradiol while resisting the anti-inflammatory effects of progesterone. It has been shown that the progesterone receptor (PR) is hypermethylated while estrogen receptor (ER) is hypomethylated in women with endometriosis (Wu, Strawn, Basir, Halverson & Guo, 2006; Xue et al., 2007). Thus, it is essential that a HCP considers the interaction of genetic, epigenetic, environmental as well as the interactions among these three environmental factors. These examples stress the importance of the *Environment* and the need for HCPs to consider and address

environmental interactions when evaluating a patient's risk of disease and to determine the appropriate therapeutic course(s).

The *Clinical Practice* construct represents the area in which genetic research is applied with the goal of positively impacting health outcomes. In this construct, HCPs, clinicians and other professionals utilize their knowledge about the genetics of disease to screen, diagnose, and prescribe therapeutic regimens based on the results their genetic information (family history and genetic test results) (Slomp, Morris, Inglis, Lehman & Austin, 2018). HCPs must be knowledgeable about availability of genetic tests, how these tests are ordered, and their accurate interpretation to ensure optimal health outcome(s) for their patients. It is also important that HCPs stay abreast of new DNA-based therapies and how these therapies are implemented.

As a part of *Clinical Practice*, patients and their families must be aware that *Environment*, including lifestyle factors, influences their genetic/genomic health. Inclusion of lifestyle highlights the importance of patients and families making informed healthcare decisions. The healthcare team also educates/counsels patients and their families on a variety of issues, e.g. the likelihood that a couple could have a child with a disease or an individual's risk of developing disease. For example, a family with a history of physical malformations and intellectual disability may undergo genetic testing for a panel of diseases, including Smith-Lemli-Opitz syndrome (Kalsner et al., 2018). Health promotion and disease prevention are other important constructs of clinical care involving genetics (Slomp, Morris, Inglis, Lehman & Austin, 2018). Patient and Family

educational needs are one example of the overlap between *Education* with *Clinical Practice*.

At the same time, genetic science and technological advances have outpaced the Ethical, Legal and Social (ELS) understanding, increasing the potential for harmful implications/impacts on an individuals and families. *Ethical, Legal and Social Implications* were identified/existed prior to the Human Genome project, as researchers were already aware that these issues would arise from the application of the new genetic knowledge (Clayton, 2003). While ELSI is recognized as being an important construct in the HCG model, it is not a focus in this dissertation research.

The fifth construct is *Education*. It is essential that everyone, from healthcare providers to the public, become knowledgeable in the basic principles of heredity and the role that an individual's deoxyribonucleic acid (DNA) plays in their overall health status. Thus, it is crucial that genetic education is required in curricula at all academic levels, beginning in the primary grades and extending through high school, community/technical college, and undergraduate education (Talwar, Tseng, Foster, Xu & Chen, 2017). Genetics is recognized as a major content area in the educational programs of healthcare professionals (REF nursing & medicine). In addition, the importance of continuing education in genetics be emphasized, as the field is expanding so rapidly. This overlap emphasizes the importance of teaching families and patients with a genetic disease diagnosis.

Chapter Two explores the effects of various point mutations on the conformational stability of the 7-dehydroxycholesterol reductase (DHCR7) protein via

computer protein modelling. The DHCR7 protein is associated with Smith-Lemli-Opitz syndrome (SLOS), a cholesterol synthesis disorder (Smith, Lemli, & Opitz, 1964). Bioinformatic and biophysical techniques were used to locate, collect and analyze publicly-available DNA and protein data using methods that discriminate between disease-causing and benign *DHCR7* mutations. Ten mutations (five pathogenic, two benign and three with unknown effects), that occur in the protein, underwent an extensive atom-by-atom molecular dynamic simulation to determine the effects of the mutations on protein conformational dynamics. In this analysis, the mutated protein was compared to the normal protein using a computer simulation program representing the actual intracellular environment where the DHCR7 enzyme resides. (Peng, Myers, Zhang, & Alexov, 2018).

Chapter Three reviews literature surrounding the role of the pro-inflammatory cytokine interleukin 17 (IL-17) and its primary cellular source, T-helper 17 (Th17) cells, in endometriosis. The IL-17 pathway and its function in the initiation of inflammation are examined. The incidence, etiology and pathogenesis of endometriosis plus available treatments and their limitations are discussed. General guidelines for diagnosis and treatment are presented (Armstrong, 2011; Journal of Obstetrics and Gynaecology Canada [JOGC], 2010). Finally, the potential for future treatments targeted to the IL-17 pathway and clinical research are explored.

Chapter Four reports original bench research that assessed the effects of the cannabinoid beta-caryophyllene (BCP) on a cell culture model of endometriosis. Beta-caryophyllene, produced by plants such as oregano and basil as well as *Cannabis sativa,*

initiates an anti-inflammatory response without the psychotropic effects of cannabis. This makes it attractive as a potential therapy to treat the chronic inflammation associated with endometriosis. Two endometrial cancer cell lines, as well as primary human endometrial cells from women with and without endometriosis, served as biological models for this research. Cells were cultured with proinflammatory factors to induce inflammation, then treated with or without beta-caryophyllene to determine effects on markers of inflammation (estrogen and vascular endothelial growth factor (VEGF)). Effects of BCP on cell proliferation and cell viability were also investigated.

Chapter Five provides a summary of the findings from the individual dissertation research studies. A discussion of the HCG model, as the foundation for these research exemplars, is presented. The limitations and significance of these studies is also discussed. Chapter Five concludes with recommendations for future research in the emerging field of healthcare genetics.

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CHAPTER TWO

COMPUTATIONAL INVESTIGATION OF THE MISSENSE MUTATIONS IN *DHCR7* GENE ASSOCIATED WITH SMITH-LEMLI-OPITZ SYNDROME

Yunhui Peng , **Rebecca Myers** , Wenxing Zhang and Emil Alexov

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Abstract: Smith-Lemli-Opitz syndrome (SLOS) is a cholesterol synthesis disorder characterized by physical, mental, and behavioral symptoms. It is caused by mutations in 7-dehydroxycholesterolreductase gene (*DHCR7*) encoding DHCR7 protein, which is the rate-limiting enzyme in the cholesterol synthesis pathway. Here we demonstrate that pathogenic mutations in DHCR7 protein are located either within the transmembrane region or are near the ligand-binding site and are highly conserved among species. In contrast, non-pathogenic mutations observed in the general population are located outside the transmembrane region and have different effects on the conformational dynamics of DHCR7. All together, these observations suggest that the non-classified mutation R228Q is pathogenic. Our analyses indicate that pathogenic effects may affect protein stability and dynamics and alter the binding affinity and flexibility of the binding site.

Keywords: Smith-Lemli-Opitz syndrome; missense mutations; DHCR7; binding free energy; folding free energy; KNN classification; molecular dynamics simulation; MM/PBSA

1. Introduction

Smith-Lemli-Opitz syndrome (SLOS) is an inherited disorder of cholesterol synthesis characterized by intellectual disability and multiple malformations, including facial and genital abnormalities and syndactyly and was first described by Smith and coworkers [1]. The reported incidence of SLOS varies widely depending on the heterogeneity of the population studied, the biochemical methods used and the alleles assessed. Current estimates of SLOS carrier frequency in Caucasian populations lie between 1% and 3% [2–4]. SLOS is more prevalent in individuals of northern and eastern European descent and is rarely described in individuals of Asian or African descent [5]. Reports that up to 80% of affected fetuses, likely those heterozygous for null mutations, die before birth and that milder cases of the disease may not be diagnosed, conceivably prevent accurate determination of frequency [6–8]. The majority of "classical" SLOS patients are compound heterozygotes with one severe null mutation and a second missense mutation which retains some enzyme functionality. Milder cases often possess two less severe missense mutations [9].

SLOS is linked to mutations in 7-dehydroxycholesterol reductase (DHCR7), which is the rate-limiting enzyme in the cholesterol synthesis pathway [10]. DHCR7 reduces the C7–C8 double bond of 7-dehydrocholesterol (7DHC), the precursor molecule to cholesterol [11]. Cholesterol, though harmful in high levels, is essential to life since it is involved in membrane structure and permeability, synthesis of steroid hormones and proper fetal development. The loss of functionality of the DHCR7 enzyme in individuals with SLOS results in a significant decrease in cholesterol levels and possibly toxic

buildup of 7DHC and other cholesterol precursors [12]. It was shown that accumulation of 7DHC in the brains of rats is associated with intellectual and learning disabilities [13,14].

In addition to its role in cholesterol synthesis, 7DHC is also required for vitamin D3 production. Exposure to sunlight cleaves the C9–C10 bond of 7DHC in the skin, resulting in vitamin D3. Vitamin D3 is essential for calcium absorption and bone health [13]. As DHCR7 activity decreases the amount of 7DHC available for vitamin D3 synthesis, there is a potential heterozygote advantage to carriers of *DHCR7* mutations, which typically decrease enzymatic activity [14,15]. This may explain the prevalence of mutations originating in areas with decreased sun exposure such as northern Europe and northeast Asia [7,16].

The *DHCR7* gene maps to chromosome 11q13.2–13.5 [17–19] and consists of nine exons with the initiation codon located in exon three. The gene is expressed in all tissues with peak expression in adrenal glands, liver and brain [17]. *DHCR7* encodes a 475 amino acid polypeptide with a molecular weight of 54.5 kDa, which is a transmembrane protein located in the endoplasmic reticulum (ER) membrane, the location of cholesterol synthesis.

The first *DHCR7* mutations were identified in 1998 by several groups and the early years of the 21st century resulted in more advanced molecular tests to rapidly identify *DHCR7* mutations [17,18]. Most mutations are identified through sequence analysis of coding exons and flanking intronic sequences [5,17]. To date, more than 160 *DHCR7* mutations have been reported [5]. The most common mutation with a prevalence of

~30% of reported SLOS patients is the IVS8AS G > C − 1 splice acceptor site mutation. This results in the inclusion of 134 base pairs of intronic sequence into the transcript and a non-functional protein. Other common mutations include T93M, W151X, V326L and R404C.

The majority of pathogenic *DHCR7* mutations occur in the highly conserved C-terminus region of the protein. In their molecular model of the DHCR7 protein, Li and coworkers predicted two overlapping binding sites: one for docking of the sterol 7DHC and one for binding of the coactivator NADPH [19]. As both binding sites are critical for proper protein function, it can be speculated that mutations affecting these areas would be most likely to result in disease. In support of this hypothesis, Waterham and Hennekam conducted a systematic review of published SLOS patients and compared genotype with phenotype [5]. They concluded that the most severely affected patients presented with two null alleles or two mutations in the 8–9 cytoplasmic loop while a milder phenotype was associated with mutations in the 1–2 loop or one mutation in the N- or C-terminus [5].

In the present study, we obtained variations in the *DHCR7* gene from online databases and modelled their effects on the corresponding protein to make predictions about SLOS phenotype. We demonstrate that structural and conservation properties are good discriminators between pathogenic and non-pathogenic mutations, while folding free energy changes (ΔΔGs) are not. This is consistent with previous observations [20] that current methodology for computing ΔΔGs are not accurate enough when applied to

membrane proteins. Furthermore, based on detailed analysis of selected mutants, we predict that the currently non-classified mutation, R228Q, is pathogenic.

2. Results and Discussion

2.1. Mapping Missense Mutations onto the 3D Structure of DHCR7 Protein

The dataset of *DHCR7* missense mutations includes three types of mutations: pathogenic, non-pathogenic and mutations of unknown effect. The mutations were visualized by mapping them onto the DHCR7 structure (Figure 2.1A). Pathogenic mutations are predominantly located in transmembrane and ligand-binding regions while non-pathogenic mutations are primarily situated outside the membrane. This observation indicates that pathogenic mutations occur at protein sites that are either buried or directly involved in protein function, which corroborates the findings of previous investigations [21–24]. To investigate the linkage between structural and evolutionary features of DHCR7 protein, we obtained the evolutionary conservation score (EC score) for each residue from multiple sequence alignment and mapped them onto the 3D structure of DHCR7 (Figure 2.1B). The transmembrane and ligand-binding regions appear to be highly conserved. Thus, most pathogenic mutations are located in highly conserved positions, while non-pathogenic mutations are less conserved. To further quantitatively assess the mutations' effects, we computed the relative solvent accessible surface area (rSASA), evolutionary conservation score (EC score) and folding free energy change ($\Delta\Delta G$) for all mutations studied in this work (Table S1). Pathogenic mutations tend to have lower rSASA values and higher EC scores compared with non-pathogenic

mutations. However, ΔΔG results show no obvious tendency to discriminate pathogenic from non-pathogenic mutations. The predictions made with different servers frequently contradict each other resulting in large standard deviation (SD) when averaging these predictions (Table S1). As DHCR7 is a transmembrane protein and recent work [20] demonstrated that current tools of ΔΔG predictions are not accurate when applied to membrane proteins, this may explain why ΔΔG fails to discriminate pathogenic from non-pathogenic mutations in this case. In addition, we also performed Polyphen predictions on all types of mutations (Table S1). Almost all the pathogenic mutations are predicted to be probably damaging by Polyphen. However, Polyphen overestimated the deleteriousness of the non-pathogenic mutations. About half of the non-pathogenic mutations were classified as possibly or probably damaging. Thus, Polyphen has limited accuracy in discriminating the pathogenic mutations from the mutations with unknown effects for this particular protein.

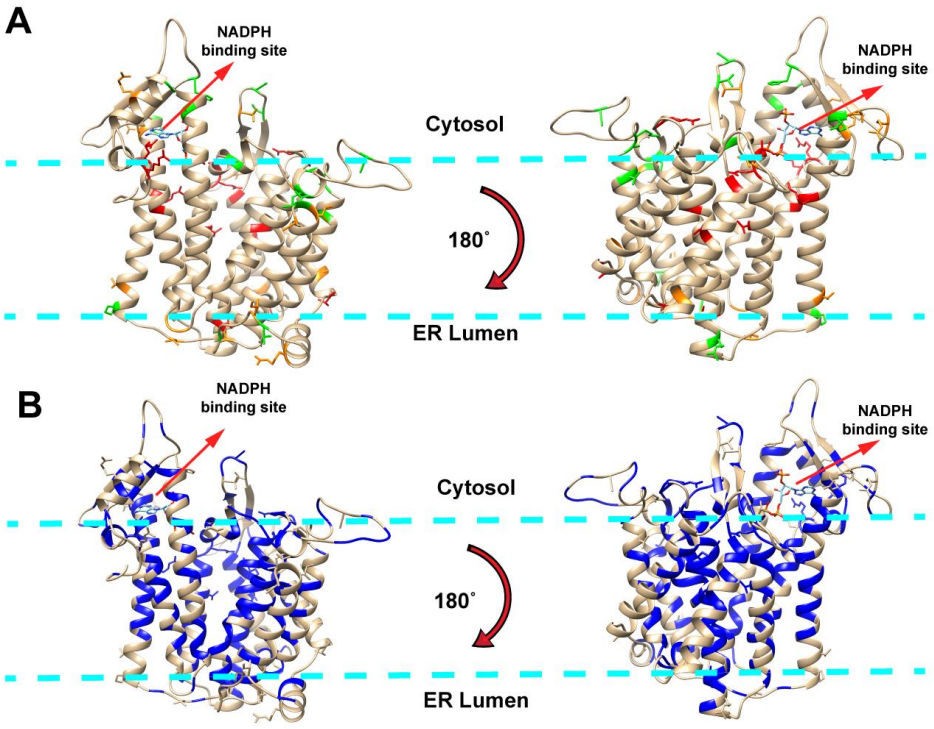

Figure 2.1. (**A**) Visualization of mutations mapped onto DHCR7 protein. Red, orange and green colored sites represent pathogenic, unknown effects and non-pathogenic mutations, respectively. The membrane boundaries are schematically shown with light blue dashed lines; (**B**) Most highly evolutionarily conserved residues mapped onto DHCR7 protein. Residues with EC score > 0.9 are marked with blue and all mutation-affected residues are shown with side chain. The membrane boundaries are schematically shown with light blue dashed lines.

2.2. Classification of the Mutations with Unknown Effects Using KNN Model

One of the goals of this study was to identify biophysical features allowing us to distinguish between pathogenic and non-pathogenic mutations, and thus to make predictions about unclassified mutations. Above, we outlined several biophysical

features, namely rSASA, EC score, PD and ΔΔG, which will be used in conjunction with the K-nearest neighbors (KNN) method (see Method section). The dataset includes 16 pathogenic mutations and 23 non-pathogenic mutations. These 39 mutations were randomly partitioned into training dataset (29 mutations) and test dataset (10 mutations) and then subjected to the KNN classifications. As the ΔΔG was shown to be less successful in distinguishing between pathogenic and non-pathogenic mutations, we performed the KNN classification with and without the ΔΔG (Table S2). The classification shows better performance without using the ΔΔG and the accuracy is 100% when K value is within 5 to 9. Here, we select $K = 7$ (the median of the K value corresponding to highest accuracy). Finally, KNN model with $K = 7$ and using properties: rSASA, EC score and PD applied to classify the mutations with unknown effects (Table 2.1). Thus, we predict that among all currently known unclassified mutations, only R228Q is pathogenic. In Table 2.1 we also compared our KNN classification results with the predictions from Polyphen. Consistent with our results, Polyphen predicted R228Q to be probably damaging. However, Polyphen gives contradictory predictions for eight additional mutations (predicted to be probably damaging), which are classified as non-pathogenic by our KNN classification. Overestimation of mutation deleteriousness was also observed when applying Polyphen to the known non-pathogenic mutations (Table S1).

Table 2.1. KNN classifications and Polyphen predictions of the mutations with unknown effects. P and N represent pathogenic and non-pathogenic mutations, respectively.

Mutation	KNN Classification	Polyphen	Mutation	KNN Classification	Polyphen
A41V	N	Benign	R228Q	P	Probably damaging
I44T	N	Benign	V330M	N	Probably damaging
A67T	N	Possibly damaging	V338M	N	Benign
I75F	N	Benign	F361L	N	Probably damaging
R81W	N	Probably damaging	T364M	N	Probably damaging
A97T	N	Possibly damaging	R367C	N	Probably damaging
V126I	N	Probably damaging	G424S	N	Probably damaging
V134L	N	Benign	G425S	N	Benign
A162V	N	Possibly damaging	R461C	N	Probably damaging

2.1 Case Study of Selected Mutations Using Molecular Dynamics (MD) Simulations

The above classification and analyses were performed using fast computational approaches and were applied to the entire dataset. We selected a subset of mutations for extensive MD simulations to investigate the possibility that pathogenic and non-pathogenic mutations have different effects on DHCR7 protein conformational dynamics. For this purpose, we selected 10 representative mutations including five pathogenic

mutations (T154R, E288K, T289I, G303R and R404C), two non-pathogenic mutations (R260Q and A452T) and three mutations with unknown effects (V134L, R228Q and F361L). These mutations are localized to different regions of protein structure. Five mutations (T154R, R228Q, E288K, T289I and G303R) are located in the transmembrane region and are buried in the membrane, two mutations (F361 and R404C) occur near the ligand-binding site and potentially affect ligand binding, and the remaining three mutations (V134, R260Q and A452T) are in neither the transmembrane region nor the ligand binding site.

Since our focus was on protein conformational dynamics, we calculated the corresponding RMSDs and RMSFs for the wild type and mutant proteins. The average RMSD data shows no obvious difference between wild type protein and proteins with non-pathogenic or pathogenic mutations. However, the average RMSF indicates some differences between the wild type and mutants. For example, in the mutant A452T, cytosol loops (CL) 2 and 4 and transmembrane domain (TM) 10 regions are more rigid compared to the wild type (Table 2.2). However, no apparent patterns were identified to differentiate pathogenic mutations and non-pathogenic mutations by simply observing the graphs. A previous study of the AGAL protein has indicated a correlation between the protein's flexibility and the severity of a mutant's pathogenicity [25]. Thus, to identify such potential correlation in DHCR7 protein, we mapped the pathogenic and non-pathogenic mutations on the average RMSF of the wild type proteins (shown in Figure S1). We observed that most pathogenic mutations are located on the low RMSF region while the non-pathogenic mutations show the opposite trend. As the low RMSF residues

are mostly transmembrane, such observed correlation is expected when majority of the pathogenic mutations are located on the transmembrane region. In addition, further analysis was performed by grouping the residues into different regions and then summing up the RMSF of residues in that region to get a region-RMSF. Based on DHCR7 protein structure information [26], residues were grouped into regions: TM1 (residues 40–60), TM2 (residues 94–115), TM3 (residues 145–164), TM4 (residues 176–191), TM5 (residues 235–256), TM6 (residues 268–288), TM7 (residues 302–326), TM8 (residues 332–352), TM9-10 (residues 408–442), CL1 (residues 116–144), CL2 (residues 198–234), CL3 (residues 289–301), CL4 (residues 354–407) and CTD (residues 443–475). The topology of the cytosol loops (CL), the C terminal domain (CTD) and transmembrane domains (TM) mapped with selected mutations are further represented for better visualization of the DHCR7 structure (Figure 2.2).

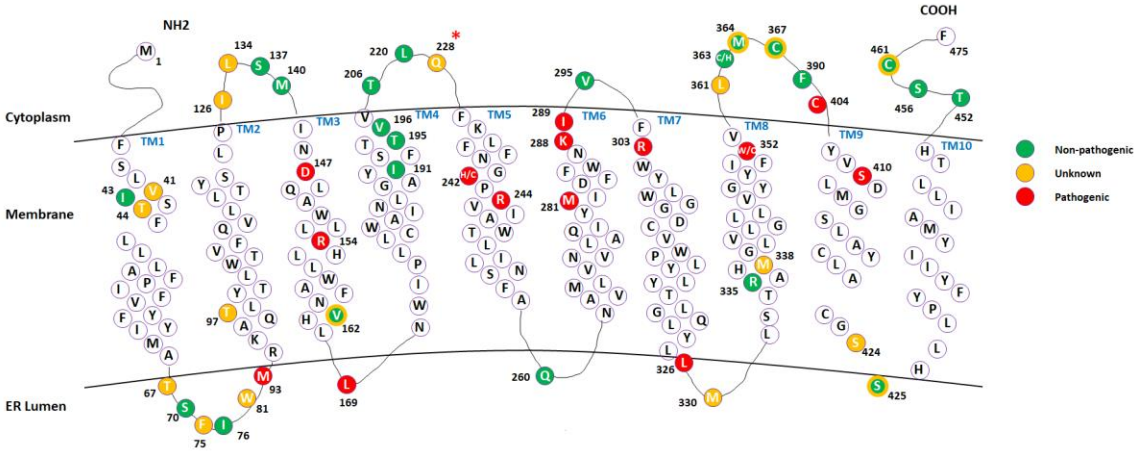

Figure 2.2. The topology of the cytosol loops (CL), the C terminal domain (CTD) and transmembrane domains (TM) in DHCR7 structure. Mutation sites are mapped with different colors according to mutation type (double color is

applied for sites with unknown and non-pathologic classification). The unclassified mutation R228Q, which we predict to be pathogenic, is highlighted with a red asterisk.

Table 2.2 shows the region-RMSFs. Pathogenic mutations tend to decrease the flexibility in the TM1, TM2 and CL2 regions and increase the flexibility in the TM7 and TM9-10 regions. Very little is known about DHCR7 function and structural changes occurring during chemical reactions, so we used the above observation to suggest an empirical formula that discriminates between pathogenic and non-pathogenic mutations, which were subjected to MD simulations (ideally, one should perform such an analysis for mutations analyzed in this manuscript, but this is too computationally demanding). For the wild type and each mutant, we sum the RMSFs of TM1, TM2 and CL2 and then subtract the RMSFs of TM7 and TM9_10 (last column in Table 2.2). We refer to this quantity as cumulative RMSF. The wild type and non-pathogenic mutants have cumulative RMSFs larger than 50 Å while all pathogenic mutants have a cumulative RMSF less than or equal to 46 Å. Among non-classified mutations, V134L is confirmed to be non-pathogenic, while R228Q and F361L show the same cumulative RMSFs as pathogenic mutations. Thus, it is encouraging to observe that R228Q is independently confirmed to be pathogenic mutation (see KNN classification above), while F361L cannot be classified with high confidence and additional investigations are reported in the next section.

Table 2.2. RMSF values per structural region (see text for details) for each of the mutants. The RMSFs are given in Å units. The last column reports the RMSF calculated as the sum of RMSFs of TM1, TM2 and CL2 subtracted by RMSF of TM7 and TM9-10. Values larger than 50 Å are underlined.

Pathogenic Missense Mutations															
	TM1	TM2	TM3	TM4	TM5	TM6	TM7	TM8	TM9_10	CL1	CL2	CL3	CL4	CTD	TM1+TM2-TM7-TM9_10+CL2
T154R	22.9	17.6	15.5	9.1	14.5	17.4	17.2	17.8	31.6	53.1	48.6	8.8	78.8	30.8	40.3
E288K	19.6	16.4	16.0	10.5	13.1	14.3	16.0	18.4	26.1	49.2	38.6	13.6	77.5	32.0	32.7
T289I	25.2	18.0	17.8	12.8	14.1	14.3	19.5	17.5	28.7	50.1	47.9	10.1	71.1	30.4	42.9
G303R	21.1	18.9	16.8	11.0	13.3	16.0	18.2	17.0	30.2	50.5	49.2	10.4	65.0	30.1	40.9
R404C	23.4	16.5	16.0	10.8	16.0	16.8	20.8	23.3	31.6	48.9	57.4	10.0	80.1	32.7	44.9
Missense Mutations with Unknown Effects															
	TM1	TM2	TM3	TM4	TM5	TM6	TM7	TM8	TM9_10	CL1	CL2	CL3	CL4	CTD	TM1+TM2-TM7-TM9_10+CL2
V134L	20.1	21.1	18.8	11.0	14.7	13.6	16.6	16.2	27.7	52.9	53.4	11.0	79.7	35.6	50.3
R228Q	17.6	17.0	15.9	8.5	13.6	13.0	15.6	16.9	27.6	53.2	54.2	10.7	75.8	36.7	45.6
F361L	19.4	17.4	14.8	9.9	14.2	14.0	18.3	16.6	28.8	54.7	50.8	11.7	74.8	33.5	40.5
Non-Pathogenic Missense Mutations															
	TM1	TM2	TM3	TM4	TM5	TM6	TM7	TM8	TM9_10	CL1	CL2	CL3	CL4	CTD	TM1+TM2-TM7-TM9_10+CL2
R260Q	19.7	18.6	15.5	9.4	12.9	14.6	15.4	17.2	24.4	58.4	52.1	11.4	79.3	28.1	50.6
A452T	20.9	19.6	17.8	10.5	13.6	16.2	16.4	17.2	26.0	55.2	52.8	8.7	66.6	30.1	51.0
Wild Type															
	TM1	TM2	TM3	TM4	TM5	TM6	TM7	TM8	TM9_10	CL1	CL2	CL3	CL4	CTD	TM1+TM2-TM7-TM9_10+CL2
WT	18.2	18.3	17.9	10.7	16.3	16.0	18.5	18.1	31.1	51.9	65.1	13.0	80.4	37.8	52.0

2.4. Analysis of Mutations' Pathogenic Effects:

2.4.1. Ligand Binding

Here, we investigated the possibility that mutations may change DHCR7 functionality by altering the binding affinity towards its ligand NADPH. For this purpose, we compared the effects of F361L (non-classified) and R404C (pathogenic mutation), both located near the NADPH binding site. It is anticipated that NADPH binding will cause structural rearrangement of the binding site and the conformational flexibility of the binding pocket is essential for proper protein function. We tested the effects of F361L and R404C on binding pocket flexibility by comparing them with the wild type protein. This was done using the MD trajectories obtained above and computing the residue cross-correlation for each trajectory with Bio3D [27]. These types of analyses were successfully used to elucidate the effects of a single mutation on the human β2-microglobulin's protein dynamics [28]. For each mutation and wild type, we calculated the average cross-correlation from three independent MD runs. Finally, the residue cross-correlation changes for mutations F361L and R404C are shown in Figure 2.3A,B, which is the subtraction of the averaged cross-correlation map between mutant and wild type proteins. Significant changes of the cross-correlation coefficient near the NADPH binding site were found for R404C, highlighted with a circle in Figure 2.2, but not for F361L.

We also performed MM/PBSA analysis to investigate the effect of mutations on NADPH binding affinity (Figure 2.3D). Mutation R404C results in a large increase of the

binding affinity by about 15 kcal/mol. As shown in the literature [21,29,30], any large deviation from wild type characteristics may be deleterious. In this case, R404C mutations contribute to disease by altering the binding affinity of NADPH. Compared to the effect of F361L, we observe that binding affinity is much less affected. This, combined with correlation analysis, allows us to speculate that F361L is a non-pathogenic mutation.

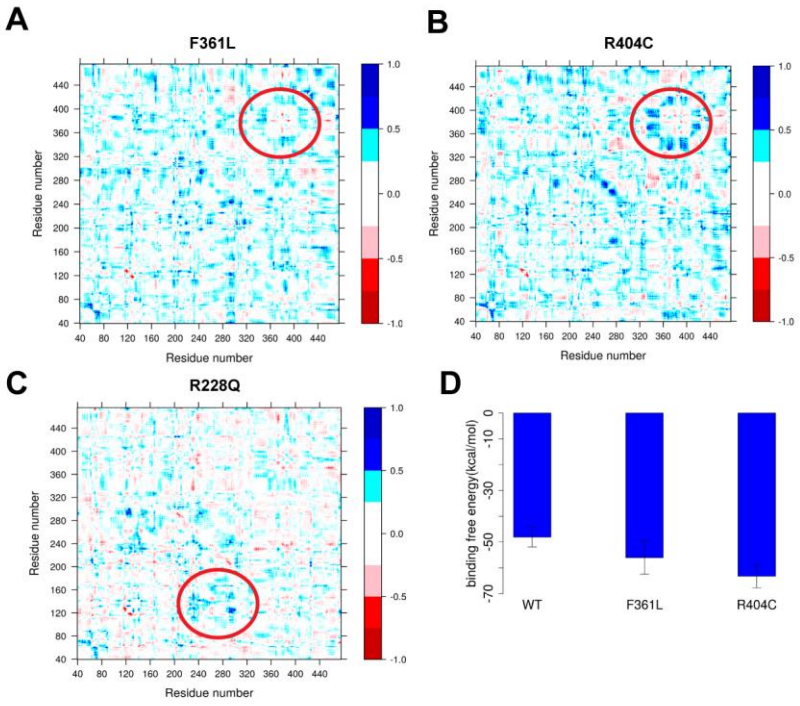

Figure 2.3. (**A**–**C**) The changes in residue cross-correlation for mutations F361L, R404C and R228Q; (**D**) NADHP binding free energy for WT and mutations F361L and R404C.

2.4.2. Protein Dynamics

We further analyzed the selected mutations including our predicted pathogenic mutation R228Q to identify other pathogenic effects on protein functionality. The residue cross-correlation analysis of R228Q (Figure 2.2C) indicates a local conformational change near the mutation site. The R228Q mutation makes the corresponding region more rigid, resulting in local flexibility changes in CL2. Changes in protein dynamics are also observed in the residue cross-correlation analysis of other pathogenic mutations such as E288K and G303R (shown in Figure S2), indicating that alterations in DHCR7 protein dynamics likely contribute to protein dysfunction.

2.5. Allele Frequency Analysis

We compared the frequency distribution of pathogenic mutations and frequently-occurring common mutations among different populations and genders. Figure 2.4A displays the top 40 *DHCR7* mutations of varying types occurring in more than 50 individuals archived in the ExAC database. At the same time, Figure 2.3B shows the distribution of pathogenic missense mutations chosen for this study within the same set of populations. The most frequently-occurring mutations in the general population are found in individuals of non-Finnish European descent followed by South Asian and African and African American descent (Figure 2.4A). Additionally, individuals of non-Finnish European and South Asian descent have the highest frequency of pathogenic mutations as shown in Figure 2.4B. African and African American populations have few cases of SLOS despite high occurrences of *DHCR7* mutations. The low occurrence and frequency of mutations in Europeans of Finnish descent is supported by the extremely low number of SLOS cases in Finland [31].

Interestingly, females in the overall ExAC population possess more *DHCR7* mutations at higher frequencies than males (Figure 2.4C), while this is an opposite for the pathogenic mutations investigated in this manuscript (Figure 2.4D), though no support for this trend has been found in the literature. One can speculate that this is linked to sex hormones and is embryo lethal, but the observation that females carry more pathogenic mutations than males should be taken with precaution.

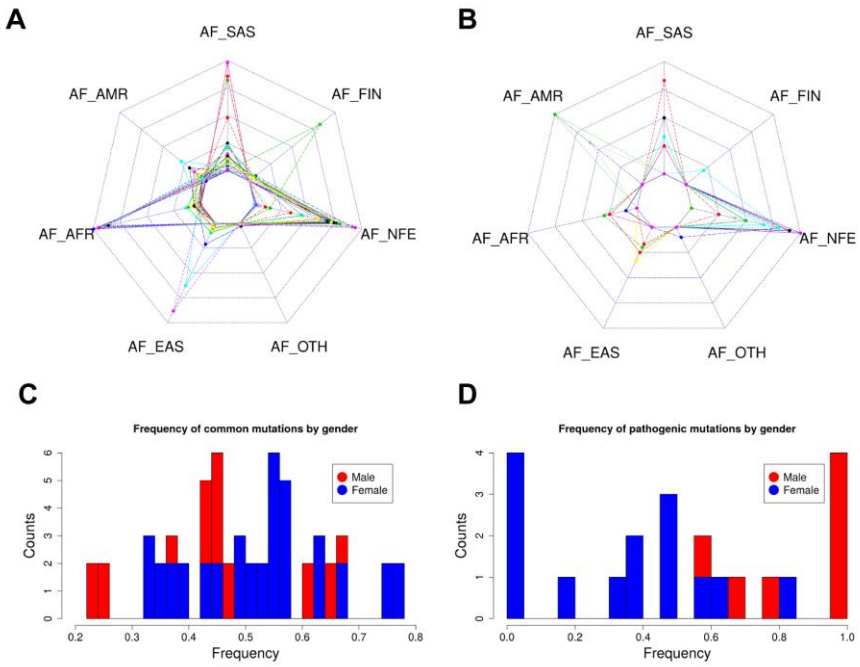

Figure 2.4. The frequency distribution of *DHCR7* mutations. AFR, AMR, EAS, FIN, NFE, SAS and OTH represent African and African American, American, East Asian, Finnish, Non-Finnish European, South Asian and other populations, respectively. (**A**) The frequency distribution among different populations of the top 40 DHCR7 mutations of varying types occurring in more than 50 individuals archived in the ExAC database; (**B**) The frequency distribution among different

populations of pathogenic missense mutations chosen for this study; (**C**) The frequency distribution in males and females of the top 40 DHCR7 mutations of varying types occurring in more than 50 individuals archived in the ExAC database; (**D**) The frequency distribution in males and females of pathogenic missense mutations chosen for this study.

3. Materials and Methods

3.1. Selection of DHCR7 Missense Variants

The missense mutations investigated in this work were selected using ClinVar [32] and ExAC [33] databases. The ClinVar database (https://www.ncbi.nlm.nih.gov/clinvar/) was queried using the search term "DHCR7". The results were further refined by missense mutations consisting of benign (2), likely benign (3), uncertain significance (30), likely pathogenic (15), pathogenic (26) and conflicting reports of pathogenicity (3) (as of 13 November 2017). The ExAC (Exome Aggregation Consortium) Browser (http://exac.broadinstitute.org/) was queried using the search term "DHCR7" and the entries were sorted by allele frequencies in descending order. The missense variants with an allele frequency greater than 0.00001, which were also classified in ClinVar were chosen for further *in silico* analysis. Of the chosen mutations, the variants defined as pathogenic or likely pathogenic in Clinvar database are classified as pathogenic mutations in this study while the others defined as uncertain significance in Clinvar database are classified as mutations with unknown effects. E288K and G303R are previously reported SLOS-causing mutations [34,35] although they are not classified as

pathogenic in the Clinvar database. Thus, E288K and G303R were treated as pathogenic mutations in this study. Overall, 16 pathogenic mutations and 18 mutations with unknown effects are classified for this study.

3.2. Selection of Non-Pathogenic DHCR7 Mutations

We first obtained the missense mutations in *DHCR7* gene from the ExAC database [33], including the whole genome sequencing data from 60,706 unrelated individuals. In total, 280 missense mutations in *DHCR7* were identified. The ExAC database also provides the corresponding allele frequency data from the 1000 Genomes Project and the NHLBI-GO Exome Sequencing Project (ESP) for each mutation. Individuals participating in the 1000 Genomes Project were all healthy while the objective of the ESP is discovery of novel genes and mechanisms contributing to heart, lung and blood disorders. As our goal was to select non-pathogenic mutations from the ExAC database, we applied the following selection criteria: (a) mutations with allele frequency >0 in the 1000 Genomes Project; (b) mutations with allele frequency of 0 in the ESP. Thus, we classified the mutations identified from the healthy population of 1000 Genomes Project but not from the ESP as non-pathogenic mutations in this study. In total, 23 non-pathogenic missense mutations were identified.

3.3. Obtaining Allele Frequency and Gender Occurrence

The allele frequency and gender data of *DHCR7* mutations were obtained from EXAC database [33]. The most recent database version was downloaded from the FTP site (http://exac.broadinstitute.org/downloads) and mutations affecting the DHCR7

protein as well as their corresponding allele frequencies and gender data were obtained. The frequency of mutation by gender is calculated by the number of carrier females or males divided by the total number of carrier individuals.

3.4. Generation of the 3D Model for DHCR7

The 3D structure of the DHCR7 protein was generated by homology modeling due to lack of an existing experimental structure. Structure of the integral membrane sterol reductase from *Methylomicrobium alcaliphilum* (PDB: 4QUV) [19] was used as a template and subjected to MODELLER [36] for homology modeling. The sequence identity between the template and DHCR7 is 37% (sequence alignment is shown in Figure S3) and thus high structural similarity was observed between the generated model and template. The model with lowest DOPE score was selected for this study and further subjected to automatic loop refinement with MODELLER [36].

3.5. Property Distance (PD)

To quantify the physical-chemical property differences between the wild type and mutant residues, we used the property distance (PD) as a parameter to quantitatively describe such changes. In this study, we describe physical-chemical properties of a particular residue using a property vector which includes two elements: hydrophobicity and charge. The hydrophobicity of the residues are taken from an experimentally determined hydrophobicity scale [37,38]. R and K carry +1 charges while E and D have −1 charges. All other residues are considered neutral. PD represents the Euclidean

distance of the property vector between the wild type and mutant residues (shown in Equation (1)). The *PD* between all types of residues are shown as a matrix in Figure 2.5.

$$PD(x,y) = \sqrt{(H(x) - H(y))^2 - (Q(x) - Q(y))^2} \quad (1)$$

where *x* and *y* represent two types of residues; *H* and *Q* are corresponding hydrophobicity and charge for a particular residue.

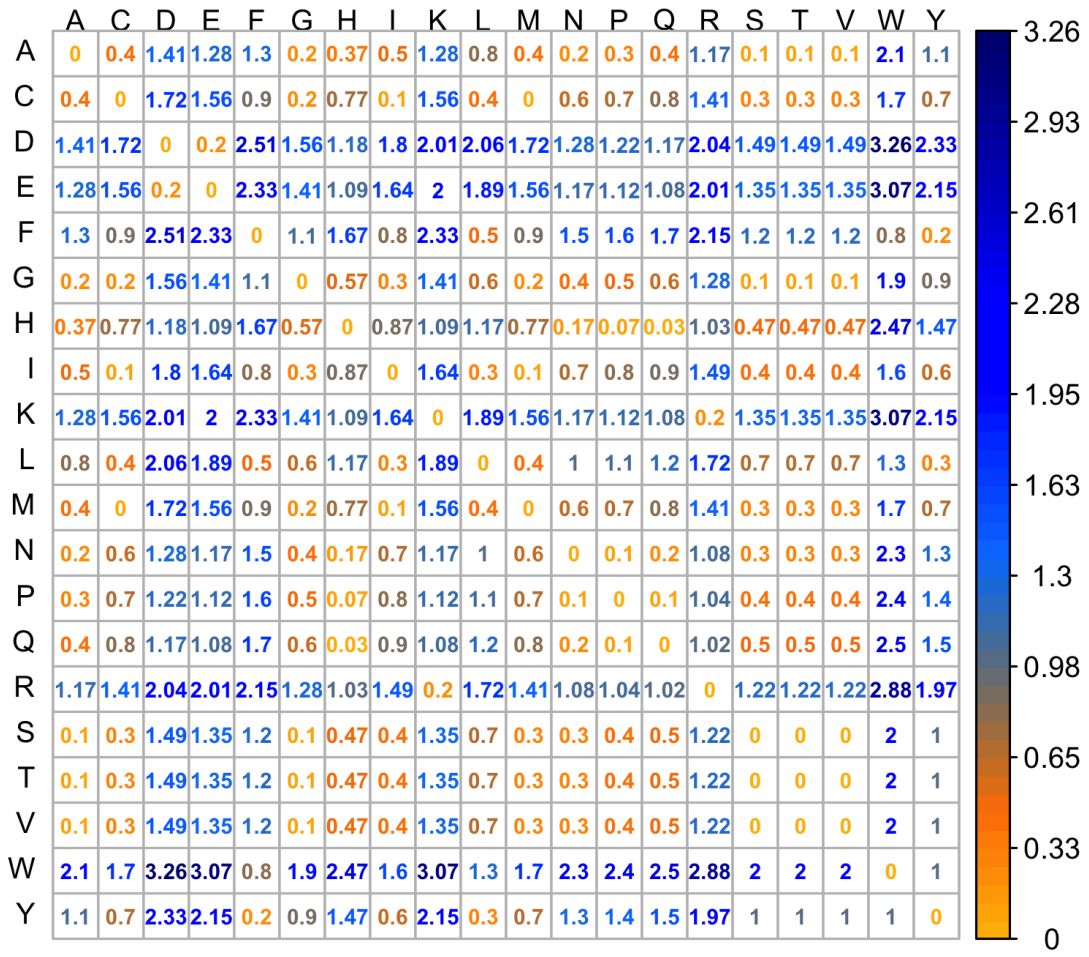

Figure 2.5. Property distance for all types of amino acid pairs.

3.6. Evolutionary Conservation Score (EC Score) Calculation

The DHCR7 sequence from 35 different species were collected from UnitProt [39] and subjected to multiple sequence alignment with the T-Coffee webserver [40]. The EC score of each residue in the human DHCR7 sequence was calculated using the multiple sequence alignment with the following equation:

$$EC\ score(i) = \frac{N(i)_{identity}}{N(i)_{total}} \quad (2)$$

where $N(i)_{identity}$ is the number of the species sharing identical residues in position *i* of the human DHCR7 sequence and $N(i)_{total}$ is the total number of the species in the multiple sequence alignment.

3.7. Folding Free Energy Change (ΔΔG) and Relative Solvent Accessible Surface Area (rSASA) Calculation

Several webservers were used to predict the effect of mutations on protein stability (folding free energy change (ΔΔG)) using the generated homology model of DHCR7 protein. The webservers used in this study include DUET [41], Eris [42], mCSM [43], SDM [44], Foldx [45] and SAAFEC [46]. The SASA were calculated using VMD [47]. As DHCR7 is a transmembrane protein, the membrane was also included when calculating the SASA. Thus, only the amino acids exposed to water were treated as exposed and the transmembrane regions were treated as buried in the calculation. The rSASA for residues were calculated using the following equation:

$$rSASA(i) = \frac{SASA(i)}{SASA(i)_{max}} \quad (3)$$

where *SASA*(*i*) is the SASA measured for particular residue *i* and $SASA(i)_{max}$ is the maximum SASA obtained for a free residue (entire residue taken off the protein).

3.8. Molecular Dynamic Simulations

The membrane-protein-ligand system was built primarily using the CHARMM-GUI [48] tools. The DHCR7 protein with ligand structure was obtained from previous homology modeling. Ten mutant (V134L, T154R, R228Q, R260Q, E288K, T289I, F361L, G303R, R404C and A452T) structures were derived from the wild type DHCR7 protein structure using VMD 1.9.3 [47] mutator package. The protein was embedded in a POPC bilayer using the CHARMM-GUI website. The protein was oriented to align with 4QUV structure in the OPM [49] database. When the oriented protein was placed into the membrane, the z axis of the protein matched the z axis of the membrane. The whole system was solvated with 0.15 M KCl. The final system was 89.13 × 89.13 × 96.64 $Å^3$ with a total of about 70,800 atoms.

Molecular dynamic simulation (MDS) was performed using NAMD2.11 [50]. The system first underwent energy minimization for 10 ps, then equilibrated through 6 cycles where harmonic constraints were applied to keep original positions of: (a) lipid head groups (force constants were gradually reduced from 5 $kcal \cdot mol^{-1} \cdot Å^{-2}$ to 0 $kcal \cdot mol^{-1} \cdot Å^{-2}$), (b) protein backbone (force constants were gradually reduced from 10 $kcal \cdot mol^{-1} \cdot Å^{-2}$ to 0 $kcal \cdot mol^{-1} \cdot Å^{-2}$), and (c) protein sidechains (force constants were

gradually reduced from 5 kcal·mol^{-1}·Å^{-2} to 0 kcal·mol^{-1}·Å^{-2}). In addition, dihedral restraints were applied to keep cis double bonds and c2 chirality (force constants were gradually reduced from 500 kcal·mol^{-1}·Å^{-2} to 0 kcal·mol^{-1}·Å^{-2}). A 1 fs timestep was used in the first few cycles and then switched to 2 fs for wild type whereas much smaller timesteps such as 0.01 fs were used for mutants to prevent restraints from failing. In the first two cycles, NVT simulation was performed and then switched to NPT simulation in the later cycles. Temperature was held at 303.15 K using a Langevin thermostat with a damping coefficient of 10 ps^{-1} and velocity rescaling thermostat. The pressure was maintained at 1 atm using a Langevin piston barostat with an oscillation period of 50 fs and a damping time constant of 25 fs. Electrostatic interactions between charged atoms were calculated using the particle mesh Ewald method. Van der Waals interactions were truncated at 12 Å with a switching function applied from 10 Å. RATTLE is used to constrain the length of all bonds involving a hydrogen atom. This stage of equilibration lasts for tens of ps to hundreds of ps. Then three 10 ns equilibration and 10 ns production runs with no constraints were performed for the wild type and each mutant. A 2 fs timestep was used. No velocity rescaling thermostat was used. Other conditions are the same as the previous stage. RMSD and root mean square fluctuation (RMSF) with the structure at the beginning of the 10 ns run as the reference structure were calculated using VMD 1.9.3.

3.9. MM/PBSA Analysis

To estimate the binding affinity of the DHCR7 protein with the ligand NADPH, we calculated the binding free energy using the MM/PBSA approach. For this purpose, we

performed three independent 20 ns MD simulations as described above. We took the frames with an interval of 20 ps from the last 10 ns and a total of 500 frames were selected from each trajectory. All ions, water and lipids were removed before MM/PBSA energy calculations. All the energy terms were averaged over 500 frames for each trajectory and the mean and standard deviation of binding free energy were calculated for wild type and mutant structures. The internal energy and van der Waals interactions were calculated using NAMD2.11b [50] by subjecting the structure to a one step equilibration at 300 K using dielectric constant = 2 for protein and = 80 for solvent. The electrostatic components of the binding free energy (Coulombic and solvation energy) were calculated by solving the Poisson Boltzmann (PB) equation using the Delphi program [51] with dielectric constant = 2 for protein and = 80 for solvent. The solvent accessible surface area (SASA) was calculated by VMD [47] with the solvent and lipid. The non-polar component of the solvation was further calculated with the following widely-used equation:

$$G_{SASA} = \alpha \cdot SASA + \beta \quad (4)$$

where α = 0.0054 and β = 0.92 kcal/mol.

3.10. K-Nearest Neighbors (KNN) classIfication

K-Nearest Neighbors algorithm was used to classify the missense mutations with unknown effects in DHCR7 protein. The dataset includes 16 pathogenic missense mutations and 23 non-pathogenic missense mutations (non-classified/unknown effect mutations were excluded). The dataset was randomly partitioned into a training dataset

(29 mutations) and a testing dataset (10 mutations). The KNN classification was performed using R program and various numbers of K values were tested to obtain the best performance.

4. Conclusions

We investigated the effects of mutations causing SLOS on the biophysical characteristics of DHCR7 protein with the goal of identifying methods allowing the discrimination of pathogenic mutations from non-pathogenic mutations. We found that pathogenic mutations are located either within the transmembrane region or are near the ligand-binding site and are highly conserved between species. In contrast, non-pathogenic mutations observed in the general population are located outside the transmembrane region and have different effects on the conformational dynamics of DHCR7. Our analyses confirmed the inability of folding free energy modeling to deliver reliable results and to be used to discriminate pathogenic from non-pathogenic mutations in membrane proteins. Future investigations may include modeling the effects of *DHCR7* mutations on melting temperature (Tm) via MD simulations conducted at different temperatures using the methodology adopted from recent work on NBD1 domain [52]. As mentioned in the work of Estacio et al. [52], the decrease of Tm may cause the protein to adopt partially misfolded states that become targeted for degradation.

In this work, using three characteristics: solvent exposure of the mutation site, residue conservation and physico-chemical descriptors, we were able to distinguish between pathogenic and non-pathogenic mutations. This observation, along with

extensive MD simulations and MM/PBSA modeling, was used to classify R228Q as a pathogenic mutation.

Taken together, these observations suggest that the non-classified mutation R228Q is in fact pathogenic. The analyses performed indicate that pathogenic effects may be of different origin, from affecting protein stability and dynamics to altering binding affinity and flexibility of the binding site.

Supplementary Materials: Supplementary materials can be found at http://www.mdpi.com/1422-0067/18/1/xx/s1.

Author Contributions: Yunhui Peng, Rebecca Myers, Wenxing Zhang and Emil Alexov collected the data and performed the computational analysis. Yunhui Peng, Rebecca Myers, Wenxing Zhang and Emil Alexov wrote the manuscript.

Conflicts of Interest: The authors declare no conflict of interest.

Abbreviations

SLOS	Smith-Lemli-Opitz syndrome
DHCR7	7-dehydroxycholesterol reductase
ER	Endoplasmic reticulum
ExAC	Exome Aggregation Consortium
PD	Property distance
ECS	Evolutional conservation score
RMSD	Root mean square deviation
RMSF	Root mean square fluctuation
SASA	Solvent accessible surface area
rSASA	Relative solvent accessible surface area
KNN	K-Nearest Neighbors
MD	Molecules dynamics
CL	Cytosol loops
TM	Transmembrane domain
ESP	Exome Sequencing Project

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CHAPTER THREE

THE EMERGING ROLE OF INTERLEUKIN 17 (IL-17) IN THE PATHOGENESIS OF ENDOMETRIOSIS

(Submitted to *Biological Research for Nursing*)

Abstract

Autoimmune and chronic inflammatory diseases such as rheumatoid arthritis, psoriasis, and multiple sclerosis have been linked to T helper 17 (Th17) cells and their cytokine, interleukin 17 (IL-17). Produced primarily by lymphocytes and acting on innate cells, IL-17 acts as a bridge between adaptive and innate immune responses. Th17 cells and IL-17 have been identified for their role in the pathogenesis of chronic inflammatory endometriosis. This narrative review will discuss the incidence, etiology and pathogenesis of endometriosis. It explores how immune cells involved with endometriosis produce IL-17, working with other cytokines to prolong the inflammatory response by stabilizing their messenger ribonucleic acid (mRNA) transcripts. The role of IL-17 to initiate the transcription of inflammatory factors through the nuclear factor-kappa B (NF-κB), mitogen-activated protein kinase (MAPK) and CCAAT/Enhancer-binding protein (C/EBP) pathways will be examined. Finally, this review will discuss current diagnosis and treatment of endometriosis, as well as future individualized treatment for women with endometriosis based on elevated IL-17 levels that target the IL-17 pathway.

Keywords: Endometriosis, interleukin 17, inflammation

Introduction

Endometriosis is an estradiol (E2)-dominant disease; the primary treatments currently consist of E2 inhibition, pain management with non-steroidal anti-inflammatory drugs (NSAIDS), and surgical interventions that include laparoscopic removal of abdominal lesions and adhesions (Greene et al., 2016; Hickey, Ballard, & Farquhar, 2014). Recently, interleukin 17 (IL-17) emerged as a potential negative mediator of inflammation in endometriosis, thus presenting as a target for novel therapies. The two-fold purpose of this narrative review is to explore literature targeting the role of IL-17 in the pathogenesis of endometriosis and to identify it as a target for potential new treatments for women experiencing this disease.

Endometriosis affects 176 million women globally (Lessey & Young, 2014). As a chronic and progressive inflammatory disorder, endometriosis is characterized by the growth of endometrial tissue outside of the uterus, typically in the pelvic cavity (ovaries, uterosacral ligaments, peritoneum, Pouch of Douglas and rectovaginal septum). These endometrial tissues, also called ectopic lesions, may grow in any place in the human body; even the lungs, brain and skin. The cells of these lesions proliferate and spread as they respond to cyclic hormonal signals, resulting in dysmenorrhea, inflammation, pelvic pain, and painful sexual intercourse. Symptoms of pleural endometriosis may include isolated chest pain, hemothorax and pneumothorax (Visouli et al., 2012). Additionally, women with this condition may also experience menstrual abnormalities and infertility (Hickey et al., 2014; Mahutte & Arici, 2002). While links between endometriosis and

infertility are not well-researched, approximately 25-50% of women with unexplained infertility also have endometriosis (Bulletti, Coccia, Battistoni, & Borini, 2010).

Etiology and Pathogenesis of Endometriosis

Endometriosis is thought to develop via retrograde menstruation, a phenomenon in which high intrauterine pressure or uterine contractions force menstrual debris containing endometrial cells, out of the fallopian tubes and into the peritoneum (Vercellini, Viganò, Somigliana, & Fedele, 2014). Although many women experience this event, only 10% of women with retrograde menstruation display the signs and symptoms of endometriosis (Ahn, Monsanto, et al., 2015; Viganò, Parazzini, Somigliana, & Vercellini, 2004). Women with endometriosis may also exhibit genetic and immunological abnormalities predisposing them to disease development (Figure 3.1. Endometriosis pathogenesis) (Ahn, Monsanto, et al., 2015).

Figure 3.1. Endometriosis Pathogenesis

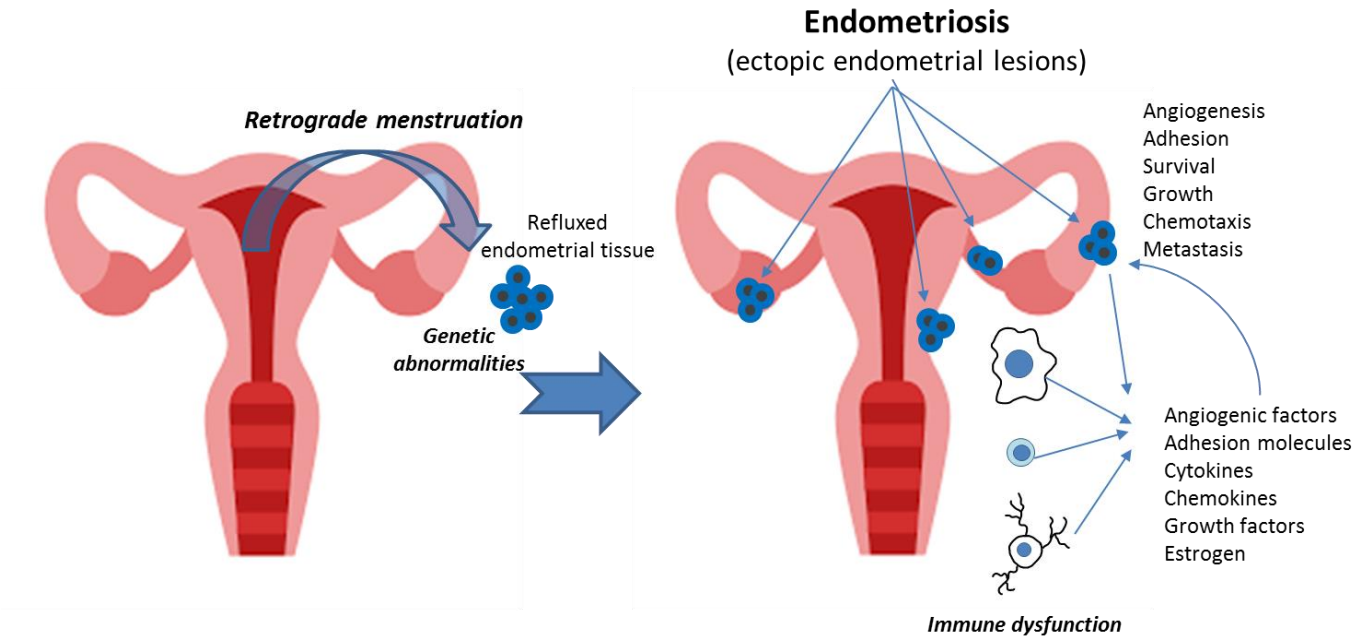

Figure 3.1. Endometriosis due to combination of retrograde menstruation, genetic abnormalities in endometrial tissue and immune dysfunction. Abnormal immune cells produce pro-inflammatory proteins that support their survival, growth and spread. Author derived.

Ectopic lesions consist of pseudo-endometrial tissues (stromal and glandular epithelial cells) permeated by dense vascular networks and surrounded by immune cells such as T lymphocytes, macrophages, and neutrophils (Ahn, Monsanto, et al., 2015; Nisolle, Casanas-Roux, Anaf, Mine, & Donnez, 1993). Additionally, ectopic lesion sites contain decreased levels of dendritic cells and natural killer cells compared to women without endometriosis (Tariverdian et al., 2009). The normal immunological response to cells in foreign locales consists of the initiation of acute inflammatory processes, coordination of the destruction of the ectopic cells, followed by resolution of inflammation. The combination of genetic abnormalities and immune dysfunction present in women at risk for endometriosis results in local immune cells supporting the lesions'

survival and growth by decreasing their cytotoxic activities and inducing a state of chronic inflammation through the production of interleukins and other pro-inflammatory cytokines (Ahn, Monsanto, et al., 2015). Both lesions and immune cells, primarily macrophages, produce matrix metalloproteinases which degrade the basement membrane and adhesion molecules permitting the anchorage of lesions to peritoneal structures. Additionally produced are angiogenic growth factors that allow lesions access to oxygen and nutrients via blood vessel formation, growth factors that allow for the survival and proliferation of ectopic tissues, and chemotactic molecules which draw additional immune cells to the site (Bacci et al., 2009; Tariverdian et al., 2009).

The presence of the macrophage-secreted cytokines tumor necrosis factor-alpha (TNF-α) and interleukin 1 beta (IL-1β) in the peritoneum induce refluxed endometrial stromal cells and peritoneal macrophages to overexpress cyclooxygenase-2 (COX-2), the rate-limiting enzyme involved in prostaglandin E2 (PGE2) synthesis. PGE2 is a critical factor regulating disease establishment and progression with levels of PGE2 higher in the peritoneal fluid of women with endometriosis than in healthy women (Wu et al., 2002). PGE2 first suppresses the phagocytic activities of macrophages and prompts them to secrete factors favorable for disease promotion, including IL-6 and transforming growth factor-beta (TGF-β) (Wu, Lu, Chuang, & Tsai, 2010). Additionally, PGE2 induces the production of angiogenic factors such as vascular endothelial growth factor (VEGF) and IL-8, stimulating the proliferation and migration of endothelial cells towards endometrial tissues to develop a vascular system in order to provide oxygen and nutrients (Wu et al., 2010). Finally, endometriosis progression is highly E2-reliant, with the stimulation of

endometrial cells by PGE2 to independently manufacture their own E2. This process promotes the production of growth factors involved in cell proliferation and survival. IL-17, produced at ectopic lesion sites, promotes inflammation and draws additional immune cells to the area by direct induction of the expression of factors involved in endometriosis, including TNF-α, IL-1β, COX-2 and PGE2, IL-6, IL-8. Additionally, IL-17 indirectly enhances the actions of TNF-α and IL-1β by stabilizing their gene transcripts (Ahn, Monsanto, et al., 2015; Bacci et al., 2009; Hirata et al., 2008, 2011).

Interleukin 17 (IL-17)

IL-17, a relatively newly discovered member of the cytokine family (Rouvier, Luciani, & Mattei, 1993), exists as six isoforms. These isoforms, IL-17A-F, are connected to the development of numerous autoimmune and chronic inflammatory conditions, such as psoriasis, rheumatoid arthritis and irritable bowel disease (Chen & Kolls, 2017). The chief immunity roles of IL-17 and its primary cellular source, clusters of differentiation 4 (CD4) + T helper 17 (Th17) cells, are to maintain mucosal defenses and to direct immune responses against extracellular bacteria such as *Klebsiella pneumoniae*, *Staphylococcus areus, and* fungi such as *Candida albicans* (Li, Casanova, & Puel, 2017). More recently, Th17 cells and IL-17 have been implicated in the pathogenesis of endometriosis. Zhang, et al. (2005) were the first to report an association between IL-17 peritoneal fluid concentration and the level of disease severity in endometriosis. Patients with mild or minimal endometriosis, particularly those with unexplained infertility, possessed significantly higher levels of IL-17 in the peritoneal fluid compared with more severe endometriosis cases (P=0.036) (Zhang, Xu, Lin, Qian,

& Deng, 2005). The authors concluded that IL-17 was essential for early disease development, inducing the production of inflammatory mediators such as IL-6, TNF-α, IL-1β, COX-2, and PGE2, all of which promote chronic inflammation and local E2 production. Additionally produced are matrix metalloproteinase 3 (MMP3) and intercellular adhesion molecule 1 (ICAM1) (Albanesi, Cavani, & Girolomoni, 1999; Zhang et al., 2005). Subsequently, Ahn, Edwards, et al. (2015) determined that removal of ectopic endometrial lesions resulted in a decrease in levels of IL-17 (Ahn, Edwards, et al., 2015).

Various immune cells involved in endometriosis produce IL-17, including activated Th17 cells, cytotoxic T-17 (Tc17) cells, gamma-delta T (γδT) cells, natural killer (NK) cells, neutrophils, and mast cells. Additionally, IL-17 targets gene expression in numerous cell types including those implicated in endometriosis, such as epithelial and endothelial cells, stromal cells, myeloid cells and immune cells (Cua & Tato, 2010; Eberl et al., 2004; Hueber et al., 2010; Kisielow, Kopf, & Karjalainen, 2008; Takatori et al., 2009; Taylor, Roy, Jr, Sun, & Howell, 2014). Acting through the heterodimeric IL-17 receptor (IL-17R) complex, IL-17 induces the production of factors related to the development and progression of endometriosis. These include: angiogenic factors such as VEGF and IL-8, which promote access to the local blood supply early in the disease process, intracellular adhesion molecules such as ICAM1, which allows endometrial cells to penetrate and grow on other tissues, and factors such as COX-2 and PGE2, which facilitate local E2 production. E2 initiates expression of factors required for the survival and proliferation of cells, as well as stimulating its own production via upregulation of

COX-2 by lesions, to perpetuate an unending cycle of inflammation (Gupta, Agarwal, Sekhon, & Krajcir, 2006). IL-17 has also been shown to also upregulate the production of IL-8 and COX-2 in endometrial stromal cells (Hirata et al., 2008, 2011). Due to their angiogenic, chemotactic and E2-producing capacities, both factors are highly implicated in the progression of endometriosis (Harada, Iwabe, & Terakawa, 2001; Ota, Igarashi, Sasaki, & Tanaka, 2001). Finally, treatment of endometrial carcinoma cells and human umbilical vein endothelial cells (HUVECs) with IL-17 revealed an increased production of angiogenic (VEGF, IL-8), pro-inflammatory (IL-1β, IL-6) and chemotactic (granulocyte-colony stimulating factor (G-CSF), C-X-C chemokine ligand 1 (CXCL1), CXCL12, CX3CL1, IL-8) factors (Ahn, Edwards, et al., 2015).

IL-17 modestly activates the production of IL-17-responsive inflammatory genes through the nuclear factor-kappa B (NF-κB), mitogen-activated protein kinase (MAPK) and CCAAT/Enhancer-binding protein (C/EBP) signaling pathways. More importantly, IL-17 works in tandem with other cytokines to stabilize messenger RNA (mRNA) and increase the lifespan of inflammatory transcripts (Figure 3.2). Upon IL-17 binding to the extracellular portion of IL-17R, the ubiquitin ligase adaptor protein NF-κB activator 1 (ACT1), which is required for activation of all IL-17 signal transduction pathways, moves to the intracellular portion of IL-17R where it attracts and ubiquinates tumor necrosis factor receptor-associated factor (TRAF) proteins. (Chang et al., 2006; Liu et al., 2009; Novatchkova et al., 2003). Activation of TRAF6 (Figure 3.2) results in the activation of the NF-κB, MAPK and C/EBP pathways and transcription of pro-inflammatory genes that code for various interleukins and other cytokines (IL-1β, IL-6,

TNF-α), chemokines (IL-8, G-CSF, CXCL1), and other factors, including pro-angiogenic VEGF and pro-estrogenic COX-2 and PGE2 (Table 1).

Figure 3.2. IL-17 Signaling

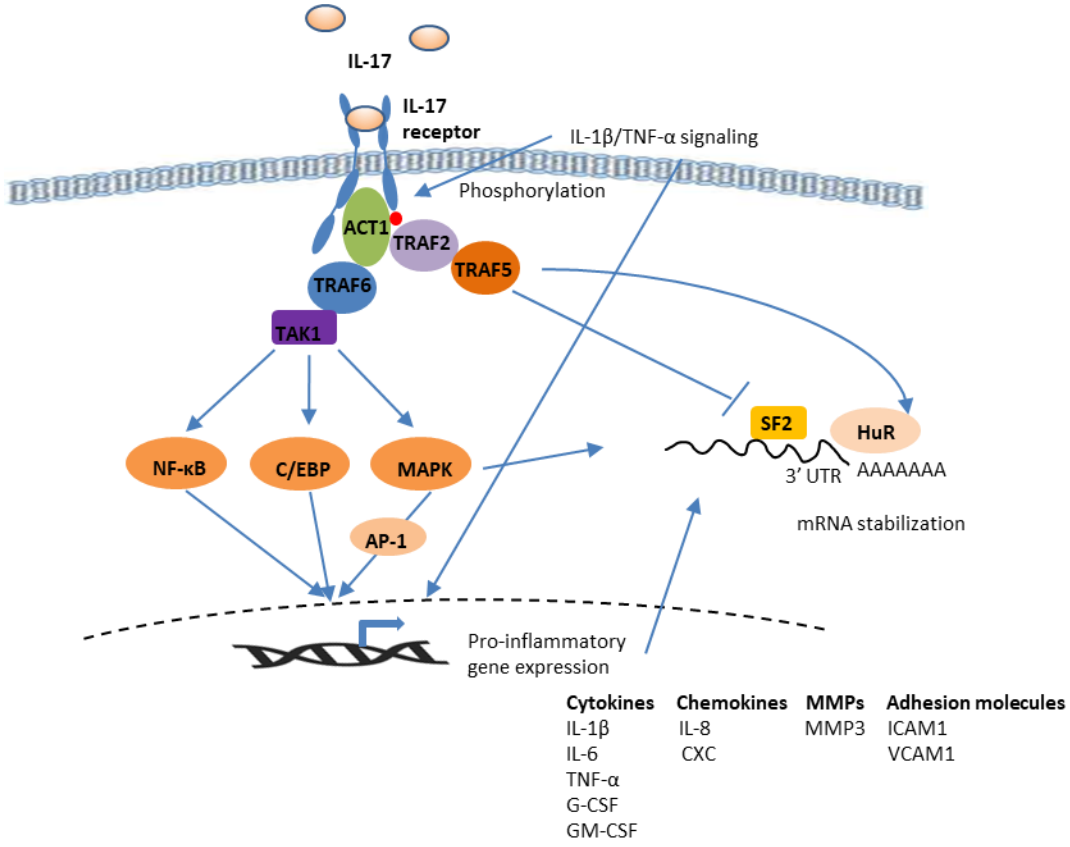

Figure 3.2. Binding of IL-17 to IL-17 receptor complex results in 1) the expression of pro-inflammatory cytokines such as IL-1β via the NF-κbB, C/EBP and MAPK pathways, or, 2) stabilizes the mRNA of other cytokines such as IL-1β or TNF-α by preventing the decay of their mRNA transcripts. Adapted with permission from: (Amatya, Garg, & Gaffen, 2017).

Table 3.1: Inflammatory Factors Involved in Disease Progression Associated with IL-17 or Th17 Activities

Cytokines	**Angiogenic factors**	**Adhesion molecules**	**Chemokines**	**E2-production factors**
TNF-α	VEGF	ICAM1	IL-8	COX-2
IL-1β	IL-8	VCAM1	G-CSF	PGE2
TGF-β			CXCL1	
IL-6			CXCL12	
IL-21			CX3CL1	
IL-22			CCR6	
IL-23			CCL20	
GM-CSF				

Note: TNF-α= tumor necrosis factor alpha; IL-1β= interleukin 1 beta ; TGF-β= tumor growth factor beta; IL-6= interleukin 6; IL-21= interleukin 21; IL-22=interleukin 22; IL-23=interleukin 23; GM-CSF= granulocyte-macrophage colony-stimulating factor; VEGF=vascular endothelial growth factor; IL-8=interleukin 8; ICAM1=intracellular adhesion molecule 1; VCAM1= vascular cell adhesion molecule 1;G-CSF= granulocyte-colony stimulating factor; CXCL1= chemokine (C-X-C motif) ligand 1; CXCL12= C-X-C motif chemokine 12; CX3CL1= chemokine (C-X3-C motif) ligand 1; CCR6= C-C motif chemokine receptor 6; CCL20= C-C motif chemokine ligand 20; COX-2=cyclooxygenase 2; PGE2= prostaglandin E2.

IL-17-mediated inflammation appears to be more potent when acting in concert with other cytokines, such as TNF-α, IL-1β, IL-22 and interferon gamma (IFN-γ) (Beringer, Thiam, Molle, Bartosch, & Miossec, 2017; Chabaud, Fossiez, Taupin, & Miossec, 1998; Gaffen, Jain, Garg, & Cua, 2014; Liang et al., 2006; van Nieuwenhuijze et al., 2014). The cooperation between IL-17 and TNF-α results in the stabilization of previously unstable TNF-α mRNA transcripts by preventing decay (Hartupee, Liu, Novotny, Li, & Hamilton,

2007; Karlsen, Borregaard, & Cowland, 2010; Miossec, 2003; Shen & Gaffen, 2008). In this process, the presence of IL-1 or TNF signaling results in the phosphorylation of ACT1, shifting priority from TRAF6 activation to TRAF2/5 activation by blocking TRAF6 binding. The ACT1/TRAF2/TRAF5 complex removes the pre-mRNA-splicing factor 2 (SF2) from the 3' untranslated region (UTR) of mRNA transcripts and recruits the mRNA stability factor human antigen R (HuR) to compete with SF2 for mRNA binding sites, thus preventing SF2-mediated RNA decay, while promoting transcript stability and translational efficacy. These processes lead to increased levels of mRNA transcripts of pro-inflammatory cytokines which are beneficial during an acute inflammatory response (such as an infection). However, without the need for active pathogen clearance or wound healing, this process may promote chronic inflammation present in such diseases as endometriosis. Indeed, Hirata et al. (2008; 2011) found that IL-17 acts synergistically with TNF-α to increase expression of pro-inflammatory factors such as COX-2 and IL-8 by endometrial stromal cells (Hirata et al., 2008, 2011). IL-17 treatment alone produced moderate increases in these factors, but the combination of TNF-α and IL-17 greatly increased levels.

T helper 17 Cells Produce IL-17

Several cell types have been shown to produce IL-17, but Th17 cells, identified in 2005, (Harrington et al., 2005; Park et al., 2005) are the primary source of IL-17 production. T helper cells are a subset of T lymphocytes, which support the immune response through the secretion of cytokines and chemokines to recruit and activate additional immune cells. There are several types of T helper cells (Th1, Th2, Th17, T

regulatory) each with distinct roles in immunity, from coordinating immune responses against pathogen-infected cells to dampening ongoing immune reactions. The normal functions of Th17 cells are to: 1) drive immunological reactions against extracellular bacteria and fungi, 2) produce anti-microbial compounds, attract neutrophils, and 3) maintain mucosal barrier immunity (Kolls & Khader, 2011; Stockinger, Veldhoen, & Martin, 2007; Ye et al., 2001). A combination of TGF-β plus IL-6 stimulation results in Th17 cell development, though other cytokines such as IL-1β and IL-13 may enhance the activation. Th17 cells (Figure 3.3. Development of IL-17-producing Th17 cells) are characterized by the expression of the transcription factor retinoic acid receptor-related orphan receptor gamma t (RORγt) and the production of IL-17, though other cytokines, including IL-21, IL-22, IL-6, TNF-α and granulocyte-macrophage colony-stimulating factor (GM-CSF) may also be secreted by Th17 cells (Gaffen, 2009).

Figure 3.3. Development of IL-17-Producing Th17 Cells

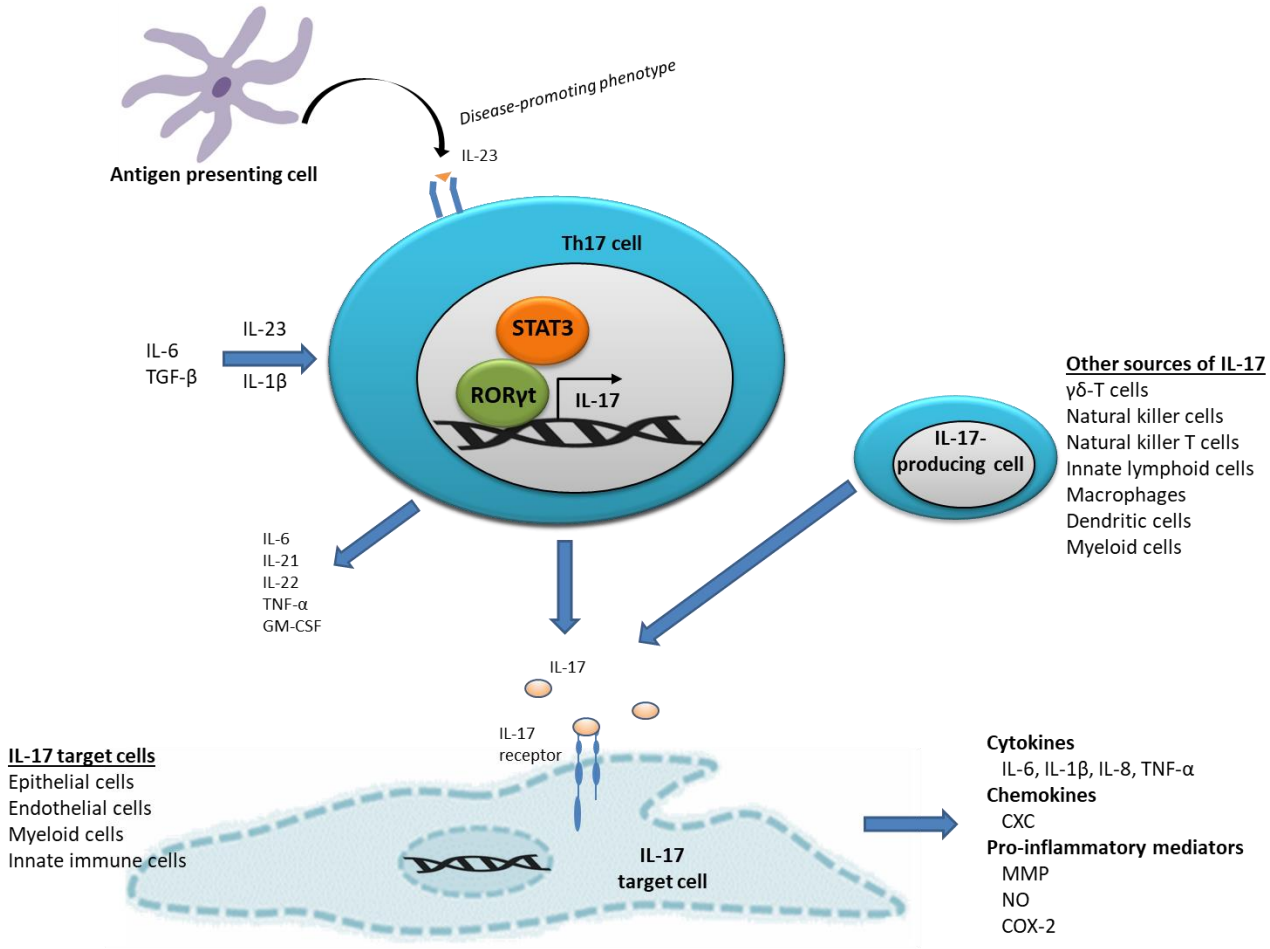

Figure 3.3. Stimulation by TGF-β and IL-6 active STAT3 and promote expression of Th17 master transcription factor RORγt. IL-23 secretion promotes disease-causing phenotype. Th17 cells may produce additional cytokines, including IL-6. Innate immune cells in endometriosis may produce IL-17. IL-17 binds IL-17 receptor complex on target cells, causing production of pro-inflammatory mediators. Author derived.

Th17 cells, while extensively linked to several autoimmune conditions, such as psoriasis (Martin et al., 2013), rheumatoid arthritis (Sarkar, 2010) and inflammatory bowel disease (Fujino et al., 2003), have not been widely investigated in relationship to endometriosis. The presence of Th17 cells at the sites of ectopic lesions was first reported in 2008 (Hirata et al., 2008). In another study, Hirata et al, 2010, corroborated the involvement of Th17 cells in endometriosis by reporting that Th17 lymphocytes near ectopic lesions express C-C chemokine receptor 6 (CCR6) while the ectopic lesions secret its ligand, the Th17 chemoattractant molecule C-C chemokine ligand 20 (CCL20), indicating that CCL20/CCR6 signaling is involved in drawing Th17 cells to ectopic lesions to further disease progression (Hirata et al., 2010).

A recent study comparing CD4+ T helper cell subsets in the blood, endometrium, and ectopic lesions of women with endometriosis found that T cell populations in lesions were skewed towards Th17 cells while endometria contained higher populations of Th1 cells (Takamura et al., 2015). There was no significant difference in Th2 ($p = 0.29$) or T regulatory ($p = 0.67$) cells between lesions and eutopic endometria. However, there were significantly more Th1 ($P=0.041$) cells in the blood of women with endometriosis compared to those without endometriosis, indicating a difference in inflammatory microenvironments both locally and systemically. Similarly, Gogacz, et al. (2016) reported that patients with severe endometriosis possessed a higher level of IL-17-producing Th17 cells in the peritoneal fluid than women with mild or minimal endometriosis (Gogacz et al., 2016).

Other researchers profiled cytokines and T helper cell subsets in the peritoneal fluid of women with varying stages of endometriosis and also found that, as previously reported, levels of IL-17, IL-6 and TGF-β as well as Th17 cells were elevated (Chang et al., 2017). Interestingly, in the more advanced cases of the disease, there were more anti-inflammatory cytokine-producing populations of Th17 cells, including IL-10 and IL-4. This indicates that as the disease progresses, the local environment begins a switch from pro-inflammatory-dominance to a balance between pro-and anti-inflammatory factors in later stages of disease progression. Thus, it appears that IL-17 promotes endometrial stromal cell angiogenesis, proliferation, and a pro-inflammatory environment while inhibiting adhesion in order to enhance the rapid spread of the disease throughout the tissues (Ahn, Edwards, et al., 2015; Chang et al., 2017). The suppressive effects of IL-10 on IL-17 in more advanced cases of endometriosis appears to result in an increase of lesion adhesions in ectopic sites, as well as deeper infiltration of endometriosis into affected tissues (Chang et al., 2017).

The ultimate determinant of whether Th17 cells will be involved in endometriosis appears to be attributed to local cytokines present during activation, particularly IL-23 (Aggarwal, Ghilardi, Xie, de Sauvage, & Gurney, 2003; Cua et al., 2003; Langrish et al., 2005). Andreoli et al. (2011) found elevated levels of IL-23, implicated in the development of pathogenic Th17 cells, in the peritoneal fluid of infertile patients with endometriosis (Andreoli et al., 2011). This suggests that IL-23 may also be an emerging factor in infertility in women with endometriosis although current research is limited at this time.

Current Diagnosis and Treatment of Endometriosis

As noted previously there is a proliferation of endometrial lesions that spread as they respond to cyclic hormonal signals, resulting in dysmenorrhea, inflammation, pelvic pain, and painful sexual intercourse. Menstrual abnormalities and infertility are also experienced by women with the diagnosis. (Hickey et al., 2014; Mahutte & Arici, 2002). While links between endometriosis and infertility are not well-researched, approximately 25-50% of women with unexplained infertility also have endometriosis (Bulletti et al., 2010).

When women present with pelvic pain and other symptoms associated with endometriosis, healthcare professionals should conduct a family history and physical examination which includes a thorough gynecologic history and examination. Tests to confirm differential diagnoses, such as nongynecological disorders causing pelvic pain (renal or urinary tract infections, irritable bowel syndrome, or sexually transmitted disease), may be indicated. Although ultrasound and other imaging modalities can detect the presence of endometrial lesions in women, definitive diagnosis of endometriosis is by histopathology, after laparoscopic or surgical removal of lesions (Armstrong, 2011).

While the patient with endometriosis is undergoing laparoscopy or surgery, the surgeon may elect to ablate, lyse or excise endometrial lesions. Surgical intervention may provide long-term pain relief, enable fertility, and/or delay surgically induced menopause via oophorectomy and hysterectomy (Journal of Obstetrics and Gynaecology Canada [JOGC], 2010). Initial medical treatment of endometriosis includes the use of oral, injectable, and intrauterine progestins, combined oral contraceptives, gonadotropin-

releasing hormone (GnRH) agonists, NSAIDs, and danazol. Since medical therapy with GnRH agonists may result in low E2 symptoms (insomnia, vaginal dryness, hot flashes, decreased libido and decreased bone mineral density), it is not unusual that low-dose hormone therapy and/or bisphosphonates and calcium supplementation is added to the medical treatment regimen (Armstong, 2011; JOCG, 2010).

The Role of IL-17 in Developing Future Treatments for Endometriosis

Currently, clinical intervention trials recruiting study participants experiencing endometrial pain include drug trials using cabergoline (a dopamine receptor agonist) with norethindrone acetate, a botulinum toxin which blocks acetylcholine and the GnRH agonists, elagolix and relugolix (Taylor et al., 2017). Recently IL-17, more specifically IL-17A, a product of the clathrin light chain A 8 (*CTLA8)* gene, is a viable drug target for treating chronic inflammatory conditions (sporatic arthritis and ankylosing spondylitis) that result from excessive levels of IL-17 (Miossec, 2017). The first inhibitor of IL-17A, secukinumab, a human monoclonal antibody (anti-IL-17A), has the indication for the treatment of moderate-to-severe psoriasis. Ixekizumab is a monoclonal antibody that neutralizes the effects of IL-17. Another monoclonal antibody that targets the IL-17A receptor, rather than the IL-17A cytokine, is broadalumab. However, the long-term use of these monoclonal antibodies has not been researched. Additionally, none of these drugs have been approved for treatment of other inflammatory disorders, including endometriosis (Campa, Mansouri, Warren & Menter, 2016; Wasilewska, Winiarska, Olsezewska & Rudnicka, 2016). Other drug candidates on the horizon that target the IL-17 pathway include anit-IL-17 nanoantibodies, dual anti-IL-17 and TNF- α inhibitors and

fynomers, engineered proteins that act as antibodies allowing for the production of bi-specific anti-TNF and anti-IL-17 A antibodies (Campa et al., 2016; Miossec, 2017; Wasilewska et al., 2016).

Conclusion

The cytokine IL-17 has been implicated in the pathogenesis of endometriosis. IL-17 directly regulates gene expression through the pro-inflammatory NF-κB, MAPK and C/EBP pathways and indirectly synergizes with other cytokines to stabilize and increase the lifespan of transient mRNA transcripts, thus prolonging an inflammatory response. IL-17 plays a role in perpetuating the cycle of E2-dominated inflammation at ectopic lesion sites, helping to create conditions suitable for the survival, growth and spread of these endometrial lesions to other parts of the body. Though primarily produced by Th17 cells, other cell types may also produce IL-17, particularly in the early stages of the disease, before adaptive immune cells (such as Th17 lymphocytes) have time to become activated. IL-17 appears to be an important factor in earlier stages of endometriosis, before conditions shift towards a more anti-inflammatory milieu.

IL-17 is a target for drug development that may reduce the inflammation and resultant pelvic pain experienced by women with endometriosis. Although many human monoclonal antibodies have been investigated and ultimately received approval to treat other inflammatory disorders, no monoclonal antibody is approved for the treatment of endometriosis. Until they gain expanded approval, drugs and therapies that only treat symptoms are available to women suffering with endometriosis.

The Authors declare that there is no conflict of interest.

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Abbreviations

ACT1:	NF-κB activator 1
AP-1:	activator protein-1
CCL:	C-C chemokine ligand
CCR:	C-C chemokine receptor
CD4+ Th cells:	cluster of differentiation 4 positive T helper
C/EBP:	CCAAT/Enhancer-binding protein
COX-2:	cyclooxygenase 2
CTLA:	clathrin light chain A
CXCL:	C-X-C chemokine ligand
E2:	estradiol
G-CSF:	granulocyte colony-stimulating factor
GM-CSF:	granulocyte-macrophage colony-stimulating factor
GnRH:	gonadotropin-releasing hormone
HuR:	human antigen R
HUVEC:	human umbilical vein endothelial cell
ICAM:	intercellular adhesion molecule
IFN-γ:	interferon gamma
IL:	interleukin
IL-17R:	IL-17 receptor
MAPK:	mitogen-activated protein kinase
MMP:	matrix metalloproteinase

mRNA:	messenger ribonucleic acid
NF-κB:	nuclear factor-kappa B
NOS:	nitric oxide synthase
NSAID:	non-steroidal anti-inflammatory drug
PGE2:	prostaglandin E2
RORγt:	retinoic acid receptor-related orphan receptor gamma t
SF2:	pre-mRNA-splicing factor 2
STAT:	signal transducer and activator of transcription
TAK:	transforming growth factor-beta-activated kinase
TGF-β:	transforming growth factor-beta
Tc17:	cytotoxic T 17
Th17:	T helper 17
TNF-α:	tumor necrosis factor-alpha
TRAF:	tumor necrosis factor receptor-associated factor
UTR:	untranslated region
VEGF:	vascular endothelial growth factor

CHAPTER FOUR

ASSESSMENT OF BETA-CARYOPHYLLENE (BCP) FOR THE TREATMENT OF ENDOMETRIOSIS USING AN *IN VITRO* APPROACH

Abstract

Endometriosis is an estrogen-dominant inflammatory condition featuring abnormal cell proliferation, angiogenesis and avoidance of apoptosis. The blood and peritoneal fluid of women with endometriosis exhibit elevated levels of estradiol (E2) and other pro-inflammatory factors including the cytokines interleukin 6 (IL-6) and IL-17 and the angiogenic mediator vascular endothelial growth factor (VEGF). Beta-caryophyllene (BCP) is a phyto-cannabinoid which selectively binds the anti-inflammatory cannabinoid receptor 2 (CB2). The effects of BCP were examined on cell proliferation, viability and VEGF expression in a cell culture model. In addition, the expression of cannabinoid receptors CB1 and CB2 were examined in two endometrial cancer cell lines and in endometrial tissue samples. Reverse transcription polymerase chain reaction (rtPCR) analysis revealed that both Ishikawa and RL95-2 cell lines express CB1, but not CB2. Thus, these cell lines are unlikely to respond to BCP stimulation. Immunohistochemistry showed that CB2 expression is upregulated in endometriosis-positive endometrial tissue compared with healthy tissue. BCP decreases IL-17-induced VEGF production in endometriosis-positive endometrial stromal cells but not when IL-6 is present. These results suggest that BCP may be a useful treatment option for endometriosis.

Keywords: Endometriosis, cannabinoids, cannabinoid receptor 1 (CB1)

Introduction

Endometriosis is a common gynecological condition estimated to affect 10% of women of reproductive age (Bulun, 2009). It is characterized by the growth of endometrial-like tissue in extra-uterine locations which respond to cyclical hormones, particularly estradiol (E2), build up, and shed into the surrounding area. (Bulun, 2009). The resulting chronic inflammatory situation may cause fatigue, pelvic pain, abnormal menstruation, and infertility (Hickey, Ballard, & Farquhar, 2014; Mahutte & Arici, 2002). Endometriosis occurs as the result of a combination of genetic, environmental and immunological factors (Berkkanoglu & Arici, 2003). Based on twin studies, endometriosis is estimated to be approximately 50% heritable (Saha et al., 2015). The remaining 50% results from environmental factors which may modify the epigenome. Increased DNA methylation of genes involved with endometriosis amelioration, such as progesterone receptor (PR) may occur (Kobayashi, Imanaka, Nakamura & Tsuji, 2014). Environmental risk factors include low birth rate, multiple pregnancies, childhood exposure to smoke and exposure to diethylstilbestrol in utero (Kvaskoff, Bijon, Clavel-Chapelon, Mesrine & Boutron-Ruault, 2013; Missmer, Hankinson, Spiegelman, Barbieri, Michels & Hunter, 2004)

Current treatments for endometriosis include estrogen-depleting therapies such as the gonadotropin-releasing hormone (GnRH) drug elagolix and oral contraceptives (H. S. Taylor et al., 2017). These therapies act to directly block ovulation or to simulate states in which ovulation does not occur, such as in pregnancy. Non-steroidal anti-inflammatory drugs (NSAIDs) are used to manage pain, and surgery may be performed to remove

ectopic growths (Greene et al., 2016; Hickey et al., 2014). However, hormone treatments often have adverse side effects, e.g.; prevent ovulation and therefore, conception, with reduction in symptoms only during actual treatment (Greene et al., 2016). Surgery often fails to remove all lesions and may lead to adhesions, sterility and other complications (Vercellini, Viganò, Somigliana & Fedele, 2014). Thus, novel therapies with fewer side effects which allow for conception and pregnancy are needed.

Beta-caryophyllene (BCP) is an essential oil present in *Cannabis sativa* (Hendricks et al., 1975) and commonly consumed spices, e.g. oregano, cinnamon and black pepper (Jayaprakasha, Rao, & Sakariah, 2003; Mockute, Bernotiene, & Judzentiene, 2001; Orav, Stulova, Kailas, & Müürisepp, 2004). BCP makes up approximately 35% of the essential oil present in cannabis (Hendricks et al., 1975). It selectively binds the G-protein-coupled cannabinoid receptor 2 (CB2), activating anti-inflammatory effects without the characteristic psychotropic effects of cannabis use (Gertsch et al., 2008). As an FDA-approved food additive, BCP has not demonstrated genotoxic or cytotoxic effects (Molina-Jasso, Álvarez-González, & Madrigal-Bujaidar, 2009).

The anti-inflammatory properties of BCP have been documented. Rodent model inflammatory diseases, such as colitis (Bento et al., 2011; Cho et al., 2015), edema (Fernandes et al., 2007), Alzheimer's (Cheng et al., 2014) and type 2 diabetes (Basha & Sankaranarayanan, 2014, 2015) have been used to demonstrate the anti-inflammatory effects of BCP. Table 4.1 compares some anti-inflammatory effects of BCP on different diseases.

Table 4.1: Effects of BCP on Inflammatory Diseases

Disease	Effector	Effect
Colitis	CB2 activation	Reduces apoptosis, cell proliferation and cell migration (Bento et al. (2011)
Multiple myeloma, breast and prostate cancer cells	Suppression of signal Transducer and activator of transcription-3 (STAT3) Upregulation of inflammatory mediator including VEGF	Prevents proliferation and induces apoptosis (C. Kim et al., 2014) Establishes circulatory system (C. Kim et al., 2014)
Edema	Downregulation of inflammatory mediators including TNF-α, PGE2 and COX-2	Decreased inflammation, likely through inhibition of the NF-κB pathway (Fernandes et al., 2007)
Alzheimer's disease	Downregulation of the inflammatory mediators TNF-α, IL-1β and COX-2	Prevented cognitive impairment (Cheng, Dong & Lui, 2014)

Note. CB2= cannabinoid receptor 2; VEGF= vascular endothelial growth factor; TNF-α= tumor necrosis factor alpha; PGE2= prostaglandin E2; COX-2=cyclooxygenase 2; IL-1β= interleukin 1 beta.

The endocannabinoid system (ECS) is involved in a variety of essential physiological processes including: food intake (Soria-Gomez et al., 2014), metabolism (Mazier, Saucisse, Gatta-Cherifi & Cota, 2015), pain sensation (Woodhams, Sagar,

Burston & Chapman, 2015) cognition (Kruk-Slomka, Dzik, Budzynska & Biala, 2017) and inflammation (Crowe, Nass, Gabella & Kinsey, 2014). Endogenous cannabinoid ligands, their receptors, and their regulating enzymes make up the ECS (Ayakannu, Taylor, Willets, & Konje, 2015). Endocannabinoids primarily exert their effects through cannabinoid receptors CB1 and CB2 (Matsuda, Lolait, Brownstein, Young, & Bonner, 1990; Munro, Thomas, & Abu-Shaar, 1993). *In vivo*, these receptors are activated by the binding of endocannabinoid ligands derived from arachidonic acid, primarily anandamide (AEA) and 2-arachidonoylglycerol (2-AG) (Devane et al., 1992; Sugiura et al., 1995). In the female reproductive system, expression of endocannabinoid ligands, their regulating enzymes and their receptors vary throughout the menstrual cycle, during implantation and during pregnancy (Battista, Bari, & Maccarrone, 2013).

Activation of the CB1 receptor is attributed to the pathophysiological effects characteristic of *Cannabis sativa* use, and is also involved in cell proliferation, apoptosis, angiogenesis and inflammation (Dhopeshwarkar & Mackie, 2014; Maccarrone et al., 2015). The CB1 receptor is primarily expressed in the brain, particularly in regions responsible for motor control, cognition and memory, and sensory perception (Gentilini et al., 2010; Kruk-Slomka et al., 2017; Morera-Herreras, Miguelez, Aristieta, Torrecilla, Ruiz-Ortega & Ugedo, 2016). CB1 is also expressed peripherally in the eye, spleen, testis and the uterus, as endometriosis lesions are derived from endometrial tissues (Aloway, Kumar, Laun & Song, 2017; Cassano, Calcagnini, Pace, De Marco, Romano & Gaetani, 2017). CB2 receptors are primarily expressed by circulating cells of the immune system and immune organs (spleen and lymph nodes), as well as the intestines, lungs,

uterus, pancreas, and skin (Casanova et al., 2003; Gardner et al., 2002; Onaivi et al., 2002; Pertwee, 1997). Activation of CB2 results in anti-inflammatory responses (Deng et al., 2015).

Taylor et al. (2010) reported an intense CB1 expression in glandular epithelium when compared with stroma, but did not find this expression to be regulated throughout the menstrual cycle. This study also found that CB2 expression was low in the early stages of the menstrual cycle, but peaked in the late proliferative phase (2010). Resuehr et al. (2012) found an increased CB1 mRNA and protein expression in the secretory phase of the cycle, regulated by progesterone (Resuehr, Glore, Taylor, Bruner-Tran, & Osteen, 2012). Resuehr et al. (2012) further demonstrated that CB1 mRNA and protein expression is significantly lower in the endometria of women with endometriosis compared to healthy controls and attributed this reduced expression to the progesterone resistance characteristic of endometriosis. This was corroborated by Sanchez et al. (2016) who found that CB1 expression was reduced in endometrial stromal cells (ESCs) in women with endometriosis compared to controls, and that this decreased expression contributes to disease-associated pain. Bilgic et al. (2017) and Sanchez et al. (2016) found that CB1 and CB2 proteins are decreased in endometriosis tissue compared to controls. Iuvone et al. (2008) reported an upregulation of CB2 on mast cells in the endometria of women suffering from endometritis compared to the endometria of healthy women (Iuvone et al., 2008). While there is little data on the expression of endocannabinoid proteins in ectopic lesions, Leconte et al. (2010) found the presence of

CB1 and CB2 receptors on cells derived from endometriotic implants (Leconte et al., 2010).

Few studies have explored the effects of BCP in or on endometriosis. Abbas, Taha, Zihlif & Disi (2013) examined the effects of BCP on a rat model of surgically-induced endometriosis with very promising results. Treatment with 10 mg/kg BCP resulted in a 50% decrease in endometriotic lesion surface area compared to controls. Furthermore, no difference in fertility between groups was observed, indicating that BCP has no negative impact on reproductive potential as is reported for many of the currently-available treatments for endometriosis (Panay et al., 2008). These researchers also found BCP induced apoptosis in both endometrial luminal epithelial cells as well as in vascular endothelial cells. Chang et al. (2014) found that BCP extracted from *Siegesbeckia orientalis* exerted an anti-proliferative effect on human endometrial RL95-2 cells (Chang et al., 2014).

The purpose of this experimental laboratory study was to characterize the presence of CB1 and CB2 receptors in two endometrial cancer cell lines and also in normal and endometriosis-positive endometrial tissues. In addition, the effects of treatment with BCP on cell proliferation, viability, estrogenicity and VEGF production in endometrial cancer cells and primary endometrial stromal cells were examined.

Materials and Methods

Ethics Approval for Human Samples

Greenville Health Systems IRB: #38040, approved on 2/23/2015. Documents provided in Appendix A.

Human Endometrium Sample Collection

Written informed consent was obtained from subjects prior to sample acquisition. Human endometrium samples were collected from two Caucasian women in their late 20's, one with endometriosis and one without endometriosis, at the time of surgery in the Greenville Health System. The presence or absence of endometriosis was confirmed during surgery. The woman without endometriosis was placed in the control group. Endometrial samples were collected by pipelle sampling and placed in isolation media consisting of Dulbecco's Modified Eagles Medium (DMEM)/high glucose media (HyClone, Logan, UT) supplemented with 5% fetal bovine serum and 2% penicillin/streptomycin amphotericin B (Lonza, Walkersville, MD).

Stromal Cell Isolation

Endometrial samples were rinsed with Hank's Balanced Salt Solution (Corning, Manassas, VA) and minced into 1-millimeter (mm) fragments with a sterile scalpel. Tissue fragments were incubated for 2.5 hours in a 1X collagenase/isolation media solution with rapid shaking on an orbital shaking platform. Digested tissue was forced through a stacked sterile wire sieve assembly with number 100 wire cloth sieve (140 μm size, Newark Wire Co., Newark, NJ), followed by a number 400 wire cloth sieve (37 μm) to isolate the stromal cells from the epithelium. The stromal cell-containing fluid was collected in a 50 milliliter (mL) falcon tube and centrifuged at 500 g for 5 minutes, the supernatant was decanted and the pellet was resuspended in isolation media. Red blood cells were removed by gently layering the cell suspension from the pellet atop 3mL Ficoll-Paque (Pharmacia, Piscataway, NJ) in a 15 mL tube. The tube was centrifuged at

400 g for 10 minutes with the brake set to zero to separate the red blood cells from the stromal cells. The milky middle stromal cell layer was carefully pipetted off and re-centrifuged at 500 g for 5 minutes. The supernatant was aspirated, and the pellet was resuspended in stromal media consisting of 1:1 Medium 199/F-12 Ham's nutrient mixture (HyClone) media supplemented with 4% heat-inactivated fetal bovine serum, 1% penicillin/streptomycin and 1 ml ITS+ (insulin, human transferrin and selenous acid) Premix Universal Culture Supplement (Corning, Tewksbury, MA) per 1000 ml. The cell suspension was added to stromal media in flasks and maintained at 37 ℃ in a 5% CO_2 humidified atmosphere.

Reagents and Cell Culture

Ishikawa cells (CRL-2923; ATCC, Manassas, VA) and RL95-2 cells (CRL-1671; ATCC) were maintained in Dulbecco's Modified Eagles Medium and Ham's F-12 nutrient mixture (DME/F-12) (1:1) (HyClone) supplemented with 10% heat-inactivated fetal bovine serum and 1% penicillin/streptomycin at 37 ℃ in a 5% CO_2 humidified atmosphere. Prior to all experiments, Ishikawa and RL95-2 cells were grown for three days in hormone-free media consisting of DMEM/F-12 (1:1) (HyClone) without phenol red supplemented with 5% heat-inactivated charcoal-stripped fetal bovine serum (JR Scientific, Inc., Woodland, CA), 1% penicillin/streptomycin and 15 mM hydroxyethyl piperazineethanesulfonic (HEPES) free acid (HyClone) as a buffering agent. Endometrial stromal cells (ESCs) were also placed in hormone-free media with 2% heat-inactivated charcoal-stripped fetal bovine serum.

Alkaline Phosphatase Assay

To screen for estrogenic activity, the alkaline phosphatase assay developed by Littlefield and colleagues (Littlefield, Gurpide, Markiewicz, McKinley, & Hochberg, 1990) was performed using Ishikawa and RL95-2 cells. Cells were switched to hormone-free medium (see above) three days prior to the experiments. The cells were washed with 1× PBS, trypsinized until cells detached and then resuspended in medium. Approximately 20,000 cells were added to each well with 200 µl medium and grown for 24 hours. The cells were then treated with medium containing specified concentrations of BCP diluted in ethanol (10^{-12}, 10^{-11}, 10^{-10}, 10^{-9} and 10^{-8}M), estradiol (E2) (10^{-8}M) or the anti-estrogen ICI 182,780 (10^{-6}M) for 72 hours. The medium was changed daily.

At the end of the treatment, the alkaline phosphatase assay was performed. Cells were washed twice with cold 1× PBS and the plates were frozen for 15 min at −140°C and then thawed to lyse the cell membranes. After thawing 50-µl of cold soluble substrate consisting of Sigma*Fast* p-nitrophenyl phosphate tablets (Sigma, St. Loius, MO) dissolved in deionized H_2O was added to each well on ice. The plates were incubated at room temperature on a plate shaker. Between 1-3 hours of incubation, the substrate solution produced a yellow color when alkaline phosphatase was present. The absorbance was read in a plate reader at 405 nanometers (nm) (Bio-Rad, Hercules, CA).

CyQUANT Cell Proliferation Assay

The effect of BCP stimulation on cell proliferation in Ishikawa and RL95-2 cells was measured using the CyQUANT Cell Proliferation Assay kit (C7026; Invitrogen, Eugene, OR). Cells were plated in triplicate on a 96-well plate at 5,000 cells/well (200µl volume/well) and treated with different concentrations of BCP diluted in ethanol with or

without estradiol (10^{-8}M). Cells were incubated for 24, 48 or 72 hours at 37°C with 5% CO_2. Treatment medium was changed at 48 hours. Post-treatment, medium was gently shaken out of the wells and the plate was blotted on clean paper towels. Plates were frozen at -70°C for a minimum of 24 hours. After thawing plates at room temperature, 200 µL of the 1X dye solution from the CyQUANT Cell Proliferation Assay kit was added to each well and incubated at room temperature for 5 minutes, protected from ambient light. Colorimetric analysis was conducted using spectrophotometry with fluorescence set at 480 nm excitation and 520 nm emission.

Trypan Blue Proliferation Assay

The effects of BCP stimulation on cell proliferation in ESCs derived from a patient with and one without endometriosis was measured using a trypan blue viability assay. ESCs were plated in triplicate at 50,000 cells/well in 6-well plates (1.5 mL volume/well). Cells were treated with BCP (10^{-8}M) or control (media plus EtOH vehicle). Cell numbers were obtained after two, four and six days of treatment. Cells were detached from wells using trypsin, collected, centrifuged, supernatant aspirated, and the pellet was resuspended in media. The solution was diluted with trypan blue which passes through the cell membrane of dead and dying cells to stain the cytoplasm blue but is excluded from viable cells whose cytoplasm remains clear. Viable cells from each replicate were counted using both chambers of a hemocytometer. The number of cells per mL was calculated using the formula: average number of cells counted * dilution factor * 10,000 * volume of media added to pellet (mL). The average number of cells/mL was calculated.

Human VEGF ELISA

The effects of BCP stimulation on ESC production of human VEGF protein was determined using the Quantikine ELISA Human VEGF Immunoassay kit (DVE00; R&D Systems, Minneapolis, MN). ESCs derived from a woman with endometriosis were plated in 6-well plates at 100,000 cells per well. Cells were treated with BCP (10^{-8}M) for 24 hours followed by IL-17 (z100465; Applied Biological Materials, Inc., Richmond, British Columbia, Canada) (50 ng/mL) alone or a combination of IL-17 (50 ng/mL) plus IL-6 (z100555; Applied Biological Materials, Inc.) (50 ng/mL) for 12 hours. The treatment media from each well (three replicates per treatment condition) was collected and frozen until analysis. The samples were thawed, and an ELISA was performed following the kit protocol. Each sample was added in duplicate.

Immunohistochemical Staining of Endometrial Tissue for CB1 and CB2

Immunohistochemical (IHC) staining for CB1 and CB2 receptors was completed on paraffin-embedded endometrial samples categorized as normal proliferative, normal mid-secretory and moderate endometriosis secretory.

Endometrial samples were deparaffinized and rehydrated in a graded alcohol series then blocked with 2% normal horse serum in PBS. Samples were incubated with anti-CB1 (10006590; Cayman Chemical, Ann Arbor, MI; dilution: 1:200) or anti-CB2 (SC-25494 Santa Cruz Biotechnologies, Santa Cruz, CA; dilution: 1:100) antibodies in 2% normal horse serum in PBS overnight at 2°C. The next day, sections were incubated with secondary antibody conjugated to horseradish peroxidase (Vector Laboratories, Burlingame, CA) for 1 hour at room temperature. Immunoreactivity was detected using

diaminobenzidine (DAB-Vector Laboratories, Burlingame, CA) then counterstained with hematoxylin and cover slipped with Permount Mounting Medium (Fisher Chemical, Waltham, MA). The slides were analyzed microscopically at 20X and 40X magnitudes and pictures were taken with a Nikon D200 camera (Nikon, Melville, NY) with microscopic lens.

7-amino actinomycin D (7-AAD) Cell Viability Assay

The effects of BCP stimulation on cell viability was measured in Ishikawa cells and primary endometrial stromal cells using 7-amino actinomycin D (7-AAD) viability dye (Beckman Coulter Life Sciences, Indianapolis, IN). Ishikawa and ESCs were plated in 6-well plates at 300,000 cells/well (2 mL volume/well) with 6 wells/treatment condition. Six-well plates were treated with different concentrations of BCP dissolved in ethanol for 24 hours. All plates were washed with PBS and cells were detached from wells with trypsin and collected into 15 mL conical tubes. The cells were centrifuged, supernatant decanted, and pellet resuspended in 1 mL PBS and quantified. Cells were diluted with PBS to a concentration of 5 million cells/mL. Five µL 7-AAD were added per well to U-bottomed 96-well plates with 5 µL PBS added to wells for unstained controls. To each well was added 100 µL sample and plates were incubated at room temperature in the dark for 20 minutes. Samples were analyzed via flow cytometry (CytoFLEX; Beckman Coulter Life Sciences).

Statistical Analyses

As each experiment was performed only once, valid statistical testing was not possible.

Results

Effect of BCP on Estrogenicity

Ishikawa cells are a model for assessing the estrogenic or anti-estrogenic potential of test compound. In the presence of E2, Ishikawa cells induce alkaline phosphatase which is assessed by spectrophometric measurement of p-nitrophenylphosphate produced above untreated control cells. A decrease in alkaline phosphatase induction indicates anti-estrogenic activity.

Figure 4.1. Dose-Response of BCP on the Alkaline Phosphatase Activity in Ishikawa Cells

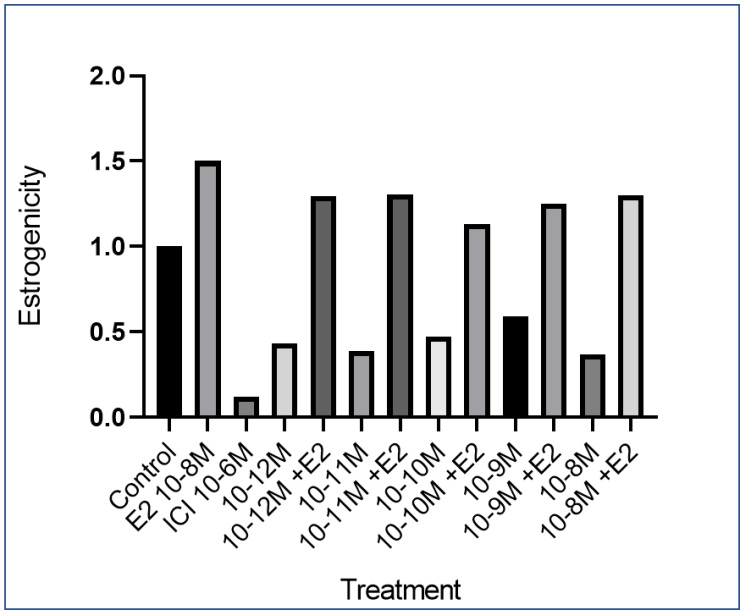

Figure 4.1. The cells were treated with BCP with or without estradiol (E2) (10^{-8}M) for 72 hours. Cells treated with the anti-estrogen ICI 182,780 (10^{-6}M) represent a negative control for alkaline phosphatase induction.

Effect of BCP on Alkaline Phosphatase Induction

BCP alone inhibited alkaline phosphatase induction of Ishikawa cells (Figure 4.1) below 50% of untreated control cells for all dosages following a 72-hour incubation.

Cells treated with estradiol (10^{-8}M) were positively responsive above untreated control cells. Simultaneous treatment of Ishikawa cells with estradiol and all BCP dosages slightly reduced alkaline phosphatase response below estradiol alone. Cells treated with the anti-estrogen ICI 182,780 (10^{-6}M) resulted in over 80% reduction of AlkP response.

Figure 4.2. Dose-Response of BCP on the Alkaline Phosphatase Activity in RL95-2 Cells

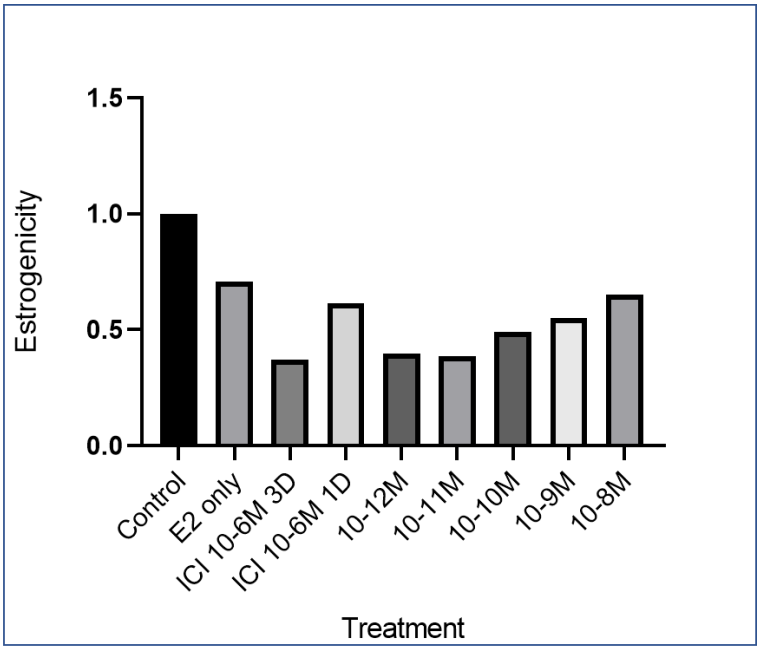

Figure 4.2. The cells were treated with BCP with or without estradiol (E2) ((10^{-8}M) for 72 Hours. Cells treated with the anti-estrogen ICI 182,780 (10-6M) represent a negative control for AlkP induction.

BCP treatment alone at all doses tested showed no effect on alkaline phosphatase induction in RL95-2 cells following 72 hours incubation (Figure 4.2). The addition of ICI 182,780 resulted in an inhibitory effect of 30%. Although RL95-2 cells are reported to be estrogen receptor positive, no stimulation of alkaline phosphatase activity was observed with the addition of E2 in the experiment.

Effect of BCP on Cell Proliferation

In Ishikawa cells (Figure 4.3), BCP alone inhibited proliferation in a dose-response pattern at 48 and 72 hours. The effects of BCP plus estradiol (10^{-8}M) were much more varied with a dose-response pattern of proliferation exhibited at 48 hours but not 72 hours. BCP inhibits proliferation of RL95-2 cells at 72 hours at concentrations of 10^{-8}M and 10^{-9}M (Figure 4.4). Interestingly, these concentrations, with estradiol added, appear to increase proliferation.

Figure 4.3. CyQUANT Proliferation Assay in Ishikawa Cells

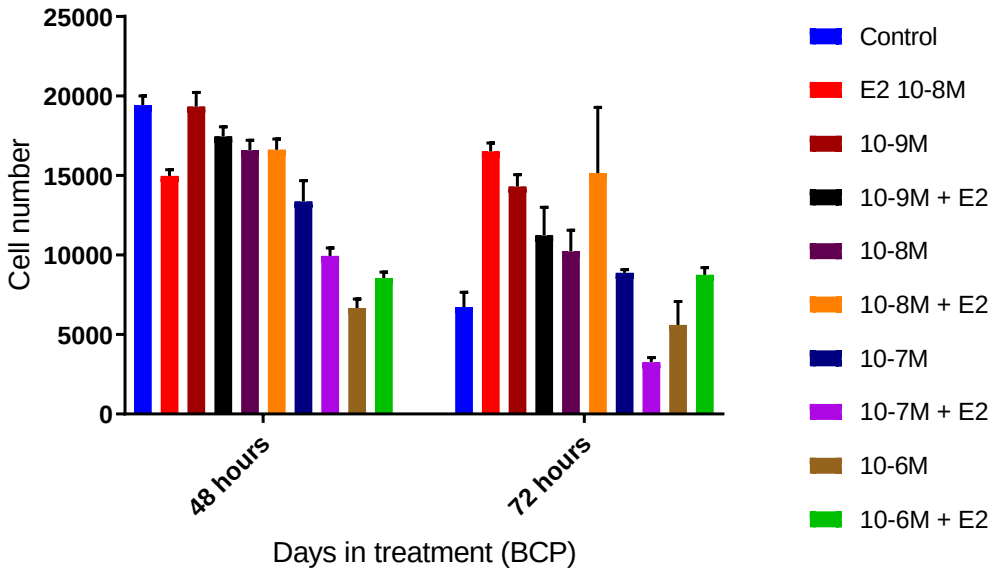

Figure 4.3. Ishikawa cells were treated with BCP and BCP plus estradiol (10^{-8}M). Control contained ethanol as vehicle.

Figure 4.4. CyQUANT Proliferation Assay in RL95-2 Cells

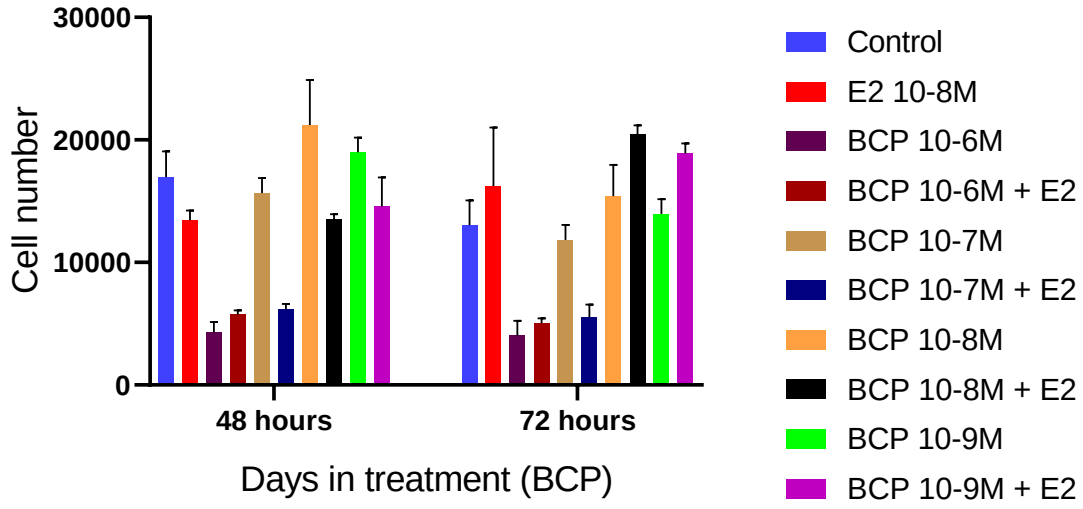

Figure 4.4. RL95-2 cells were treated with BCP and BCP plus estradiol (10^{-8}M). Control contained ethanol as vehicle.

In normal ESCs, BCP at a concentration of 10^{-8}M exhibited no significant effects on proliferation (Figure 4.5), while endometriosis-positive ESCs exhibited a significant increase in proliferation on day six of treatment (Figure 4.6).

Figure 4.5. Trypan Blue Assay in Normal ESCs

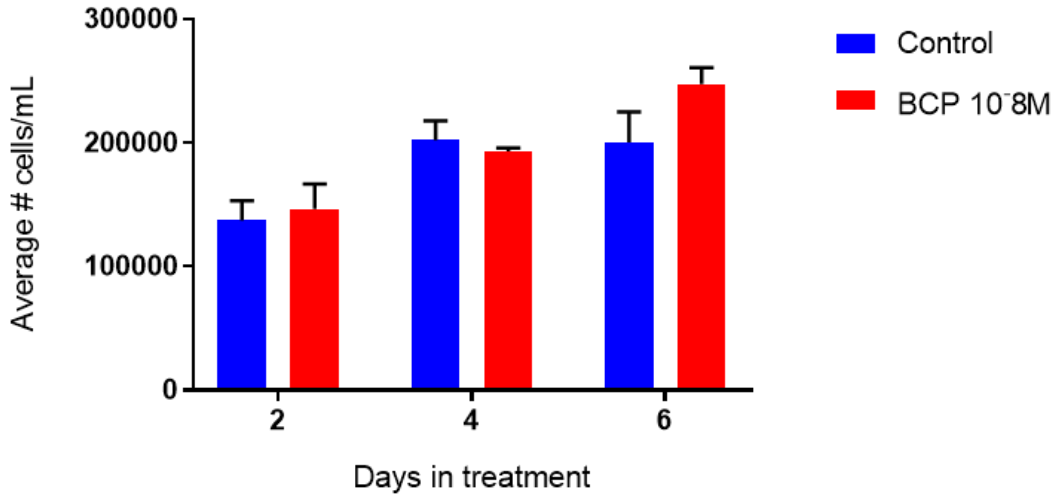

Figure 4.5. Normal ESCs were stimulated with BCP (10^{-8}M) for six days. Control contained ethanol as vehicle. Cell number obtained at two, four, and six days post-treatment.

Figure 4.6. Trypan Blue Assay in Endometriosis-Positive ESCs

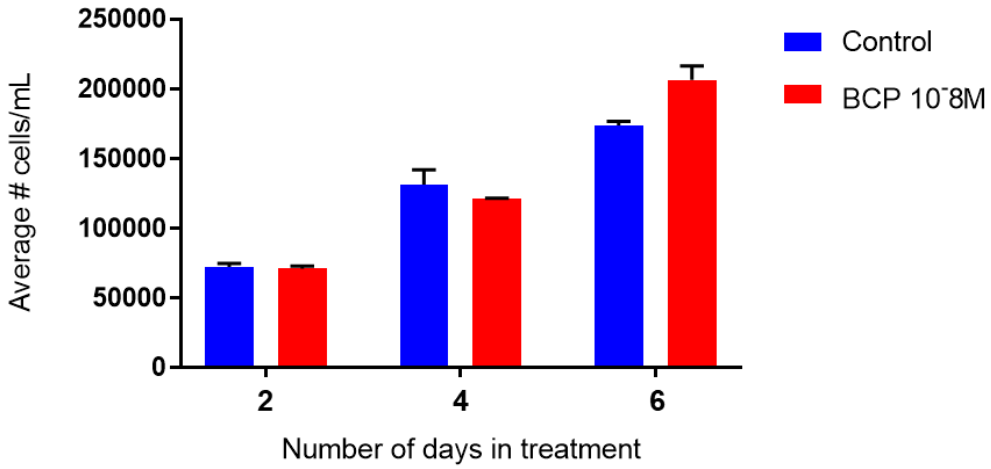

Figure 4.6. Endometriosis-positive ESCs were treated with BCP for six days. Control contained ethanol as vehicle. Cell number obtained at two, four, and six days post-treatment.

BCP (10^{-8}M) resulted in a slight percent increase in normal and slight percent decrease in endometriosis-positive ESCs on day two and a slight decrease in both on day four compared to corresponding controls (Figures 4.7 & 4.8). On day six, both cell types (normal: 23.54%; endometriosis-positive: 18.78%) demonstrated a much larger percent increase in proliferation.

Figure 4.7. Trypan Blue Assay- Comparison of Normal and Endometriosis-Positive ESCs

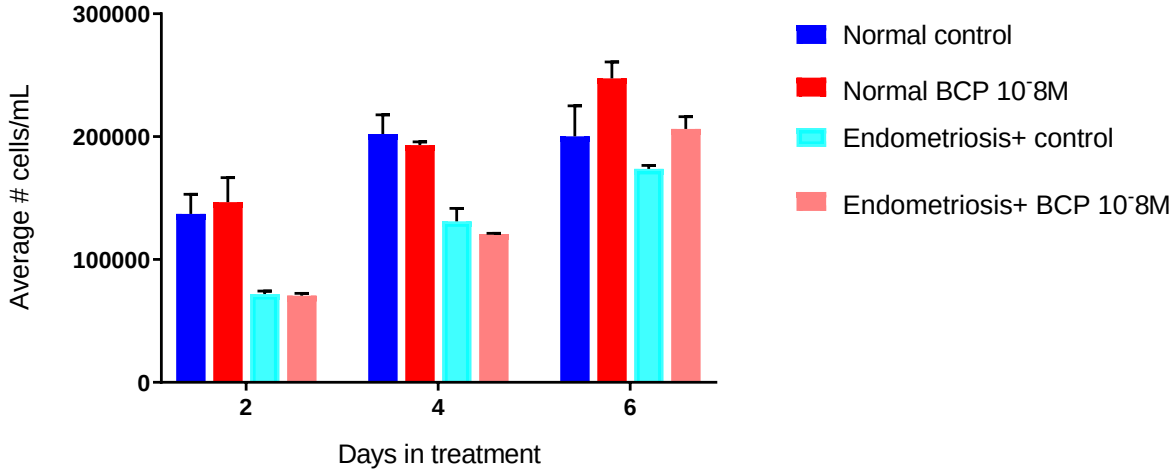

Figure 4.7. Comparison of normal and endometriosis-positive ESCs proliferation in a trypan blue proliferation assay conducted over six days. Controls contained ethanol as vehicle. Cell numbers obtained at two, four, and six days post-treatment.

Figure 4.8. Percent Change in Proliferation in Normal and Endometriosis-Positive ESCs

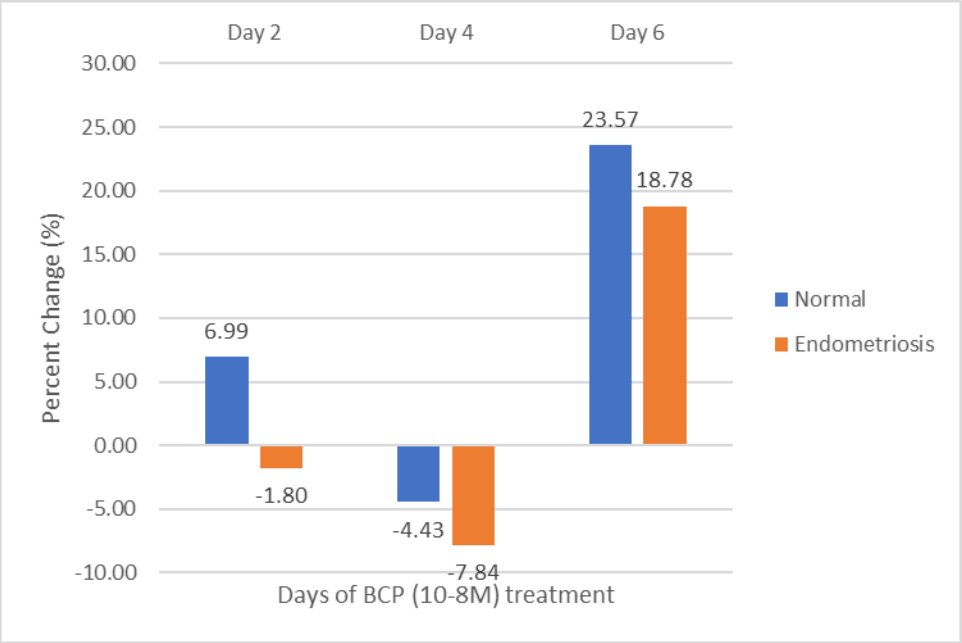

Figure 4.8. Comparison of percent change in ESC proliferation between normal cells and endometriosis-positive cells following BCP treatment. The percent change in ESC proliferation between control and BCP-treated (10^{-8}M) cells was calculated for each cell type at each time point. Positive numbers indicate an increase in proliferation of the treated cells compared to the control while negative numbers indicate a decrease in treated cell proliferation.

Effect of BCP on VEGF Production by ESCs

Pretreatment of endometriosis-positive ESCs with BCP (10^{-8}M) decreased cytokine-induced VEGF production by 31% compared to IL-17 (50 ng/mL) and by 17% compared to IL-17 (50 ng/mL) + IL-6 (50 ng/mL) (Figure 4.9). IL-17 + IL-16 stimulation increased VEGF production by 76%, IL-17 alone by 73% and IL-6 alone did not change VEGF levels compared to control. As this experiment was performed only once, valid statistical testing was not possible.

Figure 4.9. VEGF ELISA in Endometriosis-Positive ESCs

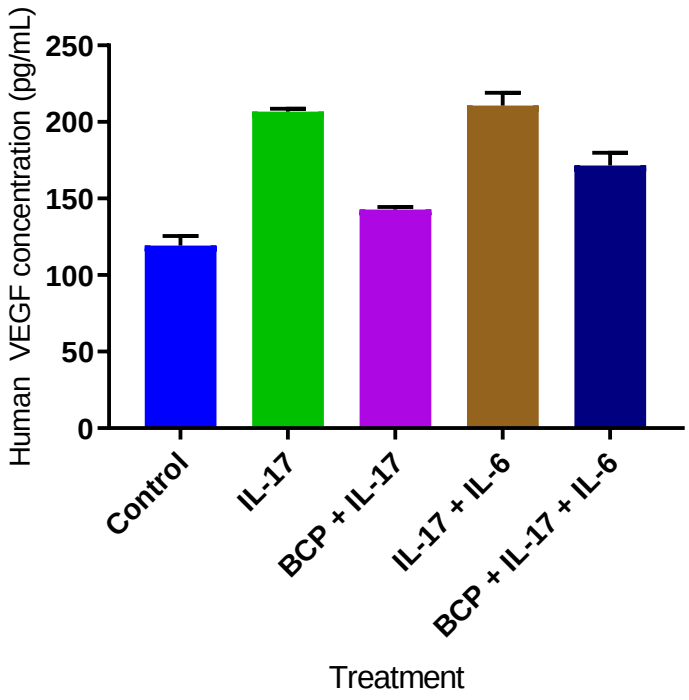

Figure 4.9. Cells were pretreated with BCP (10^{-8}M) for 24 hours followed by 12-hour cytokine treatment (IL-17, 50 ng/mL; IL-6, 50 ng/mL).

Immunohistochemistry Staining of Endometrial Tissue for CB1 and CB2

CB1.

The normal samples show that CB1 expression is high in the stroma during the proliferative phase (Figure 4.10 (A)) and decreases by the mid-secretory phase (C). Normal CB1 expression is minimal in the glands in both phases. The sample of moderate endometriosis in the secretory phase (D) exhibits intense glandular expression and moderate stromal expression, indicating that CB1 expression is upregulated in the glandular epithelium of women with endometriosis in the secretory phase.

CB2.

CB2 expression is intense in the normal stroma during the proliferative phase (Figure 4.10 (B)) and decreases by the mid-secretory phase (E). In the normal glands, CB2 expression is low in both phases. CB2 is upregulated in the secretory phase in the stroma of the endometriosis sample (F) compared to the normal secretory phase control.

Figure 4.10. Immunohistochemical Analysis of CB1 and CB2 in Endometrial Tissue Samples

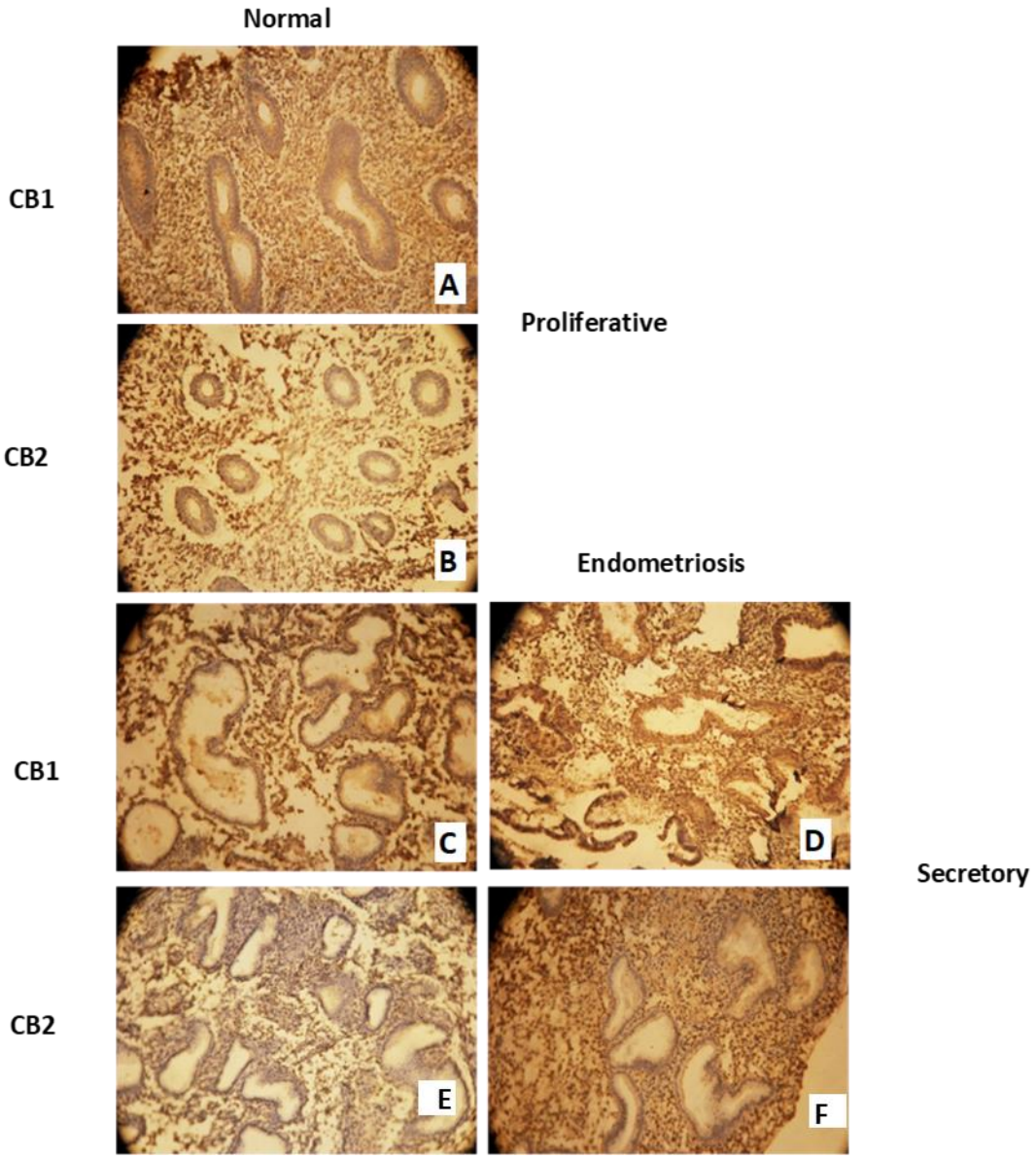

Figure 4.10. A & B: normal proliferative; C & E: normal mid-secretory D & F: moderate endometriosis, secretory

Flow Cytometry

Neither Ishikawa cell nor ESC viability differed from controls after 24 hour treatments with BCP at 10^{-8}M, 10^{-9}M and 10^{-10}M in the 7-AAD viability flow cytometry experiments (Figure 4.11). 7-AAD is a DNA-intercalating dye that is unable to enter the cell membrane of viable cells, thus only dead and dying cells are stained. The CyQUANT proliferation assays indicated that 48-72 hours is the optimal treatment time, so these experiments will be repeated using those time points. Interestingly, a distinct cell population was identified in the ESC samples with peak fluorescence between the live and dead cell populations, likely indicating a pre-apoptotic population with some level of membrane disruption (Figure 4.11). There were higher percentages of cells in this population at all concentrations compared to control.

Figure 4.11. 7-AAD Flow Cytometry Analysis of ESCs Treated with BCP

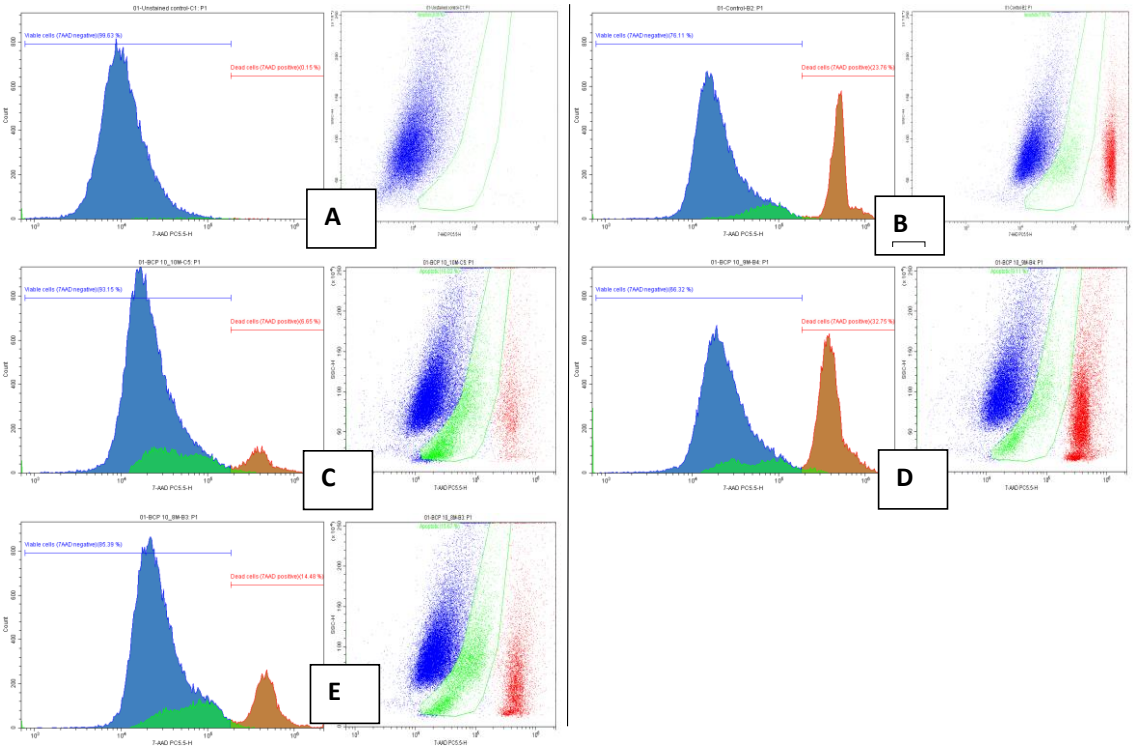

Figure 4.11. Histogram and dot-plot of 7-AAD viability analysis of ESC treated for 24 hours with BCP. Debris was eliminated using gate P1 set on the unstained control sample (A). Blue corresponds to cells not reactive to 7-AAD (viable), orange corresponds to cells fully reactive to 7-AAD (dead) and green corresponds to cells partially reactive to 7-AAD (apoptotic). (A): unstained control; (B): stained control; (C): BCP 10^{-10}M; (D): BCP 10^{-9}M; (E): BCP 10^{-8}M

Figure 4.12. Relative Expression of Cannabinoid Receptors in Ishikawa and RL95-2 Cells

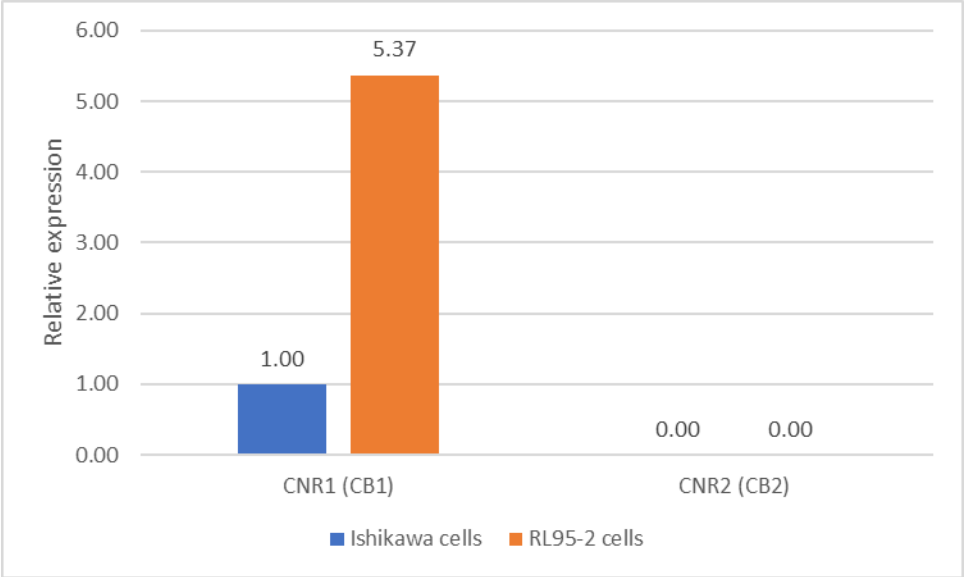

Figure 4.12. Relative expression of *CNR1*(CB1*)* and *CNR2*(CB2*)* in Ishikawa and RL95-2 cell lines.

Discussion

The purpose of this study was to examine the effects of the phyto-cannabinoid BCP on indicators of inflammation in endometriosis using a cell-culture model. Endometrial cancer cell lines Ishikawa (adenocarcinoma) and RL95-2 (adenosquamous carcinoma) were utilized as models for endometriosis. Both cell lines demonstrate markers expressed by normal endometrial epithelial cells including estrogen receptors α and β and progesterone receptor (Nishida, Kasahara, Kaneko, Iwasaki, & Hayashi, 1985; Way, Grosso, Davis, Surwit, & Christian, 1983).

rtPCR analysis of Ishikawa and RL95-2 cells (Figure 4.12) demonstrated that neither cell line expresses *CNR2* mRNA, which encodes CB2, the target receptor for BCP. This finding may explain the results from experiments employing BCP treatment

on these cell lines prior to rtPCR (Figures 4.1-4.4). While these cell lines are ineffective for testing the cannabinoid BCP due to their lack of CB2 receptor, they do express CB1. RL95-2 cells highly express CB1, making this line a potential model for future experiments using other cannabinoids exhibiting CB1-reactivity.

IL-6 and IL-17 were used in the VEGF study as both have been shown to induce VEGF expression. In this study, BCP was found to inhibit IL-17-induced VEGF expression in ESCs, but not when IL-17 was combined with IL-6. Angiogenesis, driven by VEGF, is a significant feature of endometriosis. Vascularization is normal and necessary in the endometrium for proper reproductive functions, but the vascularization of endometriosis implants allows them to survive and flourish by connecting to the local blood supply for sustenance. (McLaren, 2000; Taylor, Lebovic, & Mueller, 2002). VEGF, operating via a concentration gradient, encourages blood vessels to divide and grow towards the implant. VEGF expression is induced by cytokines through the Janus kinase (JAK)/STAT3 pathway. Cytokines of the IL-6 family, produced by local immune cells, are the primary inducers of VEGF production. IL-6 protein is produced by IL-6 gene, which maps to chromosome 7p15.3 (NCBI). Other non-IL-6 family cytokines may also be involved in VEGF production. Abnormal levels of IL-6, VEGF and aberrant STAT3 activation are reported in endometriosis (B. G. Kim et al., 2015). Further, VEGF receptors are upregulated on lesions (Di Carlo et al., 2009; Donnez, Smoes, Gillerot, Casanas-Roux, & Nisolle, 1998; Takehara et al., 2004).

IL-17 was only recently identified as a mediator of inflammation in endometriosis (Ahn et al., 2015). It is primarily produced by local Th17 cells. Alone, IL-17 is a weak

inducer of transcription of proinflammatory mediators, including VEGF, via the NFκB, MAPK and C/EBP pathways. IL-17 appears to exert potent proinflammatory effects by synergizing with other cytokines, such as IL-1β, IL-22, IFN-γ and TNF-α, all of which are elevated in endometriosis (Beringer, Thiam, Molle, Bartosch, & Miossec, 2018). IL-17 acts to stabilize the mRNA of these more powerful cytokines to increase the half-life and the likelihood of their translation and thus prolong the inflammatory response (Hartupee, Liu, Novotny, Li, & Hamilton, 2007). In line with this, Hirata et al. (2008, 2011) reported that IL-17A and IL-17F synergized with TNF-α to increase levels of COX-2 and IL-8 by ESCs (Hirata et al., 2008, 2011).

Interestingly, IL-17 alone was a greater inducer of VEGF than was IL-6 alone, which was similar to control. BCP decreased the VEGF levels of IL-17 treatment, but not when IL-6 was added. Either IL-17 and IL-6 are exhibiting synergistic effects, though there is scant literature to support that these cytokines synergize, or BCP blocks one of the pathways involved in IL-17-mediated VEGF production (NFκB, MAPK, C/EBP), but not those of IL-6 (STAT3). In contradiction to these findings, Kim et al (2013) found that BCP prevented STAT3 phosphorylation in three cancer cell lines (C. Kim et al., 2013).

Overall, the findings showed mixed results regarding the effectiveness of BCP as a therapeutic option for endometriosis. Multiple experiments using BCP were performed, but not replicates of the same experiment, so valid statistical testing was not possible. Neither of the cancer cell lines used, both of which are commonly used in endometriosis research, possess CB2 receptors, making them an ineffective model in which to test BCP. CB2 does appear to be present in endometrial tissue samples and is upregulated in

endometriosis-positive samples. BCP decreased levels of IL-17-induced VEGF in primary ESCs from a uterine tissue sample with endometriosis. Therefore, additional research using primary cell cultures is needed.

Analysis by rtPCR revealed that neither cell line expresses CB2, the receptor through which BCP acts. As BCP does appear to decrease estrogenicity and proliferation in Ishikawa cells when exogenous estradiol is not added, BCP may be working through some mechanism besides CB2, though further studies are necessary to identify what that may be.

Many experiments using BCP were performed in the study, but each was only carried out one time. Lacking adequate replication, valid statistical analysis could not be done. Endometrial stromal cells were extracted from samples of two individuals only (one endometriosis-positive, one endometriosis-negative). Both were Caucasian women in their late 20's. The small sample size prevents appropriate statistical testing and the lack of ethnic diversity among samples prevents generalizability of the results.

More research into identifying any alternate mechanisms through which BCP may act are recommended. The Ishikawa and RL95-2 cell lines do not express CB2, thus additional research should be conducted in other cell lines such as the endometrial adenocarcinoma lines HEC-1A or HEC-1B. Additionally, experiment replication allowing for statistical testing is needed.

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Abbreviations

2-AG	2-arachidonoylglycerol
7-AAD	7-amino actinomycin D
AEA	anandamide
BCP	beta-caryophyllene
CB1	cannabinoid receptor 1
CB2	cannabinoid receptor 2
C/EBP	CCAAT-enhancer-binding protein
COX-2	cyclooxygenase 2
ECS	endocannabinoid system
ELISA	enzyme-linked immunosorbent assay
ESC	endometrial stromal cell
GnRH	gonadotropin-releasing hormone
IFNγ	interferon gamma
IL	interleukin
JAK	Janus kinase
MAPK	mitogen-activated protein kinase
mRNA	messenger ribonucleic acid
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells
NSAIDs	non-steroidal anti-inflammatory drugs
PR	progesterone receptor
rtPCR	reverse transcription polymerase chain reaction

STAT3	signal transducer and activation of transcription 3
Th17	T helper 17
TNFα	tumor necrosis factor alpha
VEGF	vascular endothelial growth factor

CHAPTER FIVE

SUMMARY, LIMITATIONS AND RECOMMENDATIONS

The interdisciplinary Healthcare Genetics PhD program was developed to expose students to a broad array of genetics-focused disciplines in an effort to produce graduates prepared to tackle complex genetics-based healthcare issues. The healthcare genetics (HCG) conceptual model developed by individuals associated with the program was described in Chapter One. In this model, the field of healthcare genetics is composed of five core constructs: Clinical Practice, Education, Environment, Ethical/Legal/Social Implications (ELSI) and Research. This dissertation was guided by constructs of the HCG framework and focused on Clinical Practice, Education and Research.

Chapter Two

HCG Model Application and Significance

Chapter Two, Computational Investigation of the Missense Mutations in *DHCR7* Gene Associated with Smith-Lemli-Opitz Syndrome, employs the Research construct in which biophysics and computer modeling are applied to explore protein stability. Based on *in silico* analyses, the findings support other studies that pathogenic *DHCR7* mutations tend to occur in highly conserved and trans-membrane spanning regions of the protein. However, new results from this study suggest that the non-classified mutation *R228Q* associated with Smith-Lemli-Opitz syndrome (SLOS) is actually pathogenic.

The study in Chapter Two also identified: a) SLOS patients possessing the *R228Q* variant may exhibit more severe symptoms than other patients with the same variant; and b) the encoded protein will be unstable and less effective in its physiological role in

cholesterol synthesis. If future studies confirm that *R228Q* is, in fact, pathogenic, then HCPs in the clinical setting may use this information to help carriers of this mutation make family planning and/or personal health decisions. These two exemplars incorporated the Clinical Practice construct in this research.

Limitations

The study was based on the current published structure of the integral membrane sterol reductase from *M. alcaliphilum* with only 37% sequence identity to the human enzyme DHCR7. This experimental structure possessed the greatest homology to human DHCR7 available at the time the research was conducted. The eventual development of a structure with greater homology may further refine these research findings.

The second obstacle is that the DHCR7 is a trans-membrane protein, in which stability is difficult to predict. Membrane-embedded proteins consist of areas buried in a hydrophobic lipid bilayer with other areas exposed to water, resulting in a heterogeneous environment that is difficult to model accurately. Membrane proteins make up more than 40% of the protein structure database (Kroncke et al., 2016), however only 1% of membrane proteins have their 3D structure solved experimentally.

Future Research

Recommendations for future research include:

a) Development of novel methods to predict membrane protein dynamics due to the limitations of current methods, as noted above;

b) Online publication of the crystal structure of the human DHCR7 enzyme;

genomic studies on individuals with the *DHCR7* variant; and,

c) Further study on the symptoms associated with SLOS.

Chapter Three

HCG Model Application and Significance

Chapter Three, The Emerging Role of Interleukin 17 (IL-17) in the Pathogenesis of Endometriosis, is a review of the literature exploring the role of interleukin 17 (IL-17) and T helper 17 (Th17) cells in the common gynecological disease endometriosis. Endometriosis affects 10% of women of reproductive age, yet its variable presentation makes it difficult to diagnose. Diagnosis may take 8-12 years in some cases (Ballard, Lowton & Wright, 2006). One goal of this review is to better educate healthcare professionals about the signs and symptoms of the disease to lessen the time between a woman's onset of symptoms and diagnosis. An overview of the structure and function of IL-17, as well as a review of the role of IL-17 in endometriosis is described. Finally, some potential IL-17-targeted therapies are explored which may, in the future, provide relief for women suffering from endometriosis. This chapter aligns with the HCG constructs of Education and Clinical Practice.

Limitations

The main limitation of this chapter is its structure as a subjective narrative review, rather than a systematic review. The scope of discussion was limited by page number, so a detailed discussion of activation of the NF-κB, MAPK and C/EPB pathways by IL-17 was not included.

Future Research

Recommendations for future research include:

a) A more rigorous and comprehensive systematic review or meta-analysis of the involvement of IL-17 in endometriosis;
b) A review with inclusion of current and upcoming IL-17-targeted therapies indicated for other conditions whose therapeutic effects might be studied in endometriosis; and,
c) A more detailed review of the interactions of IL-6 with IL-17 and Th17 cells in endometriosis would be applicable, due to the role IL-6 plays in the production of IL-17 through the activation of Th17 cells.

Chapter Four

HCG Model Applications and Significance

Chapter Four, Assessment of Beta-Caryophyllene (BCP) for the Treatment of Endometriosis Using an *In Vitro* Approach, which details experiments examining the effect of the cannabinoid beta-caryophyllene (BCP) on a cell model of endometriosis. In this research, the effects of BCP on inflammatory markers was examined in two cancer cell lines (Ishikawa and RL95-2), and in primary human endometrial stromal cells (ESCs). It was anticipated that BCP, which has been shown to initiate an anti-inflammatory phenotype by selectively binding the cannabinoid receptor CB2, would decrease estrogenicity (alkaline phosphatase activity), cell proliferation, and VEGF production. This study, whose findings could inform the development of novel therapies for endometriosis, may have Clinical Practice implications. Chapter Four incorporates the HCG constructs of Research and Clinical Practice.

The study in Chapter Four identified: a) BCP downregulates alkaline phosphatase in Ishikawa cells in the absence of exogenous estradiol; b) BCP inhibits Ishikawa cell proliferation in a dose-dependent manner; c) BCP decreased IL-17-induced VEGF production in endometriosis-positive endometrial stromal cells in the absence of IL-6; and d) neither Ishikawa cells nor RL95-2 cells express CB2, the receptor through which BCP has been shown to act. As BCP does appear to decrease alkaline phosphatase activity and proliferation in Ishikawa cells when exogenous estrogen is not added, BCP may be working through some alternate mechanism. The cytokine IL-17 is weakly capable of inducing VEGF production on its own, but functions to synergize with other cytokines to stabilize their mRNA and prolong the half-lives of their transcripts. IL-17 may act in concert with IL-6 in endometriosis-positive stroma cells, though no support for synergy between IL-17 and IL-6 was identified in the literature.

Limitations

Though many experiments were performed in the study, each was replicated only one time, as directed by the mentor. This low number of replications did not allow for valid statistical testing; thus, these results serve as representations of the effects of BCP in endometriosis only. Endometrial stromal cells were extracted from samples of two individuals (one endometriosis-positive, one endometriosis-negative). The small sample size does not allow appropriate statistical testing or generalizability of the results.

Future Research

Recommendations for future research include:

a) Identification of alternate mechanisms of BCP activity as it was observed to affect estrogenicity and proliferation in Ishikawa cells, despite their lack of CB2 receptor expression;
b) Investigate the potential synergy between IL-17 and IL-6; and,
c) Replicate the experiments enough times to allow for statistical testing.

The results could suggest which mechanisms show promise for treatment of endometriosis.

References

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APPENDICES

Appendix A

Institutional Review Board (IRB) Documents

November 26, 2014

Bruce A. Lessey,
MD, PhD Attn:
Cheryl Myers, RN
University Medical Group
Department of Obstetrics and Gynecology
Greenville Health System
890 West Faris Road, Suite 470
Greenville, SC 29605

RE: IRB File # Pro00038040

Study Title: **Endometrial Biomarkers in Fertile Controls**

Items Submitted for IRB Review: **Protocol Dated 10/15/2014; Consent Form; Study Prescreening Handout**

Dear Dr. Lessey:

On November 13, 2014, the Institutional Review Board/Committee-A (IRB) of the Greenville Health System reviewed and approved the items listed above on your research study contingent upon a consent form modification. This modification was received in the IRB office on 11/20/2014 and final review/approval was given via expedited review on 11/26/2014.

Your study will **expire** on *November 12, 2015.* ***It is the investigator's responsibility to make sure the proper reapproval information is submitted to the IRB.*** This information must be submitted to the IRB in ***October 2015.***

Please keep in mind the following requirements of the Institutional Review Board:

1. All participants **must** sign a copy of the attached IRB-stamped "approved" consent form before they can be enrolled in this study. Please use this stamped "approved" consent form to make copies for each participant.
2. Only the principal investigator or co-investigator can obtain consent from the participant.
3. The participant must sign and date the consent form in the presence of a witness.
4. A report to the IRB is required at the end of the approved time period giving the results of the participants involved in the study, the status of the study and whether or not renewed approval is desired.
5. Immediate notification must be sent to the IRB of any advertisements, modification of the Form 1572, as well as all revisions, changes, or amendments to the protocol or consent form.
6. Notification must be sent to the IRB within five (5) working days of any events required to be reported by the ORCA Policy HRPP Number 16.01.
7. The investigator must be sure that all consent forms are signed, dated and witnessed and placed in the participant's study record prior to study participation. The original should be retained in the participant's study record at the clinical research site. Case histories (patient charts/records) will also document that Informed Consent was obtained prior to the subject's participation in the study.

8. A signed copy of the consent form must be given to the person signing the form and a copy placed in the medical record if the study involves any type of hospital stay.
9. Please remember to use the GHS Study Drug Request Form for all participants entered in this study, when applicable. Your cooperation in this helps the pharmacy to better serve you. This form can purchased from the Supply and Distribution Center (Karen Corwin at 455-7819), Form Number A23914.

The IRB has written procedures for the initial and continuing review of research studies; prepares written minutes of convened meetings; and retains records pertaining to the review and approval process. This is done in compliance with requirements defined in the Code of Federal Regulations (21 CFR Parts 50, 56, 312 and 812; 45 CFR Parts 46 and 164) and ICH (International Conference on Harmonisation) guidance relating to GCP (Good Clinical Practice).

Thank you for your assistance in this matter. Should you have any questions, please do not hesitate to call the IRB office at (864) 455-4984.

Sincerely,

Christopher C. Wright, MD, Chairperson
Institutional Review Board/Committee-A
Employee
Services Center
701 Grove Road
Greenville, SC 29605

CCW/ks
Attachment

GREENVILLE HEALTH SYSTEM

November 10, 2016

Bruce A. Lessey,
MD, PhD Attn:
Cheryl Myers, RN
University Medical Group
Department of Obstetrics and Gynecology
Greenville Health System
890 West Faris Road, Suite 470
Greenville, SC 29605

RE: IRB File # Pro00038040

Study Title: **Endometrial Biomarkers in Fertile Controls**

Items Submitted for IRB Review: **Protocol and Consent Form Continuing Review**

Dear Dr. Lessey:

On November 10, 2016, the Institutional Review Board/Committee-A (IRB) of the Greenville Health System reviewed your research study. Full committee approval of the above-mentioned items was given for one year.

Your study will **expire** on *November 9, 2017.* ***It is the investigator's responsibility to make sure the proper reapproval information is submitted to the IRB.*** This information must be submitted to the IRB in ***October 2017.***

The same requirements as previously outlined for you by the IRB remain in effect as long as the study is ongoing. Please refer to your initial approval letter for these requirements.

Thank you for your assistance in this matter. If you have any questions, please feel free to call the IRB Office at (864) 455-4984.

Sincerely,

Christopher C. Wright, MD, Chairperson
Institutional Review Board/Committee-A
Employee Services Center
701 Grove Road
Greenville, SC 29605

CCW/ks
Attachment

GREENVILLE HEALTH SYSTEM

October 13, 2017

Bruce Lessey, MD, PhD
Attn: Cheryl Myers, RN
University Medical Group
Department of Obstetrics and Gynecology
Greenville Hospital System University Medical Center
890 West Faris Road, Suite 470
Greenville, SC 29605

RE: IRB File # Pro00038040

Study Title: **Endometrial Biomarkers in Fertile Controls**

Items Submitted for IRB Review: **Protocol and Consent Form Continuing Review**

Dear Dr. Lessey:

On October 12, 2017, the Institutional Review Board/Committee-A (IRB) of the Greenville Health System reviewed your research study. Full committee approval of the above-mentioned items was given for one year.

Your study will **expire** on *October 11, 2018.* ***It is the investigator's responsibility to make sure the proper re-approval information is submitted to the IRB.*** This information must be submitted to the IRB in ***September 2018***.

The same requirements as previously outlined for you by the IRB remain in effect as long as the study is ongoing. Please refer to your initial approval letter for these requirements.

Thank you for your assistance in this matter. If you have any questions, please feel free to call the IRB Office at 864-455-8997.

Sincerely,

Christopher C. Wright, MD, Chairperson

Institutional Review Board/Committee-A
Employee Services Center
701 Grove Road
Greenville, SC 29605

CCW/akg

GREENVILLE HEALTH SYSTEM

September 13, 2018

Bruce Lessey, MD, PhD
Attn: Cheryl Myers, RN

RE: ***IRB File* # Pro00038040**

Study Title: **Endometrial Biomarkers in Fertile Controls**

Items Submitted for IRB Review: **Permanent Closure**

Dear Dr. Lessey:

On September 12, 2018, the Chairman of the Institutional Review Board/Committee-A of the Greenville Health System reviewed the above-mentioned item that was presented for expedited review. The Chairman accepted this as information and a copy will be kept in the IRB file.

Thank you for your assistance in this matter. Should you have any questions, please do not hesitate to call the IRB office at 864-455-8997.

Sincerely,

Christopher C. Wright, MD, Chairman
Institutional Review Board /Committee-A
Employee Services Center
701 Grove Road
Greenville, SC 29605

CCW/akg

Appendix B

Permissions for Use

Permission to use Figure 3.2. IL-17 Signaling

Request to use adapted figure from Amatya et al., 2017

3 messages

Rebecca Myers <rlmyers@g.clemson.edu> Thu, Oct 18, 2018 at 2:20 PM
To: sarah.gaffen@pitt.edu

Hello Dr. Gaffen,

I have developed a figure similar to Fig 3 (Activation of IL-17 signal transduction) in your published article "IL-17 signaling: The yin and the yang". 2017. *Trends in Immunology, 38*(5), 310–322. I would like to incorporate the figure into a chapter of my dissertation stating "adapted from Amatya, Garg, & Gaffen, 2017)" in the caption, with a citation of your paper in the references. Would this be acceptable?

Please find my version of the figure attached.

Thank you for your consideration,

Rebecca Myers, PhD (c)
Healthcare Genetics Doctoral Program
Clemson University

--
Rebecca Myers
PhD candidate, Healthcare Genetics
Clemson University
rlmyers@clemson.edu
(803) 709-1531

Adapted from Amatya et al 2017_IL-17 figure.docx
133K

Gaffen, Sarah L <sarah.gaffen@pitt.edu> Thu, Oct 18, 2018 at 2:34 PM
To: Rebecca Myers <rlmyers@g.clemson.edu>

How nice of you to ask! Yes absolutely.

Sarah

Sent from my iPhone
(Sarah Gaffen)
[Quoted text hidden]
<Adapted from Amatya et al 2017_IL-17 figure.docx>

Rebecca Myers <rlmyers@g.clemson.edu> Thu, Oct 18, 2018 at 2:37 PM
To: sarah.gaffen@pitt.edu

Great, thank you!
[Quoted text hidden]

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