THE IMPACT OF IYENGAR YOGA ON DEMANDS OF ILLNESS, COPING, AND LYMPHOCYTE NF-κB ACTIVATION IN BREAST CANCER SURVIVORS

By

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A thesis submitted in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE IN EXERCISE SCIENCE

WASHINGTON STATE UNIVERSITY SPOKANE
Clinical and Experimental Exercise Science Graduate Program

DECEMBER 2007

To the Faculty of Washington State University:

The members of the Committee appointed to examine the thesis of PAMELA ELLEN SCHULTZ find it satisfactory and recommend that it be accepted.

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Chair

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ACKNOWLEDGEMENTS

There are numerous individuals who have helped make this research project possible and bring it to completion. First and foremost, I would like to thank my advisor, Sally Blank, for the opportunity to be a part of this research project. I am grateful for the unending challenges that presented themselves, what I have learned in the process, and how I have grown both intellectually and professionally. Your guidance and mentorship has helped mold the person I am proud to be today. Thank you. I would also like to thank my committee members Mel Haberman and Kenn Daratha. Your dedication and support through this entire process has been incredible. I have enjoyed exploring and integrating into my final project, the psychosocial aspects of cancer survivorship and various data mining techniques. I could not have found a better thesis committee to learn from. Thank you. I would like to thank my fellow graduate students along with the many individuals in the Health Sciences Building who so willingly and consistently provided me with support and encouragement to do my best work possible. And finally, Mo, Judi, Nicole, and my entire fabulous family…my success is because you are a part of my life.
Thank you.

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Abstract

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December 2007

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Breast cancer survivorship is associated with changes in physical, psychological, and social aspects of well-being. Such changes may require significant cognitive and emotional adaptation. Coping strategies whether positive or negative may predict how well a survivor is able to adapt and may affect how their body responds to the illness. Integrative medicine often incorporates mind-body therapies, such as yoga, to increase overall wellbeing and physical health in breast cancer survivors. The present study was designed to investigate the relationship between Iyengar yoga practice, illness-related stressors and lymphocyte NF-κB activation in female breast cancer survivors. Females who were approximately 1 to 11 years post-diagnosis with stage I-IV breast cancer were randomly assigned to either the yoga (Y, n = 13) or control (C, n = 11) group. The eight-week yoga intervention included three yoga sessions per week. Participants completed a Demands of Illness Inventory (DOII) and Revised Ways of Coping Checklist (RWCCL) prior to (T1) and following (T2) the intervention. Blood samples were taken at T1 and T2 for use in *in vitro* unstimulated and stimulated lymphocyte NF-κB analyses.

Lymphocytes were stimulated with phytohemaglutinin (PHA), phorbol 12-myristate 12-acetate (PMA), and ionomycin. Nuclear extracts of stimulated cells were analyzed for NF-κB activation by an electrophoretic mobility shift assay. Cytosolic and nuclear extracts from unstimulated and stimulated paired aliquots were analyzed for cellular location of NF-κB by chemiluminescence. Data were analyzed by independent and paired *t*-tests, and linear regression. A global measure of the DOII revealed a significant interaction between time and group effects ($F = 5.275$, $p = 0.028$) revealing decreased DOII in the yoga group from T1 to T2. DOII was inversely correlated with overall active coping strategies ($r = -0.23$, $p = 0.05$) and "count your blessings" ($r = -0.51$, $p < 0.000$). Yoga participation was associated with a trend for decreased lymphocyte activation ($p = 0.077$). The decreased change in lymphocyte NF-κB activation was correlated with increased use of passive coping strategies ($r = 0.900$, $p = 0.03$). The results support the hypothesis that yoga participation mediates perceived demands of illness associated with breast cancer survivorship and that lymphocyte NF-κB signaling pathways are involved in physiological changes that enhance psychosocial well-being.

Key Words: breast cancer, demands of illness, coping, yoga, integrative medicine, immune function, lymphocytes, NF-κB

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CHAPTER ONE

INTRODUCTION

Breast cancer is the most common newly diagnosed cancer among woman in the United States.[1] As breast cancer survival rates improve a greater number of women have to adapt to and live with the biopsychosocial impacts of cancer survivorship. Breast cancer includes many psychological and social hardships that are directly related to diagnosis, treatment, and survivorship.[2-4] Many of the concerns present at time of diagnosis and treatment persist throughout survivorship.[2]

The relationships among psychosocial challenges of breast cancer survivorship and disease prognosis and progression remain uncertain. Several models exist linking breast cancer survivors' physical and psychological stress with coping responses.[5-11] For example, emotional and behavioral disengagement, low perceived control and fear of treatments are each associated with negative coping strategies such as avoidance and acceptance-resignation.[5, 9] On the other hand, harmony and peace are associated with seeking support and active coping, and inversely associated with avoidance.[9] Adjustment and adaptation to breast cancer include processing a wide range of stressors into directed thoughts and actions that reflect coping strategies. Instruments such as the Revised Ways of Coping Checklist measure the degree that various coping strategies are used by an individual.[12-14] Coping strategies are often classified as active coping (positive in nature) and passive/emotion-focused coping (more negative minded).[9] It is thought that adjustment through coping mediates the outcome of one's perceived stress level.

A survivor's quality of life (QOL) reflects their multidimensional perspective and attempt to restore normalcy, achieve wholeness, and re-establish a sense of purpose in life.[15] The Demands of Illness Inventory (DOII) was developed to assess individual and family adaptation to chronic illness.[16, 17] Demands are typically conceptualized as hardships or detrimental events that call for some type of coping and adaptive response. The DOII includes a selection of QOL domains related to cancer survivorship and a variety of chronic diseases such as breast cancer, diabetes, and colorectal cancer.[17, 18]

The significance of QOL in cancer survivorship warrants integration of complementary therapies into mainstream medical care to provide additional treatment for illness-related symptoms. Cancer survivors often choose to use complementary therapies to alleviate feelings of distress, and not generally because of dissatisfaction with traditional western medicine.[19] Mind-body therapy is a popular integrative modality used to induce relaxation and improve emotional well-being through healing methods that enhance the mind's interactions with bodily function.[20] Yoga is regarded as a promising method for the treatment of stress-related symptoms of chronic disease, including cancer because many yogic traditions integrated physical, mental, and spiritual elements within the practice. The results of several pilot studies support ancient beliefs regarding the health benefits of yoga practice.[21-23] In addition to emotional well-being, yoga may have a positive effect on immune function in breast cancer survivors.

Medical treatments and psychological adaptation can significantly influence immune parameters of breast cancer survivors. The immune system functions to maintain homeostasis within the body. Homeostasis is threatened by cancer initiation, its progression, and medical cancer treatment therapies. Research investigating the role of

immunity in response to cancer-related stressors is complicated by individual variability; no two individuals respond identically to the same stressor, nor does a like group of cancer survivors experience the same stressors to the same degree of potency. Nonetheless, it is acknowledged that immune cells play a part in tumor development and disease progression.[24, 25] Innate and adaptive immune cells, including lymphocytes, can foster an environment of immunoserveillance and/or immunosubversion via multiple mechanisms including the secretion of soluble factors.[26]

The transcription factor nuclear factor –kappa B (NF-κB) serves as a primary cell signaling pathway and is central to the regulation of diverse cellular immunological processes.[27] NF-κB is a collection of dimeric proteins sequestered in the cytoplasm as latent complexes bound to its inhibitor.[28, 29] NF-κB complexes are composed of homo- and hetero-dimers made up of RelA/p65, c-Rel, RelB, NF-κB1(p50/p105) and NF-κB2 (p52/p100).[28] Each family member contains an N-terminal 300-amino-acid domain (Rel homology domain) that is responsible for dimerization and DNA binding.[30]

Activation of NF-κB has been called "a sensor for smoke and stress signals" as it results from a wide range of stimuli and is involved in gene expression for hundreds of genes, including certain lymphocyte signaling pathways.[31] Mitogen stimulation is a method used to bring about lymphocyte proliferation involving activation of NF-κB. *In vitro*, it is thought mitogen stimulation of lymphocytes mimics the response of certain antigens and is therefore a practical technique to study mechanisms involved in lymphocytes activation.[32, 33]

Statement of the Problem

Breast cancer survivors deal with a multitude of stressors that cause many to seek complementary therapies in hopes of increasing their quality of life. Diagnosis, treatment and the many illness-related stressors can have a cumulative detrimental effect on immune function of survivors. As survivors seek out advice for appropriate complementary therapies, western medicine is only beginning to acknowledge and explore the benefits of these various methods, including yoga. The influence of Iyengar yoga practice on immune function is incompletely understood. To date, the effects of Iyengar yoga practice on the activation of transcription factor NF-κB in lymphocytes from breast cancer survivors is unknown. The purposes of this study were: 1) to examine the impact of Iyengar yoga on the activation of NF-κB in PHA/PMA/ionomycin stimulated peripheral blood lymphocytes from women with stage I-IV breast cancer; 2) to examine the effect of Iyengar yoga on psychosocial stressors, as measured by RWCCL and DOII, in women with stage I-IV breast cancer; 3) to examine associations among NF-κB activation and psychosocial mediators and outcomes as measured by RWCCL and DOII.

Hypotheses

The hypotheses of this study were: as compared with wait-listed controls, breast cancer survivors who participated in yoga will have 1) altered NF-kB activation in stimulated lymphocytes; and 2) increased quality of life with reduced cancer-related symptoms as determined by the RWCCL and DOII.

CHAPTER TWO

LITERATURE REVIEW

Breast Cancer Survivorship

According to the American Cancer Society, a woman in the United States has a 12.5% chance (1 in 8) of developing breast cancer in her lifetime.[1] Female breast cancer comprises the largest group of cancer survivors and is the most common site for newly diagnosed cancer cases among woman in the United States.[1] Death rates from breast cancer however have declined by an average 2.3% per year since 1990, largely attributed to earlier detection through screening, increased awareness, and improved treatment.[1] As survival rates improve however, a greater number of women have to adapt to and live with the biopsychosocial impacts of cancer survivorship.

Quality of life (QOL) concerns of breast cancer survivors often fit into one of the following domains: physical, emotional, social, sexual, and spiritual well-being. Although new issues may emerge, long-term cancer survivors are concerned with many of the same issues as at the time of diagnosis and treatment.[2] Breast cancer survivorship includes experiences such as pain, fatigue, sexual disruption, appearance and body-image concerns, emotional distress, depression, worries about insurance, worry for the future health of one's children, worry of recurrence, late effects of cancer treatment, development of second cancers, feelings of powerlessness, disappointment, futility, meaninglessness, remorse, death anxiety, and questions regarding purpose in life.[2-4]

The significance of understanding QOL and then minimizing the number of stressors that are part of chronic illness was acknowledged early by Hans Selye (mid

1900's) in his work defining the effects of stress and the resulting general adaptation syndrome (GAS).[34] Selye's GAS is a framework around which psychological factors can and do elicit a physiologic stress response in individuals with chronic physical illness.[34] Specifically, Selye examined the neuroendocrine effects and conceptualized the body's response to stress as consisting of three phases related to adaptation.[35] He proposed that different stressor stimuli of equal magnitude potency do not necessarily cause the same syndrome in different individuals, and that the same degree of stress induced by the same stimulus may provoke different lesions in different individuals.[34] Simply stated and applicable to breast cancer, survivors may share organ vulnerability, however each survivor differs in his/her response to the same biopsychosocial stressor. An individual's relationship between psychological stress and illness may be mediated through means of adjustment, perceptions of stress, and personal/social resources.

Adjustment and Adaptation to Breast Cancer Survivorship

The effectiveness and means by which one copes with the diagnosis and/or disease-related stressors of breast cancer often defines how well or poorly he/she has adjusted or continues to adapt to the disease. Coping is defined as "ongoing cognitive and behavioral efforts to manage specific external and/or internal demands that are appraised as taxing or exceeding the resources of the person"; or simply "cognitive or behavioral efforts to manage psychological stress".[36] Through a variety of identified coping strategies, researchers examine how individuals process their thoughts and actions leading to adaptation outcomes.

Coping through cognitive, emotional, or behavioral disengagement is suggested to be detrimental to long-term adjustment to breast cancer, while coping through active acceptance, seeking social support, emotional expression, and other approach-oriented coping strategies predicts diminished distress over time.[5-8, 37] Negative strategies may interfere greatest by preventing adaptive thoughts and behaviors. Survivors who use avoidance as a coping strategy or feel resigned to their fate may be less likely to use active coping strategies.[9, 38] Feelings of harmony and peace predict less frequent use of denial/avoidance as denial and avoidance were found to be inversely associated to seeking support and active coping, respectively.[9] Women who are more frequent users of active coping/social support when dealing with their cancer diagnosis report greater inner peace later in life.[9] It is believed that means of active coping with social support may help survivors find meaning through fostering engagement and emotional expression to others while also increasing feelings of self-efficacy and personal control.[9]

Perceived control, including a combination of coping strategies, self-efficacy, and personal beliefs about control, has a strong relationship to adaptation to illness in breast cancer patients.[10] Low perceived control correlates with uncertainty, fear of treatments, and overall poorer adaptation to illness.[10] Among patients with a low sense of control, active coping strategies are often not developed.[10] Instead, coping strategies such as "avoidance", "acceptance-resignation", "anxious preoccupation", and/or "fatalism" are more common and highly correlated with symptoms of anxiety and depression.[10] Patients with high perceived control on the other hand is associated with problem-focused coping and show reduced anxiety and depression.[7]

Lazarus and Folkman's theory of Stress and Coping includes eight coping strategies that individually and/or in combination, affect adaptation outcomes (quality of life).[39] Classified as either problem-focused (active) coping or emotion-focused (passive) coping, the eight strategies included: confrontive, distancing, self-controlling, seeking social support, accepting responsibility, escape-avoidance, planful problem solving, and positive reappraisal.[39] Derived from this theory, the Ways of Coping Checklist was created as an instrument to measure a variety of coping methods in response to a general range of stressors.[12, 14] Multiple versions of this instrument have been created including the original 64-item index as well as a shorter revised 42-item, 4-point Likert-type rating scale.[12-14, 39] Total scores are calculated by summing the rating for all items. Raw scores may be calculated to represent overall active and passive coping strategies as well as specific individual coping strategies incorporated into the questionnaire.[13] Although not an all inclusive list, specific coping strategies incorporated into the various versions include problem-focused, seeking social support, blames self, wishful thinking, blames others, count your blessings, avoidance, and religiosity. Determining relationships between coping strategies and adaptation to illness allows clinicians to tailor appropriate interventions to breast cancer survivors so that quality of life can be enhanced.

Assessing Quality of Life of Breast Cancer Survivors

Tools created to measure QOL allow researchers and/or clinicians to examine the impact of various interventions and treatments on overall well-being and health. QOL is best assessed from a multidimensional perspective as survivors attempt to restore normalcy, achieve wholeness, and re-establish a sense of purpose in life.[15] Numerous

instruments have been developed to quantify the QOL and the psychosocial impact of chronic illness however the value of a given instrument varies with individual need. The usefulness of a given instrument may be questionable if the scope of the tool is restricted to a single diseased population, a limited time of illness trajectory, or measures only a single construct.[17]

The Demands of Illness Inventory (DOII) was developed in theory around individual and family adaptation to chronic illness.[16, 17] Demands of illness are thought not to be identical to illness stressors, hardships, concerns, or problems, but instead appraised as difficulties and challenges.[16] Demands are typically conceptualized as hardships or detrimental events that call for some type of coping and adaptive response.[17] It is appropriate then to consider "demands" as an outcome of adjustment by which "coping strategies" may mediate.

Specific areas of interest within the DOII include personal meaning (priorities, values, and goals that change with illness; uncertainty, mortality; family susceptibility), social relationships (changes in social activities; helping others understand or accept the illness; overprotective responses), self-image (changes in physical appearance, feelings of attractiveness; impact of disfiguring surgery or treatment), monitoring treatment and symptoms (vigilance to new bodily sensations; preoccupation with symptoms; fears of recurrence, undetected metastasis, or progressive nature of the disease), and treatment issues (accommodation to regimen; treatment evaluation; relationship with providers).[17] An early version of the DOII consisted of a 125 item instrument that also integrated topics related to physical symptoms (somatic responses of illness and anxiety) and family

functioning (general systems theory aspects of decision making, adaptation, integration; partner caretaking, care of children; work or job situation).

Integrative Cancer Care for Breast Cancer Survivors

Integrative cancer care refers to complementary and/or alternative therapies implemented as part of a treatment program to improve survival, survivorship and QOL of cancer patients. Integrative cancer therapies possess "potential to contribute to improving survival as well as quality of life of cancer patients if they are integrated into a comprehensive and scientifically based program adapted to the needs of the patient's disease, stage, and biological and social individuality".[40] Integrative medicine is commonly practiced as a means to specifically reduce the symptom of psychological distress, and not reportedly used for reasons of dissatisfaction with traditional medical care.[19] Among breast cancer survivors, use of complementary therapies includes reasons such as "to assist the body's natural forces to heal", "to boost the immune system", "to increase quality of life", and "to gain a feeling of control of the cancer".[41] Breast cancer survivors that used complementary and alternative medicines reported less severe anxiety and depression symptoms as compared with survivors given treatment as usual.[41]

Mind-body therapy is a popular integrative modality used to induce relaxation and improve emotional well-being through healing methods that enhance the mind's interactions with bodily function.[20] Breast cancer survivor participation in mindfulness-based stress reduction (MBSR) programs is associated with improvements in quality of life (QOL), mood states, stress symptoms, and immune parameters such as circulating lymphocyte number and *in vitro* cytokine production.[42] Stress management techniques

including cognitive-behavioral interventions and relaxation techniques have been used for a variety of intervention motives.[43] The effectiveness of a specific intervention is often dependent on the health-outcome measure assessed. A combination of cognitive-behavioral (enhancing psychological outcomes) and muscle relaxation techniques (enhancing physiological outcomes) appears to be more effective than using a single technique in improving psychological and/or physiological health.[43]

Yoga Interventions for Cancer Survivors

Developed as part of traditional Indian medicine, yoga was formulated by ancient civilizations as a method to unite all the movements one needs for physical health with breathing and meditation practices that ensure peace of mind.[44] Derived from the root *yug* (to join), the word "yoga" has come to describe a method of discipline: to join the body to the mind and together join to the self (soul); the union between the individual self and the transcendental self.[45] Yoga combines muscle relaxation, meditation and physical training while focusing on *asanas* (postures) and *pranayama* (controlling the breath).

Yoga is regarded as a promising method for the treatment of stress-related symptoms because of its emphasis on integrating physical, mental, and spiritual elements. A study examining the effect of a cognitive-behavioral therapy (n = 19) compared to a Kundalini yoga program (n = 18) over a 4-month period showed that both interventions resulted in significant improvements of various psychological and physiological outcomes, including stress behavior, anger, exhaustion, QOL, heart rate, blood pressure, and circulating catecholamine and cortisol concentrations.[46] The purpose of the study was to investigate stress management techniques and included assumed healthy adults (male

and female) who were employees of a large financial company.[46] Preliminary evidence also supports the feasibility and efficacy of select yoga interventions as therapeutic modalities for cancer survivors, including breast cancer. Yoga practices taught in the Iyengar, Tibetan, and Hatha traditions are associated with modest improvements in sleep quality, mood, stress, cancer-related symptoms, and overall quality of life among cancer survivors.[47, 48]

Breast cancer survivors may also benefit from yoga taught as an active practice, i.e., physically challenging *asanas*. This hypothesis is based on observations by Holmes et al.[49] indicating that participation in regular moderate-intensity physical activity reduced risk of death from breast cancer. Documenting physiological benefits of yoga intervention for breast cancer survivors is important because yogic traditions differ in their approach to the yoga *asanas*. Iyengar yoga, based on the teachings of B.K.S. Iyengar, consists of an orderly and progressive method of a series of postures that are adjusted to meet the needs and physical conditions of the student.[45] Iyengar yoga incorporates the use of props (e.g., chairs, belts, blankets, blocks) to assist the practitioners in assuming the posture without strain. According to Iyengar tradition[45], specific anatomical guidelines direct the execution of the *asanas* and *pranayama*, and specific postures are adjusted so that various organs, joints, and muscles are properly positioned to facilitate physiologic changes. A pilot study investigating the effect of Iyengar yoga on the emotional states of healthy adults reported improved psychological well-being following a single yoga class.[23] Increases in positive moods, decreases in negative moods, and increases in energy levels resulted regardless of the *asanas* practiced. Specific poses affected mood differently, with back bends associated with

greater increases in positive moods and particularly so for those who were relatively hostile or depressed.[23]

Results from additional pilot studies examining the physical and psychological benefits of Iyengar yoga for breast cancer survivors commonly reveal a positive impact on mood, quality of life and stress levels.[22, 50] As healthy individuals experience similar benefits, it is unknown whether or not breast cancer survivors who may begin at a less optimal physiological and psychological status achieve any greater benefit.[50] However, improvements in illness-related symptoms including relieved joint aches and shoulder stiffness, improved body posture and body image, and increased feelings of relaxation in their daily lives were reported following an eight week Iyengar yoga intervention.[22] Repeated findings support yoga's efficacy for potential to provide both physical and psychological benefits to breast cancer survivors.[21-23, 47, 50]

Ancient eastern yogic philosophy and health benefits of yoga practice are rapidly gaining acceptance in western medicine. Scientists within the field of psychoneuroimmunology are now working toward a greater understanding of the physiological process involved in mind-body practices. Simply stated, psychoneuroimmunology is the scientific study of how the nervous system and the mind influence the immune system. As a quickly developing field of study, it is promising that future research will reveal the mechanisms behind Patanjali's classical *Yoga-Sutra* (c. 200 C.E) and the role they play in the healthy as well as the diseased.[51] Central to this effort is a developing framework for biological signaling pathways pertinent to immune function.

Introduction to the Immune Response and Cancer

The human immune response is composed of a large variety of cells and mediators which interact in a dynamic network to protect the body. Depending on past exposure and antigen specificity, the immune response is commonly divided into two subtypes: innate and adaptive immunity.[24] Recognition of a foreign pathogen elicits a response that at the cellular, tissue, and organism levels results in clearance of the pathogen or foreign matter if deemed harmful.[24, 26] Innate immunity is also thought of as the first line of defense, providing an immediate response to invading or infectious pathogens or damaged cells.[26] Adaptive immunity, also known as acquired immunity, facilitates memory-recognition and increased states of immune response to subsequent exposure with the same antigen.[26] Soluble factors such as complement, antibodies and cytokines secreted from immune cells mediate crosstalk between innate and adaptive immune cells.[24] Innate immunity typically involves an acute inflammatory response which then serves to activate and regulate adaptive immunity.[24] A dysregulated adaptive immune response such as the case in chronic inflammatory conditions may have the reverse effect and in turn mediate a chronic innate response often resulting in tissue damage.[26] Chronically unbalanced interactions between innate and adaptive immune cells leads to chronic versus acute inflammation, which can lead to increased risk of cancer development and growth,[24] with lymphocytes playing a prominent role in immune response and inflammation.[52]

Lymphocyte subsets including B cells, T cells and natural killer (NK) cells are all involved in immunosurveillance and the crosstalk between innate and adaptive immunity. In terms of immunosurveillance, for example, CD8+ cytotoxic T lymphocytes recognize

and kill tumor cells by secreting perforin which binds to major histocompatibility complex (MHC) class I receptors on tumor cells and induces apoptosis.[53] Tumor angiogenesis provides a means of growth for the carcinogenic tissue. In addition, they secrete the anti-angiogenic cytokine interferon-γ (INF-γ).[53] Activated CD4+ T cells participate in converting MHC class II macrophages, which secrete interleukin (IL) -10, into INF-γ secreting macrophages. The CD4+ T_h1 cells also secrete INF-γ while CD4+ T_h2 cells produce IL-4 which indirectly blocks tumor-angiogenesis.[53] Activated NK cells are involved in tumor lysis and apoptosis directly via a perforin-dependent manner and indirectly through cell-cell contact, for example, with dendritic cells in the presence of IL-4 and IL-13.[53] Regulated NK cell activity thus plays a key role in killing carcinogenic cells.

As the immune system functions to maintain homeostasis, it is proposed that cancer cells may escape innate and adaptive immune responses via immunoselection (selection of non-immunogenic tumor-cell-variants) or immunosubversion (active suppression of the immune response).[53] Finke et al.[25] hypothesized that two conditions exist enabling tumor cells to escape immunosurveillance and essentially thrive in inflammatory environments. The first condition occurs when lymphocytes within a microenvironment are functionally impaired.[25] The second condition requires inflammatory-cell-derived mediators secreted from activated fibroblasts, endothelial cells, and lymphocytes in response to tumor progression in the microenvironment. Lymphocytes can also secrete matrix metalloproteinases, a key component to the extracellular matrix and tumor growth.[54] In addition, tumor microenvironments are rich in lymphocyte-derived cytokines, namely tumor necrosis factor -α (TNF–α), transforming

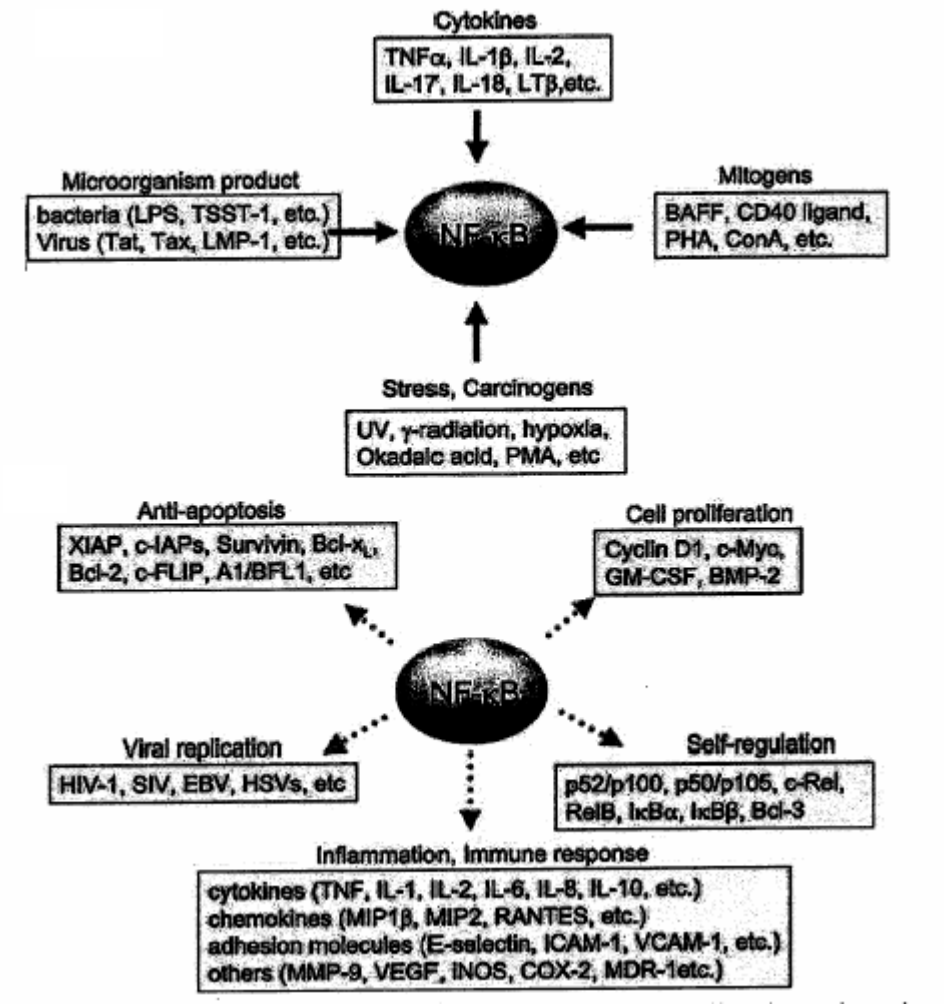
growth factor -β (TBF–β), IL-1, and IL-6, which are all mediators of cancer development.[24] Furthermore, chronically activated innate immune cells may indirectly suppress anti-tumor adaptive immunosurveillance.[24]

Nuclear Factor –kappa B (NF-κB)

Transcription factor nuclear factor –kappa B (NF-κB) is central to the regulation of diverse biological processes.[27] Within various cancer cells as well as lymphocytes, NF-κB serves as a primary cell signaling pathway. The mammalian NF-κB family consists of a collection of dimeric proteins sequestered in the cytoplasm as latent complexes bound to a member of the IκB (inhibitor of NF-κB) protein family.[28, 29] Dimeric proteins existing as both homo- and heterodimers which make up the NF-κB complexes include RelA (p65), c-Rel, RelB, NF-κB1(p50/p105) and NF-κB2 (p52/p100).[28] Each family member contains an N-terminal 300-amino-acid domain (Rel homology domain) that is responsible for dimerization, interaction with IκB's, and DNA binding to κB promoter or enhancer regions of more than 400 different target genes.[30] In addition, RelA, c-Rel and RelB also contain C-terminal transcription activation domains (TADs) which enable activation of gene expression.[30] Unless bound to a protein containing a TAD (such as Bcl-3), p50 and p52 homodimers are incapable of gene transcription, and their binding to κB sites in unstimulated cells serves to repress gene expression.[30] The IκB family, consisting of IκBα, IκBβ, IκBε, IκBγ, Bcl-3, and two precursor proteins: p100 and p105, are predominately located in the cytoplasm.[28, 29] Differing from the other IκB family members, Bcl-3 functions as a co-activator promoting activation at the site of DNA binding within the nucleus.[28]

Activation of NF-κB can occur by a diverse array of stimuli. Inflammatory cytokines, mitogens, receptor ligands, growth factors, oncogenes, bacterial products, chemicals, and cellular stress are all capable of inducing NF-κB activation and subsequently eliciting a complex range of responses[28, 31, 55] as depicted in Figure 1 below.[56] General receptors responsible for initiating cell signaling pathways include B cell receptors, T cell receptors, Toll-like receptors (TLR), IL-1 receptors, and the TNF-receptor (TNFR) superfamily of molecules (molecules other than TNF-α).[57]

Figure 1.[56]



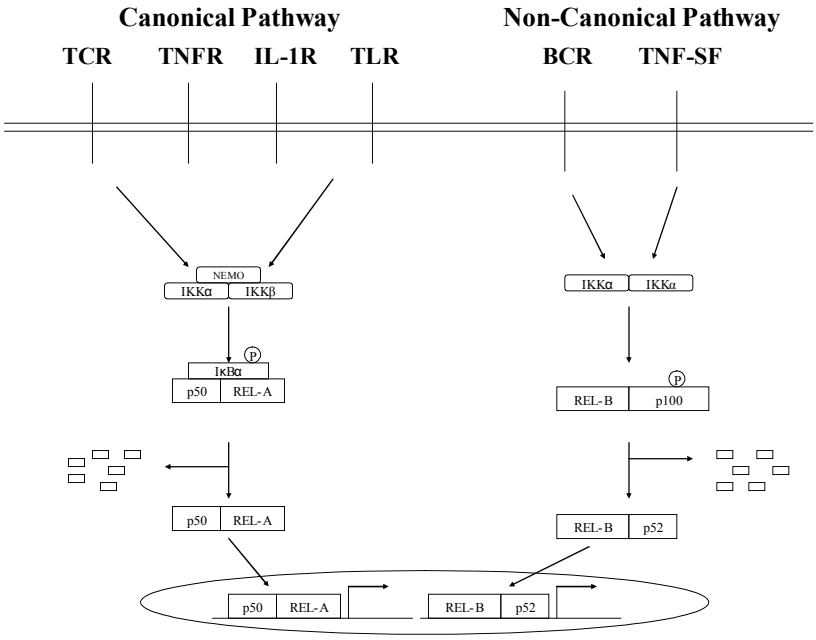
NF-κB activation pathways are generally classified as either canonical (classical) or non-canonical (alternative) depending on whether activation involves IκB degradation or p100 processing.[58, 59] A third pathway leading to NF-κB activation is sometimes referred to as the atypical pathway.[60] Although similar to the classical pathway in IκB degradation, the atypical pathway differs in the essential kinases which lead to inhibitor degradation. Ultimately however, activated NF-κB dimers are liberated from their bound inhibitor and translocate to the nucleus where NF-κB is further involved in specific gene expression activity.

The canonical, or classical, pathway is initiated by various inflammatory stimuli, including extracellular pro-inflammatory cytokines such as tumor necrosis factor (TNF)-α and IL-1, engagement of the T cell receptor (TCR) or exposure to bacterial products such as lipopolysaccharide (LPS).[60] Binding leads to sequential recruitment of various adaptor molecules to the cell membrane within the intracellular compartment which activate the IκB kinase (IKK) complex.[61] The three core subunits that make up the IKK complex consist of IKKα (also known as IKK1), IKKβ (IKK2), and IKKγ (NEMO, NF-κB essential modulator). The canonical pathway is characterized by IKKβ as being the predominant IκB kinase leading to the rapid phosphorylation of the IκBα at Ser32 and Ser 36 and subsequent ubiquitin-induced degredation by the 26S proteasome.[60] The degradation of IκB exposes the nuclear localization signal of the NF-κB family protein, leading to its nuclear translocation.[62]

The non-canonical pathway operates primarily in B lymphocytes and is stimulated through the TNFR superfamily, including receptors for B-cell activating factor (BAFF), lymphotoxins and CD40 ligand (CD40L).[58] This pathway is considered NEMO-

independent and controls the activation of complexes that consist of p100. Following the recruitment of adaptor proteins, NF-κB inducing kinase (NIK) is activated and responsible for the phosphorylation of the p100 NF-κB subunit by IKKα. Processing of p100, via ubiquitination by the 26S proteasome results in the formation of p52 and allows p52-containing dimmers to translocate to the nuclues.[28] RelB-p52 heterodimers are frequently activated as a consequence of non-canonical pathway activation and have a high affinity for a distinct subset of κB elements which may lead to the regulation of a distinct subset of NF-κB genes.[60] Figure 2 illustrates the primary difference between the canonical and non-canonical pathways, involving differing IKK/IκB/NF-κB interactions.

Figure 2.



Atypical pathways leading to NF-κB activation are believed to be IKK independent. Tyrosine-kinase-dependent pathways described as phosphorylating IκBα at Tyr42 may result from stimuli such as hypoxia and hydrogen-peroxide.[60] Treatment of

ultraviolet (UV) light or expression of the *HER2* oncogene in breast cancer cells can result in the direct phosphorylation of IκBα by casein kinase-II (CKII) at sites in its C-terminal domain.[60, 63] In both cases, IκB is either degradated or disassociated from NF-κB allowing for translocation to the nucleus and transcriptional activity.

Cell Signaling Pathways from Mitogen Stimulation

Just as NF-κB activation results from a vast array of stimuli, there are also numerous signaling pathways which link cellular stimulation to the IKK/IκB/NF-κB module. Mitogen stimulation involves fundamental signal transduction pathways leading to NF-kB activation in lymphocytes. A mitogen is a chemical or protein that can mimic actions of antigen and initiate cell mitosis, resulting in cell proliferation. As lymphocytes respond to antigen and mitogen stimuli similarly, *in vitro* mitogen stimulation provides the means to assess potential lymphocyte response to disease or other stressor.[32] Phytohemagglutinin (PHA) and phorbol-12 myristate 13-acetate (PMA) plus ionomycin are common mitogens involved in lymphocyte activation. Although PHA, PMA and ionomycin signal transduction pathways differ slightly in their primary course of action, it is believed that each is associated with protein kinase C (PKC) isoforms, which in turn activate the IKK/IκB/NF-κB cascade of events.[64, 65]

Phytohemagglutinin has a high affinity for T lymphocyte surface receptors, namely the IL-2R.[66] Activation of T cells via PHA results in cellular proliferation and increased T cell expression of IL-2 and its receptor, involved in cyclic activation.[67] Interestingly, IL-2 is a principle (autocrine and paracrine) cytokine in T cell activation and IL-2 synthesis is dependent on NF-κB activation.[65] NK cell activity also involves

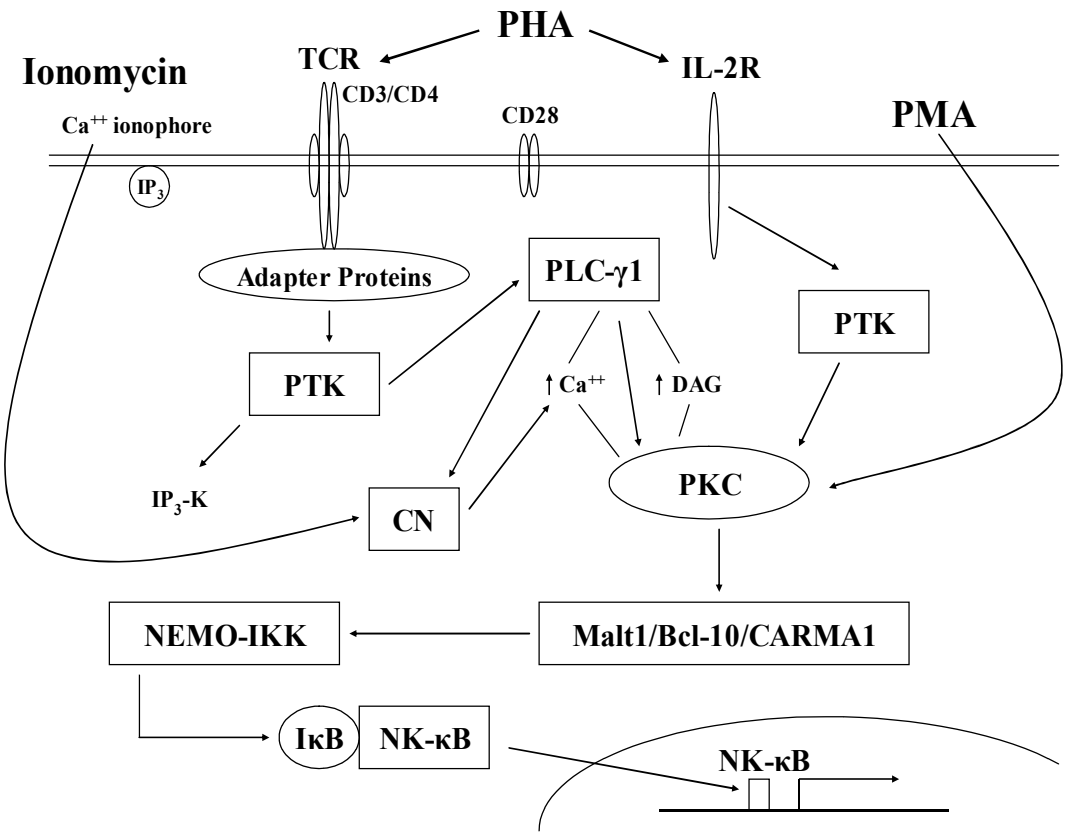
NF-κB activity induced by IL-2R signaling.[68] Exact mechanisms linking IL-2R and NF-κB activation continue to be investigated, however it is established that transduction involves upstream protein tyrosine kinases (PTK) that lead to PKC activation and NF-κB nuclear translocation.[65] Lymphocyte proliferation is also dependent on NF-κB activation however independent of IL-2 production and the IL-2 receptor.[69]

A commonly routed mitogen stimulation pathway involves the TCR complex (CD3/CD4) and CD28 in T cells and the BCR in B cells. Stimulated TCR/CD28 elicits the recruitment of PTKs as well as other adaptor proteins to the phosphorylated TCR. This action sequentially generates phospholipase C (PLC)-γ1 and inositol-3 phosphate (IP3) activity which results in the increase of intracellular calcium and diacylglycerol which stimulates PKCs.[70] PKC isoforms may differ between T and B cells[70] and dependency of calcium for activation,[71] however similarly, downstream of PKC activation, activation of the IKK complex is linked to the Carma1/Bcl10/MALT1 complex and NF-κB nuclear translocation.[70]

Plasmatic membrane content of DAG and cytosolic free calcium concentration are important second messengers which serve to activate PKC.[64] The phorbol ester, PMA, has the ability to substitute for diacylglycerol (DAG), bypassing cell-surface receptors and calcium signaling to directly activate PKC and activation of NF-κB.[67] Ionomycin, a calcium ionophore, causes an increase in intracellular calcium; thus also inducing activation and proliferation of lymphocytes via NF-κB signaling pathways.[72] Typically, PMA is used in conjunction with ionomycin for the induction of NF-κB activation in lymphocytes where calcium is required for activation of PKC.[67] Global lymphocyte stimulation using the combination of PHA, PMA, and ionomycin, integrates multiple

segments and pathways leading to NF-κB activation via various lymphocyte subtypes and cell-surface receptors. Illustrated in Figure 3 below, PHA/PMA/ionomycin signaling as discussed above proves itself as an integrative network which commonly leads to NF-κB activation. Demonstration of differences in responsiveness of peripheral blood lymphocytes to mitogen stimulation (*in vitro*) can facilitate a greater understanding of lymphocyte response to therapeutic intervention via identification of signaling pathways. Signal transduction leading to NF-κB nuclear translocation however only precedes events within the nucleus, such as gene transcription, which establishes the final cellular response.

Figure 3.[64, 73, 74]



Gene Expression and NF-κB Regulation

Gene expression associated with activated NF-κB and NF-κB-mediated transcription can be categorized into four groups based on function: inflammatory response and immuno-regulatory functions, negative regulators of NF-κB, apoptotic functions, and positive regulators of cell cycle.[28] The inflammatory response associated with NF-κB activation includes pro-inflammatory cytokines (e.g., TNF-α, IL-1, IL-6), chemokines and their receptors (e.g., MIP-1α; monocyte chemoattractant protein-1α), and adhesion molecules (e.g., VCAM-1, ICAM-1, and ELAM-1; vascular cell adhesion molecule-1, intercellular cell adhesion molecule-1, endothelial-leukocyte adhesion molecule-1).[28] Binding of TNF-α and IL-1 to their receptors strongly activates NF-κB and may generate a chronic inflammatory response if left unchecked.[30, 61] For autocrine circuits such as TNF-α/IL-1, numerous mechanisms exist to ensure appropriate control of NF-κB transcription factor activity. In addition to its direct relationship with inflammation, regulation of cell cycle and survival implicates the importance of NF-κB in cell-to-cell communication among immune cells as well as neoplastic cells.

Regulation of NF-κB activity exists at many levels. Beginning with the cell surface receptor, inhibition within a particular NF-κB signaling pathway may occur anywhere from receptor recognition to upstream and downstream signaling of IκB ubiquitination, selective interaction with co-activator and co-repressor proteins, promoter regions, post-translational modification of NF-κB and histone modifications.[28]

Following receptor recognition, NF-κB activation is tightly regulated at the level of the inhibitor. For example, inflammatory signalsomes (e.g. TNF, IL-1, various TLR ligands) are associated with a variety of kinase pathways which lead to a coordinated

IKK activation and degradation/synthesis of IκB proteins.[75] The pathway leading to IKK activation determines the IKK/IκB/NF-κB signaling module, the nature of the NF-κB response and the kinetics of NF-κB activation.[75] IκB proteins may intrinsically control intensity, duration and specificity of the NF-κB response.[28] The IκBα subunit is responsible for a negative feedback loop during short term stimulation that drives oscillations in NF-κB translocation and gene transcription events.[76] Gene transcription of IκBα is regulated itself by NF-κB activation. Present in the nucleus before shuttling back to the cytoplasm, IκBα can bind to RelA and p50 subunits and prevent DNA binding.[76] Contrary however, neither IκBβ nor IκBγ gene expression is not regulated by NF-κB and during sustained NF-κB stimulation, these subunits act to stabilize the NF-κB response.[76]

Activation of NF-κB leading to gene transcription is further regulated at the site of DNA binding. The specific κB sequence determines DNA binding affinity of the NF-κB complexes as well as the ability of the NF-κB dimmers to interact productively with cofactors.[28] A dynamic environmental transcription factor profile within the nucleus establishes combinatorial control of target gene transcription.[75] Synergistic interactions often occur between NF-κB and partner transcription factors which are time-dependent and tissue specific.[75] Specific κB site sequences not only code for specific NF-κB combinations, but also require particular allosteric conformational changes of NF-κB dimers in order to elicit gene transcription.[75] Transcriptional regulation in response to the cellular milieu mediates developmental, homeostatic and pathological events.[75, 77]

Further regulation of NF-κB-DNA binding involves the understanding of epigenetic control, chromatic structure and histone patterns. Histones consist of the core nucleosomal proteins associated with gene transcription. Genes that are actively

transcribed or repressed are associated with specific sets of histone modifications, also known as the "histone code".[75] Histone modification may result from acetylation, phosphorylation, ubiquitylation, glycosylation, or sumoylation, and provides the basis for spatial and time-dependent NF-κB-cofactor-DNA transcription.[75] For example, LPS stimulation results in biphasic recruitment of NF-κB to promoter regions as determined by histone acetylation.[28, 78] As determined in macrophages with an inflammatory stimuli, hypoacetylated promoter regions exhibited delayed NF-κB recruitment and activation of transcription compared to regions with constitutive acetylation.[78] In this model, the differing acetylation modification at promoter regions demonstrates a means by which various target genes can regulate the kinetics of NF-κB activation following upstream signaling pathways.

Relevant Biological Assays

A practical method for the determination of NF-kB activation includes nuclear protein extraction from peripheral blood lymphocytes followed by protein labeling and electrophoretic-induced protein separation. For example, sample protein labeled with a specific NF-kB/Rel oligo (single DNA strand allowing for a DNA-protein complex to form) prior to a electrophoretic-induced shift, allows for visualization techniques including chemiluminescence and fluorescence staining.[79, 80] The electrophoretic mobility shift assay (EMSA)[79] is a technique based on the observation that the migration of DNA through a nondenaturing polyacrylamide gel is hindered when protein is bound to it. Analysis of EMSA involves the identification and quantification of bands that have migrated a distance corresponding to the molecular weight of the complex.[81] Two-color

fluorescence EMSA visualization involves a short incubation period using a SYBR green stain (staining for DNA), followed by a longer incubation using a SYPRO red stain (to visualize protein). The location of the DNA-protein interactions is then determined by a visualized overlaying of the gel images in which the complex, stained both green and red, appears yellow.[80] Bands identified as yellow via image analysis can then be quantified to numerically represent activated protein, that which is actively able to bind to DNA. This method does not require radioactive isotopic labeling, pre-labeling or secondary detection methods and therefore may serve to as a novel technique that is both straightforward and accurate.

In addition to EMSA, an additional traditional method for determination and quantification of transcription factor proteins consists of a chemilumiscence ELISA assay. This method, following cytosolic and nuclear protein extraction, involves incubating the sample protein with a primary antibody which targets NF-κB with high affinity, and a secondary antibody which binds to the primary and acts as a fluorescent tag.[82] A fluorescence spectrometer is used to capture and quantitate the fluorescence emitted from the excited fluorophores once exposed to light.[82] This method is very sensitive to environmental conditions and quantity of protein. However, with adequate protein, this technique serves as a promising method for quantifying total transcription factor present in extracted protein.

Summary

Diagnosis of breast cancer can have significant physical and psychological consequences that last the duration of one's lifetime. Survivorship is a unique experience for each individual as no two people process stressful stimuli in the same manner. Active coping strategies often mediate an improved quality of life related to fewer reported demands of illness while passive coping acts in contrast. Integrative medical care provides a means for survivors to alleviate disease-associated physical ailments as well as illness-related stressors. Various pilot studies have established baseline data supporting numerous health benefits of Iyengar yoga practice among breast cancer survivors which include improved physical and emotional well-being. Psychological stress can have a suppressive effect on immune function and may impact survival of those living with a chronic disease, such as cancer. Necessary components of a healthy immune system include production and regulation of cytokines, acute phase proteins, and adhesion molecules, all of which require gene expression mediated by NF-kB. As an inflammatory transcription factor, NF-kB is often used as a biological measure of cellular stress. To data, no study has focused on the impact of Iyengar yoga on lymphocyte NF-κB activation and examined mediators of psychosocial stress simultaneously in breast cancer survivors.

CHAPTER THREE

MATERIALS AND METHODS

Sample Population

Female breast cancer survivors who were diagnosed with stages I-IV breast cancer and receiving Trastuzumab® (Herceptin) or antiestrogen or aromatase inhibitor hormonal therapy were recruited for this study. Subjects were recruited by an announcement describing the study and endorsed by the attending oncologists at Cancer Care Northwest, Spokane, WA. Participation was based on inclusion and exclusion criteria. This study was approved by the Washington State University Institutional Review Board (IRB) and written consent was obtained from each woman prior to data collection.

Participants for this study were obtained at two different occasions with differing inclusion criteria established for each. On one occasion, the following inclusion criteria were established and represent the majority of the participants in this study: a diagnosis of stage I-IV breast cancer, estrogen receptor positive, life expectancy of greater than six months, greater than or equal to eight weeks post-chemotherapy, surgery from lumpectomy or mastectomy with or without reconstruction, adequate circulating erythrocyte and leukocyte numbers, adequate kidney, liver, and cardiac function, as well as the physical and mental ability to complete the yoga training. All women received approval by their physician to participate.

Volunteers that matched the above inclusion criteria were further screened and were excluded from the study based on the following criteria: Receiving Herceptin therapy, pregnant or lactating (nursing) women, history of other neoplasm, serious

infection or immune deficiency. Women with reported alcohol or drug abuse or history of psychiatric disorders were not permitted to participate.

A smaller subgroup of participants (n = 4) was included in this study based on the following inclusion criteria: diagnosis of stage II-III ($HER2^{+}$)breast cancer with recurrence, followed by chemotherapy and Herceptin treatment, stable with minimal diseases OR diagnosis of Stage II-IV $HER2^{+}$ breast cancer but stable disease, currently receiving Herceptin treatment which may or may not be proceeding chemotherapy. These individuals were not receiving concurrent chemotherapy or radiation therapy. Surgery from a lumpectomy, mastectomy or mastectomy with reconstruction and adjuvant chemotherapy, hormonal therapy, or radiation therapy at any time in the past was allowed. Women were required to be physically and mentally capable of participation and willing to attend all yoga sessions.

Exclusion criteria for the above subgroup included women taking hormonal therapy with antiestrogen drugs and aromatase inhibitors. Additionally, all other exclusion criteria previously mentioned applied to this subgroup.

Women were randomly assigned to either the yoga (n=13) or wait-listed control group (n=11). The duration of the study was 16 weeks; divided into two eight-week sessions. During the first session, the yoga group took part in an Iyengar yoga intervention. Each week the women attended two yoga classes taught by a trained Iyengar yoga instructor. In addition, each was given an instruction sheet to practice once a week at home to make up a total of three yoga sessions per week. Immediately following the first session, the wait-listed control group crossed over and was then provided the same opportunity of yoga practice. An identical training protocol was followed for this group

and data collected during this session were combined with the first yoga group. Data, including blood samples and psychosocial assessments, were collected pre- (T1) and post-intervention (T2) for yoga and control groups. All women were required to continue on their current medical treatment regime during the course of the study.

Lymphocytes Samples

Heparinized blood samples (approximately 60 mL) were obtained by venapuncture from each participant at T1 and T2. Time of collection for each sample was between 7 am and 9 am. Participants arrived fasted with having not exercised or taken any medications that morning. Peripheral blood mononuclear cells (PBMCs) were separated by centrifugation over Histopaque separation medium (Product No. H8889, Sigma, St. Louis, MO). PBMCs were stimulated with PHA (Cat. No. 30852701, Remel Inc, Lenexa, KS), PMA (Product No. P8139, Sigma, St. Louis, MO) and ionomycin (Cat. No. 407950, Calbiochem, La Jolla, CA) in 24-well flat bottom tissue culture plates for 48 hours at 37°C and 5% CO_2. Paired aliquots of PBMCs from women receiving Herceptin treatment were also stimulated with human recombinant (r) IL-2 (Cat No. 136, NIH AIDS Research and Reference Reagent Program, Rockville, MD) or stored as unstimulated cells. Following stimulation non-adherent cells were collected, prepared for cryopreservation, and stored in liquid nitrogen until processed for protein extraction.

Thawing and Washing Procedures

Cells were removed from liquid nitrogen and immediately placed into a 37°C water bath. Thawed samples were placed on ice and washed with 25 mL of Minimum

Essential Media Eagle (MEME, Cat. No. 30-2003, ATCC, Manassas, VA) without serum, in a sterile environment. Storage vials were rinsed with MEME to ensure all cells had transferred. Centrifuge tubes, (50 mL), containing media and cells were centrifuged (CR3i, Jouan Centrifuge, S/N 400080086, LabCare America, Winchester, VA) at 250 g for 10 minutes at 4° C. Using a Pasteur pipette in a sterile environment, media was removed until approximately 1 mL was left covering the cell pellet. Cells were re-suspended in remaining media, transferred to a pre-chilled and labeled 1.5 mL eppendorf tube, and immediately placed on ice. Cellular suspensions were centrifuged (Hermle Z180M Microcentrifuge, S/N Z111131, National Labnet Co, Edison, NJ) at 1200 rpm for five minutes at 4°C. The supernatant fluid was completely removed using 100 µL pipette and the cell pellet was placed immediately on ice until further use. Washing aliquots that were paired samples of unstimulated or stimulated cells included substituting Ca^{++}/Mg^{++} free PBS (CMF) for MEME and washing twice.

Cytosolic Extraction (Procedure A)

Two 1.5 mL eppendorf tubes for each sample were pre-labeled and pre-chilled on ice. Tubes were labeled per identification plus "M" (mix) or "C" (cytosolic extract). Cytosolic extractions were performed according to the manufacturer's specifications (Nuclear Extraction Kit, Cat. No. AY2002, Panomics®, Redwood City, CA). Briefly, into the pre-chilled and "M" labeled tube, cytosolic buffer mix was prepared by adding 900 µL de-ionized distilled water, 100 µL 10x buffer A, 10 µL 100mM dithiothreitol, 10 µL protease inhibitor, and 40 µL 10% ethoxylated octylphenol. Reagents were vortexed (Fischer Vortex Genie 2, S/N2-327565, Scientific Industries, Bohemia, NY) for five

seconds. Buffer A mix (1 mL) was added to each cell pellet. Samples were vortexed vigorously for three seconds, just long enough to break the pellet. While on ice, samples incubated on a platform shaker (Classic C2, S/N 10128446 , New Brunswick Scientific, Edison, NJ) for 10 minutes at 150 rpm. Following the incubation, samples were centrifuged (GR2022, Joaun Centrifuge, S/N 30101450, LabCare America, Winchester, VA) at 15000 g for five minutes at 4°C. The supernatant fluid was completely removed, without disturbing the pellet, and transferred to the pre-chilled "C" labeled eppendorf tubes labeled to each identity. Cytosolic fractions were placed in -80°C storage. Cell pellets were placed on ice for subsequent use in nuclear extraction.

Nuclear Extraction (Procedure B)

Nuclear extractions were performed according to the manufacturer's specifications (Nuclear Extraction Kit, Cat. No. AY2002, Panomics, Inc, Redwood City, CA). Briefly, into each eppendorf tube containing a cell pellet from the preceding cytosolic extraction, 117.6 µL de-ionized distilled water, 29.4 µL 5x buffer B, 1.5 µL protease inhibitor, and 1.5 µL 100mM DTT were added in order. Sample with buffer mix was vortexed for 10 seconds on highest setting. Samples incubated on ice for three hours on a platform shaker set at 200 rpm. Following incubation, tubes were centrifuged at 15000 g for five minutes at 4°C. The supernatant fluid of the nuclear extract then underwent dialysis.

Protein Extraction (Procedure C)

Cytosolic and nuclear extractions from unstimulated and stimulated cells were performed according to the manufacturer's specifications (NE-PER® Nuclear and Cytoplasmic Extraction Reagents, Product No. 78833, Pierce Biotechnology, Rockford, IL). Briefly, ice cold cytoplasmic extraction reagent I (CER I) containing 10 µL/mL of Halt^{TM} Protease Inhibitor Cocktail (Product No. 78410, Pierce Biotechnology, Rockford, IL) and 10 mM sodium orthovanadate (Cat. No. 567540, Calbiochem, La Jolla, CA) were added to the cell pellet at a ratio of 100 µL/10 µL estimated cell volume. The tube was vortexed vigorously for 15 seconds and incubated on ice for 10 minutes. Following the 10 minute incubation, ice cold CER II was added to each tube at a ratio of 5.5 µL/10 µL estimated cell volume. The sample was vortexed vigorously for five seconds and incubated on ice for one minute. Following the incubation the tube was once again vortexed for five seconds and centrifuged in a microcentrifuge for five minutes at maximum speed (~16,000 g). The supernatant fluid, containing the cytosolic extract, was immediately transferred to pre-chilled microcentrifuge tubes and placed on ice. The remaining insoluble pellet was re-suspended in nuclear extraction reagent at a ratio of 50 µL/10 µL estimated cell volume, containing 10 µL/ml of Halt^{TM} Protease Inhibitor Cocktail and 10 mM sodium orthovanadate. The tube was vortexed vigorously for 15 seconds. The tube was incubated on ice for 40 minutes, vortexing for 15 seconds every 15 minutes. Following the incubation the tubes were centrifuged at maximum speed (~16,000 g) in a microcentrifuge for 10 minutes. The supernatant fluid containing the cytosolic or nuclear extract then underwent dialysis.

Dialysis of Samples (cells from Procedure B and C only)

Preformed in a 4°C cold room, cytosolic ($HER2^+$ only) and nuclear extracts were transferred into individual pre-chilled mini-dialysis units (Slide-A-Lyzer® MINI Dialysis Units, Product No. 69570, Pierce Biotechnology, Rockford, IL). Nuclear extract was divided into two, 75 µL fractions. Dialysis units were labeled appropriately and placed into a float (Slide-A-Lyzer MINI Dialysis Float, Product No. 69588, Pierce Biotechnology, Rockford, IL). The dialysis float was placed in one L of pre-chilled CMF covered with syran wrap secured by a rubber band, placed onto a stir plate, and mixed without a vortex overnight.

Protein Concentration

Following overnight dialysis, crude nuclear protein was concentrated from samples obtained through Procedures B. Nanosep 3K Omega® tubes (Product No. OD003C33, Pall Corporation, Ann Arbor, MI) were washed twice with 500 µL of de-ionized distilled water. Briefly, 500 µL of de-ionized distilled water was centrifuged (Hermle Z180M Microcentrifuge, S/N Z111131, National Labnet Co, Edison, NJ) at 12000 rpm for 15 minutes. Filtered water was removed to allow for the second washing. The second wash using 500 µL of de-ionized distilled water was centrifuged 17 minutes to ensure that all water had filtered, but the membrane of the filter was not spun dry. To prevent contamination of the filter, sterile pipette tips were used to remove all water from the Nanosep tube. Crude nuclear protein was transferred into the Nanosep tubes, with samples of the same identification recombined. Nanosep tubes were labeled appropriately and centrifuged (Hermle Z180M Microcentrifuge, S/N Z111131, National Labnet Co,

Edison, NJ) at 12000 rpm for 10 minutes. Tubes containing samples were centrifuged for progressively decreasing time increments which was initiated at a volume of approximately 100 µL until a final concentrated volume equated 20-25 µL. Concentrated protein was separated from membrane using a 20 µL pipette. Protein was aliquoted into two pre-chilled and pre-labeled 0.5 mL eppendorf tubes for further protein concentration determination and EMSA (6 µL and ~15-20 µL, respectively). Samples were stored at -80° C until further use. Samples obtained from Procedure R were similarly concentrated and stored until further use.

Determining Protein Concentration

Protein concentration was determined according to the bicinchoninic acid (BCA) protein assay protocol (Micro BCA™ Protein Assay Kit, Product No. 23235, Pierce Biotechnology, Rockford, IL). Bovine Serum Albumin (BSA) standards were prepared using CMF according to the manufacturer's instructions to obtain BSA concentrations of 200 µg/mL, 40 µg/mL, 20 µg/mL, 10 µg/mL, 5 µg/mL, 2.5 µg/mL, 1 µg/mL, 0.5 µg/mL and 0 µg/mL (blank). The BCA working reagent (WR) was prepared by adding 25 parts MA, 24 parts MB, and one part MC. The final volume of WR was equivalent to the sum of 150 µL for each standard concentration performed in triplicate plus 150 µL for each sample. Into a 96-well microplate, standard was added in triplicate and included 150 µL of WR plus 150 µL CMF. Wells containing sample protein included 150 µL WR, 147 µL CMR, and three µL protein. Wells were mixed by pipetting up and down several times after the addition of sample protein. The plate was covered with a labeled lid and incubated for two hours at 37°, 5% CO_2. Following incubation, the plate was read at

562nm on the spectrophotometer (Spectra max plus, S/N 02696, Molecular Devices, Sunnydale, CA). The protein concentration for each unknown was calculated. This procedure is able to detect protein in samples ranging from 0.5-20 μg/mL.

Determination of NF-κB Activation for Samples Obtained from Procedures A & B

Nuclear protein NF-κB was determined using EMSA and a dsDNA sequence specific to NF- κBp65 (Probe set for NF-κBp65, Cat. No. AY1367P, Panomics, Inc., Redwood City, CA) according to manufacturer's protocol with slight modification (EMSA "Gel-Shift" Kit, Cat. No.UM033103, Panomics, Inc., Redwood City, CA). A 6.0% polyacrylamide gel was prepared according to Panomics™ protocol. Briefly, into a sterile 50 mL centrifuge tube: 1.0 mL 10x TBE (Cat. No. 161-0733, BioRad Laboratories, Hercules, CA), 4.0 mL 30% acrylamide/bis solution (Cat. No. 161-0156, BioRad Laboratories, Hercules, CA), 625 μL 80% glycerol solution (Product No. 4750, OmniPur, Em Science, Lawrence, KS), 14.375 mL de-ionized water, 300 μL 10% ammonium peroxodisulphate (Cat. No. 161-0700, BioRad Laboratories, Hercules, CA) and 20 μL Temed (Cat. No. 161-0800, BioRad Laboratories, Hercules, CA) were added in order. Solution was vortexed on highest setting for 5-10 seconds and immediately transferred to gel casting apparatus (Cat. No. 165-3313, BioRad Laboratories, Hercules, Ca) using a sterile transfer pipette. A 10-well comb was inserted between glass plates containing solution and then set aside to allow the gel to solidify.

Nuclear protein samples were prepared according to Panomics™ protocol with slight modification. Briefly, into a pre-labeled and pre-chilled sterile 0.5 mL eppendorf tube: 2.0 μL 5x binding buffer and 1.5 μg nuclear protein were added. Samples incubated

on ice for five minutes. Following incubation, 1.0 μL NF-kBp65 probe and enough distilled water to bring final volume to 10.0 μL were added. Samples incubated in a water bath at 15.5°C for 30 minutes. During this time the solidified gel was transferred to a electrophoresis cell box (BioRad Mini Protean 3, Cat. No. 154-8004, BioRad Laboratories, Hercules, CA) and pre-run at 120 V for 10 minutes in pre-chilled 0.5x TBE buffer, at 4°C.

Immediately following incubation, 10 μL of sample was loaded into each well of gel, in duplicate, with adjacent T1 and T2 samples for each participant. Lanes 1 and 10 were not loaded with protein sample. The last lane of each gel was loaded with solution consisting of 9 μL distilled water plus 1.0 μL loading dye. Electrophoresis was carried out at 120 V for approximately 90 minutes, or until the lane containing loading dye reached ~1.0 cm from the bottom of the gel.

Minimizing exposure to light, concentrated SYBR Green I EMSA gel stain (Product No. S7563, Molecular Probes, Inc., Eugene, OR) was thawed at room temperature. SYBR Green I stain solution was prepared by adding 5.0 μL of SYBR Green I to 50 mL of 0.5xTBE solution. Solution was mixed on a platform shaker protected from light, for five minutes. Top right corner of gel was marked for orientation purposes. Gel was placed into SYBR Green I stain solution and incubated for 20 minutes on the platform shaker with continuous gentle agitation (50 rpm), protected from light. Each gel was washed twice in 150 mL of distilled water for approximately 10 seconds and placed into a lidded plastic container for easy transport and temporary storage. Individual gels were visualized immediately following staining period using digital photography (Model No. 2.3.1, Alpha Innotech Corporation, San Leandro, CA) and UV-

transillumination. Each image was acquired with UV-light at an excitation of 302nm and an appropriate filter for SYBR Green for time increments lasting 0.25, 0.5, 1, 2, 3.5, 5, and 10 seconds.

Following visualizations of SYBR Green stains, gels were carefully added to 50 mL of SYPRO ruby EMSA gel stain (Product No. S12000, Molecular Probes, Inc., Eugene, OR). Gels incubated overnight (12-15 hours) with continuous gentle agitation (50 rpm) on the platform shaker, protected from light. Gels were individually de-stained in 50 mL of 10% methanol (Cat. No. MX0485-3, EMD, San Diego, CA) 7% acetic acid (Product No. 9507, J.T. Baker, Phillipsburg, NJ) solution for three hours. Gel was washed twice in 150 mL of distilled water for ~10 seconds and placed into a plastic container with lid for easy transport and temporary storage. Gel was visualized immediately following staining period using UV-light at an excitation of 302 nm and an appropriate filter for SYBR Ruby for time increments lasting 0.25, 0.5, 1, 2, 3.5, 5, and 10 seconds. Placement of gel onto visualizing plate matched specific markings which corresponded with placement of the respective green stain. Image analyses of both stains were performed using ChemiImager^{TM} 5500 software (Alpha Innotech Corporation, San Leandro, CA).

Determination of cellular NF-κB location for Samples obtained by Procedure C

Nuclear and cytosolic NF-κB was determined using a chemiluminescence ELISA assay according to manufacturers instructions (EZ-Detect^{TM} NF-κB p65 Transcription Factor Kit, Product No. 89859, Pierce Biotechnology, Rockford, IL). Prepared Working Binding Buffer (50 μL) was added to each well followed by the competitor duplex to the

appropriate wells. For the determined protein concentrations of each sample, nuclear protein (5 μg) or cytosolic protein (10 μg) was loaded in duplicate into each well. The plates were incubated with mild agitation for one hour at room temperature. Following the incubation, the contents were emptied and the plate was tapped dry (five times) on paper towel. The plate was washed three times with 1X Wash Buffer (200 μL) and tapped dry (five times) on paper towel between each wash. Primary antibody (100 μL) was added to each well and the plates were incubated for one hour at room temperature, without agitation. The plate contents were emptied, tapped dry (five times) on paper towel and washed four times with 1X Wash Buffer (200 μL). Luminol substrate working solution (100 μL) was added to each well and immediately read using microbeta plate reader (1450 Microbeta Wallac Jet Liquid Scintillation and Luminescence Counter, S/N 4502011, Perkin Elmer life science, Boston, MA). Nuclear NF-κB was calculated by subtracting baseline values for each sample. A limitation of this assay is that the amount of bound versus unbound NF-κB cannot be detected, therefore it is unable to distinguish between the amount of free NF-κB or the NF-κB bound to IκB (within the cytosol) or the κB binding site (within the nucleus).

Psychosocial/Quality of Life Assessment

Quality of Life Assessment Questionnaires measuring a multitude of psychosocial variables were administered at T1 and T2 for each participant. Participants were given a booklet of questionnaires and asked to complete and return the booklet in a pre-paid postage envelope within the next few days. Specific instructions were provided for each

questionnaire. The booklet of questionnaires included the following: Background Questionnaire, Diagnosis and Treatment Questionnaire, Facit (Version 4)[83], Cancer Symptom Checklist[84], Demands of Illness Inventory- short form[16, 17], Short-form McGill Pain Questionnaire[85], The Coherence Scale[86], Cancer Self-Efficacy[87], Revised Ways of Coping Checklist[12], Hospital Anxiety and Depression Scale[88], Interpersonal Support Evaluation List[89], and Godin Leisure-Time Exercise Questionnaire[90]. Only the Demands of Illness Inventory and Revised Ways of Coping Checklist were utilized within the scope of this thesis and therefore, the focus of the psychosocial components of the study will be limited to these instruments.

Statistical Analyses

All data were analyzed using the Statistical Package for Social Sciences (SPSS), version 15.0. Change within measured variables over the course of the intervention period (delta) was calculated for each participant. Normality was tested on all variables. Independent *t*-tests were used to compare mean differences of T1 and T2 within groups and between groups, as well as to compare delta between groups. Paired *t*-tests were used to determine difference between T1 and T2 within subjects. Significance was determined at $p<0.05$. Self-organizing maps (SOM) were used as a multi-variate statistical tool to represent topological relationships of the participants' responses to the psychosocial assessments and the measurement and change of NF-κB activation. Numerous combinations of variables were analyzed. SOM analysis is presented without discussion in Appendix C.

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APPENDIX A

THE IMPACT OF IYENGAR YOGA ON DEMANDS OF ILLNESS, COPING AND LYMPHOCYTE NF-κB ACTIVATION IN BREAST CANCER SURVIVORS

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ABSTRACT

Breast cancer survivorship is associated with changes in physical, psychological, and social aspects of well-being. Such changes may require significant cognitive and emotional adaptation. Coping strategies whether positive or negative may predict how well a survivor is able to adapt and may affect how their body responds to the illness. Integrative medicine often incorporates mind-body therapies, such as yoga, to increase overall wellbeing and physical health in breast cancer survivors. The present study was designed to investigate the relationship between Iyengar yoga practice, illness-related stressors and lymphocyte NF-κB activation in female breast cancer survivors. Females who were approximately 1 to 11 years post-diagnosis with stage I-IV breast cancer were randomly assigned to either the yoga (Y, n = 13) or control (C, n = 11) group. The eight-week yoga intervention included three yoga sessions per week. Participants completed a Demands of Illness Inventory (DOII) and Revised Ways of Coping Checklist (RWCCL) prior to (T1) and following (T2) the intervention. Blood samples were taken at T1 and T2 for use in *in vitro* unstimulated and stimulated lymphocyte NF-κB analyses. Lymphocytes were stimulated with phytohemaglutinin (PHA), phorbol 12-myristate 12-acetate (PMA), and ionomycin. Nuclear extracts of stimulated cells were analyzed for NF-κB activation by an electrophoretic mobility shift assay. Cytosolic and nuclear extracts from unstimulated and stimulated paired aliquots were analyzed for cellular location of NF-κB by chemiluminescence. Data were analyzed by independent and paired *t*-tests, and linear regression. A global measure of the DOII revealed a significant interaction between time and group effects ($F = 5.275$, $p = 0.028$) revealing decreased DOII in the yoga group from T1 to T2. DOII was inversely correlated with overall active

coping strategies ($r = -0.23$, $p = 0.05$) and "count your blessings" ($r = -0.51$, $p < 0.000$). Yoga participation was associated with a trend for decreased lymphocyte activation ($p = 0.077$). Decreased lymphocyte NF-κB activation was correlated with increased use of passive coping strategies ($r = 0.900$, $p = 0.03$). The results support the hypothesis that yoga participation mediates perceived demands of illness associated with breast cancer survivorship and that lymphocyte NF-κB signaling pathways are involved in physiological changes that enhance psychosocial well-being.

INTRODUCTION

Breast cancer is the most common newly diagnosed cancer among woman in the United States.[1] As breast cancer survival rates improve a greater number of women have to adapt to and live with the biopsychosocial impacts of cancer survivorship. Women with breast cancer are challenged with many psychological and social hardships relating to diagnosis, treatment, and survivorship.[2-4] The relationships between these psychosocial challenges and disease prognosis and progression remain undefined.

Several models exist linking breast cancer survivors' physical and psychological stress with coping responses. [5-11] Accepting the fate of being diagnosed with breast cancer and then managing life thereafter includes processing a wide range of stressors into directed thoughts and actions that reflect coping strategies. Instruments such as the Revised Ways of Coping Checklist (RWCCL) measure the degree that various coping strategies are used by an individual.[12-14] Coping strategies are often classified as active coping (positive in nature) and passive/emotion-focused coping (more negative minded).[9] It is thought that adjustment through coping mediates the outcome of the perceived stress level resulting from breast cancer.

Integrative medical therapies are commonly used to treat psychosocial/emotional distress related to breast cancer survivorship.[15-18] Diagnosis, treatment and many illness-related stressors can, independently and in combination, negatively affect immune function of survivors. As survivors seek out advice for appropriate complementary therapies, western medicine is only beginning to acknowledge and explore the benefits of these various methods, including yoga. Several studies investigating yoga interventions indicate modest improvements in physical and psychological cancer-related symptoms

and overall quality of life among cancer survivors, and particularly, those associated with the Iyengar yoga tradition.[19-23] The influence of Iyengar yoga practice on immune function however is incompletely understood.

The present investigation was designed to examine the relationship between Iyengar yoga therapy, illness-related stressors and lymphocyte NF-κB activation in female breast cancer survivors. The transcription factor, NF-κB plays a central role in lymphocyte cell signaling of diverse immunological responses. Located in the cytosol of unstimulated cells, NF-κB dimers are found bound to an inhibitor complex and are considered to be inactive. Under conditions of cellular stress, NF-κB is liberated from its inhibitor and translocates to the nucleus where it is able to bind to DNA and signal for gene transcription.[24] Activation of NF-κB has been called "a sensor for smoke and stress signals" as it results from a wide range of stimuli and is involved in gene expression of more than 400 genes, including certain lymphocyte signaling pathways and the inflammatory response.[25] To date, the effects of Iyengar yoga practice on the activation of NF-κB in lymphocytes from breast cancer survivors is unknown. This study is part of a larger ongoing research project previously published.[21] Illness-related stressors within this study were considered with respect to: 1) coping strategies used to deal with the stressors and 2) the outcome of stress reported on the Demands of Illness Inventory (DOII)[26].

The purposes of this study were: 1) to examine the effect of Iyengar yoga on coping and psychosocial stressors, as measured by RWCCL and DOII, in women with stage I-IV breast cancer; 2) to examine the impact of Iyengar yoga on the activation of NF-κB in PHA/PMA/ionomycin stimulated and unstimulated peripheral blood

lymphocytes; and 3) to examine associations between NF-κB activation, coping as a psychosocial mediator of illness stressors, and demands of illness as the stressor outcome variable. The hypotheses of this study were: as compared with wait-listed controls, breast cancer survivors who participated in yoga will have reduced cancer-related illness stressors, stressors will be mediated by coping strategies, and altered NF-κB activation in stimulated and unstimulated lymphocytes when baseline (pre-yoga) measures are compared to measures obtained eight weeks later (immediately post-yoga).

METHODS

Participants

Volunteers for this study were recruited from female breast cancer survivors (stage I-IV) who were patients at Cancer Care Northwest, Spokane, WA. Participants were either estrogen receptor positive and receiving anti-estrogen/aromatase inhibitor hormonal therapy, or HER2 receptor positive and receiving Herceptin therapy. Additional inclusion and exclusion criteria based on diagnosed receptor type were previously described.[27] The women were asked to continue on their current medication regimen throughout the duration of the study. All women received approval from their physician to participate and a written consent was obtained from each woman prior to data collection. This study was approved by the Washington State University Institutional Review Board.

Study Design

From a total of 140 women who volunteered for the study, 29 women were eligible to participate. Participants were randomly assigned to either the yoga or control group. An Iyengar yoga intervention was implemented for eight weeks. Each week included three yoga sessions: two 90-minute Iyengar yoga classes taught by a trained Iyengar yoga instructor, and one home practice designed weekly by the yoga instructor. Data collection occurred at two times, pre- (T1) and post-intervention (T2), which included psychosocial questionnaires and a 60 mL venous blood sample.

Psychosocial Measures

Psychosocial questionnaires incorporated socio-demographic information, illness related stressors, and coping strategies. Illness related stressors were assessed using the Demands of Illness Inventory (DOII) – short form (women's version) developed by Woods, Haberman, and Packard.[26] This 68-item questionnaire was adapted from the original 125-item instrument that measures illness-related demands (problems, concerns, burdens and hardships) in multiple domains (personal meaning, social relationships, self-image, monitoring symptoms, and treatment issues). For each item (demand) the respondents' choose the extent they had experienced the demand as a result of their illness or medical treatment (0 = not at all, 1 = a little bit, 2 = somewhat, 3 = quite a bit, 4 = very much). Item number 67 and 68 (single item overall assessments) asked the respondents to rate the burden and hardship imposed by their illness and treatment at the present time and since the time of the original diagnosis, respectively, on a scale of 1 = no hardship at all, to 10 = the worst hardship imaginable. Reliability coefficients for

internal consistency reported are between 0.96-0.97 for the overall tool with domain coefficients ranging from 0.78-0.95.[28]

Coping strategies were assessed using the Revised Ways of Coping Checklist developed by Vitaliano et al.[12, 13] Participants were asked to identify and write a short statement describing a major problem they were experiencing at the present time. Following this statement 57 items representing ways of dealing with the problem (specific cognitive and behavioral coping strategies) were listed and respondents were asked to indicate the appropriate frequency of use for each item (N/A = not applicable, 0 = never used, 1 = rarely used, 2 = sometimes used, 3 = regularly used (4 – 5 times per week). Various methods of coping were identified in subscales taken from this questionnaire and included problem-focused coping, seeking social support, blames self, wishful thinking, blames others, religiosity, counting your blessings, and avoidance. Active coping strategies, reported as positively related to both physical and psychological well-being included the subscales of problem focused coping, seeking social support, and counting your blessings.[29] Passive coping strategies, thought to have negative social and health outcomes, included the subscales of blames self, wishful thinking, blames others, and avoidance.

Lymphocyte NF-κB Cellular Localization and Activation

Lymphocyte NF-κB was evaluated as a biological indicator of stress and lymphocyte responsiveness. Lymphocyte samples were prepared from peripheral blood mononuclear cells (PMBCs) separated from heparinized blood samples by centrifugation over Histopaque separation media (Sigma, St. Louis, MO). PMBCs were stimulated with

PHA (Remel Inc, Lenexa, KS), PMA (Sigma, St. Louis, MO) and ionomycin (Calbiochem, La Jolla, CA) in 24-well flat bottom culture plates for 48 hours at 37°C and 5% CO_2. Following stimulation, non-adherent cells were collected, prepared for cryopreservation, and stored in liquid nitrogen until processed for protein extraction. Prepared aliquots of unstimulated PBMCs were also stored in liquid nitrogen.

Procedure A. Thawed samples were washed in Minimum Essential Media Eagle (MEME, ATCC, Manassas, VA), centrifuged at 250 g for 10 minutes at 4°C, re-suspended in media, and centrifuged at 1200 rpm for five minutes at 4°C. Supernatant fluid was then removed and the cell pellet was placed immediately on ice. Nuclear extraction was performed according to the manufacturer's specifications (Nuclear Extraction Kit, Panomics®, Redwood City, CA) including overnight dialysis. Following dialysis, crude nuclear protein was concentrated using Nanoset 3K Omega tubes (Pall Corporations, Ann Arbor, MI). Protein concentration for each sample was determined according to the bicinchoninic acid (BCA) protein assay protocol (Micro BCA™ Protein Assay Kit, Pierce Biotechnology, Rockford, IL).

Procedure B. Paired aliquots of stimulated and unstimulated samples were thawed and washed as described in Procedure A. Cytosolic and nuclear fractions from these samples were extracted according to manufacturer's specifications (NE-PER® Nuclear and Cytoplasmic Extraction Reagents, Pierce Biotechnology, Rockford, IL). Samples were also dialyzed overnight and protein concentration was determined using the BCA assay as described above.

Procedure C. Nuclear protein extracted in Procedure A was analyzed for NF-κB activation using electrophoretic mobility shift assays (EMSA) and a double-stranded

DNA sequence specific to NF-κBp65 (Probe set for NF-κBp65, Panomics Inc., Redwood City, CA) according to the manufacturer's protocol with slight modification (EMSA " Gel-Shift Kit, Panomics Inc., Redwood City, CA). Visualization of probe and protein were carried out with SYBR Green and SYPRO Ruby stains (Molecular Probes Inc., Eugene, OR) and UV light. Image analyses of both stains were performed using ChemiImager^{TM} 5500 software (Alpha Innotech Corporation, San Leandro, CA)

Procedure D. Cellular location of nuclear and cytosolic NF-κB obtained in Procedure B was determined using a chemiluminescence ELISA assay according to manufacturers instructions (EZ-Detect^{TM} NF-κB p65 Transcription Factor Kit, Pierce Biotechnology, Rockford, IL). A stimulation index relating the cellular location of NF-κB in paired aliquots of stimulated and unstimulated cells was calculated using the following formula:

$$\frac{\text{(nuclear NF-κB post-stimulation)} - \text{(nuclear NF-κB pre-stimulation)}}{\text{(nuclear NF-κB pre-stimulation)}} \times 100\%$$

Statistical Analysis

All data were analyzed using the Statistical Package for Social Sciences (SPSS), version 15.0. Change within measured variables over the course of the intervention period (delta) was calculated for each participant. Normality was tested on all variables. Pearson Correlation, Spearman Rank Order Correlation, ANOVA, and independent and paired *t*-tests were conducted to examine appropriate relationships. Significance was determined at $p<0.05$. A trend was determined at $p<0.10$.

RESULTS

Characteristics of the Sample

Demographic and illness-related characteristics of the total sample and intervention groups are shown in Table 1. The mean age of the participants was 58.7 years, ranging from 45 to 73 years. Time since diagnosis ranged from 8 to 129 months (mean 53 months) and the majority of the participants were either diagnosed with stage I (54%) or stage II (29%) breast cancer. All of the women were Caucasian. Fifty percent of participants were married and the majority (92%) of the women had children. There was a wide range of highest achieved education and annual household income, ranging from some high school to a master's degree and less than $10,000 to more than $75,000, respectively. The majority (85%) of the participants had at least fifty percent or more of medical expenses covered by health insurance over the past year.

Relationship of Yoga, Illness-related Stressors, and Coping

Figure 1 compares the scores obtained at T1 and T2 on the Demands of Illness Inventory for a single item "at the present time, rate the overall burden of your illness and treatment." Analysis of variance revealed that there was a significant interaction between time and group ($F = 5.275$, $p = 0.028$) regarding the overall rating of burden and hardship (DOII item #67). Women who participated in yoga reported no change in overall burden over time, whereas in the control group, demands of illness significantly increased ($p = 0.002$) from T1 to T2. The difference between groups at T1 was not significantly different. The change (T2 score minus T1 score) in total demands (summed score for all

66 single items) and in the single item of overall burden "at the present time" were significantly different ($p = 0.032$, $p = 0.004$, respectively) between the yoga and control groups (Table 2). The yoga group reported a reduction in total demands of illness from T1 to T2 (T1 mean = 75.2; T2 mean = 61.0). Total DOII reflects a range of scores from 0 to 264. The actual range of scores for entire sample for both T1 and T2 was 17 to 191.

The use of various coping strategies was weakly associated with reported demands of illness. An inverse trend was observed between the increased use of active coping strategies and a decrease in overall burden of illness "at the present time" ($r = -0.229$, $p = 0.050$) from T1 to T2. The specific method of active coping, referred to as "counting your blessings" was inversely correlated with the single item of overall demands "at the present time" ($r = -0.511$, $p < 0.000$). This signifies that the overall burden of illness and treatment decreased with increasing use of "counting your blessings" coping strategies. The frequency of coping strategies measured by the RWWCL for both the yoga and control group at T1 and T2 are shown in Table 3. Coping strategies utilized were not significantly different between the yoga and control group.

Figure 2 demonstrates NF-κB detection by chemiluminescence and EMSA staining. Within this figure, lane 1 represents chemiluminescence, lane 2 represents EMSA Green, lane 3 represents EMSA Ruby, and lane 4 and 5 represent EMSA Ruby of the same participant at T1 and T2. Shown in Figure 2, lanes 4 and 5, quantified NF-κB activation decreased from T1 to T2. Overall, yoga participation was associated with a trend ($p = 0.077$) for decreased lymphocyte NF-κB activation from T1 to T2 (Figure 3; n = 5 yoga, 3 control). Among yoga participants, percent change in NF-κB activation was

positively correlated with increased use of passive coping strategies ($r = 0.900$, $p = 0.03$) (Figure 4).

From the subset of samples from yoga participants analyzed for cellular NF-κB localization, the nuclear to cytosolic ratio increased in unstimulated cells (T1, 1.17 ± 0.57; T2, 1.41 ± 0.66) and decreased in stimulated cells (T1, mean ± SD, 1.10 ± 0.46; T2, 0.85 ± 0.22). Following *in vitro* stimulation, the stimulation index of yoga participants decreased approximately 33.3%. This represents diminished nuclear translocation of NF-κB in response to *in vitro* stimulation and may be associated with yoga participation.

DISCUSSION

The purpose of this study was to examine if Iyengar yoga participation would decrease demands of illness and treatment, enhance active coping methods, and alter NF-κB activation in lymphocytes from breast cancer survivors. The results of this study indicate that eight weeks of active yoga practice stabilized demands of illness reported by breast cancer survivors as compared with increased demands reported among women who did not practice yoga. Coping strategies used among the participants in this study did not differ between the yoga and control group. However, a greater frequency of active strategies and "count your blessings" active strategies used was related to fewer reported demands (stressors) over an eight week period. Yoga participation altered NF-κB activation as a trend was observed for decreased lymphocyte NF-κB activation in yoga participants following the intervention. This change in lymphocyte NF-κB activation correlated with increased use of passive coping methods by yoga participants from T1 to T2.

Overall, the results indicated that the yoga intervention was successful in reducing illness-related stressors reported among female breast cancer survivors. These results are in agreement with Michalsen et al.,[30] who reported a three-month Iyengar yoga intervention demonstrated significant improvements in perceived stress, anxiety, well-being, vigor, fatigue, and depression among emotionally distressed but otherwise healthy women who participated compared to wait-listed controls. Furthermore, the findings of the present study support the work of others delineating the benefits of Iyengar yoga therapy on the physical and psychological well-being of breast cancer survivors.[21, 31] Thus, the present findings contribute to an accumulating body of evidence supporting the efficacy of Iyengar yoga practice in attenuating psychosocial stressors related to the burden and hardship of breast cancer survivorship.

Behavioral and cognitive coping strategies used among the cancer survivors in efforts to master, tolerate, or reduce (mediate) illness-related demands were examined in this study using the RWCCL. Strategies of active and passive coping were identified based on the questionnaires designated subscales. Understanding coping methods utilized by a particular sample or individual allows for a better understanding and prediction of adjustment to survivorship. In the present study, active coping was identified as being inversely related to the overall rating of DOI "at the present time". As strategies of active coping increased, the reported overall perception of demands decreased. This is consistent with previously published literature relating active coping methods such as problem-focused coping, seeking support, and spirituality, to positive outcomes such as lower life stress in women adapting to breast cancer.[5, 29]

Results from this pilot study also indicated an association between negative (passive) coping and lymphocyte NF-κB activation, a biological marker of stress central to the immune response.[24, 32-36] As the percent change in lymphocyte NF-κB activation decreased, more passive coping strategies were reported as being used by the yoga participants. A reduction in NF-κB activation demonstrates a modification in immune response that coincides with greater usage of passive coping strategies. Passive coping is related to negative social and health outcomes. Coping methods such as avoidance, blaming others and wishful thinking have been shown to be maladaptive to chronic illness. Passive coping, similar to emotion focused coping, has been associated with greater distress, lower quality of life and increased pain perception.[8, 9] The general category of emotion-focused coping is related to more emotional distress and poorer adjustment in medical patients.[12-14] Collectively, the findings of this study indicate that the use of active coping, active "count your blessings" strategies, and passive coping in combination improve immune response, as indicated by either a trend in NF-κB deactivation (for active coping strategies) or a statistically significant deactivation in NF-κB (for passive coping).

Lymphocytes play a key role in many immune responses and are regulated by a multitude of signaling molecules, including the transcription factor NF-κB.[32, 37] Activated NF-κB is closely linked to inflammatory processes. It is believed that inflammatory processes not only promote tumor development, but are also related to many negative psychological processes, including the perception of pain and depressive symptoms.[38-40] In this study, yoga participants who reported decreased overall demands of illness over

the course of yoga therapy also showed a trend for decreased lymphocyte NF-κB activation.

Translocation of NF-κB to the nucleus as well as the transcription of target genes after cellular stimulation, is largely attributed to degradation of inhibitory proteins (IκBs) that sequester the inactive form of NF-κB in the cytoplasm.[24] Additional preliminary data as part of the present study indicated that nuclear NF-κB localization in unstimulated cells was indirectly related to the NF-κB stimulation index, indicating that greater nuclear localization of NF-κB in unstimulated cells was associated with less translocation of NF-κB to the nucleus in response to *in vitro* activation. It is interesting to note that lymphocytes from yoga participation exhibited a trend for greater nuclear NF-κB localization in unstimulated cells at T2 versus T1. Nuclear localization of NF-κB however does not always imply sequential gene transcription. Transcriptional activation is dynamically regulated including, but not limited to, its conformational specificity, recruitment of various co-activators, and time-dependent interactions.[37, 41] In addition, nuclear NF-κB can undergo nucleocytoplasmic shuttling. For example, NF-κB translocation into the nucleus can induce transcription of its inhibitor IκBα, which in turn is able to bind to nuclear NF-κB and shuttle out of the nucleus to the cytosol. Therefore, greater nuclear NF-κB localization within unstimulated cells does not singly reflect lymphocyte activation.

Triggering of NF-κB pathways can be investigated by measuring NF-κB activation through its binding to DNA oligo nucleotides, such as conducted in this study (Procedure C). We observed a trend for decreased NF-κB activation in stimulated lymphocytes from breast cancer survivors following yoga participation, which

corresponded with a reduction of nuclear NF-κB localization in stimulated cells from T1 to T2. The stimulation index, reflecting NF-κB localization, also decreased following yoga participation indicating that *in vitro* stimulation was less capable of translocating additional NF-κB into the nucleus. The implicated health benefit related to this down-regulation of nuclear NF-κB activity warrants further investigation. Nevertheless, decreased lymphocyte NF-κB activation was associated with fewer reported demands of illness and improved quality of survivorship by yoga participants.

Conclusions

In conclusion, Iyengar yoga therapy may be an effective treatment for attenuating psychosocial stressors related to breast cancer survivorship. Yoga participation decreased demands of illness scores and was associated with increased use of passive coping in those women exhibiting decreased lymphocyte NF-κB activation. It is likely that molecular signaling influenced by psychological and physical stress responses involves cellular activation of NF-κB and that yoga may be used as an integrative medical therapy to alleviate the illness and treatment-related stressors associated with breast cancer survivorship. Further research is warranted in order to delineate the biological relevance of increased nuclear NF-κB expression in unstimulated cells that subsequently demonstrate reduced nuclear activation response to stimulation. We postulate that lymphocyte NF-κB activation may be an important biomarker for monitoring physical and psychological illness-related symptoms in breast cancer survivorship.

The authors gratefully acknowledge the women who volunteered to participate in the study and the yoga instruction by Jeri Hudak, Certified Iyengar Yoga instructor and

Registered Yoga Teacher (RYT), Alison Rubin, RYT, and Jackie Kittel, OTR, RYT. We thank Jorming Goh for his assistance in laboratory data collection. This research was supported by Cancer Prevention and Research Center, WSU, the University of Washington Center for Women's Health and Gender Research, Hugger-Mugger Yoga Products, and Trans American Spinning Mills, Inc.

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APPENDIX B

LIST OF TABLES

Table 1. Demographic Characteristics of Intervention Groups*

Characteristics	Yoga Group	Control Group
Current Age (yr)	57.1 (8.5)	60.8 (7.6)
Time since Diagnosis (mo)	48.3 (32.2)	61.0 (28.9)
Stage of Breast Cancer		
I	8	5
II	4	3
III	1	0
IV	2	1
Marital Status		
Single	1 (8%)	0 (0%)
Married	6 (50%)	4 (50%)
Divorced	3 (25%)	2 (25%)
Widowed	2 (17%)	2 (25%)
Children		
0	2 (13.3%)	0 (0%)
1	2 (13.3%)	1 (11.1%)
2	6 (40%)	3 (33.3%)
3	5 (33.3%)	4 (44.4%)
4	0 (0%)	1 (11.1%)
Education		
Some High School	1 (8%)	1 (12.5%)
High School	5 (42%)	1 (12.5%)
2-year College or Technical School	3 (25%)	4 (50%)
College Graduate	2 (17%)	1 (12.5%)
Master's Degree	1 (8%)	1 (12.5%)
Doctorate	0 (0%)	0 (0%)
Yearly Income		
Less than $10,000	1 (8%)	1 (12.5%)
$10,000 - $19,999	2 (17%)	1 (12.5%)
$20,000 - $29,999	1 (8%)	1 (12.5%)
$30,000 - $39,000	2 (17%)	0 (0%)
$40,000 - $49,999	2 (17%)	3 (37.5%)
$50,000 - $74,999	3 (25%)	1 (12.5%)
$75,000 or more	1 (8%)	1 (12.5%)
Medical Expenses covered by Health Insurance		
All or most all	4 (33.3%)	3 (37.5%)
More than ¾ but less than all	6 (50%)	2 (25%)
Between ½ and ¾	0 (0%)	2 (25%)
Between ¼ and ½	1 (8.3%)	1 (12.5%)
Less than ¼	1 (8.3%)	0 (0%)

* Values given as mean (SD) or no. (%)

Table 2. Change in DOII from pre- to post-intervention				
Score	Group	Mean	SD	p-value
DOII sum	Yoga	-16.97	20.31	p = 0.032
	Control	-1.33	12.14	
DOII #67	Yoga	-0.71	1.38	p = 0.004
	Control	2.11	2.09	

Table 3. Frequency of Coping Strategies Pre- and Post-Intervention

		T1		T2	
Coping Strategy	Group	Mean	SD	Mean	SD
Problem Focused	Yoga	32.1	6.4	35.3	10.0
	Control	31.9	7.2	32.2	10.5
Seeks Social Support	Yoga	12.3	3.4	12.6	5.2
	Control	9.8	4.3	10.7	5.5
Blames Self	Yoga	6.4	3.3	3.3	1.8
	Control	9.3	5.0	5.5	5.0
Wishful Thinking	Yoga	13.8	5.9	11.3	5.6
	Control	15.0	7.0	14.5	5.9
Blames Others	Yoga	9.8	5.0	9.8	8.4
	Control	11.7	9.3	8.8	6.2
Religion	Yoga	6.2	2.9	6.5	2.2
	Control	4.7	1.5	4.0	1.6
Count Your Blessings	Yoga	13.8	4.2	15.8	2.1
	Control	15.0	1.0	11.8	4.3
Avoidance	Yoga	16.4	6.7	11.2	4.3
	Control	16.3	6.0	15.0	9.0
Active (sum)	Yoga	59.4	15.8	60.8	10.8
	Control	62.7	7.0	56.5	14.8
Passive (sum)	Yoga	46.4	17.7	35.7	17.7
	Control	48.3	27.0	43.8	24.4

List of Figures

Figure 1. Interaction between Time and Group for Present DOI

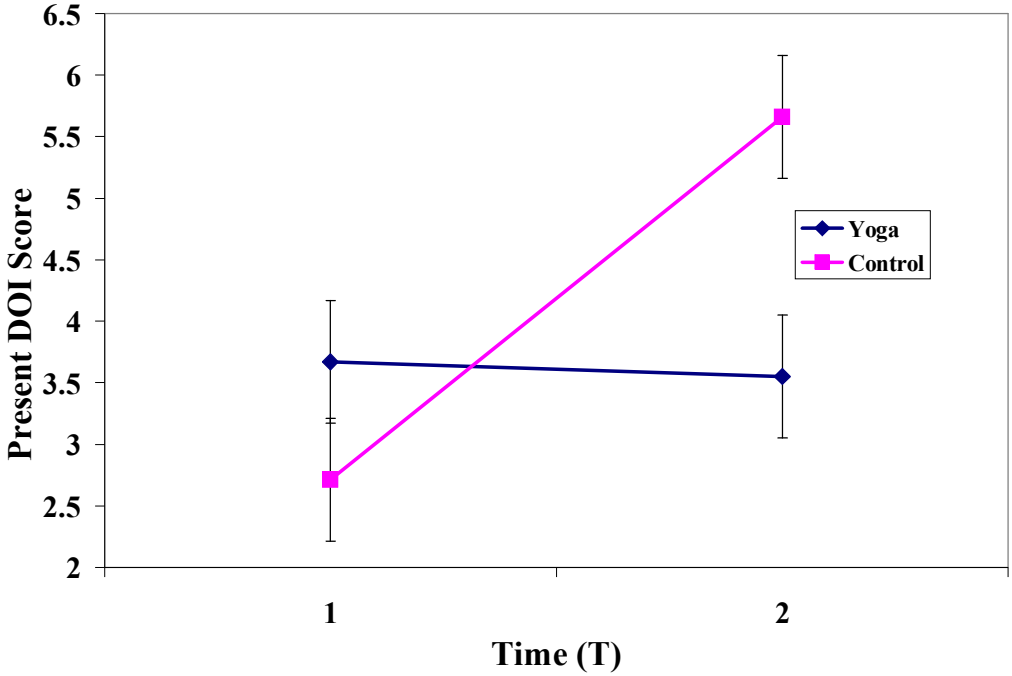


Fig. 2 EMSA for NF-κB Activation

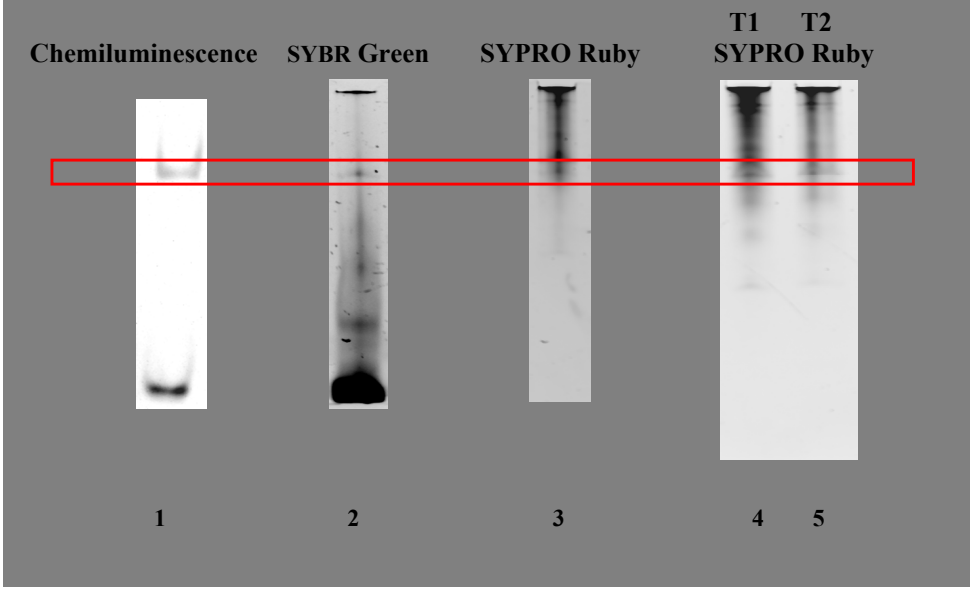


Figure 3. Lymphocyte NF-kB Activation

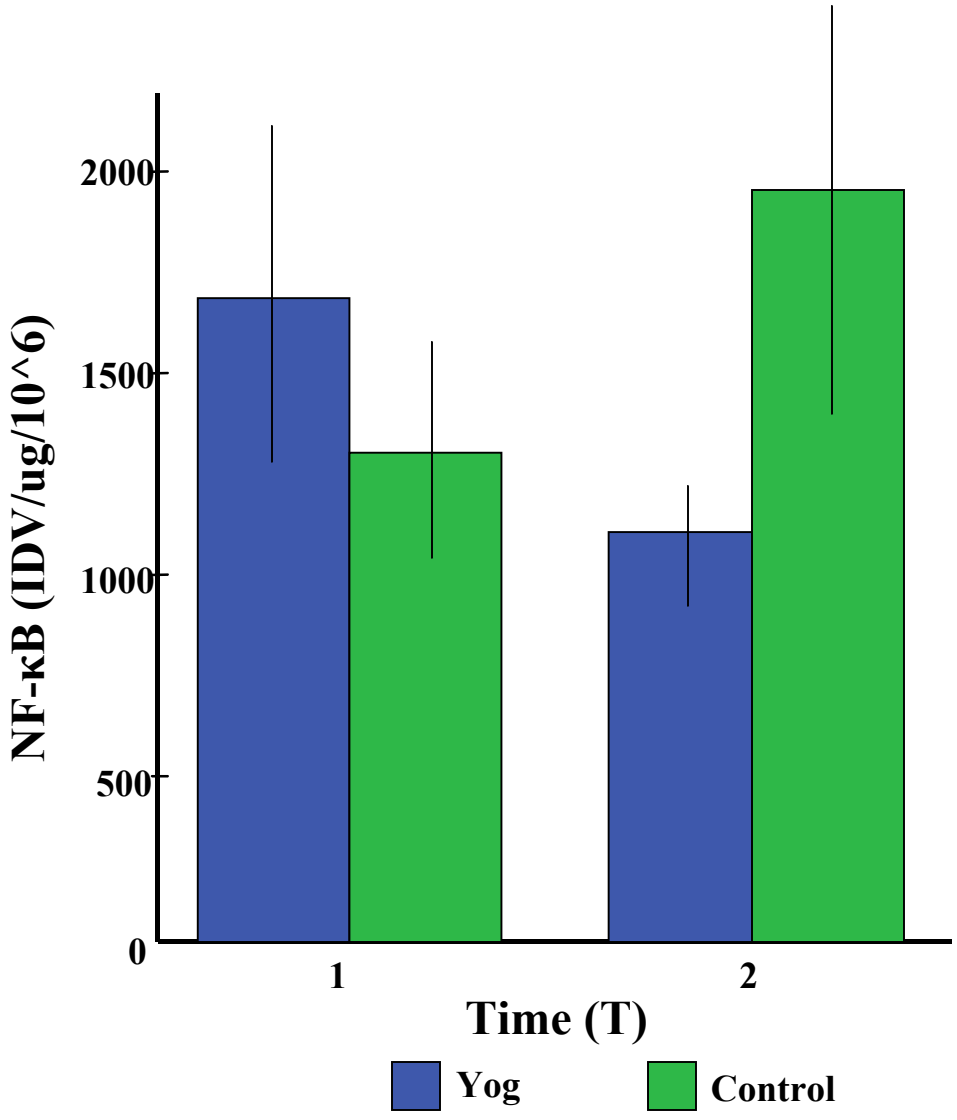
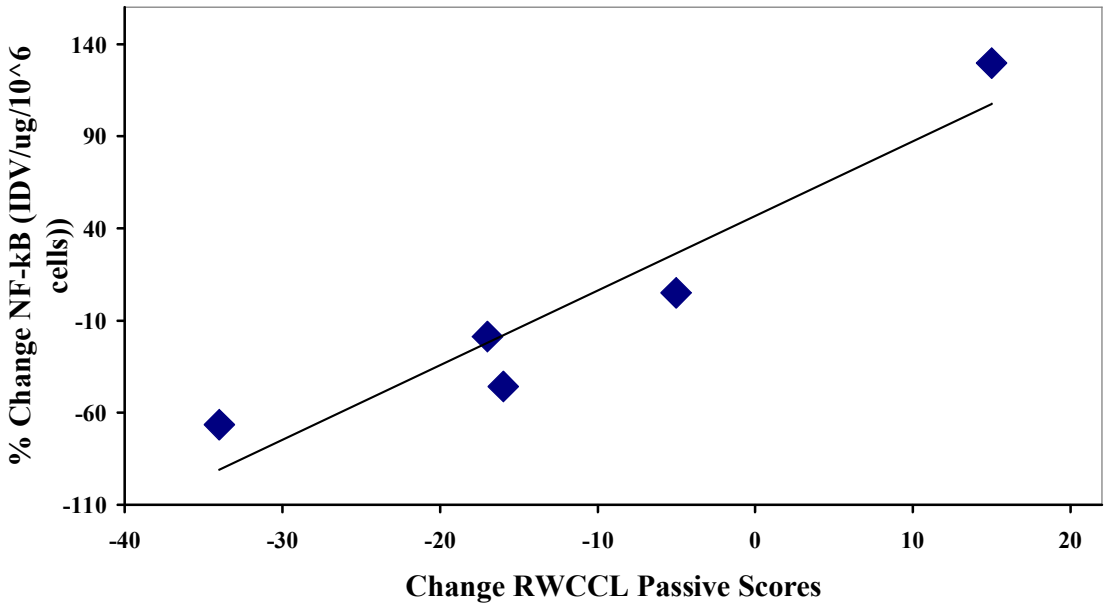


Figure 4. Change in NF-kB Activation is Correlated with Passive Coping Scores from Yoga Participants



APPENDIX C

Self-Organizing Maps

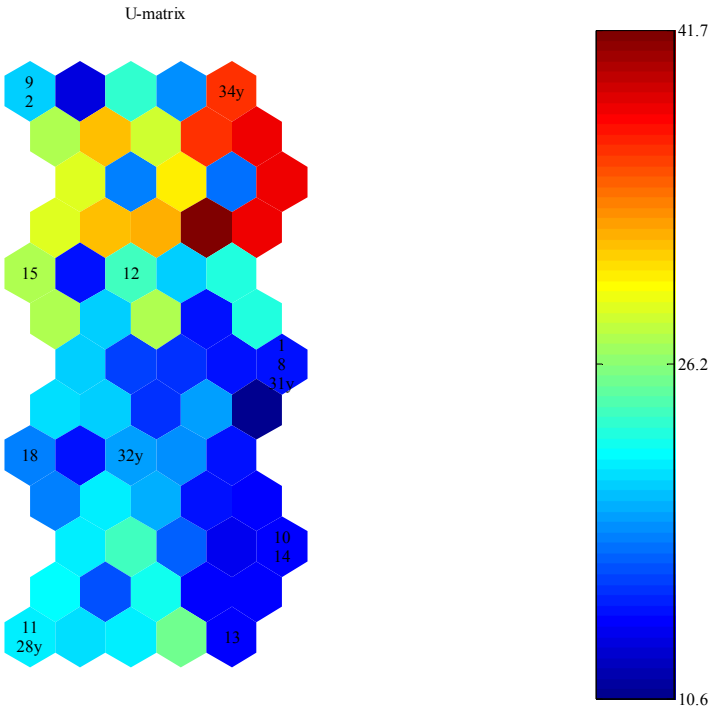
The final analysis of this paper utilizes self-organizing maps (SOMs) as a qualitative tool to interpret a multi-dimensional data set comprised of all the variables collected from each participant as part of the larger study of which this project was a component. Using MATLAB 5 (Mathworks, Inc.) SOM toolbox, relationships between NF-κB activation and/or natural killer (NK) cell activity and the multiple psychosocial variables collected are presented in cluster structure. Developed by Teuvo Kohonen, SOM is a data mining technique that clusters high-dimensional data based on unsupervised learning of neural networks. Resulting from algorithms based on Euclidean distance and the similarity of weighted vectors, the end product SOM displays a unified distance matrix (U-matrix) and individual component planes. The interpretation of the U-matrix associated with each SOM identifies the presence of any clustered participants, indicated by a high concentration of adjacent blue patches. The color scale corresponding to the U-matrix represents distance and how similar each participant is from one another, blue being very similar and red being more different. The location of the U-matrix cluster (if present) can then be transferred to the individual component maps to identify the magnitude of reported scores for each respective corresponding component. The color scale of each component map represents a range of reported scores specific to that questionnaire, blue representing numerically lower scores and red areas representing greater scores.

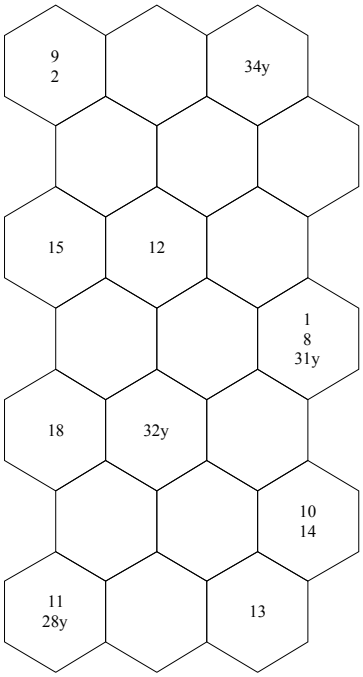
The purpose of the following SOMs is an attempt to illustrate: a) a baseline (T1) profile of the yoga participants in this study, b) a profile representing change of each

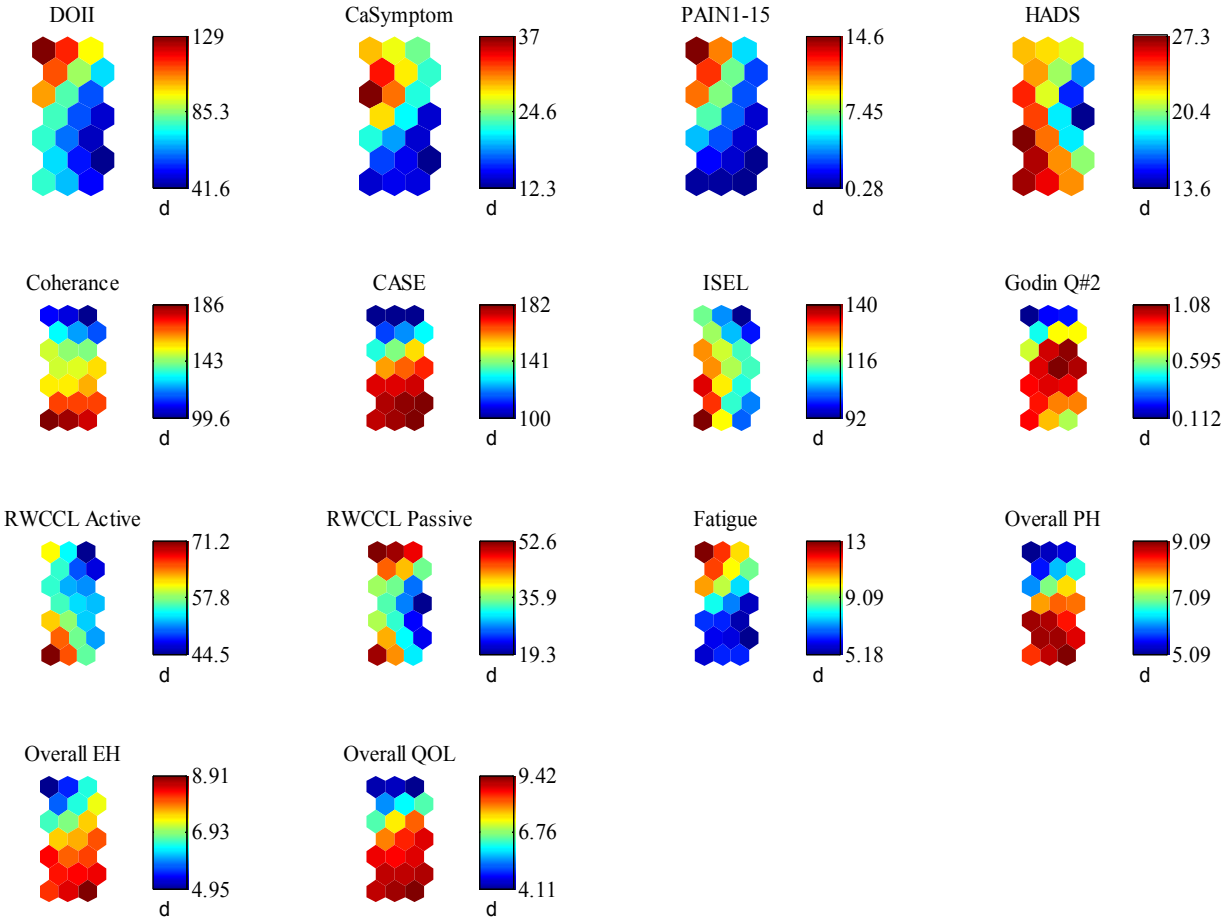
variable, and c) a baseline profile of the yoga participants who similarly expressed decreased lymphocyte NF-κB activation following the intervention. SOM datasets incorporated into the maps include either aggregated questionnaire scores or a larger collection of subscales taken from each questionnaire. The questionnaires included in this complete data set include: Functional Assessment of Chronic Illness Therapy (FACIT), Demands of Illness Inventory (DOII), McGill Pain Questionnaire (Pain), the Coherence Scale (Coherence), Cancer Self-Efficacy Scale (CASE), Revised Ways of Coping Checklist (RWCCL), Hospital Anxiety and Depression Scales (HADS), Interpersonal Support Evaluation List (ISEL), and Godin Leisure-Time Exercise Questionnaire (Godin). In addition, fatigue (taken from the sum of fatigue-related items throughout the questionnaires) as well as single item questions of overall physical health, overall emotional health, and overall quality of life are incorporated into selected SOM data sets.

The intent for inclusion of this analysis has been reserved for future publications under the primary authorship of S.E. Blank and M.R. Haberman with discussion focused on the global perspective of specific psychosocial variables most associated with the effect of yoga on immune function and quality of life in breast cancer survivors. Preliminary findings suggest that breast cancer survivors who responded positively to the yoga intervention (exhibited decreased lymphocyte NF-κB activation associated to decreased demands of illness and increased use coping strategies) shared baseline similarities of reported fatigue, self-efficacy, coherence, depression, pain, menopausal symptoms, emotional and physical well-being, and demands of illness. Future research may lead to identifying specific physical and psychosocial characteristics common to breast cancer survivors predicted to benefit maximally from yoga practice.

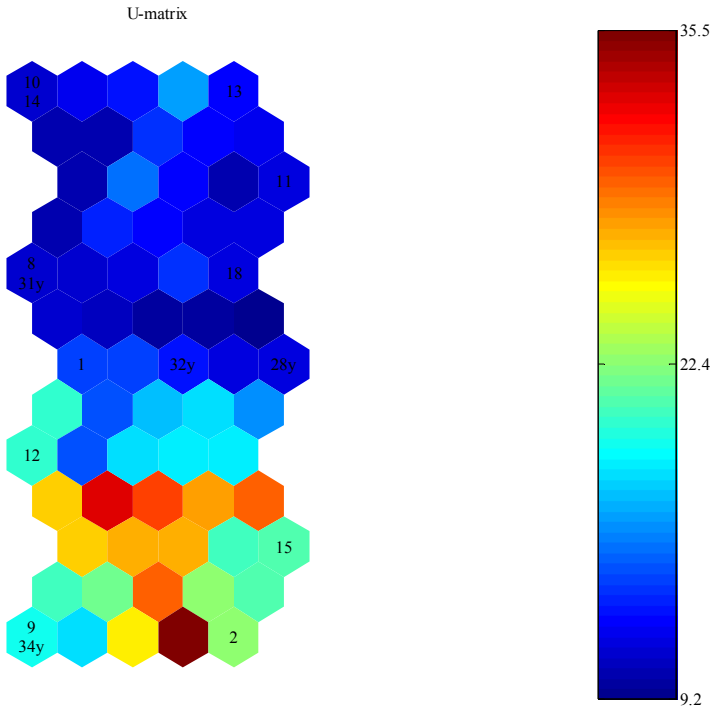
Yoga, T1, aggregates

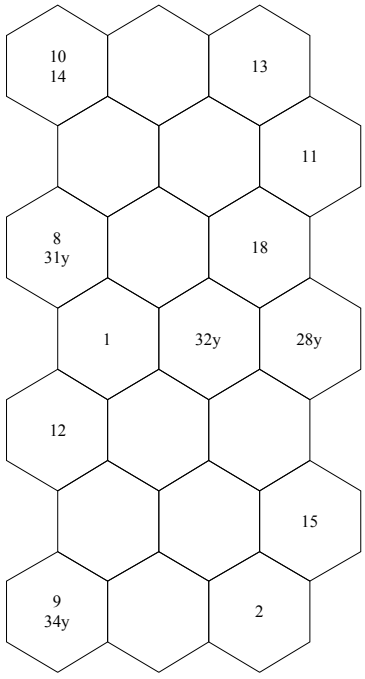


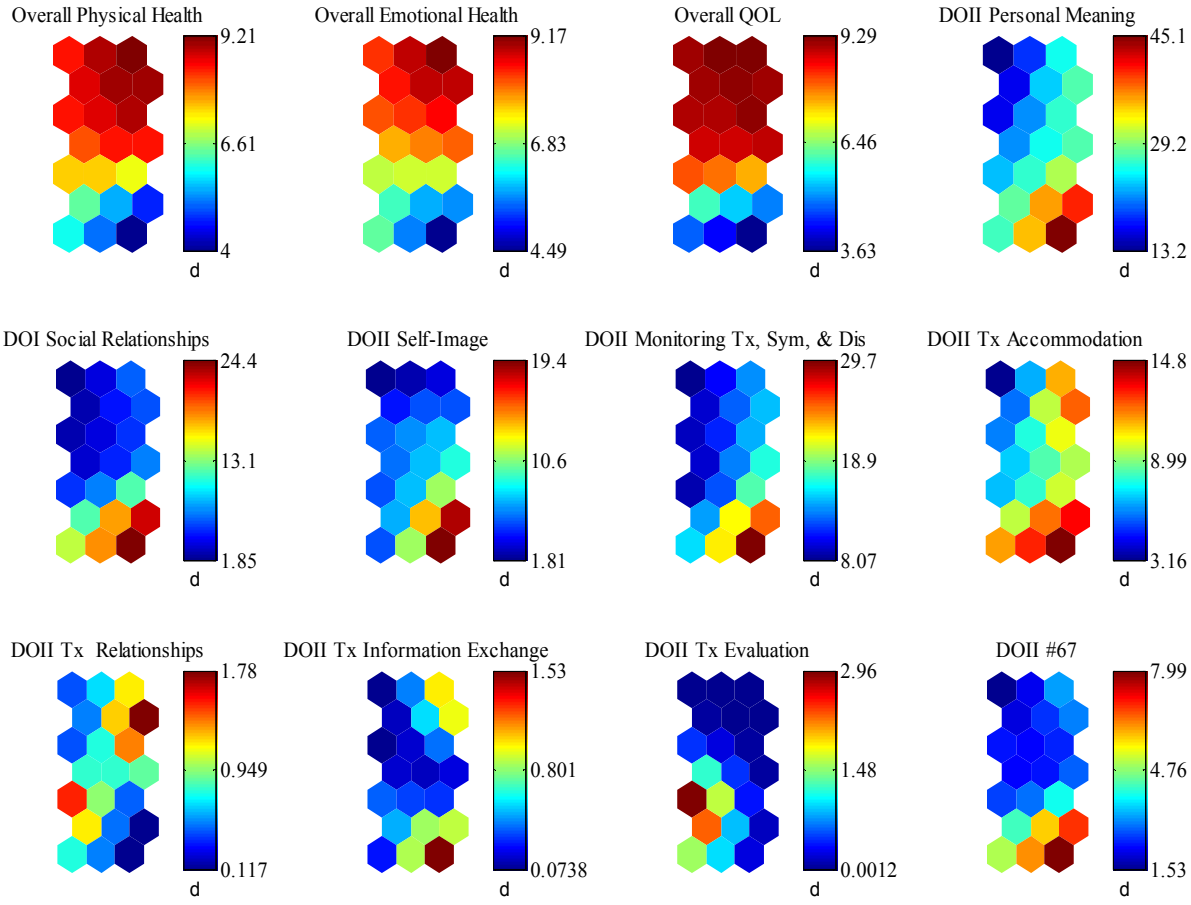



DOII
129
85.3
41.6
d
CaSymptom
37
24.6
12.3
d
PAIN1-15
14.6
7.45
0.28
d
HADS
27.3
20.4
13.6
d
Coherance
186
143
99.6
d
CASE
182
141
100
d
ISEL
140
116
92
d
Godin Q#2
1.08
0.595
0.112
d
RWCCL Active
71.2
57.8
44.5
d
RWCCL Passive
52.6
35.9
19.3
d
Fatigue
13
9.09
5.18
d
Overall PH
9.09
7.09
5.09
d
Overall EH
8.91
6.93
4.95
d
Overall QOL
9.42
6.76
4.11
d

Yoga, T1, subscales






Overall Physical Health
9.21
6.61
4
d
Overall Emotional Health
9.17
6.83
4.49
d
Overall QOL
9.29
6.46
3.63
d
DOII Personal Meaning
45.1
29.2
13.2
d
DOI Social Relationships
24.4
13.1
1.85
d
DOII Self-Image
19.4
10.6
1.81
d
DOII Monitoring Tx, Sym, & Dis
29.7
18.9
8.07
d
DOII Tx Accommodation
14.8
8.99
3.16
d
DOII Tx Relationships
1.78
0.949
0.117
d
DOII Tx Information Exchange
1.53
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0.0012
d
DOII #67
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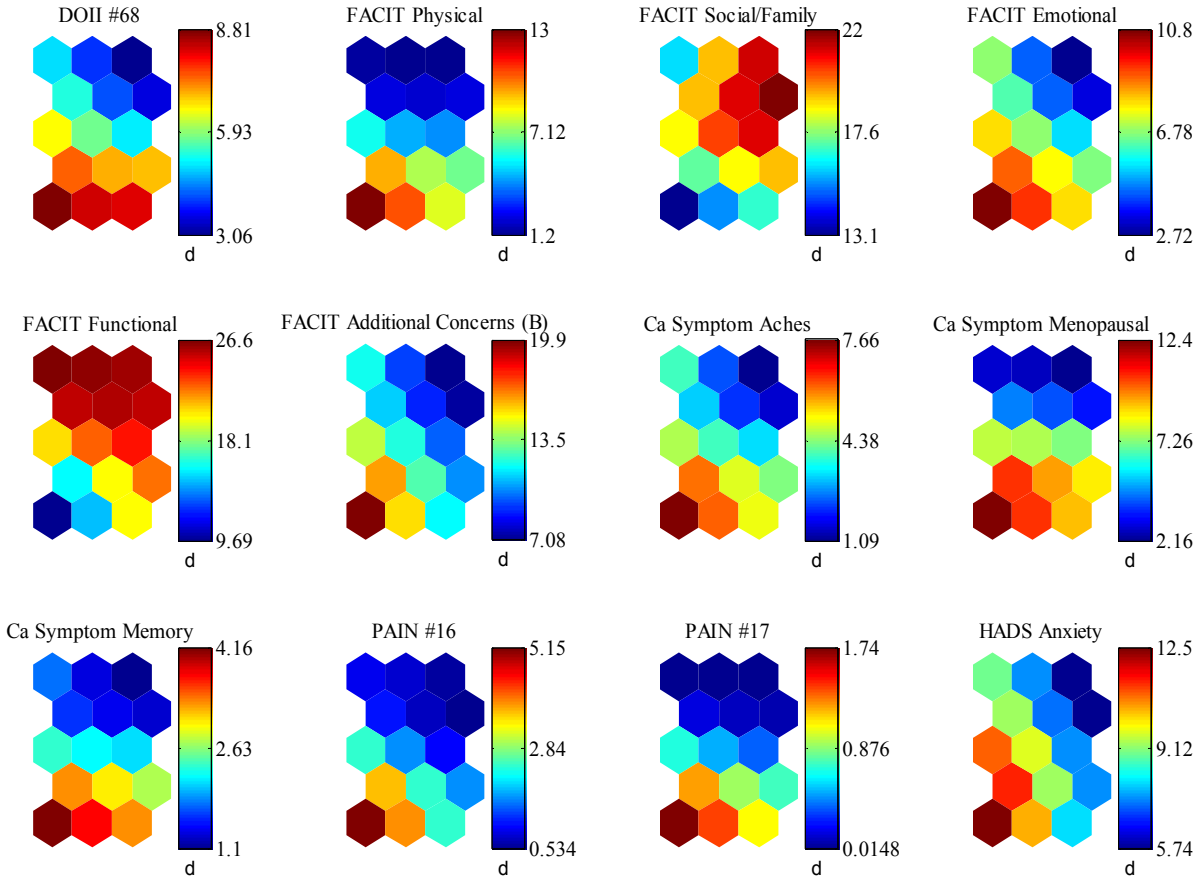
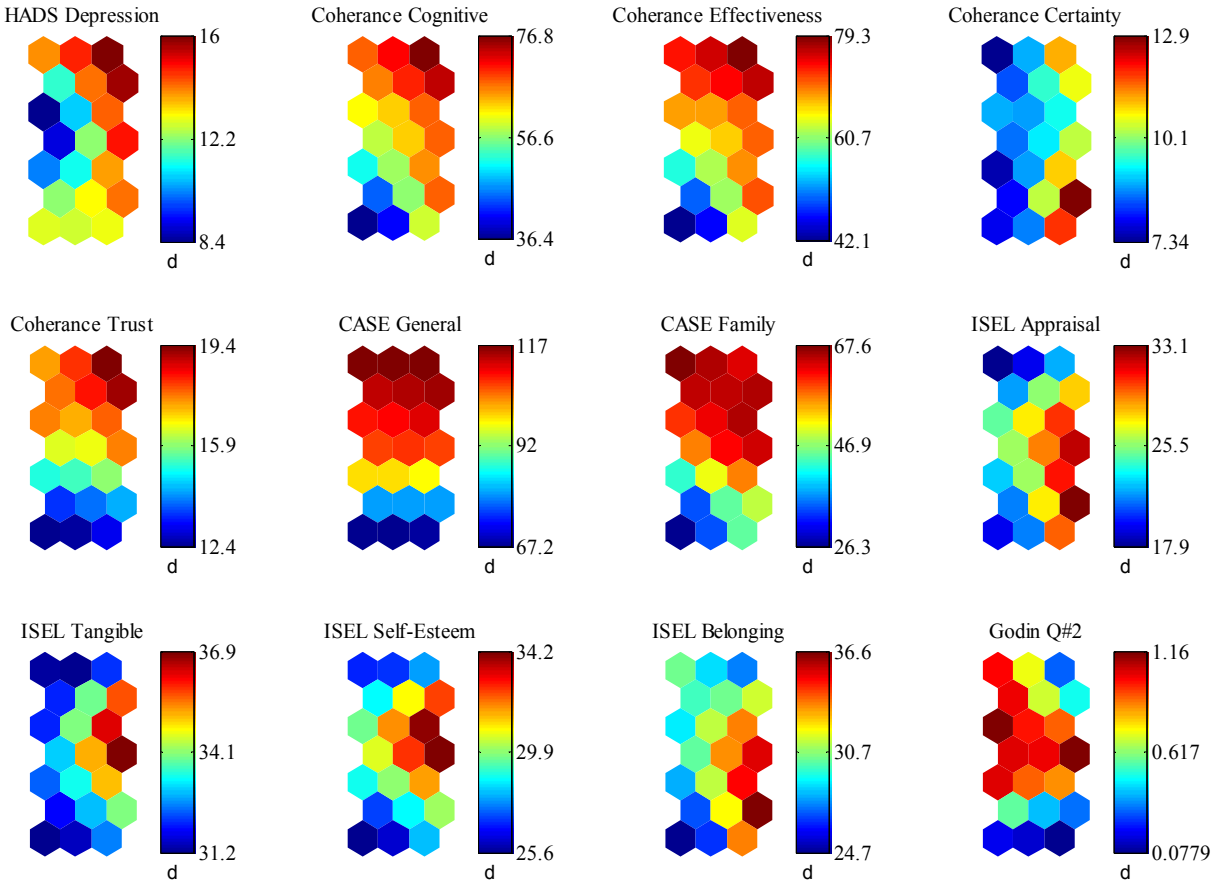
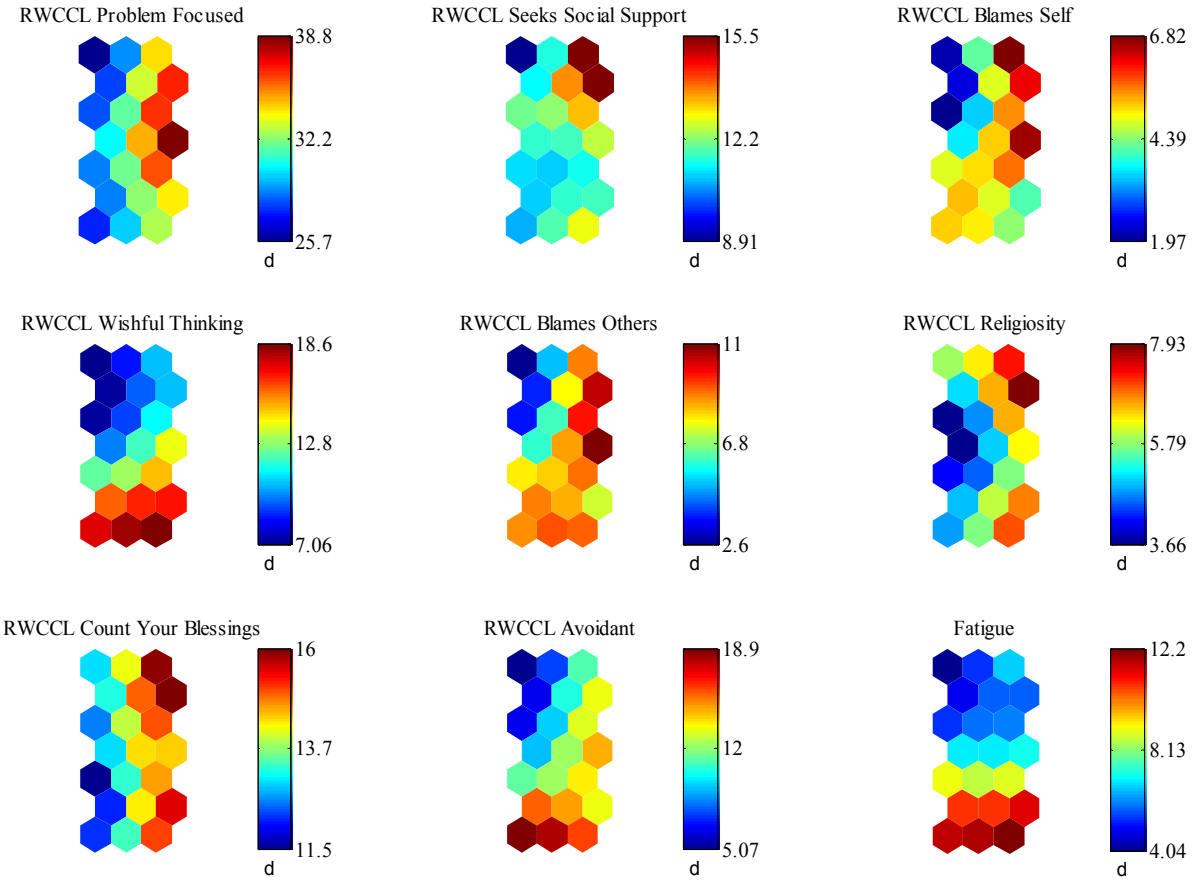
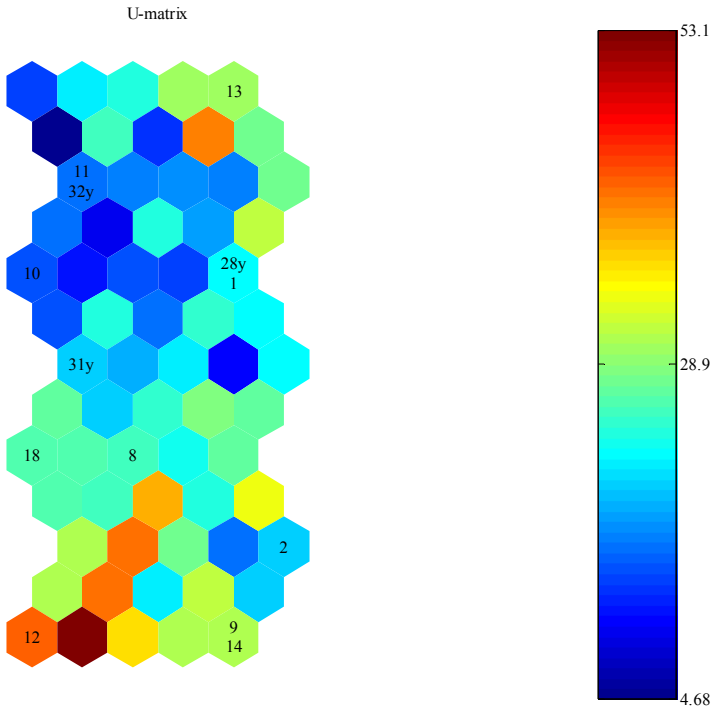
4.76
1.53
d

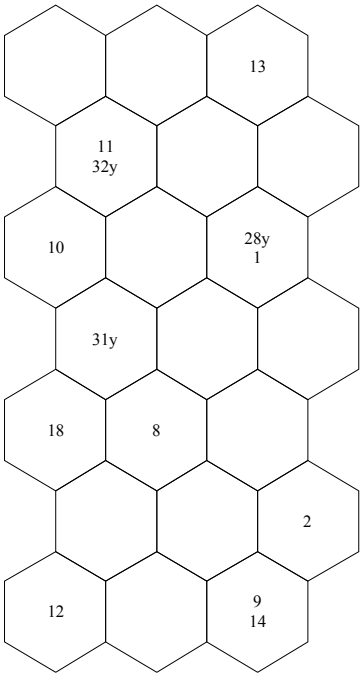

DOII #68
8.81
5.93
3.06
d
FACIT Physical
13
7.12
1.2
d
FACIT Social/Family
22
17.6
13.1
d
FACIT Emotional
10.8
6.78
2.72
d
FACIT Functional
26.6
18.1
9.69
d
FACIT Additional Concerns (B)
19.9
13.5
7.08
d
Ca Symptom Aches
7.66
4.38
1.09
d
Ca Symptom Menopausal
12.4
7.26
2.16
d
Ca Symptom Memory
4.16
2.63
1.1
d
PAIN #16
5.15
2.84
0.534
d
PAIN #17
1.74
0.876
0.0148
d
HADS Anxiety
12.5
9.12
5.74
d

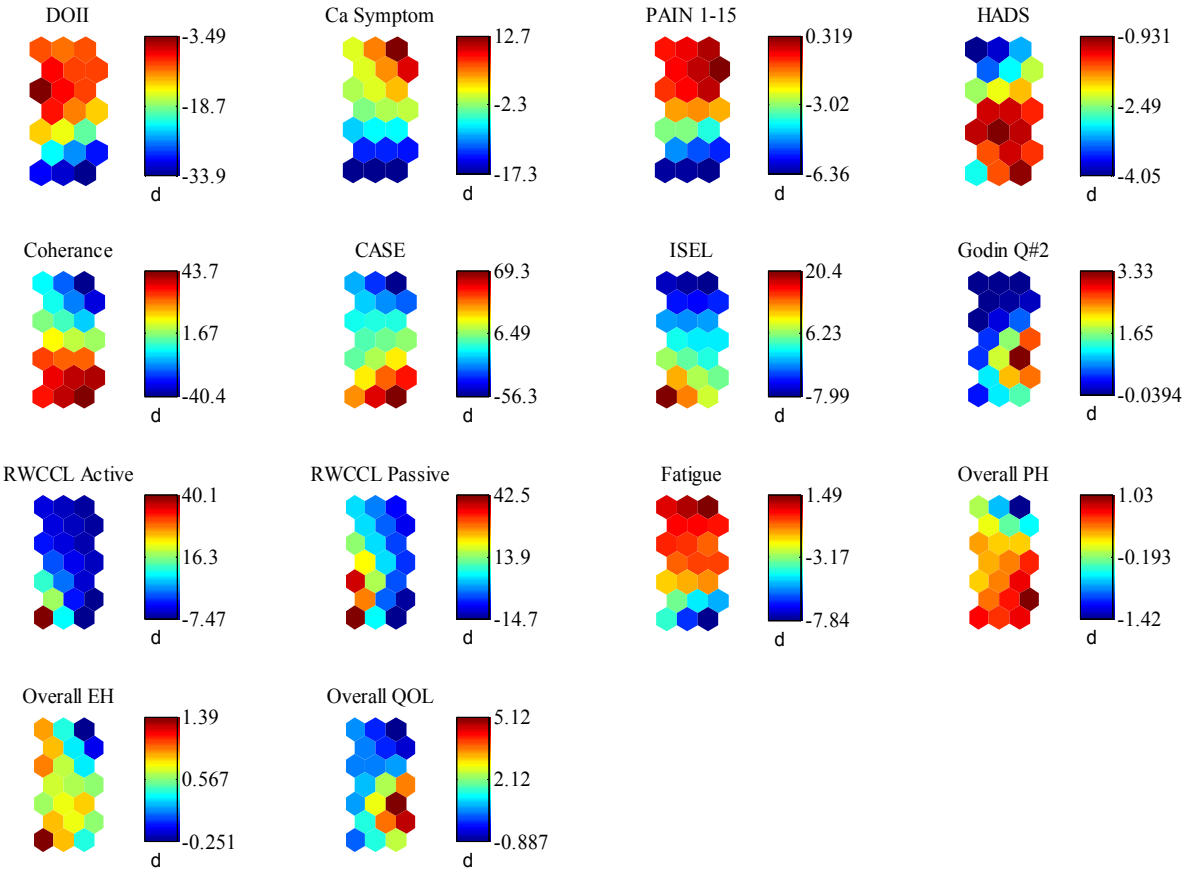

HADS Depression
16
12.2
8.4
d
Coherance Cognitive
76.8
56.6
36.4
d
Coherance Effectiveness
79.3
60.7
42.1
d
Coherance Certainty
12.9
10.1
7.34
d
Coherance Trust
19.4
15.9
12.4
d
CASE General
117
92
67.2
d
CASE Family
67.6
46.9
26.3
d
ISEL Appraisal
33.1
25.5
17.9
d
ISEL Tangible
36.9
34.1
31.2
d
ISEL Self-Esteem
34.2
29.9
25.6
d
ISEL Belonging
36.6
30.7
24.7
d
Godin Q#2
1.16
0.617
0.0779
d


RWCCL Problem Focused
38.8
32.2
25.7
d
RWCCL Seeks Social Support
15.5
12.2
8.91
d
RWCCL Blames Self
6.82
4.39
1.97
d
RWCCL Wishful Thinking
18.6
12.8
7.06
d
RWCCL Blames Others
11
6.8
2.6
d
RWCCL Religiosity
7.93
5.79
3.66
d
RWCCL Count Your Blessings
16
13.7
11.5
d
RWCCL Avoidant
18.9
12
5.07
d
Fatigue
12.2
8.13
4.04
d

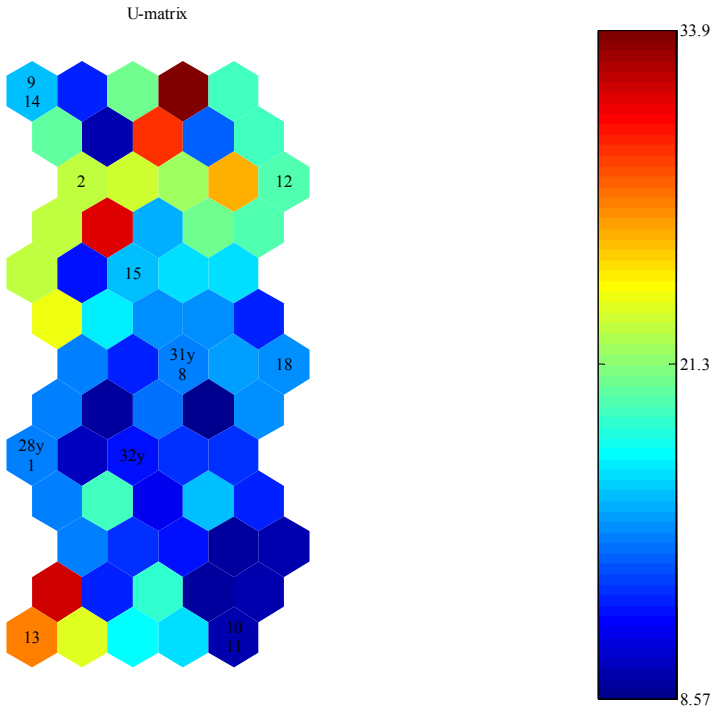
Yoga, change, aggregates

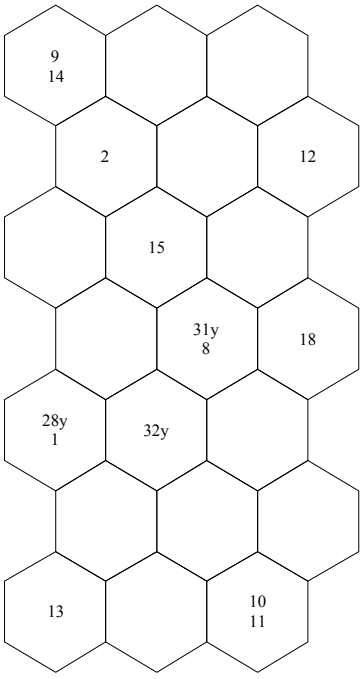


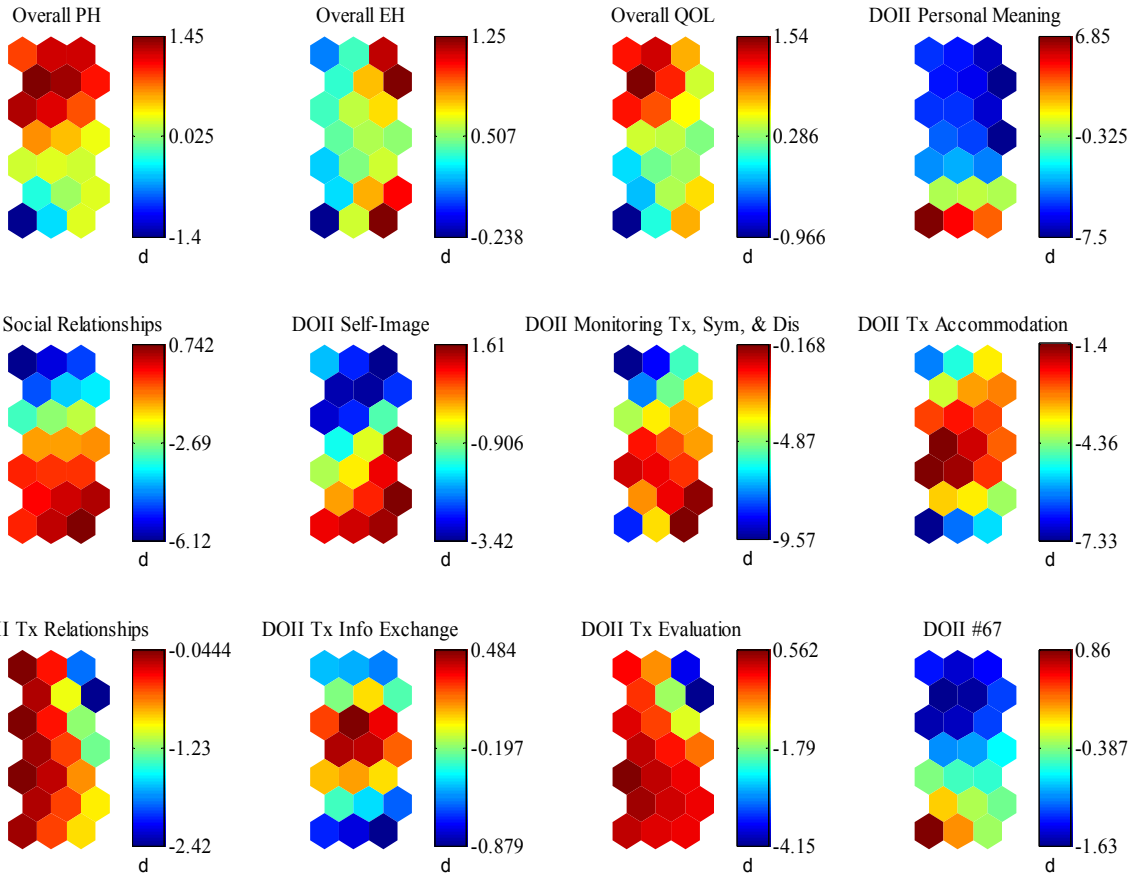


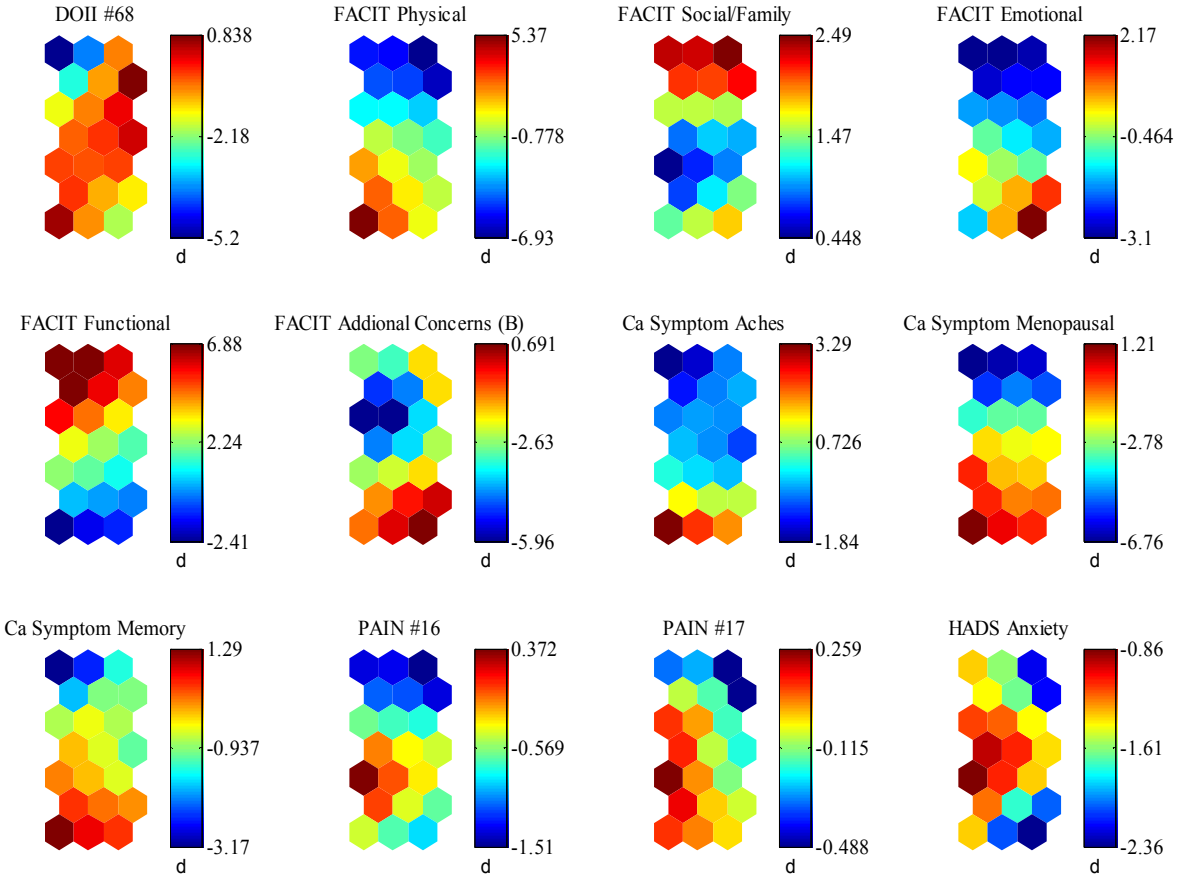

DOII
-3.49
-18.7
-33.9
d
Ca Symptom
12.7
-2.3
-17.3
d
PAIN 1-15
0.319
-3.02
-6.36
d
HADS
-0.931
-2.49
-4.05
d
Coherance
43.7
1.67
-40.4
d
CASE
69.3
6.49
-56.3
d
ISEL
20.4
6.23
-7.99
d
Godin Q#2
3.33
1.65
-0.0394
d
RWCCL Active
40.1
16.3
-7.47
d
RWCCL Passive
42.5
13.9
-14.7
d
Fatigue
1.49
-3.17
-7.84
d
Overall PH
1.03
-0.193
-1.42
d
Overall EH
1.39
0.567
-0.251
d
Overall QOL
5.12
2.12
-0.887
d

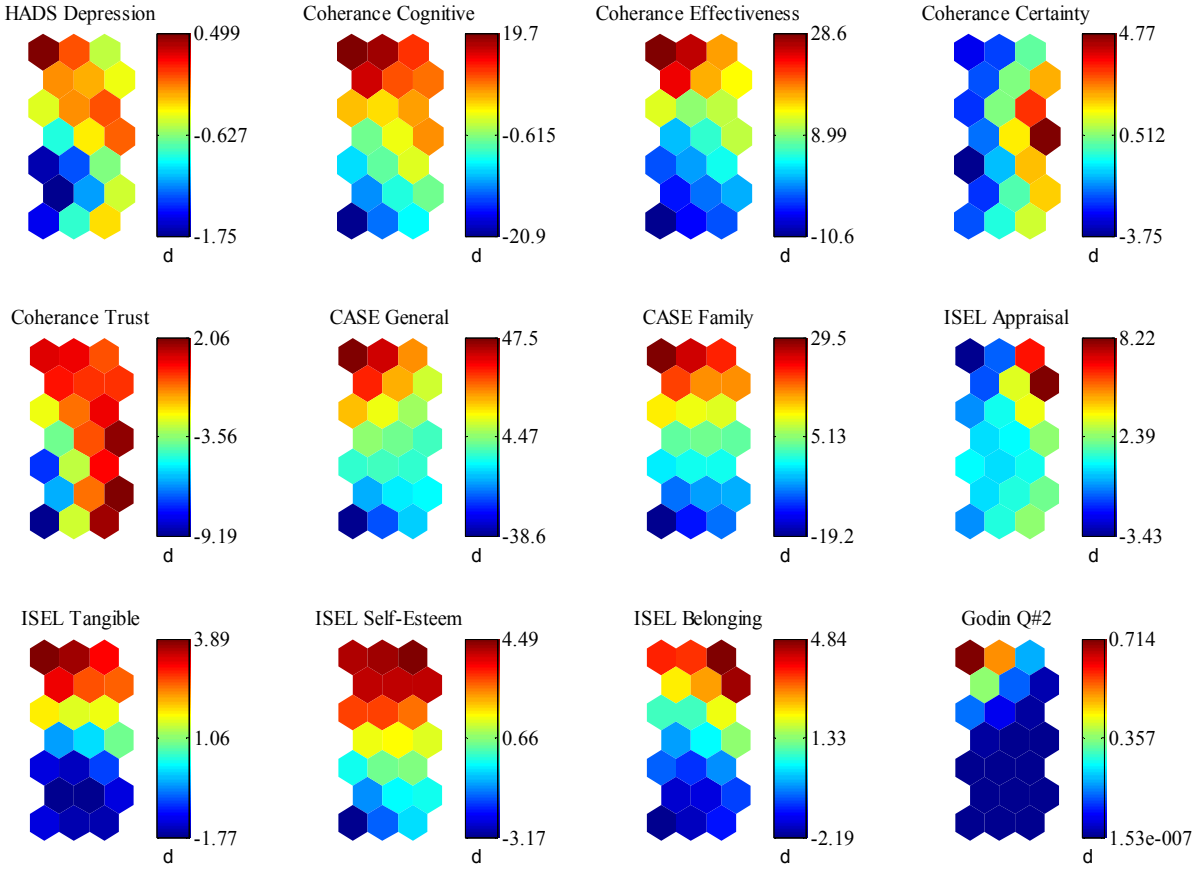
Yoga, change, subscales



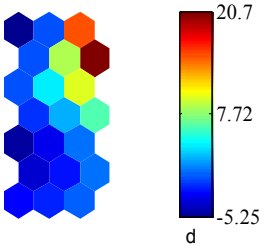



Overall PH
1.45
0.025
-1.4
d
Overall EH
1.25
0.507
-0.238
d
Overall QOL
1.54
0.286
-0.966
d
DOII Personal Meaning
6.85
-0.325
-7.5
d
DOII Social Relationships
0.742
-2.69
-6.12
d
DOII Self-Image
1.61
-0.906
-3.42
d
DOII Monitoring Tx, Sym, & Dis
-0.168
-4.87
-9.57
d
DOII Tx Accommodation
-1.4
-4.36
-7.33
d
DOII Tx Relationships
-0.0444
-1.23
-2.42
d
DOII Tx Info Exchange
0.484
-0.197
-0.879
d
DOII Tx Evaluation
0.562
-1.79
-4.15
d
DOII #67
0.86
-0.387
-1.63
d

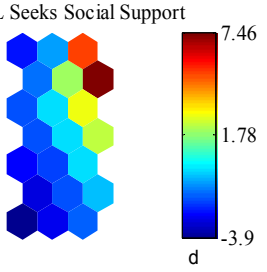

DOII #68
0.838
-2.18
-5.2
d
FACIT Physical
5.37
-0.778
-6.93
d
FACIT Social/Family
2.49
1.47
0.448
d
FACIT Emotional
2.17
-0.464
-3.1
d
FACIT Functional
6.88
2.24
-2.41
d
FACIT Addional Concerns (B)
0.691
-2.63
-5.96
d
Ca Symptom Aches
3.29
0.726
-1.84
d
Ca Symptom Menopausal
1.21
-2.78
-6.76
d
Ca Symptom Memory
1.29
-0.937
-3.17
d
PAIN #16
0.372
-0.569
-1.51
d
PAIN #17
0.259
-0.115
-0.488
d
HADS Anxiety
-0.86
-1.61
-2.36
d


HADS Depression
0.499
-0.627
-1.75
d
Coherance Cognitive
19.7
-0.615
-20.9
d
Coherance Effectiveness
28.6
8.99
-10.6
d
Coherance Certainty
4.77
0.512
-3.75
d
Coherance Trust
2.06
-3.56
-9.19
d
CASE General
47.5
4.47
-38.6
d
CASE Family
29.5
5.13
-19.2
d
ISEL Appraisal
8.22
2.39
-3.43
d
ISEL Tangible
3.89
1.06
-1.77
d
ISEL Self-Esteem
4.49
0.66
-3.17
d
ISEL Belonging
4.84
1.33
-2.19
d
Godin Q#2
0.714
0.357
1.53e-007
d

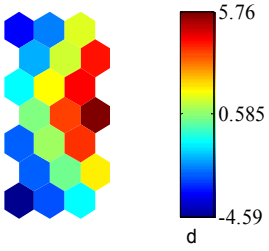
RWCCL Problem Focused



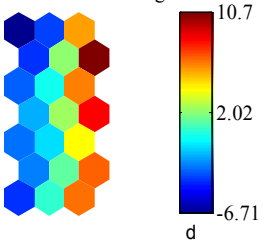
RWCCL Seeks Social Support



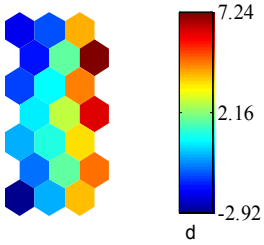
RWCCL Blames Self



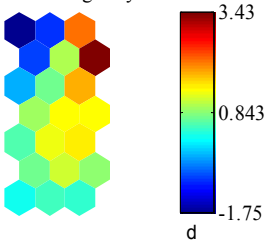
RWCCL Wishful Thinking



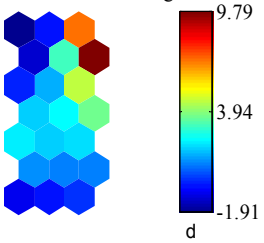
RWCCL Blames Others



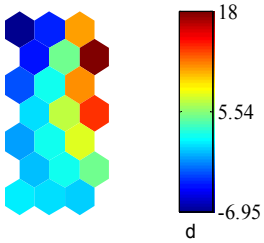
RWCCL Religiosity



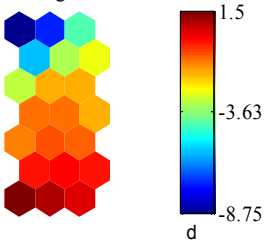
RWCCL Count Your Blessings



RWCCL Avoidant



Fatigue



NF-κB Responders, Baseline Selected Psychosocial Subscales