EFFECTS OF MACULAR CAROTENOID SUPPLEMENTATION ON BEHAVIORAL, NEUROTROPHIC, AND NEUROINFLAMMATORY FACTORS RELATED TO PSYCHOLOGICAL STRESS

by

NICOLE TRESSA STRINGHAM

(Under the Direction of Philip V. Holmes)

ABSTRACT

Purpose: Oxidative stress and systemic inflammation are associated with chronic psychological stress, and play a major role in stress-induced neural atrophy. There is a wide body of literature linking oxidative and inflammatory stress with a reduction in both neurotrophic factors and psychological stress resilience. We investigated the potential for the dietary carotenoids lutein, zeaxanthin, and mesozeaxanthin (collectively the macular carotenoids [MC]), which have high antioxidant / anti-inflammatory capacity, to reduce psychological stress, serum cortisol, and the pro-inflammatory cytokines TNF-α, IL-6, and IL-1β. Levels of brain-derived neurotrophic factor (BDNF – associated with neural plasticity) and behavioral data regarding emotional and physical well-being were also obtained.

Methods: 59 young (18-25 yrs.), healthy subjects participated in a double-blind, placebo-controlled supplementation trial. Subjects were randomly assigned to one of three groups: placebo, 13 mg, or 27 mg / day total MCs. Fasting blood draws were conducted between 9-11 am. Serum cortisol, BDNF, TNF-α, IL-6, IL-1β, and serum
antioxidant potential (AOP) were assessed via ELISA. The retinal concentration of MCs (termed macular pigment optical density [MPOD]) was determined via customized heterochromatic flicker photometry. Behavioral data were obtained via questionnaire.

Results: Supplementation with MCs reduced psychological stress, serum cortisol, and measures of suboptimal emotional and physical health (p < 0.05 for all), versus placebo. BDNF, MPOD, and AOP all increased significantly versus placebo in both treatment groups as well (p < 0.05 for all). IL1-β decreased significantly versus placebo in the treatment groups (p < 0.05). No significant changes in any parameter were found for the placebo group.

Conclusions: Supplementation with the MCs had several significant effects, including reductions in psychological stress, cortisol, IL1-β, and symptoms of suboptimal emotional and physical health. Significant increases were seen in serum BDNF, MPOD, and AOP. These findings illustrate the impact that reduction in systemic and local oxidative stress can have on many aspects of health and performance. Determining the basis (whether systemic or a more central), and neurophysiological pathway(s) for these effects is a question that warrants further study. Future research directions also include examining effects downstream from those described in the present studies, such as cognitive performance.

INDEX WORDS: Lutein, Zeaxanthin, Macular Carotenoids, Psychological Stress, Brain Derived Neurotrophic Factor (BDNF), Inflammatory Cytokines
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by

NICOLE TRESSA STRINGHAM
BS, University of Utah, 2005
MS, University of Georgia, 2013

A Dissertation Submitted to the Graduate Faculty of The University of Georgia in Partial
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NICOLE TRESSA STRINGHAM

Major Professor: Philip V. Holmes
Committee: Gaylen Edwards
            Anne Shaffer

Electronic Version Approved:

Suzanne Barbour
Dean of the Graduate School
The University of Georgia
May 2016
DEDICATION

I dedicate this dissertation to Jim Stringham—my partner in research, discussion, science, life, and “crime.” Without his undying dedication to my education, I would not be where I am—here—able to share with you my contribution to the scientific community.
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I would like to thank my advisor, Philip V. Holmes for his helpful comments, support, and patience throughout this process. His expertise, experience, and ability to help me think about how these mechanisms may work were invaluable to interpretation and analysis of my research.

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CHAPTER 1
INTRODUCTION AND LITERATURE REVIEW

Macular Carotenoids in Neural Tissue

The carotenoids lutein (L) and zeaxanthin (Z) are naturally-occurring pigments found primarily in leafy-green vegetables, such as spinach and kale (Sommerburg et al. 1998), and have many healthful benefits throughout the body (e.g. Johnson, 2002), including reducing the risk of developing age-related macular degeneration (AMD; Richer et al. 2004), increasing several skin health parameters (Roberts et al. 2009), and reduction of atherosclerotic plaques (Kailora et al. 2006). They are not synthesized de novo, and are therefore obtained solely from dietary sources or supplements. Those who have diets rich in leafy greens, or supplement with sufficient L and Z, tend to have higher blood and tissue concentrations of these carotenoids (Ciulla et al. 2001; Bone et al. 2003). Although rare, trace amounts of the Z isomer mesozeaxanthin (MZ) are present in the diet in various parts of the world – it is found in 21 species of fish, shrimp and sea turtles, as well as eggs (due to supplementation of chicken feed) in California and Mexico (Maoka et al. 1986; Nolan et al. 2013). Although relatively rare in the diet, MZ is biochemically converted from L within the retina (Neuringer et al. 2004), where it is then concentrated in the most vulnerable location of the retina, the central fovea. In terms of dietary response, the body appears to recognize MZ, as it is readily deposited in the retina when taken in supplement form (Bone et al. 2007; Loughman et al. 2012). L, Z, and MZ have exceptional antioxidant capacity (Krinsky et al. 2003), which enables them to
protect bodily tissues against damaging free-radical oxygen. This is especially important in areas of high metabolic demand such as cardiovascular tissue, the retina, and brain, where oxidative byproducts of normal metabolic function can cause DNA and tissue damage (Cocate et al. 2015).

As alluded to above, L, Z, and MZ are deposited in the retina, where they are collectively referred to as macular pigment (MP). The optical density of MP (MPOD) is positively associated with several important functions related to both ocular health (noted above) and visual performance, including increased visual processing speed (Hammond & Wooten, 2005). On average, subjects with higher MPOD are able to maintain visibility of a flickering light at higher frequencies than those with lower MPOD (who see the light as a stable, solid disc of light). In other words, those subjects with higher concentrations of L, Z, and MZ in their retinas have faster visual systems; this ultimately can manifest as faster overall cognitive processing speed (Feeney et al. 2013). High MPOD also substantially improves visual performance in bright light environments (i.e. glare). These effects include reduced visual discomfort in bright light (Stringham et al. 2003; 2004; 2011), increased ability to see through glare (Stringham and Hammond, 2007; 2008), and decreased photostress recovery time (recovering a visual target after exposure to an extremely bright light; Stringham and Hammond, 2007; 2008; Stringham et al. 2011).

More recently, MPOD has been associated with better cognitive function in people over 50 – subjects with higher MPOD (which is correlated to brain levels of L and Z – Vishwanathan et al. 2013) perform better on cognitive tasks related long-term memory and decision-making (Feeney et al. 2013). Additionally, in a recent study of
deceased centenarians (those who had lived to over 100 years of age), Johnson et al. (2013) found that brain concentrations of L were significantly higher than any other carotenoid, especially in areas that serve cognitive function, such as the frontal and temporal lobes. This suggests not only that L is very important to brain function during the aging process, but also (based on the areas into which it is deposited) that L is important in preserving high-level cognitive function.

A promising new research direction for L, Z, and MZ involves their potential role in preventing the onset, or slowing the progression, of cognitive decline. As noted above, in several studies, people over 50 years of age performed significantly better on cognitive tasks as a function of MPOD. This idea was recently investigated by Nolan et al. (2014) in a study of early-stage Alzheimer’s disease patients versus normal, age-matched controls. The Alzheimer’s patients were shown to have significantly lower macular carotenoid concentrations than the control subjects. This finding suggests that, as in AMD, perhaps the macular carotenoids are preventing cumulative damage over the lifespan that can, if left unchecked, produce neural damage that ultimately leads to impairment. In a follow-up study (Nolan et al. 2015), Alzheimer’s disease patients were found to respond positively in the retina to macular carotenoid supplementation, which suggests that the body maintains the ability to absorb and use these carotenoids in neural tissue well into old age, and that they may offer some potential benefit in increased concentrations. With regard to neurodegenerative disease, supplementation of L has been found to slow or even stop the progression of AMD (Richer et al. 2004). It appears therefore that the macular carotenoids have not only long-term protective effects on tissues, but may also have acute beneficial effects as well. A trial to determine the effects
of L, Z, and MZ supplementation on the progression of Alzheimer’s disease is currently underway (Nolan, personal communication).

**Psychological Stress and the Diet**

The conspicuous deposition of L and Z in the brain, coupled with their well-known antioxidant and anti-inflammatory functions is suggestive of several potential neural roles for these carotenoids. The aforementioned preservation of cognitive function into old age would appear to be a manifestation of protection of neural tissue against cumulative oxidative stress / inflammation over the lifespan. In terms of evolutionary biology, however, the accumulation of L and Z in the brain must have afforded some survival advantage earlier in life; certainly before or during the human reproductive cycle. Oxidative stress and inflammation in the brain are associated with a multitude of poor outcomes in young adults, including excessive psychological stress (Srivastava and Batra, 2014) and depression (Michel et al. 2012). Moreover, the specific regions of the brain in which L and Z are deposited maintain relatively high metabolism and concomitant oxygen tension; it is well known that oxidative stress and inflammation significantly reduce the efficiency of metabolic processes (see e.g. Moiseyev et al. 2010). Reduction in metabolic efficiency of regions of the brain serving cognitive and emotional functions would presumably be maladaptive. This idea, coupled with the paucity of these carotenoids in the modern human diet (see Johnson et al. 2010) suggests that there may exist subclinical issues associated with deficiencies of L and Z in the brain.

Benton (2013) posited that effects of minor nutritional deficiencies would manifest first as subclinical disruption of brain function, given the complexity and metabolic demands of the brain. Psychological stress, experienced by a large portion of
the population (APA, 2011), could certainly be described as indicative of a disruption of normal brain function. In general support of this idea, there have been recent human and animal studies that report stress-reducing effects of supplementation of specific nutrients, such as curcumin (Sciberras et al. 2015), alpha tocopherol (Lodhi et al., 2014), and docosahexaenoic acid (DHA; Keenan et al. 2014). In each case, supplementation appears to lead to reduced psychological stress and physiological parameters of stress (e.g., blood cortisol). Additionally, Long and Benton (2013) conducted a meta-analysis of studies on effects of vitamin and mineral supplementation on stress and mood in subclinical populations, and found a general trend towards stress reduction and improvement in mood. This effect was most pronounced in studies that examined supplements containing relatively high levels of B-vitamins; this may have been due to the fact that vitamin B deficiency (esp. B-12) is fairly common (Allen, 2009), and it is therefore likely that a much greater number of people maintain a subclinical B-vitamin deficiency. 

Supplementation with B vitamins would presumably help to meet metabolic and other physiological needs that may serve to ultimately reduce psychological stress. By contrast, Camfield et al. (2013) determined that ingestion of a daily multivitamin for 16 weeks was ineffective at reducing salivary cortisol and relieving psychological stress, compared to placebo, in healthy young participants. A closer examination of the aforementioned studies that found psychological benefit reveals the possibility that results may have been influenced by sample effects, or perhaps specific conditions used in the study. For example, Keenan et al. (2014) examined the effects of DHA supplementation (versus placebo) on perceived stress in low-income, pregnant African American women. Although the finding of reduced stress with DHA supplementation is both statistically
and practically significant, one could argue that the sample used in this study is far too specific to responsibly extend the results to other populations. Additionally, the finding could have been influenced by subject characteristics, such as very high baseline perceived stress or perhaps significantly low baseline DHA status. In the Sciberras et al. (2015) investigation, the effects of curcumin supplementation on physiological and psychological parameters of stress were examined in eleven recreational athletes. In experimental subjects, supplementation began three days prior to an intense, 2-hr cycling trial. Although most of the physiological parameters (e.g. IL-6) were not modulated by curcumin supplementation, the athletes taking curcumin reported significantly less psychological stress between trials compared to placebo. As the authors note, it is difficult to have absolute confidence in results obtained from a relatively small sample, especially in an outcome (psychological stress) that is notoriously variable among people.

Support for the idea that general dietary patterns can influence psychological parameters such as stress and depression was found by El Ansari et al. (2014) in a large (n = 3,706), generally healthy population of college-aged adults. Perceived stress level and depressive symptoms were found to exhibit a significantly inverse relationship with consumption of healthy foods such as fresh fruits and vegetables. Conversely, consumption of “junk” food was associated with increased perceived psychological stress. Although the correlative findings prevent inference of causation, there is a basis for the idea that healthy foods can reduce psychological stress: consumption of antioxidants. It has been shown that systemic oxidative stress is induced by psychological stress (e.g. in medical students; Srivastava and Batra, 2014), and it appears that reduction of systemic oxidative stress significantly reduces indicators of
psychological stress (via alpha-tocopherol administration: Smitha and Mukkadan, 2014; and by lutein: Yajima et al., 2013). Oxidative stress therefore appears to be a significant product of psychological stress, and a factor in the genesis of psychological stress. Increasing systemic and local concentrations of L, Z, and MZ would seemingly hold much promise in both cases.

**Brain, Behavior, and the Immune system**

The immune, endocrine, central, and autonomic nervous systems do not operate independently of each other, but rather are intimately linked via elaborate signaling pathways (Brebner et al. 2000). As in the case of physiological or psychological stress, homeostatic mechanisms actively seek to restore functional balances among these systems. The dependence of bodily systems upon each other, and the idea of homeostatic balances can be illustrated on a microcosmic level by oxidative stress and its relationship with inflammation. At optimal functional levels, oxidation and inflammation operate to protect the body from spreading infection and antigens (Nathan and Shiloh, 2000). Moreover, oxidation and inflammation (although generally associated with negative outcomes) can contribute to efficiency of metabolic processes (Vielma et al. 2010). Due to the astounding complexity of interaction between the various physiological aspects of body systems, a consistent, favorable balance is difficult to achieve. This is especially true in the case of oxidative stress. If oxidative processes are left unchecked by antioxidant mechanisms such as L, Z, and / or MZ, oxidative stress occurs (Valko et al. 2007). Prolonged, excessive oxidative stress can cause devastating, often irreversible damage to neural tissues such as the retina (e.g. Beatty et al. 2000) and brain (e.g., Floyd and Hensley, 2002). Moreover, prolonged oxidative stress promotes pro-inflammatory
responses (Nakanishi and Wu, 2009), which can exacerbate damage and result in the creation of more reactive oxygen species (ROS; Afonso et al. 2006).

In addition to their antioxidant capacity, L, Z, and MZ have been found to have anti-inflammatory properties (Li et al. 2012); namely, they break the chain of inflammation by exerting their antioxidant effects. As with the deleterious effects of excessive oxidative stress, inflammation is also associated with the pathogenesis of several diseases. In fact, there is a curious correspondence between the diseases in which oxidative stress and inflammation are involved. For example, type II diabetes (Esposito et al. 2002), AMD (Klein et al. 2014), and atherosclerosis (Hulsmans and Holvoet, 2010) are all significantly impacted by both oxidative stress and inflammation. Additionally, inflammation is associated with a myriad of “sickness behaviors,” including lethargy, anhedonia, and in some cases anxiety or depression (Anisman and Merali, 1999).

Markers of inflammation, such as the pro-inflammatory cytokines IL-6, IL1-β, and TNF-α, are generally elevated in cases of inflammatory stress (Brebner et al. 2000), and therefore provide a means to assess inflammatory status either systemically, or in local tissues.

As outlined above, there is much evidence for a significant link between oxidative stress and pro-inflammatory cytokines, and their collective relationship with generally negative outcomes. These negative outcomes can be extended to include effects on neural function. From a broad perspective, neuroinflammation is associated with cognitive impairment (Lee et al. 2008; Pistell et al. 2010). Mechanistically, much of cognitive performance is due to synaptogenesis and neurogenesis – both of which are promoted by the neurotrophin brain-derived neurotrophic factor (BDNF; e.g. Cotman et
al. 2007). Because oxidative stress and inflammation have been shown in several studies to reduce BDNF levels (Berk et al. 2011), the connection between reduced cognitive performance and oxidative stress / inflammation seen in previous studies may be driven by a reduction in BDNF.

Based on the available evidence, there exists a logical set of connections between oxidative stress, inflammation, behavior, and neural outcomes. In several cases (noted above), these connections form positive feedback loops, which can exacerbate and accelerate disease processes. The antioxidant capacity and localization of the carotenoids L, Z, and MZ (if consumed in adequate quantities), would appear to offer a mode of therapy that could potentially interrupt the cycle of oxidation and inflammation, and thereby promoting optimal function and health.

**Purpose of the Study**

The purpose of the studies described in this dissertation is to evaluate effects of L, Z, and MZ supplementation on behavioral, neurotrophic, and neuroinflammatory factors related to psychological stress. Based on the evidence presented thus far in the manuscript, L, Z, and MZ (via their antioxidant capacity, anti-inflammatory action, and localization of tissue deposition), have strong potential to positively influence these outcome measures. Studies such as these have not been conducted previously, and represent a novel approach to addressing the various negative outcomes of psychological stress.
CHAPTER 2

SUPPLEMENTATION WITH MACULAR CAROTENOIDS REDUCES
PSYCHOLOGICAL STRESS, SERUM CORTISOL, AND SUBOPTIMAL
SYMPTOMS OF PHYSICAL AND EMOTIONAL HEALTH IN YOUNG ADULTS

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Abstract

Purpose: Oxidative stress and systemic inflammation are the root cause of several deleterious effects of chronic psychological stress. We hypothesize that the antioxidant and anti-inflammatory capabilities of the macular carotenoids (MCs) lutein, zeaxanthin, and mesozeaxanthin could, via daily supplementation, provide a dietary means of benefit.

Methods: 59 young healthy subjects participated in a 12-month, double-blind, placebo-controlled trial to evaluate the effects of MC supplementation on blood cortisol, psychological stress ratings, behavioral measures of mood, and symptoms of sub-optimal health. Subjects were randomly assigned to one of three groups: placebo, 12 mg, or 24 mg / day total MCs. All parameters were assessed at baseline, 6-, and 12-months. Serum MCs were determined via HPLC, serum cortisol via ELISA, and macular pigment optical density (MPOD) via customized heterochromatic flicker photometry. Behavioral data were obtained via questionnaire.

Results: Significant baseline correlations were found between MPOD and Beck Anxiety scores (r = -0.28; p = 0.032), MPOD and Brief Symptom Inventory scores (r = -0.27; p = 0.037), and serum cortisol and psychological stress scores (r = 0.46; p < 0.001). Supplementation for 6 months improved psychological stress, serum cortisol, and measures of emotional and physical health (p < 0.05 for all), versus placebo. These outcomes were either maintained or improved further at 12 months.

Conclusions: Supplementation with the MCs significantly reduces stress, cortisol, and symptoms of suboptimal emotional and physical health. Determining the basis for these effects, whether systemic or a more central (i.e. brain) is a question that warrants further study.
Introduction

The basis for all stress responses is the disruption of some homeostatic set point, be it physical, physiological, or psychological (McEwen & Wingfield, 2009). Physiologically, stress is associated with activation of autonomic and endocrine systems (Chrousos et al., 2000; diAbsi et al., 2000), which involves limbic, hypothalamic, and brainstem circuits (e.g. Mitra & Sapolsky, 2008). Chronic activation of these systems may manifest as anxiety disorders (Mitra et al., 2005; Mitra & Sapolsky, 2008) or other stress-related disorders such as depression (Rainnie et al., 2004). Indeed, the link between anxiety and chronic activation of the limbic structures such as the amygdaloid complex has been well established (e.g., Adamec et al., 2005). Psychologically, the basis of anxiety and depression may involve heightened vulnerability to stress or the inability to cope with life stressors (Cole et al., 1998) and appears to be mediated by dysregulation in cortico-limbic circuitry (Duman & Monteggia, 2006).

The convergence of physiological, psychological, and neurological data in the case of stress, anxiety, and depression is compelling. In fact, the basis for the Depression Anxiety Stress Scales 21 (DASS-21; Henry & Crawford, 2005) is the “tripartite model,” in which anxiety and depression are both related to psychological stress: Anxiety arises out of physiological hyperarousal, whereas depression arises from low positive affectivity, both being impacted by the negative affect brought on by stress. It appears therefore that although anxiety and depression are somewhat discrete phenomena, they both share a common root in psychological stress (Pittenger & Duman, 2008; Mitra et al., 2005). This idea is further supported by comorbidity rates exceeding 50% (Kessler et al., 1996).
It has been suggested that dietary differences could modulate susceptibility to stress (Tannenbaum et al., 2003). Benton (2013) noted that effects of minor nutritional deficiencies would manifest first as subclinical disruption of brain function, given the complexity and metabolic demands of the brain. In general support of this idea, there have been recent human and animal studies that report stress-reducing effects of supplementation of specific nutrients, such as curcumin (Sciberras et al., 2015), alpha tocopherol (Lodhi et al., 2014), and docosahexaenoic acid (DHA; Keenan et al., 2014). In each case, supplementation appears to lead to reduced psychological stress and physiological parameters of stress (e.g., blood cortisol). Additionally, Long and Benton (2013) conducted a meta-analysis of studies on effects of vitamin and mineral supplementation on stress and mood in subclinical populations, and found a general trend towards stress reduction and improvement in mood.

A recent report by El Ansari et al. (2014) on a large (n = 3,706), generally healthy population of college-aged adults examined, via survey, dietary patterns and stress / depressive symptoms. They found a significant relationship between consumption of healthy foods (fresh fruits, salads, and cooked vegetables) and reduced perceived psychological stress. Conversely, consumption of “junk” food was associated with increased perceived psychological stress. Given that these findings are correlations, one could suggest that stress may have caused junk food consumption, or perhaps that consumption of healthy food is simply indicative of a pattern of behavior that is associated with higher socioeconomic status, and that this relationship may mediate the relationship between diet and lower stress. There is, however, a physiological rationale to account for how healthy foods may reduce psychological stress: consumption of
antioxidants. It has been shown that systemic oxidative stress is induced by psychological stress (e.g. in medical students; Srivastava and Batra, 2014), and it appears that reduction of systemic oxidative stress significantly reduces indicators of psychological stress (via alpha-tocopherol administration: Smitha and Mukkadan, 2014; and by lutein: Yajima et al., 2013). Of particular relevance to the present study, Yajima et al. reported that lutein (L) supplementation produced an anxiolytic-like effect in mice exposed to constant illumination stress. Taken together, these findings suggest a role for dietary antioxidants in reducing psychological stress.

Carotenoids such as L comprise a fairly large proportion of dietary antioxidants for humans with a reasonably healthy diet that includes daily consumption of fruits and vegetables (Reboul et al. 2007). Along with L, two other yellow-orange carotenoids, zeaxanthin (Z), and mesozeaxanthin (MZ) are deposited in rich concentration in the central retina, where they form the macular pigment (MP; e.g. Bone and Landrum, 2010). MP is most dense in the metabolically intense central retina (fovea), where its powerful antioxidant (Krinsky et al., 2003) and high-energy short-wave light filtration (Snodderly et al., 1984a) properties appear to protect the macula from acute damage (Ham et al., 1978), protect against cumulative damage resulting in age-related macular disease (Snodderly, 1995), and maintain visual sensitivity over a lifetime (Hammond et al., 1998). MP is strictly derived via diet, and so a person’s level of MP is dependent upon their consumption of foods that contain these carotenoids; for example, dark leafy-green vegetables such as kale and spinach are excellent sources of L (Humphries & Khacik, 2003).
L and Z also accumulate in the brain (Craft et al., 2004; Vishwanathan et al., 2013), where they may influence cognitive performance, especially in aged individuals (Johnson et al., 2008; Feeney et al., 2013; Vishwanathan et al., 2014). In a manner apparently similar to the retina, L and Z cross the blood-brain barrier and accumulate in the brain regions that maintain relatively high metabolism (e.g. frontal and occipital lobes, and hippocampus), and are therefore at higher risk for oxidative stress and inflammation (Gemma et al., 2007). Importantly, MPOD has been shown to be significantly correlated with brain levels of L and Z (Vishwanathan et al., 2013), which suggests similar mechanisms of uptake, and supports the idea that there is preferential deposition of these powerful antioxidants / anti-inflammatories in neural tissues that maintain high metabolism and therefore concomitant oxygen tension and potential for oxidative stress and inflammation.

There were two goals of the present study: 1) To determine, in healthy young adults, the relationship at baseline between MPOD and psychological stress level, serum cortisol, and symptoms of suboptimal emotional and physical health, and 2) To determine the effect of 12 months’ L, Z, and MZ supplementation on the aforementioned parameters. Once deposited in retinal tissue, L and Z (the two primary dietary components of macular pigment [Bone et al., 1988]) are quite stable in the absence of high oxidative stress, e.g. such as that brought on by smoking (Hammond et al., 1996) or diabetes (Scanlon et al., 2015). Therefore, a person’s macular pigment level is generally thought to reflect their lifelong consumption of L and Z. The baseline assessment of MPOD and psychological stress, and physical / emotional health status would thereby enable the analysis of potential cumulative effects of diet on these outcome
parameters. By contrast, the 12-month supplementation trial enabled the analysis of potential acute effects of macular carotenoid supplementation.

**Methods**

Fifty-nine subjects participated in this 12-month, double-blind, randomized, placebo-controlled supplementation trial. Subjects were generally healthy, college-aged (18-25, mean = 21.5 yrs.; 27 Male / 32 female) non-smokers with a BMI < 27. Subjects were instructed to maintain their current diet; those that were planning on changing their diet (for whatever reason) were excluded from consideration for the trial. In consideration of macular pigment testing, all subjects had uncorrected or contact lens-corrected visual acuity of 20/20 or better in the test (right) eye, and had no current or previous history of ocular pathology. Subjects were recruited from the population of students at the University of Georgia in Athens, Georgia. Informed consent was obtained from each subject and the study adhered to the tenets of the Declaration of Helsinki. The study was approved by the Institutional Review Board of the University of Georgia.

Several parameters were assessed over the course of the study, including retinal status of macular carotenoids, serum cortisol, serum lutein, serum zeaxanthin isomers. Symptoms of suboptimal health, psychological health, and emotional health were assessed via questionnaire (see Table 2.1 for a summary of questionnaires used in the study and order of administration). All measures were taken at baseline, 6 months and 12 months. Laboratory visits included (in order): blood draw, questionnaire completion, and vision testing.
**Macular Carotenoid (MC) Supplementation**

Subjects were randomly assigned to one of three groups: placebo, n = 10; 13 mg/day MC, n = 24; or 27 mg/day MC. Pills were brown colored, soft gelatin capsules, with L, Z, and MZ suspended in safflower oil. Independent analysis indicated that the 13 mg supplement contained 10.86 mg lutein / 2.27 mg zeaxanthin isomers, and the 27 mg supplement contained 22.33 mg lutein / 4.70 mg zeaxanthin isomers. Placebos contained no L or Z isomers. All reported values were within +/- 5% variability. Subjects were instructed to ingest one pill with a meal (preferably lunch or dinner) every day. Compliance was ensured with weekly phone calls and pill counts.

**Measurement of Macular Pigment Optical Density (MPOD)**

The concentration of macular carotenoids in the central retina (MPOD) was assessed with a non-invasive, perceptual task called heterochromatic flicker photometry (HFP). A densitometer (Macular Metrics Corp., Rehoboth, MA) described by Wooten et al. (1999) was used for this purpose. The densitometer, detailed measurement procedures, and the principle of HFP have been fully described in earlier publications (e.g. Wooten et al., 2005; Stringham et al., 2008). Briefly, subjects are presented with two superimposed lights that are temporally alternated in square-wave counterphase. This gives the subject an impression on a flickering disc of light. The peak (550 nm) of the spectral composition of one of the lights is chosen to bypass the absorption of MP, and the other (460 nm) is strongly absorbed by MP. The subject’s task is to adjust the relative radiance of the two lights until a percept of no flicker is achieved. All other factors being equal, a subject that requires more short-wave (i.e., 460 nm) relative to middle-wave (i.e., 550 nm) light to achieve null flicker has higher MPOD. This task is performed for the
locations of interest within the fovea, which presumably contain MP, and for a reference location in the parafovea that does not (about 7° eccentricity). To obtain a measure of MPOD at a given test locus, the logarithmic ratio of short- to middle-wave radiance (for null flicker) at the reference location is subtracted from the corresponding logarithmic ratio found at the test locus.

**Blood collection**

Fasting blood was collected between 9am-11am, by a licensed phlebotomist, at baseline, 6-month, and 12-month visits. Subjects’ whole blood was collected into a serum separator vacutainer tube (SST) via venipuncture. Blood was allowed to clot for 30 minutes at room temperature before centrifugation for 15 minutes at 1000 x g. Serum was then removed and stored in microvials at -20 C until analysis.

**High-Performance Liquid Chromatography (HPLC)**

Sample extractions and analyses were completed under yellow light. Serum proteins were precipitated with an equal volume of ethanol (1% BHT) containing the internal standard, trans-β-apo-8'-carotenal. After centrifugation, samples were extracted three times with n-hexanes, mixing and centrifugation. Organic layers were pooled and evaporated to dryness with nitrogen and re-suspended in the mobile phase. An Agilent 1200 series HPLC system consisting of a quaternary pump with degasser, autosampler, thermostated column compartment, UV–Vis diode array detection (DAD) with standard flow cell, and 3D ChemStation software (Agilent Technologies, Santa Clara, CA, USA) was employed for the chromatography. A reversed-phase YMC C30 column (4.6 × 250 mm, 5-µm particle size) was utilized. A stepwise elution consisting of mobile phase A (95% methanol) and mobile phase B (methyl tert-butyl ether) from 15 to 85% B
over a 27-min period at a flow rate of 1 mL/min was employed. A volume of 100 µL was injected for each of the serum samples. Detection wavelengths were \( \lambda = 447 \text{ nm (L) and 450 nm (Z isomers).} \)

*Enzyme-linked immunosorbent assay (ELISA)*

Serum was diluted and processed according to the manufacturer's instructions for the Parameter Cortisol Human ELISA kit (KGE008, R&D Systems, Minneapolis MN, USA). Wells were read at 450 nm (MiniReader MR590, Dynatech Instruments Inc, Santa Monica CA, USA), averaged across duplicates, and a curve of best fit was used to calibrate to standards. Cortisol concentration data are reported as ng/mL. All coefficient of variability values were under 10%.

*Psychological Stress Measure (PSM-9)*

Subjects’ psychological stress level was assessed via questionnaire with the 9-item Psychological Stress Measure (PSM-9; Lemyre and Lalande-Markon, 2009).

*Brief Symptom Inventory (BSI)*

Subjects’ current psychological distress was assessed with the Brief Symptom Inventory (BSI; Derogatis & Melisaratos, 1983), a 53-item, self-report instrument developed from the longer SCL-90-R.

*Beck Anxiety Inventory (BAI)*

Subjects’ symptoms of anxiety were assessed with the Beck Anxiety Inventory (BAI; Beck et al., 1988), a 21-item self-report instrument that is validated for measuring severity of anxiety.
Beck Depression Inventory (BDI)

Subjects’ symptoms of depression were assessed with the Beck Depression Inventory (BDI; Beck et al., 1961), a 21-item self-report instrument that is validated for measuring severity of depression.

General Health Status

The number of physical symptoms of suboptimal health was determined via self-report questionnaire, using the 25-item Suboptimal Health Status Questionnaire (SHSQ-25; Yan et al., 2009).

Statistical Analysis

Graphs and statistical analysis, including descriptive statistics, Pearson product-moment correlations, dependent-samples t-tests, and Repeated-Measures ANOVA were generated using Origin software (Northampton, MA, USA).

Results

At baseline, significant correlations were determined between MPOD and BAI scores (r = -0.28; p = 0.032 - see Figure 2.1), MPOD and BSI scores (r = -0.27; p = 0.037 - see Figure 2.2), and between serum cortisol and PSM-9 scores (r = 0.46; p < 0.001 - see Figure 2.3). Marginally significant correlations were determined at baseline for MPOD and serum cortisol (r = -0.202; p = 0.124), MPOD and psychological stress (r = -0.218; p = 0.10), and MPOD and symptoms of suboptimal health (r = -0.22; p = 0.092). See Table 2.2 for a summary of baseline and supplementation effects for all behavioral measures. After
six months of macular carotenoid supplementation, repeated-measures ANOVA revealed that there were no significant changes from baseline in any parameter for the placebo group. It was found for 13 mg/day group however, that MPOD (p < 0.001) was significantly higher (see Figure 2.4), and serum cortisol (p < 0.001 - see Figure 2.5), BSI scores (p = 0.005), and number of suboptimal health symptoms (p = 0.0012) were significantly lower compared to baseline. The 27 mg/day group was found to significantly increase in MPOD (p < 0.001 - see Figure 2.4), and decrease significantly for the BSI (p = 0.009), BAI scores (p < 0.001), psychological stress (p = 0.05), serum cortisol (p = 0.01 - see Figure 2.5), and number of suboptimal health symptoms (p < 0.001).

Similar to 6-month measures, there were no significant changes determined in any measure in the placebo group at 12 months. For the 13 mg/day group, a significant increase from 6 months to 12 months was found for MPOD (p < 0.001 - see Figure 2.4), and significant decreases were determined for BSI scores (p = 0.002), BAI scores (p = 0.013), psychological stress (p = 0.018), serum cortisol (p = 0.0037 - see Figure 2.5), and number of suboptimal health symptoms (p = 0.007). Comparing 6- and 12-month measures, the 27 mg/day MC increased significantly in terms of MPOD (p = 0.0087 - see Figure 2.4), and decreased significantly for the BSI (p = 0.013), the BAI (p = 0.038), serum cortisol (p = 0.037 - see Figure 2.5), and symptoms of suboptimal health (p = 0.041). A significant decrease in scores on the PSM-9 and BDI were determined at 12 months for the 27 mg/day MC group when compared to baseline (p = 0.05 and 0.025, respectively).
Repeated-measures ANOVA determined that serum L and Z isomers increased significantly after 6 months of supplementation for both active supplement groups (p < 0.001; see Figures 2.6 & 2.7, respectively) versus placebo, and maintained an apparent steady-state level at 12 months. As can be seen in Figure 2.6, the steady-state L serum level was found to be roughly 2.25 µg / mL for the 13 mg/day MC group, and 3.25 µg / mL for the 27 mg/day MC group. The placebo group remained at a concentration of approximately 0.25 µg / mL throughout the 12-month study period. The change in serum concentration of Z isomers was also found to be significant at 6 months (p < 0.001) and, as in the case of L, maintained the 6 month level through 12 months (see Figure 2.7). From Figure 2.7 it can be seen that the Z isomer steady-state level for the 13 mg/day MC group was 0.37 µg / mL, and 0.47 µg / mL for the 27 mg/day MC group. The placebo group remained at a concentration of roughly 0.10 µg / mL throughout the study.

As noted above, MPOD increased significantly from baseline at 6 months, and from 6 months to 12 months in both 13- and 27- mg/day MC groups (see Figure 2.4). Despite double the amount of carotenoid in the 27 mg/day MC group’s supplement (27 mg vs. 13 mg), retinal response across the study period was virtually identical for both groups.

In terms of change in measures over the 12-month study period, the relationship between increases in MPOD and decreases in serum cortisol was found to be significant (r = -0.454; p < 0.001; see Figure 2.8). This same relationship was found for psychological stress, where increases in MPOD were significantly related to reduced PSM-9 scores (r = 0.398; p = 0.002 – see Figure 2.9). This kind of relationship with
change in MPOD was not found for the other behavioral measures. There were, however, nearly significant relationships determined between the change in symptoms of suboptimal health and psychological stress (p = 0.08), and cortisol (p = 0.07), respectively. The finding of a relationship between cortisol and suboptimal health symptoms was also determined by Yan et al. (2012), using the same scale (SHSQ-25) as the present study.

**Discussion**

Given the results of this study it appears that there is a significant role for diet, specifically the macular carotenoids, in reducing stress and improving symptoms of both physical and emotional health. Although similar improvements were determined for all outcome measures over the course of the study in both active supplement groups, measures of stress (serum cortisol and PSM-9) were the only measures that were related directly to increases in MPOD. The mechanism for the stress reduction effects appears therefore to be related to the accumulation of the macular carotenoids in the retina (and presumably the brain). As for the measures related to mood (BAI, BDI, BSI) and physical health (SHSQ-25), there was a clear benefit of supplementation with the macular carotenoids, but the improvements were not directly related to the change in MPOD. If retinal / brain deposition of L, Z, and MZ, doesn’t account for the improvements in physical / emotional health symptoms, then it would seem plausible that changes in systemic (i.e. serum) carotenoid levels could explain the effects. But changes in serum carotenoid levels weren’t directly related to changes in physical / emotional health symptoms. There are several possible reasons for this. It may be that differences in systemic oxidative stress and inflammation among participants served to modify serum
carotenoid levels in such a way as to mask any relationship between mood / health scales and serum carotenoid concentration. Alternatively, retinal and brain carotenoid transport efficiency differs substantially between individuals (Bone & Landrum, 2010), and this may have impacted serum levels in a non-systematic way. Lastly, serum carotenoid concentrations for a supplementation trial such as the present study tend to saturate by about 12 weeks of daily supplementation (Meagher et al., 2013; Stringham & Stringham, 2016). Therefore, correlations involving analysis of change would be limited, due to the fact that our subjects probably reached serum saturation long before their second measure (6 months). Whatever the case, the effects found in our study are consistent with either systemic or neural elevation of macular carotenoids. Based on our supplementation data for serum and MPOD, it appears that deposition in neural tissues requires a consistent, relatively elevated serum concentration of L, Z, and MZ – that the placebo group (which did not exhibit improvements in any outcome parameter) did not increase in either serum or MPOD speaks convincingly to this point.

MPOD response to supplementation in both active supplement groups was very similar, despite the higher dose supplement containing roughly double the amount of carotenoids. Serum response was about 30% higher for the 27 mg/day MC group, which indicates that the additional carotenoids either remained higher in serum, or were deposited in other tissues, such as skin or adipose tissue. Another possibility is that, despite random assignment, participants assigned to 13 mg/day MC group tended to (overall) respond more favorably in the retina, compared to those in 27 mg/day MC group. Variability in retinal response to supplementation with retinal carotenoids has been shown previously (e.g., Bone & Landrum, 2010). Moreover, retinal response
typically is found to increase somewhat linearly with increased dose (Bone & Landrum, 2010; Stringham & Stringham, 2016). Although the results of these previous studies are difficult to reconcile with the present results, retinal response was nevertheless robust in both active supplement groups.

Taken together, the cross-sectional and supplementation-trial data make a strong case for the involvement of L, Z, and MZ in psychological stress levels and physical / emotional health symptoms. In terms of stress and physical health, given the well-established relationship between psychological stress and compromised immune function, it is quite possible that the reduction in suboptimal health symptoms over the period of the study is an effect subsequent to the reduction in stress seen with supplementation. Support for this possibility is provided by the baseline relationship between psychological stress level and number of suboptimal health symptoms (r = 0.415; p = 0.0011 - see Figure 2.10). The nearly significant relationships between change in symptoms of suboptimal health and change in both serum cortisol (r = 0.24; p = 0.07), and change in psychological stress (p = 0.08) over the study period is further evidence for this idea.

Cortisol is the effector hormone of the hypothalamic-pituitary-adrenal (HPA) axis (the “stress” axis), and widely considered to be an excellent physiological marker for psychological stress (Chrousos, 1995). We determined a marginally significant relationship between MPOD and serum cortisol at baseline (p = 0.124), and a strongly significant relationship between change in MPOD and change in serum cortisol for all subjects over the study period (r = 0.454; p < 0.001). As noted above, however, the effect of cortisol reduction was not related to serum carotenoid response. In other words,
a subject’s blood response was somewhat independent of cortisol reduction over the study period. This apparent discrepancy could be explained by the fact that the effect of stress reduction is driven by the neural (presumably brain) deposition of these carotenoids, and that this deposition may lead to modulation of the HPA axis. Although systemic use of L, Z, and MZ, and their deposition in non-neural tissues (e.g. skin; Heinrich et al. 2006) is important in many ways to health and maintenance of function, it appears that the effects of reduced serum cortisol and perceived psychological stress is dependent upon these carotenoids being deposited in neural tissue. Based on the literature, however, it is plausible that (despite no direct relationship) reduced psychological stress and cortisol may ultimately lead to improvements in emotional health measures such as those that were found in our study.

That such specific nutrients are able to confer substantial and meaningful effects over a relatively short time period could be interpreted in several ways. First, it could be that human beings were meant to consume significantly more foods (e.g. leafy-green vegetables) that contain these carotenoids than is currently the case (see Johnson et al., 2010). Our serum data from the baseline measure of our entire sample are indicative of low intake (overall) of L, Z, and MZ. Indeed, data from the National Health and Nutrition Examination Survey (NHANES, 2003, as cited in Johnson et al. 2010) indicate that Americans in the age range (19-30 yrs.) corresponding to our subjects’ general age range consume a paltry 1.5 mg of L and Z daily. At such low levels of consumption, the body may use any available carotenoid for more immediate, systemic purposes (e.g. inflammation, or oxidative stress) rather than depositing it in tissues such as the retina or brain (where our data suggest stress-reducing effects). Perhaps our intervention simply
brought serum macular carotenoids, MPOD (and brain carotenoid) levels up to a point that facilitated relatively “normal” function. In terms of psychological stress and cortisol, this point can be argued not only from the standpoint of the intervention, but also the cross-sectional analysis, where subjects with higher levels of MPOD were found to have marginally significantly lower psychological stress levels and serum cortisol (see Table 2.2).

In addition to low baseline dietary intake of L, Z, and MZ, another consideration for our findings is the level of stress experienced by the study participants. Our subjects were young and healthy, but nevertheless experienced relatively high levels of psychological stress, and reported a fair number of suboptimal physical and emotional health symptoms. Stressful situations most often noted by subjects were struggles with coursework (e.g. worrying about grades), relationship problems, and worrying about money. It may be the case that college students experience higher-than-average stress (and subsequent negative health symptoms) than the overall population. If the macular carotenoids serve a function of reducing serum cortisol and stress, then it follows logically that supplementation in individuals experiencing relatively high levels of stress would produce acute benefits.

As with any study, caution should be exercised before extending these results to other populations. Although there are advantages in terms of experimental control to studying a fairly homogeneous group, it can limit external validity. Our subjects were similar along many dimensions, including age, BMI, education level, and current life status (i.e. college student); our findings may therefore hold true for this group, but may not extend to others. Additionally, it may be tempting to interpret the behavioral data
(BAI, BDI, and BSI) as evidence for the ability of macular carotenoid supplementation to reduce anxiety or depression. None of our subjects were diagnosed with depression or an anxiety disorder. Our results simply suggest that supplementation with the macular carotenoids can reduce symptoms (however few) of anxiety and/or depression. In order to address other populations (e.g. clinically anxious or depressed individuals), additional studies would need to be conducted. In the future, we hope to investigate the effects characterized in the present study in subjects with different lifestyle and dietary habits, in different age groups, and different socioeconomic backgrounds.
Table 2.1. Summary of self-report questionnaires used during the course of the study.

<table>
<thead>
<tr>
<th>Instrument</th>
<th>Items</th>
<th>Outcome measure</th>
<th>Range of Scores</th>
<th>Cronbach's α</th>
<th>test/retest reliability</th>
<th>Published</th>
<th>Order</th>
</tr>
</thead>
<tbody>
<tr>
<td>Psychological Stress Measure 9 (PSM-9)</td>
<td>9</td>
<td>Stress in general population</td>
<td>9-72</td>
<td>0.95</td>
<td>.68-.8</td>
<td>Lemyre and Tessier, 2003</td>
<td>5</td>
</tr>
<tr>
<td>Brief Symptom Inventory (BSI)</td>
<td>53</td>
<td>Current psychological distress</td>
<td>0-212</td>
<td>.71-.85</td>
<td>.68-.91</td>
<td>Derogatis and Melisaratos, 1983</td>
<td>1</td>
</tr>
<tr>
<td>Beck Anxiety Inventory (BAI)</td>
<td>21</td>
<td>Severity of anxiety</td>
<td>0-63</td>
<td>0.92</td>
<td>0.75</td>
<td>Beck et al., 1988</td>
<td>2</td>
</tr>
<tr>
<td>Beck Depression Inventory (BDI)</td>
<td>21</td>
<td>Severity of depression</td>
<td>0-63</td>
<td>0.86</td>
<td>0.93</td>
<td>Beck et al., 1961</td>
<td>3</td>
</tr>
<tr>
<td>Suboptimal Health Status Questionnaire</td>
<td>25</td>
<td>Suboptimal Health Status</td>
<td>25-125</td>
<td>0.93</td>
<td>.89-.98</td>
<td>Yan et al., 2009</td>
<td>4</td>
</tr>
</tbody>
</table>
Table 2.2. Cross-sectional relation of each self-report measure (PSM-9, BSI, BAI, BDI, and SHSQ-25) to MPOD at baseline. Mean scores and standard deviations for each supplementation group at baseline, 6-, and 12- months.

<table>
<thead>
<tr>
<th>Measure:</th>
<th>Psychological Stress Measure (PSM-9)</th>
<th>Brief Symptom Inventory (BSI)</th>
<th>Beck Anxiety Inventory (BAI)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>r = - 0.218; p = 0.10</td>
<td>r = - 0.27; p = 0.037</td>
<td>r = - 0.28; p = 0.032</td>
</tr>
<tr>
<td>Time point:</td>
<td>Baseline</td>
<td>6-months</td>
<td>12-months</td>
</tr>
<tr>
<td></td>
<td>Mean</td>
<td>SD</td>
<td>Mean</td>
</tr>
<tr>
<td>0 mg/day MC</td>
<td>26.7</td>
<td>6.43</td>
<td>29.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>13 mg/day MC</td>
<td>31.83</td>
<td>7.38</td>
<td>31.38</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>27 mg/day MC</td>
<td>31.44</td>
<td>10.24</td>
<td>27.76</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Measure:</th>
<th>Beck Depression Inventory (BDI)</th>
<th>Suboptimal Health Status Questionnaire (SHSQ-25)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>r = 0.078; p = 0.671</td>
<td>r = 0.22; p = 0.092</td>
</tr>
<tr>
<td>Time point:</td>
<td>Baseline</td>
<td>6-months</td>
</tr>
<tr>
<td></td>
<td>Mean</td>
<td>SD</td>
</tr>
<tr>
<td>0 mg/day MC</td>
<td>5.3</td>
<td>8.01</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>13 mg/day MC</td>
<td>4.83</td>
<td>3.57</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>27 mg/day MC</td>
<td>4.2</td>
<td>4.03</td>
</tr>
</tbody>
</table>

\* p < 0.05 compared to baseline
\( b \) p < 0.05 compared to 6-months
Figure 2.1. Baseline correlation between BAI scores and MPOD. Dotted line least-squares fit to data.
Figure 2.2. Baseline correlation between BSI scores and MPOD. Dotted line least-squares fit to data.

$r = -0.27; p = 0.037$
Figure 2.3. Baseline correlation between serum cortisol (ng/mL) and PSM-9 scores. Dotted line least-squares fit to data.
Figure 2.4. MPOD at the 30’ retinal locus, for all groups, at baseline, 6 months, and 12 months. Mean +/- SEM. Asterisk denotes statistical significance, $p < 0.05$. 
Figure 2.5. Serum cortisol in all groups, as a function of time in the study. Reported as percent change from baseline. Means +/- SEM plotted for baseline, 6-month, and 12-month measures. Asterisk denotes statistical significance, p < 0.05.
Figure 2.6. Serum lutein concentration as a function of time in the study, for all groups. Means +/- SEM for baseline, 6-month, and 12-month measures. Asterisk denotes statistical significance, p < 0.05.
Figure 2.7. Serum zeaxanthin concentration as a function of time in the study, for all groups. Means +/- SEM for baseline, 6-month, and 12-month measures. Asterisk denotes statistical significance, p < 0.05.
Figure 2.8. Change in serum cortisol over the 12-month study period as a function of change in MPOD over the same time period.

Dotted line least-squares fit to data.
Figure 2.9. Change in PSM-9 score over the 12-month study period, as a function of change in MPOD over the same time period.

Dotted line least-squares fit to data.

$r = -0.398; p = 0.002$
Figure 2.10. Baseline SHSQ-25 scores (higher scores = greater number of suboptimal health symptoms), as a function of baseline PSM-9 scores. Dotted line least-squares fit to data.
CHAPTER 3

EFFECTS OF MACULAR CAROTENOID SUPPLEMENTATION ON BRAIN-DERIVED NEUROTROPHIC FACTOR AND PRO-INFLAMMATORY CYTOKINES

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2 Stringham, N.T., Stringham, J.M., and Holmes, P.V. To be submitted to *Brain, Behavior, and Immunity.*
Abstract

Purpose: Oxidative and inflammatory processes play a major role in stress-induced neural atrophy. There is a wide body of literature linking oxidative and inflammatory stress with a reduction in both neurotrophic factors and psychological stress resilience. We investigated the potential for the dietary carotenoids lutein, zeaxanthin, and mesozeaxanthin (the “macular carotenoids” [MC]), which have high antioxidant / anti-inflammatory capacity, to increase brain-derived neurotrophic factor (BDNF) and reduce concentrations of the pro-inflammatory cytokines TNF-α, IL-6, and IL-1β.

Methods: 59 young (18-25 yrs.), healthy subjects participated in a 6-month, double-blind, placebo-controlled trial to evaluate the effects of MC supplementation serum BDNF, TNF-α, IL-6, and IL-1β. Serum antioxidant potential (AOP) and the retinal concentration of MCs (termed macular pigment optical density [MPOD]), were also measured. Blood was obtained via fasting blood draws. All parameters were assessed at baseline and 6 months. Subjects were randomly assigned to one of three groups: placebo, 13 mg, or 27 mg / day total MCs. BDNF and the cytokines were assessed via ELISA, and MPOD via customized heterochromatic flicker photometry.

Results: BDNF, MPOD, and AOP all increased significantly versus placebo in both treatment groups over the 6 month study period (p < 0.05 for all). By contrast, IL1-β decreased significantly versus placebo in both treatment groups (p = 0.0036 and p = 0.006, respectively). In terms of changes over the course of the study, significant relationships were determined for BDNF and IL1-β (r = -0.47; p < 0.001), and for BDNF
and TNF-α (r = -0.41; p = 0.0014). No significant changes in any parameter were found for the placebo group.

Conclusions: 6 months of daily supplementation with at least 13 mg of MCs significantly reduces serum IL1-β, and significantly increases serum BDNF, MPOD, and AOP. Although increases in AOP and MPOD were not directly related to decreases in IL-1β and BDNF, it is reasonable to suggest that the effects on IL-1β and BDNF are due to the increase in systemic and perhaps local (neural) antioxidant / anti-inflammatory capacity in our treatment groups. The significant relationships between the change in BDNF and both IL-1β and TNF-α over the course of the study suggest that regular consumption of MCs can interrupt the inflammatory pathway that leads to reduction of BDNF.

Introduction

Oxidative stress occurs when oxidation caused by reactive oxygen species (ROS) outpaces the body’s endogenous and exogenous antioxidant capacity. Prolonged, excessive oxidative stress can cause devastating, often irreversible damage to neural tissues such as the retina (e.g., Beatty et al., 2000) and brain (e.g., Floyd and Hensley, 2002). Moreover, prolonged oxidative stress promotes pro-inflammatory responses (Nakanishi and Wu, 2009), which can exacerbate damage and result in the creation of more ROS (Afonso et al., 2006). This positive feedback loop can result in the cumulative damage that is manifest in several age-related diseases, such as age-related macular degeneration (Liang & Godley, 2003), atherosclerosis (Bonomini et al., 2008), and certain forms of cancer (Valko et al., 2006). At relatively low levels, however, ROS can
promote optimal physiological function via their role in glial and neuronal signaling pathways (Valko et al., 2007). Additionally, ROS such as superoxide radicals (at low / moderate levels) can protect cells by mounting a defense against infectious agents (Nathan and Shiloh, 2000). The body’s ability to maintain an advantageous “redox homeostasis” therefore appears to be crucial to protecting tissues and promoting optimal function (Droge, 2002; Bouayed and Bohn, 2010).

Endogenous antioxidant agents (e.g. glutathione and superoxide dismutase) have been shown to be inadequate in maintaining this balance (Valko et al., 2007). Moreover, corticosteroids generated from the stress response, decrease the effectiveness of endogenous antioxidant systems (McIntosh et al., 1998). Exogenous, diet-derived antioxidants, such as vitamins C and E and the carotenoids lutein and zeaxanthin, supplement the endogenous antioxidant system, and play a key role in maintaining this delicate equilibrium. In fact, the two systems have been shown to act synergistically to re-establish oxidative homeostasis; an example of this is the regeneration of Vitamin E by glutathione to prevent lipid peroxidation (Bouayed et al., 2009).

In addition to cellular damage and potential for the development of age-related disease, oxidation and inflammation can lead to deficits in synaptogenesis and dendritic spine density (Calabrese et al., 2014), which impairs neuroplasticity. Brain-derived neurotrophic factor (BDNF), the neurotrophin most often associated with neuroplasticity (e.g. Bramham and Messaoudi, 2005; Cotman et al. 2007), exhibits an inverse relationship with inflammatory status (Raetz and Whitfield, 2002). The pro-inflammatory cytokines, IL-6 and TNF-α, which are derived centrally by microglia (in addition to peripheral sources), have been shown specifically to attenuate neuroplasticity...
via reduction of BDNF (Garcia-Bueno et al., 2008). For example, exposure to uncontrollable stress leads to a chronic elevation in brain and serum levels of these pro-inflammatory cytokines (Johnson et al., 2002), which leads to the aforementioned reduction in BDNF (Garcia-Bueno et al., 2008; Pittenger & Duman, 2008), and compromised cognitive capacity (Aimone et al., 2014). Additionally, in rats injected with IL1-β, BDNF mRNA levels were significantly reduced in the hippocampus (Lapchak et al., 1993). The hippocampus, given its roles in learning and memory, and its capacity for neurogenesis (Aimone et al., 2014) is considered a principle brain structure in terms of neuroplasticity (Schmidt-Hieber et al., 2004); the finding that neuroinflammatory status can directly impact BDNF levels within the hippocampus illustrates the dependence of neuroplasticity on neural health.

As noted above, one way that the body promotes neural health and optimal neural efficiency is through the use of endogenous and exogenous antioxidant systems. Of relevance to the present study is the potential for exogenous, diet-derived antioxidants to enhance BDNF levels, via reduction of inflammation. The diet-derived xanthophyll carotenoids lutein (L), zeaxanthin (Z) and mesozeaxanthin (MZ) are exceptional antioxidants (Krinsky et al., 2003), and have been shown to exhibit meaningful anti-inflammatory action (Tian et al., 2013; Firdous et al., 2015). Indeed, recent experiments have revealed that L decreases microglia-derived IL1- β and TNF-α, and that this anti-inflammatory action involves in inhibition of the intracellular transcription factor NFκ-B (Wu et al., 2015). The reduction of inflammation associated with increased L could serve to moderate the many negative effects of neuroinflammation, including the reduction in BDNF production. As a preliminary basis for this idea, in a rat model of diabetic
retinopathy, administration of L resulted in maintenance of BDNF levels in the retina; those rats receiving only vehicle showed significant deficits in BDNF (Seki et al., 2004).

L, Z, and MZ accumulate in the macula of the retina in primates as macular pigment (Snodderly et al., 1984 b); concentrations are referred to as macular pigment optical density (MPOD). L and Z also accumulate in the brain (Craft et al., 2004; Vishwanathan et al., 2013), where concentrations have been shown to be associated with cognitive performance, especially in aged individuals (Johnson et al., 2008; Feeney et al., 2013; Vishwanathan et al., 2014). In a manner similar to the retina, L and Z cross the blood-brain barrier and accumulate in the brain regions that maintain relatively high metabolism (e.g. frontal and occipital lobes, and hippocampus), and are therefore at higher risk for oxidative stress and inflammation (Gemma et al., 2007). Importantly, MPOD has been shown to be significantly correlated with brain levels of L and Z (Vishwanathan et al., 2013), supporting the idea that there is preferential deposition of these powerful antioxidants / anti-inflammatories in neural tissues that maintain high metabolism and potential for oxidative stress and inflammation. If these carotenoids reduce inflammation locally in structures such as the hippocampus, supplementation with L, Z, and MZ may serve to reduce local inflammation and potentially enhance BDNF levels. Importantly, L, Z, and MZ are obtained solely via diet, and recent data suggest that Americans consume very little of the foods that contain these carotenoids (Johnson et al., 2010). Supplementation with even modest amounts of L, Z, and MZ may therefore have acute, meaningful effects on oxidative, inflammatory, and BDNF status. To this end, we supplemented our participants with L, Z, and MZ (versus placebo) for 6 months,
and examined the effect on MPOD, BDNF, systemic antioxidant potential, and the pro-
inflammatory cytokines TNF-α, IL-6, and IL-1β.

**Methods**

Fifty-nine subjects participated in a double-blind, randomized, placebo-controlled macular carotenoid supplementation trial. Subjects were healthy, college-aged (18-25, mean = 21.5 yrs.; 27 male / 32 female) non-smokers with a BMI < 27. Subjects were instructed to maintain their current diet. In consideration of macular pigment testing, all subjects had uncorrected or contact lens-corrected visual acuity of 20/20 or better in the test (right) eye, and had no current or previous history of ocular pathology. Subjects were recruited from the population of students at the University of Georgia in Athens, Georgia. Informed consent was obtained from each subject and the study adhered to the tenets of the Declaration of Helsinki. The study was approved by the Institutional Review Board of the University of Georgia.

*Macular Carotenoid (MC) Supplementation*

Subjects were randomly assigned to one of three groups: placebo (n = 10), ~13 mg total MC / day group (n = 24), or ~27 mg total MC / day (n = 25). Pills were brown colored, soft gelatin capsules, with L, Z, and MZ suspended in safflower oil. Independent analysis indicated that the 13 mg supplement contained 10.86 mg lutein / 2.27 mg zeaxanthin isomers, and the 27 mg supplement contained 22.33 mg lutein / 4.70 mg zeaxanthin isomers. Placebos contained no L or Z isomers. All reported values were within +/- 5% variability. Subjects were instructed to ingest one pill with a meal every day. Compliance was ensured with weekly phone calls and pill counts.
Measurement of Macular Pigment Optical Density (MPOD)

The concentration of macular carotenoids in the central retina (MPOD) was assessed with a non-invasive, perceptual task called heterochromatic flicker photometry (HFP). A densitometer (Macular Metrics Corp., Rehoboth, MA) described by Wooten et al. (1999) was used for this purpose. The apparatus, procedures, and the principle of HFP have been fully described in earlier publications (e.g. Wooten et al., 2005; Stringham et al., 2008). Briefly, subjects are presented with two superimposed lights that are temporally alternated in square-wave counterphase—giving the subject the perception of a flickering disc of light. The peak (550 nm) of the spectral composition of one of the lights is chosen to bypass the absorption of MP, and the other (460 nm) is strongly absorbed by MP. The subject’s task is to adjust the relative radiance of the two lights until a percept of no flicker is achieved. All other factors being equal, a subject that requires more short-wave (i.e., 460 nm) relative to middle-wave (i.e., 550 nm) light to achieve null flicker has higher MPOD. This task is performed for the locations of interest within the fovea, which presumably contain MP, and for a reference location in the parafovea that does not (about 7° eccentricity). To obtain a measure of MPOD at a given test locus, the logarithmic ratio of short- to middle-wave radiance (for null flicker) at the reference location is subtracted from the corresponding logarithmic ratio found at the test locus. Measurements of MPOD were taken at baseline and 6 months.

Blood collection

Fasting blood was collected between 9am-11am, by a licensed phlebotomist, at baseline and 6-month visits. Subjects’ whole blood was collected into a serum separator vacutainer tube (SST) via venipuncture. Blood was allowed to clot for 30 minutes at
room temperature before centrifugation for 15 minutes at 1000 x g. Serum was then removed and stored in microvials at -20 C until analysis.

*Enzyme-linked immunosorbent assay (ELISA)*

Concentration of BDNF, IL-6, IL-1β, and TNFα, in serum obtained from baseline and 6 month visits, was determined via solid-phase sandwich ELISA. All wells for each marker were read at 450 nm (MiniReader MR590, Dynatech Instruments Inc, Santa Monica CA, USA), averaged across duplicates, and a curve of best fit was used to calibrate to standards. All coefficient of variability values were under 15%. Specific methods for each serum marker is as follows:

**BDNF:** Serum was diluted and processed according to the manufacturer's instructions for the Quantikine, Human BDNF ELISA kit (DBD00, R&D Systems, Minneapolis MN, USA). BDNF concentration data are reported as pg/mL.

**IL-6:** Serum was processed according to the manufacturer's instructions for the Quantikine, Human IL-6 ELISA kit (KGE008, R&D Systems, Minneapolis MN, USA). IL-6 concentration data are reported as pg/mL.

**IL-1β:** Serum was diluted and processed according to the manufacturer's instructions for the Quantikine, Human IL-1β ELISA kit (DLB50, R&D Systems, Minneapolis MN, USA). IL-1β concentration data are reported as pg/mL.

**TNF-α:** Serum was diluted and processed according to the manufacturer's instructions for the Quantikine, Human TNF-α ELISA kit (DTA00C, R&D Systems, Minneapolis MN, USA). TNF-α concentration data are reported as pg/mL.
Colorimetric Microplate Assay

Total antioxidant capacity of serum obtained from baseline and 6 month visits, was determined via colorimetric microplate assay. Serum was diluted and processed according to the manufacturer's instructions for the Colorimetric Microplate Assay for total antioxidant power (Product No. 430710, Neogen Corporation, Lexington KY, USA). Microplate wells were read at 450 nm (MiniReader MR590, Dynatech Instruments Inc, Santa Monica CA, USA), averaged across duplicates, and a curve of best fit was used to calibrate to standards. All coefficient of variability values were under 15%. Antioxidant capacity data are reported in Trolox equivalents.

Statistical Analysis

Graphs and statistical analysis, including descriptive statistics, Pearson product-moment correlations, dependent-samples t-tests, and Repeated-Measures ANOVA were generated using Origin software (Northampton, MA, USA).

Results

As confirmation that our experimental and control groups were reasonably homogeneous, one-way ANOVAs determined no differences at among groups at baseline with regard to any of the variables of interest (p > 0.40 for all). In terms of relationships among variables at baseline, significant correlations were found between all inflammatory cytokines (see Table 3.1). Baseline BDNF was not found to correlate significantly with any other outcome measure, although it was marginally related to subjects’ baseline MPOD (p = 0.094; see Figure 3.1). A one-way ANOVA determined that MPOD increased significantly (F = 3.55; p = 0.023) versus placebo in the 13 mg /
day MC (0.106 OD increase) and the 27 mg / day MC (0.12 OD increase) – see Figure 3.2. In both the 13 mg / day MC and 27 mg / day MC groups, paired-samples t-tests showed that BDNF increased significantly after the 6-month supplementation period (p = 0.014, and p = 0.04 respectively). BDNF in the placebo group was found to decrease. See Figure 3.3 for a graphical representation of the change in BDNF over 6 months for each of the study groups. Antioxidant potential was found to increase significantly in 13 mg / day MC group (t = -3.41; p = .0024) and the 27 mg / day MC group (t = -3.66; p = 0.00124 – see Figure 3.4). In terms of the cytokines tested, IL-1β was found to significantly decrease in both the 13 mg / day MC (t = 3.25; p = 0.0036) and 27 mg / day MC groups (t = 2.99; p = 0.006 – see Figure 3.5). TNF-α was not found to significantly change over the 6-month study period in any group, but changes between placebo versus treatment groups were markedly different (see Figure 3.6). These differences failed to reach statistical significance (p = 0.13), but are noteworthy. The differences also indicate that TNF-α increased (albeit very slightly in both supplementation groups) over the study period. No statistically significant changes were determined for IL-6, although there was a general trend of decrease with increasing macular carotenoid dose.

With regard to the amount of change in variables over the course of the study, significant correlations were found between BDNF and both IL1-β (r = -0.47; p < 0.001 – see Figure 3.7) and TNF-α (r = -0.42; p = 0.0012 – see Figure 3.8). The change in IL1-β was also correlated with TNF-α (r = 0.41; p = 0.0014) and IL- 6 (r = 0.26; p = 0.048). There were no significant correlations found between changes in MPOD or AOP and changes in other variables.
Discussion

Based on the results of this study, 6 months of supplementation with the macular carotenoids, at levels of at least 13 mg / day, significantly reduces IL-1\(\beta\) and significantly increases BDNF, MPOD, and AOP. Although increases in AOP and MPOD were not directly related to decreases in IL-1\(\beta\) and BDNF, it is reasonable to suggest that the effects on IL-1\(\beta\) and BDNF are due to the increase in systemic and perhaps local (neural) antioxidant / anti-inflammatory capacity in our treatment groups. The significant relationships between the change in BDNF and both IL-1\(\beta\) and TNF-\(\alpha\) over the course of the study suggest that regular consumption of MCs can interrupt the inflammatory pathway that leads to the reduction of BDNF.

That our findings were produced in a population of young, healthy adults raises a couple of points. First, the diet of “young, healthy adults” may not actually be sufficiently healthy. Specifically (based on our results), the level of dietary carotenoids such as L is apparently are suboptimal. This observation is not new – data from NHANES (2003) indicate that young adults consume only 1 mg / L per day (as cited in Johnson et al., 2010). Secondly, our results may hold some promise for unhealthy populations, or perhaps those with systemic, pro-inflammatory disease such as diabetes. Previous data show that significantly low retinal BDNF levels precipitate diabetic retinopathy in rat models of diabetes, and that administration of BDNF therapy rescues retinal neurons from degeneration (Seki et al., 2004). If a simple dietary modification can produce meaningful effects such as this, the benefit to society would be significant. The connection between diet, the immune system, and the brain represents an exciting new area of research. Planned future studies include evaluation of cognitive
performance, to determine the potential for macular carotenoid supplementation to improve aspects of cognition (e.g., verbal memory and verbal fluency) that are related most strongly to neuroplasticity.
Table 3.1. Baseline correlation matrix for serum markers.

<table>
<thead>
<tr>
<th>Serum Marker</th>
<th>BDNF</th>
<th>IL-6</th>
<th>IL-1β</th>
<th>TNFα</th>
<th>AOP</th>
</tr>
</thead>
<tbody>
<tr>
<td>BDNF</td>
<td>1</td>
<td>-0.023</td>
<td>-0.061</td>
<td>-0.081</td>
<td>-0.023</td>
</tr>
<tr>
<td>IL-6</td>
<td>-0.023</td>
<td>1</td>
<td>0.340*</td>
<td>0.418*</td>
<td>-0.131</td>
</tr>
<tr>
<td>IL-1β</td>
<td>-0.061</td>
<td>0.340*</td>
<td>1</td>
<td>0.378*</td>
<td>0.075</td>
</tr>
<tr>
<td>TNFα</td>
<td>-0.081</td>
<td>0.418*</td>
<td>0.378*</td>
<td>1</td>
<td>-0.106</td>
</tr>
<tr>
<td>AOP</td>
<td>-0.023</td>
<td>-0.131</td>
<td>0.075</td>
<td>-0.106</td>
<td>1</td>
</tr>
</tbody>
</table>

* p < 0.05
Figure 3.1. Baseline relationship between BDNF and MPOD at 30’ eccentricity. Dotted line least-squares fit to data.
Figure 3.2. Increase in MPOD between baseline and 6 months for placebo and treatment groups. Asterisk denotes statistical significance, p < 0.05.
Figure 3.3. Change in serum BDNF between baseline and 6 months for placebo and treatment groups. Asterisk denotes statistical significance, p < 0.05.
Figure 3.4. Change in serum antioxidant potential from baseline to 6 months for placebo and treatment groups. Asterisk denotes statistical significance, $p < 0.05$. 
Figure 3.5. Change in serum IL-1β between baseline and 6 months for placebo and treatment groups. Asterisk denotes statistical significance, p < 0.05.
Figure 3.6. Change in serum TNF-α between baseline and 6 months for placebo and treatment groups.
Figure 3.7. Relationship between the change in BDNF and change in IL-1β from baseline to 6-months. Dotted line least-squares fit to data.
Figure 3.8. Relationship between the change in BDNF and change in TNF-α from baseline to 6-months. Dotted line least-squares fit to data.
CHAPTER 4
DISCUSSION

The results presented in these studies illustrate the broad spectrum of influence that oxidative stress has across bodily systems. Additionally, the findings underscore the correspondence between neurophysiological mechanisms and behavior. Serum markers from endocrine, immune, and neural systems were significantly affected by macular carotenoid supplementation, and these markers often corresponded to changes in behavior, including reduction in psychological stress and symptoms of suboptimal emotional and physical health.

As outlined in the Introduction section, oxidation and inflammation play a major role in the pathogenesis of a number of disease processes. Several of these diseases (e.g. AMD, atherosclerosis, and Alzheimer’s disease) are age-related, and reflective of long-term, cumulative damage. Our studies, however, showed acute effects in young, healthy subjects. This is important for a couple of reasons: 1) it suggests that a diet that includes the macular carotenoids (and other components of healthy foods) may have beneficial effects on several aspects of neurophysiological health and performance, even in young subjects, and 2) due to the deposition and stability of the macular carotenoids in vulnerable neural tissues, the robust response to the supplements seen in our study may result in protection against long-term, cumulative damage in tissues (e.g. retina, hippocampus) in which these carotenoids are deposited.
Given the antioxidant properties of L, Z, and MZ, one could reasonably conclude that the stabilization of free-radical oxygen species is involved mechanistically in the effects presented in these studies. This conclusion may be superficial, however. Recent data suggest that neuroinflammation is largely mediated by microglial transcription of NF-κB, a protein complex that controls pro-inflammatory cytokine production (Braiser, 2006), and can therefore act as a precursor to IL-6, IL-1β, and TNF-α (Delpech et al., 2015). Reactive oxygen species have indeed been shown to activate NF-κB (Chandel et al., 2000), and may therefore trigger the neuroinflammatory cascade. Importantly, with regard to our results, L has been shown to inhibit NF-κB (Wu et al., 2015), thus interrupting the neuroinflammatory cascade (see Figure 4.1 for neuroinflammatory cascade, and Figure 4.2 for proposed effects of L/Zi). Although quenching of free radical oxygen is perhaps the primary mechanistic step, it appears that the consequential reduction in transcription of NF-κB by microglia effectively breaks the chain of neuroinflammation.

Much of the current focus of studies on L, Z, and MZ is on aging populations (e.g. Johnson et al., 2008; Vishwanathan et al., 2013; Kelly et al., 2015). Given the effects seen in the eye (with regard to AMD), and the preliminary evidence for improved cognition / slowed cognitive decline in those taking macular carotenoid supplements, this is not surprising. The studies conducted for this manuscript, however, produced results in young, healthy subjects, which indicates the apparent importance of consistent dietary consumption of these carotenoids in order to maintain or improve aspects of neural, endocrine, immune, and psychological health and function. Another crucial point to consider with regard to dietary consumption of the macular carotenoids (and
xanthophylls in general [Demmig-Adams and Adams, 2006]) is their remarkable ability to remain stable while deactivating free-radical oxygen. This means that, if appreciable amounts of L, Z, and MZ are incorporated into cell membranes (e.g. in the retina or brain), they may be reliably functional for perhaps several decades (in the absence of extreme oxidative stress). By logical extension, several age-related diseases that are precipitated by cumulative oxidative damage may be delayed significantly or perhaps avoided by inclusion of the macular carotenoids in the diet while young.

The results presented in these studies raise a number of questions that may be answered with future research. The effect of increased BDNF in our active supplement groups, for example, warrants investigation of potential cognitive performance enhancement in young subjects. Additionally, effects of increased AOP and reduced parameters of inflammation relative to placebo indicate that supplementation with the macular carotenoids may provide some benefit to those with inflammatory conditions other than those noted previously (e.g. AMD, Alzheimer’s disease), such as arthritis, eczema, or perhaps multiple sclerosis. Indeed, the apparent ability of the macular carotenoids to modulate inflammatory aspects of the immune system represents a foundation upon which a productive research program could be built.

Lastly, the effects of cortisol and psychological stress reduction seen with macular carotenoid supplementation are perhaps the most meaningful findings presented in this dissertation. Future studies of these phenomena should include different age groups, life status, dietary and lifestyle habits, life experience (e.g., military experience or not), and socioeconomic backgrounds. Psychological stress is found in nearly all walks of life, but sources and levels of stress can be radically different, depending on the individual
situation. These differences could potentially impact the effects and potential benefits of L, Z, and MZ supplementation, and warrant further study.
Figure 4.1. Neuroinflammatory cascade in response to a stressor. ROS: reactive oxygen species.
Figure 4.2. Interruption of neuroinflammatory cascade by L/Zi via quenching of reactive oxygen species (ROS).
REFERENCES


66. Lemyre L, Lalande-Markon MP. Psychological Stress Measure (PSM-9): integration of evidence-based approach to assessment, monitoring, and


