The Effect of Vitamin C Supplementation on sICAM-1 in Asthmatic Study Participants

by

Lindsay Gnant

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Graduate Supervisory Committee:

Carol Johnston, Chair
Karen Sweazea
Yung Chang

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ABSTRACT

The common cold is a significant cause of morbidity world-wide, with human rhinovirus infections accounting for a majority colds suffered each year. While the symptoms of the common cold are generally mild and self-limiting, vulnerable populations such as individuals with asthma can experience severe secondary complications including acute asthma exacerbation which can result in severe morbidity. Most human rhinovirus types utilize Intercellular Adhesion Molecule-1 (ICAM-1) as a receptor to enter cells and initiate infection. Expression of this cell-surface protein is elevated in the respiratory tract of asthma patients. The theoretical basis for this research is the observation that plasma measures of the soluble form of Intercellular Adhesion Molecule-1 (sICAM-1) decrease in response to vitamin C supplementation. As rhinovirus infection occurs in the upper respiratory tract, the primary aim of this study was to evaluate change in sICAM-1 concentration in nasal lavage of asthmatic individuals in response to vitamin C supplementation. Otherwise healthy asthmatic adults between the ages of 18-65 years who were not currently using steroidal nasal sprays, smoking, or actively training for competitive sports were recruited from a university community and surrounding area to participate in an 18-day double-blind randomized placebo-controlled supplement study with a parallel arm design. 13 subjects were stratified based on age, gender, BMI and baseline plasma vitamin C level to receive either 500 mg vitamin C twice daily (VTC, n=7) or placebo (PLC, n=6). Biochemical measures included nasal lavage sICAM-1, plasma sICAM-1, plasma histamine, and plasma vitamin C. Survey measures included Wisconsin Upper Respiratory Symptom Survey-21 to assess colds, Daytime Symptom Diary Scale to assess asthma symptoms,

and measures of diet quality including a vitamin C food frequency questionnaire and Rapid Eating Assessment for Participants. No between group comparison of means reached significance (Mann-Whitney U test, $p>0.05$). Nasal lavage sICAM-1 levels were decreased in VTC group by 37% at study day 4, although this finding did not reach significance. Findings in this study can be used to develop future investigations into the response of nasal lavage sICAM-1 to vitamin C supplementation.

DEDICATION

I dedicate this work to my family for their enthusiastic support in all my endeavors.

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I would like to acknowledge my mentor, Dr. Carol Johnston, for inspiring me to strive for my goals in science and cheering me all the way to the finish.

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CHAPTER 1

INTRODUCTION

The common cold is one of the most significant causes of morbidity world-wide, with human rhinovirus infections accounting for 50-80% of all colds endured by people each year (Mackay, 2008, 297-320). While the symptoms associated with the common cold are generally mild and resolve within a few days, vulnerable populations can experience severe disease such as lower respiratory infection or serious secondary complications (Linsuwanon et al., 2009, 115-121; Message et al., 2008, 13562-13567). Acute asthma exacerbation is a serious complication of rhinovirus infection and can result in hospitalization and in extreme cases, fatality (Denlinger et al., 2011, 1007-1014; Ozcan et al., 2011, 888-893; Gavala, Bertics and Gern, 2011, 69-90).

Human rhinoviruses (HRV) are small non-enveloped viruses that belong to the family Picoronaviridae (Dreschers et al., 2007, 181-191). Three distinct species and over 100 serotypes of HRV have been identified to date (Palmenberg, Rathe and Liggett, 2010, 1190-9). Over 90% of HRV species use intercellular adhesion molecule -1 (ICAM-1) as a cellular receptor to dock to cells of the nasal epithelium and initiate infection (Bella and Rossmann, 2000, 291-297). ICAM-1 exists both on the surface of cells and as a soluble protein found in serum and secretions from epithelial tissues. This receptor is expressed by a variety of cell types in response to inflammation and functions primarily as an adhesion molecule for leukocytes, allowing neutrophils and other cell types to localize to sites of injury or infection. ICAM-1 expression is increased in nasal

epithelial cells in response to HRV infection, representing a potential mechanism for enhanced viral replication within infected tissues (Whiteman et al., 2003, 11954-11961). This hypothesis is supported by *in vitro* experiments where anti-ICAM-1 antibodies are used to block viral access to the ICAM-1 receptor. In one example, Whitmann and colleagues observed that viral titers recovered from cell culture supernatants decreased in a dose-dependent fashion in response to increasing concentrations of anti-ICAM-1 antibody (2003).

ICAM-1 is commonly over-expressed in individuals with asthma (Bella and Rossmann, 2000, 291-297; Bianco et al., 2000, 339-345; Ciebiada, Gorska-Ciebiada and Gorski, 2011, 660-666). Baseline ICAM-1 expression has been reported to be 3 times higher in asthmatic patients compared to healthy controls when measured immunohistochemically in cultured nasal epithelium and 4.7 times higher when ICAM-1 expression was induced in tissue culture incubated with whole blood from asthmatic patients (Bianco et al., 2000, 339-345; Horvathova, Jahnova and Gazdik, 2001, 17-30). It has been previously suggested that the elevated levels of ICAM-1 present on the respiratory epithelium of individuals with asthma might account for the increased incidence of rhinovirus infection in this population (Bianco et al., 2000, 339-345).

It is widely accepted that vitamin C can reduce the severity and duration of the common cold, but the impact of vitamin C on preventing the common cold is less clear (Douglas et al., 2007, CD000980). Vitamin C has antihistamine properties, which likely account for some of the observed reduction in symptoms when vitamin C is taken at the onset of a cold (Johnston, 1996, 189-213; Subramanian et al., 1973, 1671-1673).

Additionally, the antioxidant properties of vitamin C may reduce cold symptoms by protecting tissues from oxidative damage that results from virus infection and immune response (Uchide and Toyoda, 2011, 2032-2052).

Interestingly, supplementation with vitamin C has been shown to decrease ICAM-1 expression in a variety of in vitro and in vivo studies (Jain et al., 2010, 1371-1380; Vincent et al., 2009, 254-262; Murphy et al., 2005, 181-190; Scott et al., 2005, 138-144; Tahir et al., 2005, 302-306; Son et al., 2004, 1073-1079; Rayment et al., 2003, 339-345).

There are a number of studies that address HRV infection in individuals with asthma (Denlinger et al., 2011, 1007-1014; Ozcan et al., 2011, 888-893; Gavala, Bertics and Gern, 2011, 69-90). Several studies specifically evaluate the relationship of elevated ICAM-1 expression to HRV infection in asthmatic subjects (Denlinger et al., 2011, 1007-1014; Ozcan et al., 2011, 888-893; Gavala, Bertics and Gern, 2011, 69-90). Separate studies have observed a decrease in ICAM-1 levels in response to vitamin C supplementation (Jain et al., 2010, 1371-1380; Son et al., 2004, 1073-1079; Rayment et al., 2003, 339-345). One study by Papi and colleagues (2002) identified a dose-dependent reduction in ICAM-1 expression in response to the antioxidant glutathione in HRV-infected respiratory epithelial cells. To our knowledge, no study has addressed the observed reduction in ICAM-1 following supplementation with vitamin C as a potential mechanism to reduce risk of rhinovirus infection in asthmatic patients.

The primary purpose of this study is to evaluate the effect of vitamin C supplementation on sICAM-1 concentration in nasal lavage and serum in adult subjects with asthma recruited from a university community as part of a preliminary feasibility

study. A secondary aim of this study is to assess participant cold symptoms and serum histamine levels during the study period. We hypothesize that supplementation with vitamin C will reduce levels of sICAM-1 both in nasal lavage and serum as well as reduce cold symptoms and decrease serum histamine among study participants.

Definition of Terms

The common cold: A general term for a mild upper respiratory illness that is caused by a number of unrelated viral pathogens. Symptoms typically include sore throat, nasal congestion, cough and fatigue and resolve spontaneously within 3-7 days after initiation.

Human rhinovirus (HRV): An icosahedral non-enveloped virus that belongs to the Picoronaviridae family of viruses. The HRV capsid is composed of 4 viral proteins and encloses a single-stranded positive-sense RNA viral genome. HRV is the most frequent etiologic agent of the common cold.

Intercellular adhesion molecule-1 (ICAM-1): Also known as CD54, ICAM-1 is a member of a class of cellular adhesion molecules that is expressed by a number of tissue types including epithelial tissues and some lymphocytes. ICAM-1 is a glycoprotein with five immunoglobulin domains and consists of extracellular, transmembrane and intercellular regions.

Soluble Intercellular adhesion molecule-1 (sICAM-1): sICAM-1 is a soluble glycoprotein that is identical to the extracellular region of ICAM-1. sICAM-1 is thought to be derived from epithelial and endothelial tissues by shedding (proteolytic cleavage) of the extracellular region of ICAM-1. However, sICAM-1 may also be derived from pathway via alternate splicing of the ICAM-1 gene transcript.

Histamine: Histamine is an amine derived from the amino acid histidine. It is an important bioactive substance involved in a number of physiological processes. Histamine participates in the inflammatory response by stimulating local vasodilation and increasing capillary permeability. Other physiological functions attributed to histamine include promoting bronchial smooth muscle constriction and stimulating gastric secretions. In the central nervous system, histamine acts as a neurotransmitter.

Asthma exacerbation: Commonly known as an asthma attack, asthma exacerbation is an acute decrease in peak expiratory volume and lung function related to constriction of the bronchioles through local inflammation and smooth muscle contraction. Severe asthma exacerbation can be a medical emergency depending on the acuity of symptoms and responsiveness to treatments such as bronchodilators.

Immunohistochemical staining: A technique for quantifying subcellular components on tissue slides using labeled antibodies. Specific labeled antibodies are

used to stain cells and expression of subcellular components is quantified by the intensity of the staining.

Delimitations

The study participants were adults aged 18 to 65 years old who have been diagnosed with asthma by a physician and consider themselves to have asthma currently. Both men and women were recruited for participation in this study. Volunteers were recruited for study participation from a university community using fliers and e-mail distribution lists.

Limitations

This study has been designed as a preliminary feasibility study to evaluate the effect of a vitamin C supplementation protocol on sICAM-1 concentration in nasal lavage and plasma of asthmatic study participants. This study is limited by small sample size. Power analysis was not completed because the primary outcome measure, change in sICAM-1 concentration in nasal lavage in response to a dietary intervention, has not been previously reported in the literature. Currently there is no clinically useful diagnostic test to identify HRV infection; therefore cold symptoms were assessed by self-report questionnaire.

CHAPTER 2

REVIEW OF LITERATURE

The Common Cold

Significance and economic impact. The term "common cold" refers to a group of mild and typically self-limiting symptoms that include nasal congestion, sore throat and cough that are caused by a viral infection of the upper-respiratory tract (Rollinger and Schmidtke, 2011, 42-92). As one of the most common illnesses, colds account for significant morbidity world-wide with a large associated economic impact. In the United States alone, the common cold is estimated to cost $25 billion annually in lost productivity and $17 billion in direct medical costs (Bramley, Lerner and Sarnes, 2002, 822-829; Fendrick et al., 2003, 487-494). Recent estimates suggest as many as 214 million lost workday equivalents are a result of the common cold each year, with about 2 million lost workdays due to caring for sick children (Rollinger and Schmidtke, 2011, 42-92).

Current treatment. There is currently no specific treatment for the common cold (Rollinger and Schmidtke, 2011, 42-92). Typical medical treatment focuses on alleviation of symptoms and includes nasal decongestants, analgesics, fluids and rest (Rollinger and Schmidtke, 2011, 42-92). Developing a vaccine against the common cold is considered an ineffective strategy because there are many viruses that cause the symptoms of the common cold. Over 200 different viruses can cause the symptoms of the common cold while the most important of these viruses has more than 100 antigenic types (Bella and Rossmann, 2000, 291-297; Rollinger and Schmidtke, 2011, 42-92;

McKinlay, 2001, 477-481). While the symptoms of the common cold may create misery and suffering in affected individuals, a cold poses very little risk of serious illness in most people. Since otherwise healthy individuals with colds experience a mild illness that spontaneously resolves in a short period of time, any new therapy developed to treat the common cold must have an excellent safety profile to ensure an acceptable risk to benefit ratio (McKinlay, 2001, 477-481). Given the significant challenges of developing a vaccine or specific therapy to "cure the common cold," the low risk of complications for generally healthy individuals, and the high standard of safety that must be met, development of a new specific treatment for the common cold is unlikely in the near future.

The Human Rhinovirus. While a number of different viral pathogens can cause the symptoms of the common cold, human rhinovirus (HRV) is the most clinically important and is responsible for 50% to 80% of colds (Poland and Barry, 2009, 2245-2246). HRVs are small non-enveloped viruses consisting of a single, positive sense strand of RNA within an icosahedral capsid (Dreschers et al., 2007, 181-191). As a member of the Picoronaviridae family of viruses, HRV is a diverse group that includes three distinct species and over 100 identified serotypes (Palmenberg, Rathe and Liggett, 2010, 1190-9). Despite this variety, most HRVs use the same receptor to gain access to target cells, intercellular adhesion molecule-1 (ICAM-1). Within the HRV-A and HRV-B species, there are at least 88 virus isoforms that use ICAM-1 as a receptor. When a viral particle binds to ICAM-1, it is taken into the cell by endocytosis. The mechanistic details of how the viral RNA molecule leaves the resulting endosome to begin the process

of viral replication are not completely resolved. A current theory proposes a process that is mediated by the interaction of the viral capsid proteins with ICAM-1 in the low pH conditions of late endosomes (Fuchs and Blaas, 2010, 281-297).

The remaining 11 viruses within the HRV-A species bind to the very low density lipoprotein (VLDL) receptor. HRV-C is the third and most recently identified species. At least 11 viruses belong to this species, but a receptor has not been identified for this group (Palmenberg, Rathe and Liggett, 2010). HRV-C does not replicate in commonly used cell culture lines and has been grown with only limited success in sinus tissue culture, potentially accounting for its recent discovery. Both genetic sequence comparisons and *in vitro* studies of HRV-C using anti-ICAM-1 and anti-VLDL antibodies and support the conclusion that HRV-C has a distinct receptor specificity from other HRV species (Bochkov and Gern, 2012, 485-494). Despite its recent characterization, HRV-C is both genetically diverse and prevalent within groups that have been sampled, suggesting that it is not a newly emergent group, but that it has been present in the human population for some time (Lee et al., 2012, 886-891; Mackay et al. 2012).

Seasonality and severity. While HRV infection occurs throughout the year, the rate of infection follows a seasonal pattern, with the highest rate occurring in the fall and spring (Brownlee and Turner, 2008, 67-71). Lee and colleagues (2012) used a prospective cohort design to characterize factors that affect severity of illness from HRV infection among children enrolled in the childhood origins of asthma (COAST) study at birth. Enrollment criteria for this study included one or more parents with a history of

allergic sensitization such as asthma or positive skin test and birth at 37 weeks gestation or greater. A total of 285 infants were followed for 12 months. Study measures included nasal lavage samples taken during scheduled study visits at 2, 4, 6, 9, and 12 months of age, irrespective of whether the child was showing signs of illness. Additional nasal lavage samples were collected either by parents or clinic staff each time a child had symptoms of a respiratory illness. Severity of illness was classified according to a respiratory symptom scorecard. All nasal lavage samples were screened for 20 common respiratory viruses and samples that tested positive for HRV were further analyzed and typed using serological and genetic techniques (Lee et al., 2012, 886-891).

Lee and colleagues' (2012) findings confirmed prior characterizations of the seasonality of HRV infection and demonstrated relationships between HRV type and severity of respiratory illness in infants during the first year of life. Most infants in this cohort, 72.3%, had at least one nasal lavage sample positive for HRV infection during the first 12 months of life with the rate of infection three times higher in Fall (September, October) and Spring (March) compared to other seasons. The authors demonstrated a relationship between severity of illness and month of infection, finding that more moderate to severe illnesses occurred with HRV infection in the months of December to February compared with the months of April to August. Severity of illness was strongly related to HRV virus type. Both HRV-A and HRV-C infections were seven times more likely to be associated moderate to severe illness than HRV-B (Lee et al., 2012, 886-891).

In a similar prospective cohort study, Mackay and colleagues (2012) enrolled 234 healthy children five years of age or younger and followed them for a year to characterize

the diversity and clinical impact of HRV species circulating in preschool-aged children. Nasal swabs were collected by parents from participants who showed symptoms of viral upper respiratory infection. Specimens were evaluated for the presence of HRV and other common respiratory viruses using conventional and reverse transcription polymerase chain reaction methods. All HRV-positive samples were further characterized using genetic techniques. A seasonal pattern of illness was observed, similar to the findings of other studies, with peaks in spring and fall. However, no association between virus species and clinical outcomes was identified (Mackay et al., 2012).

In this investigation, Mackay and colleagues (2012) did not report a score of subjective illness severity but measured incidence of co-occurring fever, otitis media or wheeze. There was a low incidence of wheeze reported in this study with 6 cases of virus-associated wheeze out of 563 episodes of acute upper respiratory illness. The authors speculate that the low rate of wheeze in this cohort was related to exclusion criteria of history of asthma or prior wheeze. One of the significant findings of this study is the diversity of HRV species circulating in the study population over the course of one year. A total of 74 different HRV species were identified within the study cohort. Repeated HRV infection was common, with a mean rate of 1.8 HRV infections per child. While no study participants were infected with the same virus type twice in the study period, the same virus species may have been detected more than once in different cold episodes. HRV-A and HRV-C were the most common species accounting for 41.5% and 47.5% of genotyped samples, respectively (Mackay et al., 2012).

A notable difference between the two studies by Lee and MacKay is that asthma diagnosis or history of wheeze were used as exclusion criteria by MacKay's group, whereas Lee and colleagues enrolled infants with increased risk for developing asthma based on parents' medical history. The two groups also differed in measures of the impact of HRV infection in study participants. Lee's group used a subjective assessment of illness severity based on symptoms while MacKay and colleagues used clinical outcome measures such as fever or ear ache to assess illness severity. Fever and ear ache are frequent complaints within a preschool age group, but not classical symptoms of the common cold (i.e., nasal congestion, runny nose, cough, etc.).

Pathogenesis. HRV infection is spread by contact. Viral particles are transferred by the hands from a contaminated surface to the nose or eyes. If HRV is transferred to the eyes, the virus reaches the nasal epithelium through the lacrimal duct (Kirchberger, Majdic and Stockl, 2007, 1-10). Once introduced into the nose, viral particles are swept to the adenoid at the back of the nasopharynx through the action of ciliated epithelium. HRV then binds to specific receptors to enter cells and initiate infection (Rollinger and Schmidtke, 2011, 42-92; Kirchberger, Majdic and Stockl, 2007, 1-10). The primary site of infection is the ciliated epithelial tissue of the nasopharynx, although HRV infection has also been detected non-ciliated epithelium and in the lower respiratory tract (Rollinger and Schmidtke, 2011, 42-92; Kirchberger, Majdic and Stockl, 2007, 1-10). The incubation period following exposure before cold symptoms emerge is short, lasing only 10-12 hours (Rollinger and Schmidtke, 2011, 42-92). Viral shedding peaks two to

three days following initial infection and declines immediately following the peak (Dreschers et al., 2007, 181-191).

The symptoms of a cold are thought to result from an inflammatory process initiated by infected host epithelial cells and not from tissue damage caused by HRV infection. This theory of HRV pathogenesis has emerged from a number of observations at the tissue and cellular level. Interestingly, HRV replicates in only 10% of the cells in an infected tissue (Kirchberger, Majdic and Stockl, 2007, 1-10). This rate of infection seems insignificant compared to the severity of symptoms experienced by many cold sufferers. Also, nasal biopsy specimens from individuals infected with HRV are typically negative for the histopathological changes that are consistent with significant tissue damage (Dreschers et al., 2007, 181-191). Nasal secretions from HRV infected individuals contain a number of inflammatory cytokines including IL-6 and IL-8. These cytokines are powerful chemoattractant signals that promote the infiltration of neutrophils and other leukocytes to the site of infection and further exacerbate local inflammation (Kirchberger, Majdic and Stockl, 2007, 1-10).

Immune system interactions. Some estimates suggest that a typical U.S. adult experiences 2.5 colds per year on average (Fendrick et al., 2003, 487-494). Given the high prevalence of HRV, more than half of these upper respiratory illnesses are likely a result of HRV infection (Poland and Barry, 2009, 2245-2246). In a study where volunteers were experimentally infected with a strain of HRV-A, Barclay and colleagues (1989) observed a specific antibody response in serum and nasal secretions 1-2 weeks after inoculation which remained in most participants for a year or longer. This response

did not confer lasting protection from HRV re-infection in all participants as 2 out of 14 subjects were re-infected when challenged with the same strain one year after the initial infection. However, the volunteers appeared to have reduced the severity of illness in subsequent infections (Barclay et al., 1989, 659-669). Other investigators have shown that multiple infections by different strains of virus in the same HRV species during a single year is not uncommon (Mackay et al., 2012).

The high diversity among HRVs (> 100 serotypes) is one factor that accounts for weak immune protection. A recent investigation by Niespodziana and colleagues (2012) revealed an additional immune evasion property of HRV. The authors evaluated the antibody response to each of the 4 HRV capsid proteins, designated VP1-VP4, in the blood of children with confirmed HRV infection. Subjects in this investigation were 56 children with naturally acquired upper respiratory illness recruited from physician offices. HRV infection was confirmed by polymerase chain reaction in 48 of the 56 enrolled study participants. Serum samples were evaluated for antibody response to each of the viral proteins (Niespodziana et al., 2012, 1001-1008).

While the subjects produced some antibodies to each of the 4 viral proteins, the strongest antibody response was directed against VP1, the viral capsid protein that interacts with the ICAM-1 receptor. The investigators mapped the epitope specificity of the anti-VP1 antibodies in the sera of study participants and found the strongest response was to a 20-amino acid fragment at the n-terminus of the VP1 protein. In the complete viral capsid, this portion of VP1 is not exposed except during un-coating when VP1 is bound to ICAM-1. The major finding of this study is that most of the anti-HRV

antibodies produced by the participants were not capable of neutralizing HRV particles. This observation lead the authors to conclude one reason for recurrent HRV infection is a misdirected antibody response (Niespodziana et al., 2012, 1001-1008).

An *in vitro* model of HRV pathogenesis has been developed by Hosoda and colleagues (2002) to explore some facets of the effect of HRV on inflammatory signaling by immune cells. Their findings indicate HRV infection primes mast cell and basophil cell lines to produce significantly more histamine, interleukin-4 (IL-4), interleukin-6 (IL-6) and interleukin-8 (IL-8) when stimulated with phorbol myristate acetate (PMA) than uninfected controls (Hosoda et al., 2002, 1482-1491). PMA is a compound commonly used in cell biology experiments to stimulate cytokine production in cytokine-secreting cells. The actions of histamine are associated with many common cold symptoms such as nasal congestion or runny nose (DeFranco, Locksley and Robertson, 2007, 302-317). Individual cytokines have many functions and act on multiple cell types in a coordinated and redundant fashion. Many of the principal effects of IL-4, IL-6, and IL-8 are closely related to the symptoms and pathophysiology of HRV infection. IL-4 stimulates differentiation of helper T cells and also plays a role in allergy and asthma. IL-6 is a pro-inflammatory cytokine that recruits monocytes to an area of inflammation. IL-8 recruits granulocytes including neutrophils to the site of inflammation and promotes histamine release in surrounding tissues (DeFranco, Locksley and Robertson, 2007, 302-317). Hosoda and colleagues' (2002) model of HRV infection is different from a natural HRV infection in that cultured mast cell and basophil cell lines were modified in order to be infected with the HRV virus. Physiological HRV infection occurs in nasal epithelial

cells, not leukocytes. This experimental model is not a true-to-life representation of *in vivo* HRV infection. However, it is suggestive with respect to the ability of HRV to promote modulation of inflammatory response.

HRV modulates the expression of host genes related to immune function. Whiteman and colleagues (2003) evaluated the effect of HRV infection in cultured human bronchial epithelial cells on the production of both soluble ICAM-1 (sICAM-1) and membrane bound ICAM-1. Unique mRNA transcripts were observed for each of the ICAM-1 proteins. HRV infection induced an increase in ICAM-1 protein expression at the cell surface 2.5 times greater than the basal rate and an increase in ICAM-1 mRNA expression 2 times greater than the basal rate. The amount of sICAM-1 recovered from culture supernatants varied in a time-dependent manner in control cultures. However, no sICAM-1 protein was recovered from culture supernatants in HRV infected cell cultures. The rate of sICAM-1 mRNA expression was reduced to half of the basal rate in HRV infected cell cultures (Whiteman et al., 2003, 11954-11961).

Intercellular Adhesion Molecule-1

Overview. ICAM-1, also known as CD54, is a member of the immunoglobulin superfamily that is expressed on the surface of many cell types, ranging from lymphocytes such as tissue monocytes and dendritic cells to surface tissues like vascular endothelium and mucosal epithelium (Long, 2011, 5021-5023). In endothelium, ICAM-1 functions to provide an adhesion site for lymphocytes in the blood steam, allowing lymphocytes to migrate from the blood to a site of inflammation. Similarly, ICAM-1

expression on the surface of epithelial cells allows lymphocytes to localize to a site of inflammation and enter affected tissues through diapedesis (Bella and Rossmann, 2000, 291-297).

Structure and Ligands. The structure of ICAM-1 consists of cytoplasmic, transmembrane and extracellular regions (Witkowska and Borawska, 2004, 91-98). The cytoplasmic region of ICAM-1 is anchored to the actin cytoskeleton and this connection to the cytoskeleton is necessary for ICAM-1 to stimulate some components of the immune system (Long, 2011, 5021-5023). The extracellular region is heavily glycosylated and consists of five immunoglobulin (Ig) domains (Bella and Rossmann, 2000, 291-297). Immune cells recognize and bind to distinct ICAM-1 Ig domains through two different β-integrin receptors: leukocyte function-associated antigen (LFA-1) and macrophage-1 antigen (Mac-1) (Bella and Rossmann, 2000, 291-297). ICAM-1 can exist on the cell surface either as a monomer or as a dimer. The dimerized form of ICAM-1 exhibits the highest binding affinity for LFA-1 (Witkowska and Borawska, 2004, 91-98).

A soluble form of ICAM-1 (sICAM-1) circulates in the blood and can be detected in a number of different body fluids and secretions. sICAM-1 is similar to ICAM-1, except that it lacks the transmembrane and intercellular domains of membrane-bound ICAM-1 (Witkowska and Borawska, 2004, 91-98). Two mechanisms for sICAM-1 production have been identified. Champagne and colleagues (1998) described a mechanism of proteolytic cleavage of ICAM-1 at the membrane surface mediated by human neutrophil elastase. This mechanism represents the shedding of ICAM-1 from the

cell surface and agrees with the observation that sICAM-1 concentration in culture supernatants is related to the amount of ICAM-1 expression on the cell surface (Witkowska and Borawska, 2004, 91-98). Distinct mRNA transcripts coding for both forms of ICAM-1 have been observed in cell culture. Whiteman and colleagues (2003) identified unique mRNA transcripts for ICAM-1 and sICAM-1 and showed that gene expression can be independently regulated for each protein.

ICAM-1 is exploited as a receptor by three different pathogens. *Plasmodium falciparum*, the parasite that causes malaria, directs infected blood cells to adhere to the endothelial surface of deep capillary beds using ICAM-1. Binding of infected red blood cells to capillary walls may be a method of immune evasion, but it also contributes to the virulence of the malarial parasite by creating blockages in blood vessels (Bella and Rossmann, 2000, 291-297). Some members of the Coxsackievirus A genus as well as the major subgroup of HRVs use ICAM-1 as a receptor to gain entry into epithelial cells. Coxsackievirus A, like HRV, belongs to the Picoronaviridae family and *Enterovirus* genus. Various Coxsackie A virus species cause illnesses including the common cold, hand, foot and mouth disease, and viral meningitis (Rossmann et al., 2000, 239-247). A number of virus species within this genus require the interaction of other factors and receptors in addition to ICAM-1 to initiate infection (Newcombe et al., 2003, 3041-3050).

Although there are some variations in the precise mechanisms, i.e. additional co-factors or co-receptors, both viruses are thought to bind to ICAM-1 in a similar way described as the canyon hypothesis. The three-dimensional structure of the viral capsids

are very similar and have a small depression, or canyon, at the icosahedral vertices which receives the portion of ICAM-1 that is most distal from the cell membrane (Rossmann et al., 2000, 239-247).

Factors effecting ICAM-1 Expression. ICAM-1 may be constitutively expressed at a low rate in some tissues, but increases dramatically during infection or injury (Fuchs and Blaas, 2010, 281-297). Mast cells and macrophages produce inflammatory cytokines at the site of damage including interleukin-1 (IL-1) and tumor necrosis factor-α (TNF-α). Tissue cells rapidly translocate ICAM-1 to the cell surface when exposed to TNF-α or IL-1, acting as a target for recruitment of additional lymphocytes (DeFranco, Locksley and Robertson, 2007, 22-53). sICAM-1 levels increase in a number of disease states, particularly in conditions involving inflammation such as rheumatoid arthritis, atherosclerosis, and hypertension (Witkowska and Borawska, 2004, 91-98). Increased ICAM-1 expression has been detected in the respiratory epithelium of patients with atopic (allergy-induced) asthma (Bianco et al., 2000, 339-345).

Drugs with anti-inflammatory properties decrease ICAM-1 expression. Valera and colleagues (2011) used tissues cultured from nasal polyp biopsy specimens to assess the effect of the corticosteroid drug fluticasone propionate on markers of inflammation, including ICAM-1. Tissue from nasal polyp biopsies was selected as a model system because nasal polyps form as part of a severe chronic inflammation of the upper respiratory tract and are often treated with corticosteroid nasal sprays. Nasal polyp tissues express ICAM-1 at a high rate constitutively. Fluticasone propionate decreased ICAM-1 gene expression and sICAM-1 protein level in a dose dependent manner (Valera

et al., 2011, 605-610). A similar study by Sabatini and colleagues (2003) assessed the effect of two different corticosteroid drugs on markers of inflammation in a cell culture model of lung tissue. In this study, human fetal lung fibroblast cells were exposed to TNF-α to stimulate production of inflammation-related proteins. The authors compared relative effects of mometasone furoate and dexamethasone on inflammatory markers. A dose-dependent decrease in ICAM-1 was seen with both drugs in the TNF-α stimulated cell cultures. Treatment at the highest concentration of mometasone furoate had a powerful effect, lowering ICAM-1 expression below the constitutive level of ICAM-1 expression in unstimulated cells (Sabatini et al., 2003, 287-297).

Human rhinovirus. During HRV infection, the expression of ICAM-1 increases significantly in nasal epithelium within the first 24 hours after infection in both *in vitro* and *in vivo*. *In vitro* experiments have shown a complete suppression of sICAM-1 release in culture supernatants from HRV infected cells (Whiteman et al., 2003, 11954-11961). In experimentally induced HRV infection in human volunteers, the level of sICAM-1 detected in nasal secretions has been observed to increase slightly, but appears to lag behind the increase in membrane-bound ICAM-1 and increase to a lesser extent (Winther et al., 2002, 131-136). The increase in ICAM-1 expression that occurs in response to HRV infection represents a mechanism that favors increased viral replication and spread of viral infection within a tissue. In contrast to ICAM-1, sICAM-1 has anti-viral properties and suppresses HRV replication (Witkowska and Borawska, 2004, 91-98). Viral particles that are docked to sICAM-1 have fewer binding sites available to attach to ICAM-1 at the cell surface and may be less able to cause infection. HRV is able

to modulate the expression of ICAM-1 within nasal epithelial cells in a way that promotes viral replication. Observations in cultured bronchial epithelial cells indicate that HRV is able to alter the levels ICAM-1 messenger RNA to increase the expression of membrane-bound ICAM-1 and decrease the release of sICAM-1 (Papi and Johnston, 1999, 9707-9720; Whiteman et al., 2003, 11954-11961). These changes may promote increased viral replication through increased binding to ICAM-1 and decreased host defenses through suppression of sICAM-1.

Dietary factors that influence ICAM-1 expression. Dietary factors modulate the expression of both sICAM-1 and ICAM-1. Cell culture models of ICAM-1 expression have shown a reduction in ICAM-1 in response to various dietary antioxidants in stressed cells. In one such experiment, Mo and colleagues (2003) used cultured endothelial cells in an investigation of phytochemicals and inflammation. Human umbilical endothelial cells were first stimulated with TNF-α to produce ICAM-1. The effect of alginate (present in seaweed), allicin (present in garlic) and vitamin C on TFN-α stimulated cultures was assessed by ELISA. The investigators measured a significant and dose-dependent decrease in TFN-α stimulated ICAM-1 production in response to all three compounds, with the greatest percent inhibition achieved by vitamin C. Addition of these three compounds to unstimulated cells did not significantly alter the baseline ICAM-1 expression (Mo et al., 2003, 244-251).

The influence of dietary factors on ICAM-1 has been investigated in a number of human trials involving supplements and dietary interventions. In a randomized, double-blind, crossover study, Rayment and colleagues (2003) investigated the effect of vitamin

C supplementation on monocyte ICAM-1 expression in healthy adults. The investigators recruited apparently healthy men between the ages of 20 and 45 who were non-smokers, had no history of heart disease and were not currently taking supplements. Study participants were supplemented with 250 mg of vitamin C daily for six weeks. Monocyte ICAM-1 protein expression was measured using flow cytometry while ICAM-1 gene expression was measured by PCR. Plasma sICAM-1 level was measured using ELISA (Rayment et al., 2003, 339-345). For analysis, study participants were grouped by baseline vitamin C status into a low vitamin C group (LOC) with plasma levels < 50 μM and an above average vitamin C group (HIC) with plasma levels ≥ 50 μM. Both groups demonstrated an increase in plasma vitamin C after 6 weeks of supplementation, but the LOC group demonstrated the greatest increase with plasma vitamin C levels 2-fold greater than baseline. The LOC group showed the greatest decrease in ICAM-1 gene expression and had significantly lower plasma sICAM levels. Monocyte ICAM-1 levels were not significantly reduced in either group following supplementation. The HIC group did not show a significant decrease in ICAM-1 gene expression in response to vitamin C supplementation (Rayment et al., 2003, 339-345).

Because of its role in inflammation, ICAM-1 has been investigated in relation to diseases that have an inflammatory component, such as atherosclerosis and heart disease. Several studies have explored the effect of antioxidant nutrients on ICAM-1 as an intervention in inflammatory disease states. In one investigation of exercise-induced endothelial dysfunction in patients with intermittent claudication, intravenous vitamin C infusion (50 mg/minute for 20 minutes) reduced an exercise-induced increase in plasma

sICAM-1 (Silvestro et al., 2002, 277-283). In another study with subjects at high risk for cardiovascular disease, investigators developed a dietary intervention involving walnut-enriched meat products (Canales et al., 2011, 703-710). The rational for using walnuts as part of a dietary intervention related to prior findings that walnut-enriched meat products improved the antioxidant status of study participants at high risk for heart disease. The findings from this study showed a significant decrease in plasma sICAM-1 following the 5-week intervention, highlighting the effect of antioxidant-rich whole foods on plasma sICAM-1 (Canales et al., 2011, 703-710).

Vitamin C Overview

Antioxidant function. Vitamin C (ascorbic acid) is the most significant aqueous antioxidant in human serum. Ascorbic acid carries out its antioxidant function by donating a hydrogen atom to an oxidizing species and interrupting the chain of oxidative reactions. In this process, ascorbic acid is converted first to an intermediate ascorbate free radical which is then converted to dehydroascorbate. Dehydroascorbate can be recycled back to ascorbic acid or may form 2,3-diketo-l-gluconic acid which cannot be recycled to ascorbic acid and is excreted after further degradation (Asard, May, and Smirnoff, 2004, 139-152). In serum, vitamin C travels freely as ascorbic acid and oxidized dehydroascorbate is taken up by red blood cells via GLUT glucose transporters for recycling (Combs, 2012, 233-261).

Enzymatic co-factor functions. Vitamin C is also an essential co-factor for the function of a number of enzymes that are dependent on reduced copper or iron for their

catalytic function, i.e. Cu^{+}-dependent monooxygenases and Fe^{2+}-dependent dioxygenases (Linster and Van Schaftingen, 2007, 1-22). Physiological functions such as collagen synthesis, tyrosine metabolism, carnitine synthesis and epinephrine/norepinephrine synthesis all depend on vitamin C for the function of at least one enzyme in their respective metabolic pathways. Scurvy is the disease that results from vitamin C deficiency and has various symptoms that can be attributed to dysfunction of these enzymatic pathways (Asard, May, and Smirnoff, 2004, 159-169). Some of the most readily apparent symptoms of scurvy result from defects in collagen synthesis and include swollen, bleeding gums, tooth loss, bruising, hemorrhage, bone malformation and joint pain. Individuals affected by scurvy also exhibit fatigue, weakness, apathy and malaise which are thought to result from defects in the synthesis of the neurotransmitters epinephrine and norepinephrine as well as defects in carnitine synthesis which is necessary for the oxidation of fatty acids for energy.

Dietary Reference Intake. The current dietary reference intake for vitamin C established by the Institute of Medicine's Food and Nutrition Board (2000) recommend an intake of 75 mg per day for adult women and 90 mg per day for adult men. This recommendation is based on maximizing vitamin C concentration in neutrophils and minimizing urinary ascorbic acid excretion. The tolerable upper limit is set to 2,000 mg daily based on minimizing gastrointestinal distress and osmotic diarrhea that can occur with ingestion of high supplemental doses (> 3,000 mg) of vitamin C although high supplemental doses of vitamin C from 2,000-4,000 mg per day are considered relatively non-toxic (Food and Nutrition Board, Institute of Medicine, 2000; Johnston, 1999, 71-

77). Doses of vitamin C that optimize physiologic function *in vivo* may be considerably higher than DRI levels. For example, when Heinz bodies and lipid peroxidation were used as measures of oxidative stress in plasma, supplemental doses of vitamin C between 500 and 1000 mg daily provided maximal protection in healthy adults. Higher doses of vitamin C at the level of 2000 mg daily maintained the level of benefit achieved at the 500-1000 mg level, but did not confer additional benefit (Johnston and Cox, 2001, 623).

Vitamin C Status in the United States. Several factors including age, sex, smoking and socioeconomic status were associated with serum vitamin C concentration in a nationally representative sample of the United States population. Overall, the prevalence of vitamin C deficiency in the 2003-2004 National Health and Nutrition Examination Survey (NHANES) was 7.1% (Schleicher et al., 2009, 1252-1263). The demographic groups with the lowest mean serum vitamin C concentration were smokers, men between 20 to 59 years of age and individuals with low income. Population sub-groups with the highest mean serum vitamin C concentration included children between 6 and 11 years old and adolescents between 12 and 19 years old and females 12 years of age and older (Schleicher et al., 2009, 1252-1263).

Historical Controversies in Vitamin C Research. In 1970, respected scientist and two-time Nobel Laureate Linus Pauling published his book *Vitamin C and the Common Cold*. Pauling advocated supplemental doses of vitamin C in amounts ranging from 1 to 10 grams daily and asserted that high-dose supplemental vitamin C could extend an individual's life by between 2 and 6 years. This publication was not well accepted by the scientific community, generating considerable criticism (Bing, 1971, 1506-1506; Hodges,

1971, 532). Following the publication of *Vitamin C and the Common Cold*, several studies were undertaken to assess whether Pauling's claims had scientific merit (Anderson, 1975, 498-504; Karlowski et al., 1975, 1038-1042). However, the controversy surrounding Pauling's dramatic claims with respect to vitamin C was so significant that it may have impacted contemporary researchers' interpretations of vitamin C supplementation trials. For example, Karlowski and colleagues (1975) conducted a randomized, placebo-controlled trial of the effect of vitamin C supplementation on common cold symptoms among National Institutes of Health employees in 1975. In this trial, the authors observed no significant reduction in the frequency of colds, but there was a dose-dependent reduction in the duration and severity of cold symptoms, which the authors attributed to the placebo effect (Karlowski et al., 1975, 1038-1042). Later authors who reviewed the Karlowski trial have argued that attributing the observed dose-dependent reduction in the duration of colds to a placebo effect was not consistent with the study findings (Hemilä, 1996, 1079-1084).

Vitamin C and the Common Cold. Currently, there is general agreement that vitamin C can decrease the severity and duration of the common cold, but the evidence is less clear with respect to prevention. For example, when writing for the Cochrane Collaboration, Hemilä and colleagues (2010) concluded that prophylactic vitamin C supplementation did not reduce the frequency of colds but did reduce duration and severity of colds. However, a number of individual trials have shown a preventative effect of vitamin C for the common cold in healthy volunteers (Van Straten and Josling, 2002, 151-159; Sasazuki et al., 2006, 9-17). Since "the common cold" is a general term

that describes an illness that can be caused by a number of unrelated pathogens, some of these observed differences in effect may be related to differences in the cause of cold symptoms. Also, different subject populations tend to show different effects of vitamin C on cold prevention. For example, studies in participants who are subject to high levels of stress show a preventative effect of vitamin C against cold incidence. In one such study, Peters and colleagues (1993) compared ultra-marathon (>42 km) runners to age-matched non-running controls in a double-blind placebo-controlled trial. Both groups were supplemented with 600 mg vitamin C daily for 21 days prior to an ultra-marathon race and then assessed for cold symptoms during the 14-day period following the race. In this sample, 68% of runners receiving placebo reported cold symptoms following the race compared to 33% in the supplement group ($p < 0.01$). Runners in the supplement group and matched controls who reported cold symptoms also experienced a shorter duration of colds compared to placebo control (Peters et al., 1993, 170).

Asthma Overview

Prevalence and impact. The most recent report from the US Centers for Disease Control found that the overall prevalence of asthma in the US population had increased by 12.3% between 2001 and 2009, bringing the most current estimates of asthma prevalence in the US to 8.2% (Zahran et al., 2011, 547). This prevalence rate represents about 24.6 million people affected in 2009. The authors found that some demographic groups bear a disproportionate burden of asthma compared to the general population. This study found that the prevalence of asthma was significantly greater for children than

adults. Among children, boys (11.3%) and non-hispanic blacks (17%) had the highest prevalence. Asthma prevalence was greater among individuals with household incomes below the federal poverty level with an overall prevalence of 11.6%. Children in poor households had an asthma prevalence of 13.5% (Zahran et al., 2011, 547). Given the high prevalence of asthma, findings of significant cost to society from this disease are not surprising. Using data from the Medical Expenditure Panel Survey 2002-2007, Barnet and Nurmagambetov (2011) estimated the incremental cost of asthma at $56 billion. The authors attributed $3.8 billion to lost productivity from asthma morbidity and $2.1 million to mortality related to asthma in 2009 dollars (Barnett and Nurmagambetov, 2011, 145-152).

Pathophysiology. Asthma is a chronic disorder characterized by airway hyperreactivity, reversible airway obstruction and specific histopathological changes resulting from inflammatory airway remodeling (Maddox and Schwartz, 2002, 477-498). Hyperreactivity refers to the inflammatory response of the airway to environmental factors such as pet dander or pollen that do not produce a response in healthy individuals. Symptoms can vary, but typically include cough, wheeze and shortness of breath. While there are various etiologies of asthma, atopic or allergy-induced asthma is one of the best characterized manifestations of asthma. Immunologically, atopic asthma results from a dysregulated T helper 2 cell response (Jacobsen et al., 2007, 18-26). The normal function of T helper 2 cells is to coordinate epithelial barrier immunity against invading arthropod and helminth pathogens (DeFranco, Locksley and Robertson, 2007, 274-277). T helper 2 cells orchestrate the responses of B cells, eosinophils, basophils, mast cells and

macrophages as well as epithelium and smooth muscle in a coordinated response to hinder the ability of blood-sucking worms or biting insects to feed. In response to a specific antigen, stimulated T helper 2 cells secrete IL-4, IL-13, IL-5 and IL-9, each of which has a specific effect in stimulating a barrier immunity response. IL-4 and IL-13 activate macrophages and B cells, stimulating the B cells to differentiate in to IgE secreting plasma cells. IL-4 and IL-13 act on epithelial tissues to increase mucin production and on smooth muscle to increase contractility. IL-9 is responsible for attracting basophils and mast cells and enhancing their survival while IL-5 promotes recruitment and enhances survival of eosinophils (DeFranco, Locksley and Robertson, 2007, 22-53).

The smooth muscle contraction and localized edema of a well-controlled T helper 2 cell response is effective in dislodging worms and impairing the ability of biting insects to feed. However, in atopic asthma T helper 2 cells initiate an inflammatory response to benign allergens in the airway that results in vascular edema and smooth muscle contraction within the bronchioles, leading to a restricted airway. T helper 2 cells release inflammatory cytokines and chemokines which in turn increase expression of adhesion molecules in the local vascular endothelium drawing lymphocytes of hematopoietic origin such as neutrophils, mast cells and eosinophils (Maddox and Schwartz, 2002, 477-498).

The T helper 2 response generates a powerful chemoattractant stimulus for eosinophils. Eosinophils are one of the primary leucocytes associated with asthma symptoms. Eosinophil counts in bronchial lavage and eosinophil granule proteins have

been correlated with severity of asthma symptoms and eosinophils are the primary cellular infiltrate found in the lung tissue of asthma patients (Maddox and Schwartz, 2002, 477-498). Eosinophils enter the lung parenchyma by an integrin-mediated process which involves cell rolling or loose adhesion via P- or E-selectin, followed by firm adhesion between integrin and ICAM-1. After firm adhesion to the vascular endothelium, eosinophils leave the circulation through extravasation or diapedesis and migrate into the lung tissue (DeFranco, Locksley and Robertson, 2007, 118-151). Histological examination of asthmatic lung tissue reveals eosinophils localize immediately below the respiratory epithelium and above the smooth muscle layer (Maddox and Schwartz, 2002, 477-498).

Asthma and Diet. Various studies have related dietary factors to asthma in both adults and children. Dietary antioxidants including vitamin C, vitamin E, beta carotene and selenium have been related to asthma incidence and pulmonary function. Hu and Cassano (2000) used both dietary and serum markers of antioxidant status when assessing pulmonary function in an NHANES II data set. Spirometry measurements following the American Thoracic Society standards were collected as part of the NHANES medical exam. The authors used forced expiratory volume as their primary assessment of pulmonary function. Forced expiratory volume measures the maximum volume of air that can be exhaled by a subject under forced conditions. This measure is not specific to asthma since any condition that impacts lung function such as chronic obstructive pulmonary disease or cystic fibrosis also affects spirometry measurements. Additionally, asthmatics may have normal spirometry values when they are not experiencing an acute

exacerbation. Using multiple linear regression analysis, Hu and Cassano observed a positive association between pulmonary function and intake of vitamin C, vitamin E and beta carotene after controlling for total calories. Higher serum levels of these antioxidants were also associated with improvements in lung function (Hu and Cassano, 2000, 975-981).

A systematic review and meta-analysis of 40 studies relating dietary intake and serum vitamin A, C and E to asthma outcome measures concluded that vitamin A and vitamin C status was significantly lower in individuals with asthma (Allen, Britton and Leonardi-Bee, 2009, 610). The findings of this meta-analysis indicated that vitamin A intake was significantly lower in subjects with asthma compared to non-asthmatic individuals. Interestingly, subjects with severe asthma had lower vitamin A intake that subjects with mild asthma. With respect to vitamin C, dietary intakes and serum status in the lowest quartiles was associated with greater risk of asthma. The authors also found that vitamin E was not related to asthma in general, except that subjects with severe asthma had significantly lower intakes of vitamin E than subjects with mild asthma (Allen, Britton and Leonardi-Bee, 2009, 610). The findings of this meta-analysis differ from the results from Hu and Cassano (2000) analysis of NHANES II data with respect to vitamin E. One possible explanation for this discrepancy is that Hu and Cassano used a general assessment of lung function and the authors of this meta-analysis were looking specifically at studies that measured asthma outcomes.

While most of the evidence linking certain dietary factors to asthma is taken from observational studies, some interventions have investigated the effects of nutrients and

food components on asthma. Tecklenburg and colleagues (2007) conducted a randomized, placebo-controlled cross-over supplementation study to evaluate the effect of a high-dose vitamin C supplement (1500 mg daily) on exercise-induced asthma symptoms in adult asthmatic subjects. Subjects who received vitamin C had significant reductions in their post-exercise drop in forced expiratory volume compared to subjects receiving placebo (Tecklenburg et al., 2007, 1770-1778). In a separate study, Nadi and colleagues (2012) designed a double-blinded, placebo-controlled parallel-arm trial involving 60 adult asthmatic patients to assess the effect of a 1000 mg vitamin C supplement on severity of asthma symptoms assessed by spirometry. At the end of a month-long trial, leukocyte vitamin C had significantly increased in the supplement group, but no benefit of vitamin C was seen in spirometry parameters (Nadi et al., 2012, 233-238). Writing for the Cochrane collaborative, Kaur and colleagues (2009) reviewed the effectiveness of vitamin C supplementation as a treatment for asthma in randomized controlled trials. Only 9 mostly small studies met the inclusion criteria, yielding a total of 330 subjects. The authors concluded that there is insufficient evidence to recommend vitamin C as a treatment for asthma at the current time (Kaur, Rowe and Arnold, 2009).

Respiratory Viruses and Asthma. Early childhood upper respiratory viral infections have been strongly implicated in the etiology of asthma although the question of whether childhood respiratory viral infections cause asthma is still a matter of discussion in the literature. In support of this hypothesis, HRV infection is generally limited to the upper respiratory tract, but can cause lower respiratory infection in vulnerable populations such as infants and small children. Lower respiratory infection in

infancy has been associated with later development of asthma (Kim and Gern, 2012, 116-121). Recurrent lower respiratory viral infections may damage developing lungs and promote over-sensitization of the immune system that leads to the development of asthma. However, it is unclear whether there is a separate predisposing factor to both asthma and severe childhood viral infections or whether the changes in the lung tissue that result from chronic lower respiratory infections can be a precipitating cause of later development of asthma (Kim and Gern, 2012, 116-121).

While the contribution of early rhinovirus infection in children to the later development of asthma is still a matter of debate, it is well accepted that HRV infection in asthmatic individuals can lead to potentially serious asthma exacerbations. HRV infection increases asthma exacerbations through a variety of mechanisms. HRV has been shown to increase the proinflammatory response of airway smooth muscle *in vitro* (Oliver et al., 2006, 71). Human airway smooth muscle cells were isolated from both asthmatic and non-asthmatic volunteers by Oliver and colleagues (2006) and experimentally infected with HRV. The researchers assessed the inflammatory response of asthmatic and normal respiratory smooth muscle using ICAM-1 cell surface expression, cytokine production and IL-6 transcription. Cultured respiratory smooth muscle cells from asthmatic volunteers exhibited a distinct HRV-specific inflammatory response compared to cells from non-asthmatic subjects. Cells from asthmatic subjects secreted significantly more IL-6 than cells from non-asthmatic subjects. Additionally, IL-6 transcription was under different regulatory control, with the two cell groups

responding differently to the same experimental stimulus for IL-6 production (Oliver et al., 2006, 71).

HRV infection also affects epithelial cell cultures derived from asthmatic patients differently than epithelial cells derived from normal individuals. Wark and colleagues (2005) compared the innate immune response of bronchial epithelial cells derived from normal and asthmatic volunteers *in vitro* during experimental infection with HRV. Cells derived from asthmatic patients had diminished apoptotic response compared to cells derived from normal volunteers. This diminished response supported higher rates of viral replication and promoted a higher over-all viral load. An important factor in the apoptotic response to viral infection is interferon-β, which was observed to be deficient in the cells from asthmatic patients. The normal apoptotic response was restored when interferon beta was added to the culture medium (Wark et al., 2005, 937-947).

Asthma and ICAM-1. ICAM-1 expression is elevated in individuals with asthma. A number of studies have replicated evidence for elevated ICAM-1 in asthmatics through a variety of experimental techniques. In an investigation of a dietary antioxidant supplement and markers of inflammation, Horvathova and colleagues (2001) incubated human umbilical endothelial cells with whole blood taken from healthy and asthmatic donors and measured the resultant ICAM-1 expression using flow cytometry. Induced ICAM-1 expression was 4.7-fold greater in asthmatics versus healthy controls (Horvathova, Jahnova and Gazdik, 2001, 17-30).

Asthmatic individuals have a higher level of ICAM-1 expression in nasal epithelium than non-asthmatic study participants. Bianco and colleagues (2000) recruited

13 volunteers with atopic asthma and 11 non-atopic, apparently healthy controls for a study aimed at characterizing ICAM-1 expression. The authors evaluated the response of ICAM-1 expression to both grass pollen allergen and HRV infection, which are common environmental triggers for asthma exacerbation. Subjects were non-smokers who were not currently using steroidal nasal sprays and who did not have a recent history of a cold. The study participants supplied specimens in the form of nasal brushings. Three asthmatic patients provided nasal polyp biopsy specimens. Nasal epithelial cells were cultured and surface ICAM-1 expression was evaluated using immunoenzymatic labeling protocol and semiquantitative scoring by a blinded, independent observer (Bianco et al., 2000, 339-345).

The baseline ICAM-1 expression of the asthmatic study participants was significantly greater than healthy controls. Exposure to clinically relevant allergens significantly increased ICAM-1 expression in nasal epithelium taken from asthmatic participants, but had no effect on healthy controls. Nasal polyp biopsy specimens expressed ICAM-1 at a very high rate. The amount of ICAM-1 expression in these cells did not increase in response to allergen exposure. *In vitro* infection with HRV was performed on nasal epithelial cells from healthy controls and on epithelial cells from nasal polyp biopsy. Cells from healthy controls showed an increased ICAM-1 expression following HRV infection, but nasal polyp biopsy cells did not. Given that the nasal polyp epithelial cells showed a marked elevation in ICAM-1 expression relative to cells from both asthmatic and healthy study participants and did not respond to stimulus from allergen exposure or HRV infection, the authors speculated that nasal epithelial cells

taken from nasal polyps might already be expressing ICAM-1 at a maximal rate (Bianco et al., 2000, 339-345).

Individuals with asthma have significantly higher levels of circulating sICAM-1 than non-asthmatic individuals. Ciebiada and colleagues (2011) evaluated the relationship of plasma sICAM-1 to asthma and allergy in an observational study. In total, 192 volunteers participated in this study. Subjects were divided into groups based on allergy and asthma status as follows: atopic asthma (63), non-atopic asthma (27), allergic rhinitis (64), non-allergic rhinitis (17), and healthy control (21). Recent infection, smoking or serious comorbid health conditions were among the exclusion criteria. Study measures included detailed medical history, skin prick tests for allergy, pulmonary function tests, asthma symptom score as well as serum sICAM-1 measured by ELISA. A portion of study participants in both asthma groups were taking corticosteroid drugs for management of asthma symptoms (Ciebiada, Gorska-Ciebiada and Gorski, 2011, 660-666). Serum sICAM-1 level for the two groups of asthmatic participants was similar and was significantly higher than other study groups. The two rhinitis groups were similar to each other and had serum sICAM levels that were intermediate to the asthmatic groups and to the healthy control (Ciebiada, Gorska-Ciebiada and Gorski, 2011, 660-666). The combined observations of several studies indicating that asthmatic individuals express higher levels of both ICAM-1 and sICAM-1 than non-asthmatic individuals is consistent with the pathophysiology of chronic and acute inflammation associated with asthma (Ciebiada, Gorska-Ciebiada and Gorski, 2011, 660-666).

CHAPTER 3

METHODS

Subject Selection

Asthmatic men and women aged 18 to 65 were recruited from a university community for participation in this pilot study. Volunteers were solicited through e-mail using departmental ListServ and flyers distributed throughout the university campus. Individuals who responded to recruiting advertisements were directed to an online survey to determine if they met the medical history, dietary and physical activity requirements for study participation (Appendix C). Eligible individuals were contacted by e-mail to schedule an initial study visit.

Subjects were excluded from study participation for regular oral or intranasal steroid use or for daily use of dietary supplements containing more than 60 mg of vitamin C. Subjects using inhaled steroidal and non-steroidal asthma medications were eligible to participate. Actively training endurance athletes or individuals engaged in significant vigorous structured exercise on five or more days per week were also excluded. Subjects who felt ill with symptoms consistent with a cold as assessed by participant interview were not eligible to start the trial. This study has been approved by Arizona State University's Institutional Review Board and all participants provided written informed consent prior to enrollment in the study (Appendix A).

Study Design

This 18-day pilot study followed a randomized, double-blinded, placebo-controlled parallel arm design. Participants were required to appear at the Arizona State

University nutrition laboratory facility on the Downtown Phoenix campus for a total of 4 visits; 1 screening visit and 3 study visits as described in the study timeline (Appendix B). Participant height, weight, nasal lavage and postprandial blood sample (no food or drink except water within the last 5 hours) was obtained during the initial screening visit. Participants also completed a Rapid Eating Assessment for Participants questionnaire (REAPS), Medical History Questionnaire, a food frequency questionnaire to assess dietary vitamin C intake, WURSS-21 and Asthma Daytime Symptom Diary Scale (DSDS).

Subjects who meet the inclusion criteria were stratified by age, gender and serum vitamin C level and were randomized to either the experimental group or control group. The experimental group received a 500mg vitamin C capsule twice daily (morning and evening) for a total of 1000mg of vitamin C daily while the control group received a wheat flour placebo capsule, identical in appearance to the vitamin C capsule, twice daily. Subjects in both groups were instructed to avoid drinking fruit juices during the 18-day study period.

Study visits involving collection of a fasting blood sample were scheduled in the morning, between 7:00 and 9:00 am. At the first study visit, all participants were given a booklet containing a study calendar with a daily check-list and copies of the WRSS-21 and DSDS instruments to record daily cold and asthma symptoms. Study participants also received either supplement or placebo, each in opaque packaging marked with the subject number based on randomization to either control or experimental group. Participants were instructed to begin taking the capsules twice daily, beginning the

morning after leaving the nutrition laboratory. Participants were asked to return in 4 days (study day 4) to provide a nasal lavage sample.

On day 18 of the study, post-intervention measures including fasting blood samples (no food or beverages aside from water for 8 hours) and nasal lavage specimens were collected, along with study booklets and remaining supplement or placebo capsules. Participant compliance with study protocol was assessed by reviewing study calendar check list completion, counting remaining capsules, and by evaluating serum vitamin C levels.

The primary outcome measure of this study was sICAM-1 in nasal lavage. Secondary outcome measures included serum sICAM-1, serum histamine and cold and asthma symptoms assessed by questionnaire.

Nasal Lavage Collection

Nasal lavage was performed following the technique described by Winther and colleagues (2002). Briefly, the subject's head was placed in a hyperextended position and 5 ml of isotonic saline was instilled into each nostril and retained for 5 to 10 seconds. The saline was then expelled through the nose into a cup by tipping the head forward over a collection container. Samples were centrifuged to remove mucus contamination and stored at -80°C for up to 6 months. Samples were subjected to no more than 2 freeze-thaw cycles prior to analysis. Study participants provided nasal lavage specimens on three occasions during the study period.

Laboratory Analyses

sICAM-1 was measured in both nasal lavage and serum using a sandwich enzyme-linked immunosorbent assay technique with a commercially available kit following manufacturer's protocol (Invitrogen Corporation, Camarillo, CA). Protocol is available at http://tools.lifetechnologies.com/content/sfs/manuals/KHS5411_revA11.pdf. The sensitivity for detection of sICAM-1 in plasma was 0.33 ng/mL. The manufacturer's expected value in serum was estimated in 50 healthy volunteers as 230.3 ± 47.4 ng/mL.

Serum histamine was quantitatively acylated and analyzed using a commercially available competitive enzyme-linked immunosorbent assay (ALPCO Diagnostics, Salem, NH). Manufacturer's protocol can be accessed at www.alpco.com/pdfs/17/17-hishu-e01.pdf. The sensitivity for detection of histamine in plasma was 0.12 ng/mL, with an expected range of <1 ng/mL in healthy adults.

Plasma vitamin C was assessed using the 2,4-dinitrophenylhydrazine technique (Jacob, Skala and Omaye, 1987, 818-826). Immediately following specimen collection, 1 ml aliquots of plasma were mixed with 1 ml of ice-cold 10% trichloroacetic acid and centrifuged (3500 x g, 0˚C). Supernatants were stored at -80˚C and analyzed for vitamin C concentration within 7 days of specimen collection according to protocol (Appendix E). Subjects provided blood samples for vitamin C assessment at the screening visit and on study day 18. Blood samples for determination of sICAM-1 and histamine concentration were collected on study day 1 and study day 18.

Survey Measures

Wisconsin Upper Respiratory Symptom Survey—21. The Wisconsin Upper Respiratory Symptom Survey-21 (WURSS-21) is a validated tool for assessing the symptoms of the common cold (Barrett et al., 2009, 76-96). The instrument consists of 21 questions that evaluate symptom severity, functional impairment and global illness severity. The questions are evaluated on a magnitude scale with a score of 0 corresponding to the absence of symptoms and 7 indicating the most severe symptoms. The questionnaire is scored by summing the ratings from the first and second sections for an assessment of symptom severity and functional impairment respectively, and summing the scores of all sections for a global assessment of illness severity. A higher numerical score corresponds to greater severity of cold symptoms. A cold was defined as reported in previous publications (Johnston, 2014, 2572-2583). A study participant was considered to have a cold when upper respiratory symptoms reported on the WURSS-12 yielded a cold symptom score of 5 or greater on the first 10 questions of the survey. This score represents any combination of the presence of several mild symptoms, moderate and mild symptoms together or at least one symptom of moderate or greater severity. A single cold episode was defined as a period of consecutive days with a cold symptom score greater than or equal to 5. An episode was counted as a single cold if a period of three or fewer days with symptom scores less than 5 separated two days with a cold symptom score of 5 or greater. This measure was completed daily by the subjects during the study period.

Asthma Daytime Symptom Diary Scale. The Daytime Symptom Diary Scale (DSDS) is a validated questionnaire used to assess frequency and inconvenience of asthma symptoms (Santanello et al., 1997, 646-651). The survey instrument consists of four questions that assess symptom severity and degree of interference with daily activities using a seven-point scale representing the magnitude of symptoms. Asthma symptom scores are calculated as the mean score of the four survey questions. This measure was completed daily by the subjects during the study period.

Statistical analysis

All data is reported as means ± SD. Data analysis was performed using IBM SPSS Statistics release 21.0.0 (IBM Corporation, Armonk, New York). Data was tested for normal distribution and $\log_{10}$-transformed to achieve normality when necessary. When parametric tests were utilized, outliers greater than three standard deviations from the mean were excluded from analysis. Non-parametric tests were used for all data that were not normally distributed. Statistical significance is indicated by p values less than or equal to 0.05. Blinded conditions were maintained until all data was tabulated and prepared for statistical analysis.

CHAPTER 4

RESULTS

Baseline Characteristics

Rolling recruitment took place from April to June of 2013 with subjects completing the 18-day study from May to July of 2013. Of the 56 survey respondents who were screened for study participation, 12 were disqualified for failing to meet inclusion criteria and 44 were contacted to schedule a screening appointment (**Figure 1**). Among 44 initially qualified respondents, 31 individuals declined further participation and 13 volunteers were enrolled in the study, five of whom were men. The volunteers were screened, stratified by age, gender, and plasma vitamin C concentrations and randomly assigned by coin toss to either the placebo group (PLC) or the vitamin C group (VTC). Of the 13 initially enrolled subjects, one subject who had been randomized to the VTC group dropped out of the study. A second subject who had been randomized to the PLC group was excluded from some analysis due to failure to complete study questionnaires and failure to provide a nasal lavage specimen on study day 4. Of the study participants who completed the trial, 6 subjects were in the VTC group and 6 were in the PLC group.

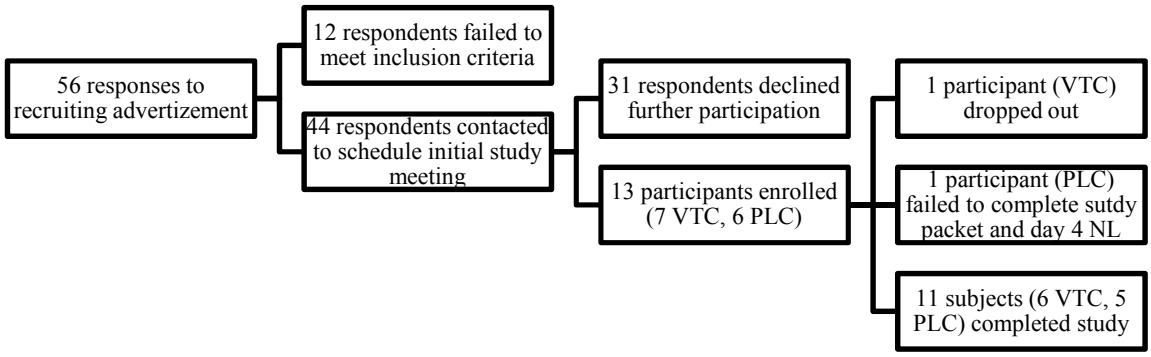

Figure 1. Subject Recruitment

The groups did not differ significantly in gender, medication use, age, BMI, body fat percentage, REAP-S diet quality score, physical activity (METS/week) or plasma vitamin C concentration at baseline (**Table 1**). The participants ranged in age from 22 to 55 years although the mean age was similar in both groups (30.5±9.6 in VTC and 33.8±11.8 in PLC). The current recommended dietary allowance (RDA) for vitamin C as set by the National Institutes of Medicine Food and Nutrition Board (2000) is 90 mg/day for non-smoking men and 75 mg/day for non-smoking women aged 19 years and older. Baseline mean dietary vitamin C intake in both groups exceeded the RDA levels for age and gender. On average, study participants' baseline dietary vitamin C intake was 3.5 times greater that the RDA level for women and 1.2 times greater that the RDA level for men. The US age-adjusted mean plasma vitamin C for adults aged 20 years or older has been reported elsewhere as 86.2 mg/dL (Schleicher et al., 2009, 1252-1263). Baseline plasma vitamin C for both the VTC and PLC groups was lower than the national average.

To compare asthma medication use between VTC and PLC groups, subjects were divided into two categories based on the type of medications used to treat asthma. Subjects who used daily inhaled steroidal medications to control asthma were placed in one category ("daily"). In this study, all subjects who used daily preventative treatment also reported using bronchodilator medications as needed to treat asthma exacerbation, i.e. rescue inhaler. Subjects who did not use a daily preventative treatment but reported using only a rescue inhaler as needed or who reported using no prescription medications were placed in a second category ("as needed"). A Chi-square test for independence indicated no significant association between group assignment (VTC or PLC) and asthma medication use, χ^2=0.066, p=0.797.

Table 1. Baseline Characteristics by Group [a]

Characteristic	Vitamin C (n=7)	Placebo (n=6)	*p*
Gender (male/female)	3/4	2/4	0.725 [b]
Asthma Medication Use (daily/as needed)	3/4	3/3	0.797 [b]
Age (years)	30.5±9.6	33.8±11.8	0.518
BMI (kg/m^2)	26.5±3.7	23.8±5.5	0.199
Body Fat (%)	31.7±10.4	24.9±8.2	0.086
Dietary Vitamin C (mg/day)	171.8±93.3	239.2±279.5	0.775
Diet Quality Score	30.6±4.1	30.8±3.3	1.00
METS (kcal·kg^{-1}·week^{-1})	39.4±39.7	52.2±34.3	0.520
Plasma Vitamin C (mg/dL)	0.52±0.20	0.59±0.26	0.568

a. Values are mean ± SD with the exception of gender. There were no significant differences between groups (p>0.05, Mann-Whitney *U* test).
b. Chi-Square test

Biochemical Measures

There were no significant differences between VTC and PLC groups in any plasma biochemical indices (**Table 2**). Both the VTC and PLC groups showed an

increase in plasma vitamin C level on study day 18 compared to baseline. A larger, but not significant increase was measured in the VTC group (0.33±0.59 vs. 0.10±0.22, $p>0.05$). Plasma histamine concentrations showed a slight, non-significant increase in both groups compared to baseline (VTC group 1.47±3.02 vs. PLC group 1.89±4.11). Plasma sICAM-1 concentrations decreased from baseline for both groups, however the difference between groups was not significant (VTC group -23.07 ±9.32 vs. PLC group -30.85±10.76, $p>0.05$).

There were no significant mean differences between groups in nasal lavage sICAM-1 (**Table 3**). The mean nasal lavage sICAM-1 concentration was lower for the VTC group compared to the PLC group on study day 4 (-1.51±0.76 vs. 3.33±2.81, $p>0.05$), although these differences were not significant. The mean nasal lavage sICAM-1 concentrations were similar for both groups on day 18 (1.58±0.95 vs. 1.72±0.80, $p>0.05$). There was a strong, significant correlation between baseline plasma sICAM-1 and nasal lavage sICAM-1 on study day 18 (Spearman's rho =0.637, $p=0.026$) for both groups. Nasal lavage sICAM-1 levels at baseline were significantly correlated with nasal lavage baseline sICAM-1 on study day 18 (Spearman's rho =0.753, $p=0.005$). Other correlations between nasal sICAM-1 measures and plasma sICAM-1 measures were not significant.

Table 2. Plasma biochemical indices at day 1 and day 18 [a]

	Day 1	Day 18	Δ Day 1 vs Day 18	*p* [b]
Plasma Vitamin C (mg/dL)				
VTC	0.52±0.20	0.90±0.47	0.33±0.59	0.873
PLC	0.59±0.26	0.69±0.11	0.10±0.22	
Plasma Histamine (ng/mL)				
VTC	1.04±0.31	2.51±3.28	1.47±3.02	0.749
PLC	1.41±0.82	3.30±4.42	1.89±4.11	
Plasma sICAM-1 (ng/mL)				
VTC	88.67±9.14	66.4±9.95	-23.07±9.32	0.262
PLC	91.50±19.26	60.65±9.94	-30.85±10.76	

a. VTC (Vitamin C group) n=6, PLC (Placebo Group) n=6, values are mean ± SD
b. There were no significant differences between groups (p>0.05, Mann-Whitney *U* test).

Table 3. Nasal lavage sICAM-1 at day 1, day 4 and day 18 [a]

	Day 1	Day 4 [b]	Day 18	Δ Day 1 vs Day 4	*p* [c]	Δ Day 1 vs Day 18	*p* [c]
VTC	2.39±1.24	1.51±0.76	1.58±0.95	-0.88±0.95	0.223	-0.93±1.07	0.873
PLC	2.63±0.67	3.33±2.81	1.72±0.80	0.63±2.65		-0.91±0.53	

a. Values are mean ± SD, VTC (Vitamin C group) n=6, PLC (Placebo Group) n=6 for all measures except Day 4
b. VTC n=6, PLC n=5 for Day 4 only
c. There were no significant differences between groups (p>0.05, Mann-Whitney *U* test)

Survey Measures

There were no significant differences between VTC and PLC groups in cold or asthma symptoms (**Table 4**). Cold symptoms were assessed daily using Wisconsin Upper Respiratory Symptom Survey-21 (WURSS-21) (**Figure 2**). Total cold symptom score was calculated as the sum of 18 days. The total cold symptom score for the VTC group was slightly greater than for the PLC group, but this difference was not significant (VTC 64.2±66.3 vs. PLC 48.4±44.3, p >0.05). Number of colds and total cold days were

similar in both groups (Number of colds VTC 0.5±0.5 vs. PLC 0.8±0.8, total cold days VTC 3.3±5.0 vs. PLC 2.8±3.7, p >0.05).

Table 4. Analysis of Cold and Asthma Symptoms Over 18 Days [a, b]

	VTC (n=6)	**PLC (n=5)**	***p***
Total Cold Symptom Score [c]	64.2±66.3	48.4±44.3	0.602
Number of Colds [c]	0.5±0.5	0.8±0.8	0.545
Cold Days [d]	3.3±5.0	2.8±3.7	0.848
Total Asthma Symptom Score [e]	12.9±11.8	17.9±10.5	0.602

a. VTC (Vitamin C group) n=6, PLC (Placebo Group) n=5
b. Values are mean ± SD. There were no significant differences between groups (p>0.05, Mann-Whitney U test).
c. Colds assessed using WURSS-21; units are arbitrary measure of magnitude of symptoms
d. Assessed using WURSS-21; Number of days where cold symptom score was >5 (definition of "cold")
e. Asthma symptoms assessed using DSDS; units are units are arbitrary measure of magnitude of symptoms

Participants reported asthma symptoms daily during the study period using Daytime Symptom Diary Scale (DSDS) (**Figure 3**). The DSDS consists of 4 questions that assess frequency, severity and impact of asthma symptoms on daily activities where each item is rated on a seven-point scale from 0 (no symptoms) to 6 (very severe). Daily asthma symptom scores were calculated by taking the mean daily score of survey questions. Total asthma symptom score was calculated as the sum of 18 daily scores. Scores are arbitrary units representing a magnitude of symptom severity. Total asthma symptom scores were lower for the VTC group compared to the PLC group; however this difference was not significant (VTC 12.9±11.8 vs. PLC 17.9±10.5, p> 0.602). There was

no significant correlation between total cold symptom score and total asthma symptom score, or between survey measures and biochemical measures.

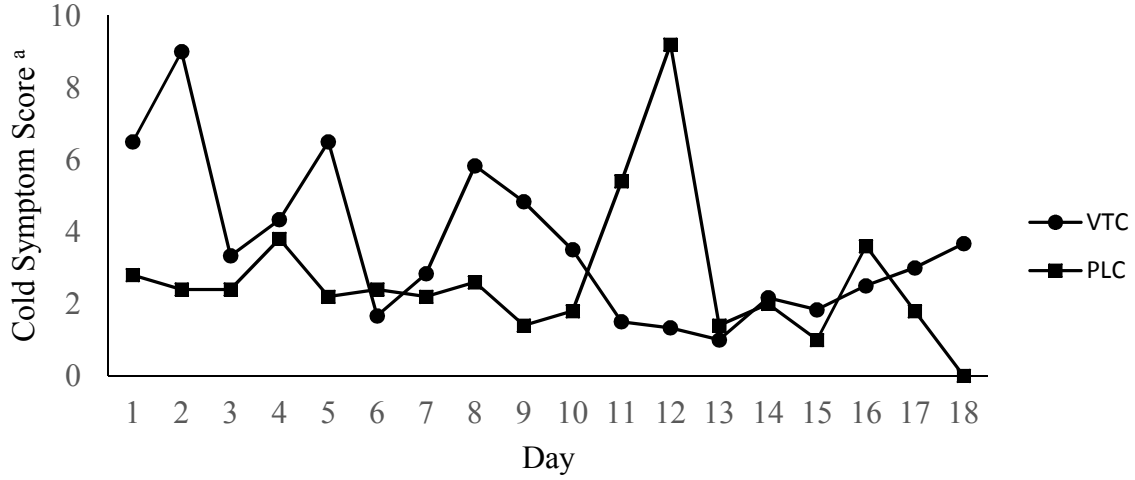

Figure 2. Comparison of daily cold symptom scores by group
a. Colds assessed using WURSS-21, units are arbitrary measure of magnitude of symptoms. VTC (Vitamin C group) n=6, PLC (Placebo Group) n=5

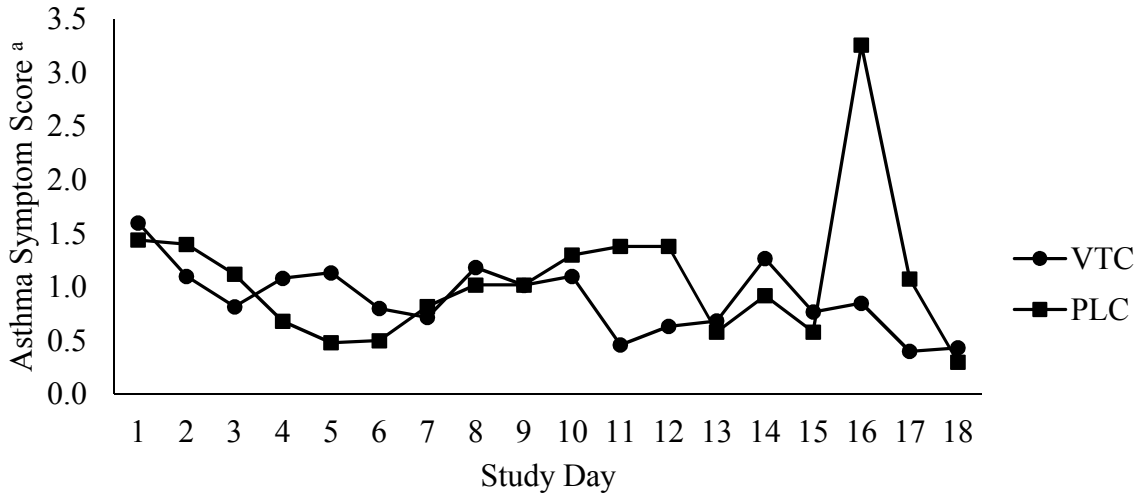

Figure 3. Daily asthma symptom score
a. Daily asthma symptoms assessed using Daytime Symptom Diary Scale. Units are arbitrary measure of magnitude of symptoms. VTC (Vitamin C group) n=6, PLC (Placebo Group) n=5

Compliance Measures

Participant compliance with study protocols was high. Overall, there was a 95.2% compliance rate for subjects taking pills as determined by the number of pills returned at the final study visit and self-report of missed doses. With the exception of one subject who failed to return the study questionnaire packet, 98.9% of daily study questionnaires within the study packet were completed.

CHAPTER 5

DISCUSSION AND CONCLUSION

Major Findings

No mean comparisons reached a level of significance in this study. It is possible that these findings are related to a genuine lack of response in the experimental group, or it may be related to other factors, such as the small number of study participants in the VTC and PLC groups. Several observations from this trial support the conclusion that sample size was too small to detect an effect from the intervention, if an effect existed. For many measures in this study, the standard deviation was large, indicating high inter-individual variability. Given that the number of subjects in both the VTC and PLC groups was 6 for most measures, individual variation may have exerted undue influence in the analysis.

Prior investigations into plasma vitamin C level in response to vitamin C supplementation informed the design of the current study with respect to level of supplementation and expected participant response (Johnston, 1999, 71-77; Johnston and Cox, 2001, 623-627; Johnston 2014, 2572-2583). As expected, the VTC group showed a mean increase in plasma vitamin C level on study day 18 compared to baseline (n=6, 0.90±0.5, Δ 0.33±0.6). This finding agrees with a high rate of participant compliance in taking supplements. However, participants in the PLC group also showed a small increase in plasma vitamin C level over the study period (n=6, 0.69±0.1, Δ 0.10±0.2) and a comparison of means revealed no significant difference between groups (p=0.873). This result is not consistent with the expectation that plasma vitamin C level would be

significantly greater for subjects receiving 1000 mg of vitamin C daily than for subjects receiving a wheat flour placebo. Subjects in both groups were instructed to not to make any changes to their usual dietary habits over the course of the 18-day study period, with the exception of avoiding fruit and vegetable juices. The observation that the mean plasma vitamin C level increased the PLC group suggests that subjects did alter their diets somewhat, even if these changes were not intentional.

Subjects in this study had a mean plasma vitamin C level that was lower than the US age-adjusted mean (VTC + PLC 0.55 mg/dL vs. US 0.86 mg/dL) (Schleicher et al., 2009, 1252-1263). This agrees with findings of previous studies that report an association between low plasma vitamin C status and asthma. Interestingly, subjects in this study had low plasma vitamin C levels despite reporting a relatively high dietary intake of vitamin C. Other authors have reported that low dietary vitamin C intakes were associated with the greatest risk of having asthma (Allen, Britton and Leonardi-Bee, 2009, 610). This group of study participants appears to have adequate dietary vitamin C intake, but lower than average vitamin C status.

Plasma sICAM-1 measures were not significantly different between groups at the end of the study period. Based on findings from Rayment and colleagues (2003) and others, plasma sICAM-1 was expected to be lower in the VTC group than the PLC group at the end of the study. It is possible that subject plasma vitamin C levels did not increase enough in the VTC group to induce a change. There is no established reference range for sICAM-1 in plasma. The manufacture's kit provides an expected value of 230.3 $\pm$ 47.4 ng/mL, based on a sample of 50 apparently healthy volunteers. Other investigators who

measured plasma sICAM-1 using ELISA report a wide range of values from 120 ng/mL to a 507.6 ng/mL in both healthy and diseased study populations. Canales and colleagues (2011) reported a baseline value of 507.6 ± 165.0 ng/mL among study participants who were at high risk for developing heart disease. Rayment (2003) and colleagues observed baseline levels of plasma sICAM-1 of 470±30 ng/mL among otherwise healthy subjects with lower than average serum vitamin C. Witkowska (2004) reviewed a number of studies measuring sICAM-1 in plasma in relationship to various disease states. In one of the studies reviewed by Witkowska, patients with angina had a baseline plasma sICAM-1 of 120±10 ng/mL. Another study reported a baseline plasma sICAM-1 of 373.27±183.5 ng/mL among heart transplant patients with graft rejection. The sICAM-1 values measured in the present study range from 64.4 to 121.6 ng/mL, with a mean value 89.89 ng/mL. It may be that these values are not directly comparable between studies due to variation in laboratory methods.

Based on the hypothesis that sICAM-1 levels in the nasal epithelium would be related to sICAM-1 levels in plasma, the sICAM-1 level in nasal lavage was expected to decrease in the VTC group following vitamin C supplementation. The mean nasal lavage sICAM-1 level was lower in the VTC group on study day 4 (-1.51±0.76 vs. 3.33±2.81, $p>0.05$), but the difference was not significant between VTC and PLC groups. Because of dilution from lavage fluids, nasal sICAM-1 levels were expected to be lower than plasma levels, and this expectation was met. Assessing sICAM-1 in lavage fluids has been previously reported in the literature. For example, Marguet and colleagues (2000) assessed sICAM-1 by ELISA in bronchoalveolar lavage specimens obtained from

children with airway disease. The range of values in this study was fairly wide, but the median value was 106 ng/mLwith an interquartile range of 94-257 ng/mL. Winther and colleagues (2002) measured nasal lavage sICAM-1 in a study of experimental HRV infection in healthy volunteers. Since the authors only reported their data for nasal lavage sICAM-1 graphically, exact values were not available. However, it is clear that there was a wide variation in sICAM-1 levels as the data was plotted on a logarithmic scale from 1 to100 ng/mL. The mean baseline value was between 1 and 10 ng/mL in Winther and colleagues' report. The mean baseline nasal lavage ICAM-1 value in this study was 2.5 ng/ mL, and appears generally similar to the observations made by Winter and colleagues. In this study plasma sICAM-1 level at baseline was significantly correlated with nasal sICAM-1 on study day 18. This suggests a relationship between endothelial shedding of ICAM-1 detected in blood and epithelial shedding of ICAM-1 detected in nasal lavage.

Limitations

This study has a number of limitations. One limitation is small sample size. Power analysis could not be completed for this research because the primary outcome measure of this study, change in sICAM-1 concentration in nasal lavage in response to a dietary intervention, has not been previously reported in the literature. It was one aim of this study to collect data to make power analysis calculations possible for future studies. Another limitation of this research is reliance on subjective self-report of symptom severity for both asthma and cold symptoms. Although an objective measure of asthma

symptoms exists, i.e. spirometry, pulmonary function tests, these measures were not included as outcomes in this study. An objective measure of HRV infection could have contributed to the strength of this study, however there is no clinically useful diagnostic test to easily identify HRV infection and a validated survey was used to identify presumptive colds based on symptoms. The subjects in this study were free-living volunteers and many measures relied on participant self-report without the ability to independently verify participant statements. Subjective measures, such as ratings of perceived symptom severity can vary widely from individual to individual.

Strengths

Strengths of this study include a robust experimental design. As a double-blind, placebo-controlled parallel arm clinical trial, this study was designed to address cause and effect research questions. Significant care was taken to maintain blinded conditions throughout the investigation. The study was not un-blinded until all data were scored and prepared for statistical analysis. Asthma patients, the target population for this study, have been identified as a group with poor vitamin C status compared to the general population. The asthmatic participants in this study had lower baseline plasma vitamin C than the general population. With minor exceptions, subjects appeared to have very good compliance with study protocols, taking 95.2% of study capsules and completing 98.9% of study questionnaires.

Conclusion and Application

This study was designed as a preliminary feasibility trial to assess the impact of vitamin C supplementation on sICAM-1 in nasal lavage and plasma of asthmatic study

participants. The primary end point of this study, change in sICAM-1 in nasal lavage, has not previously been measured in response to a dietary intervention. While the power of the statistical analysis in this study was limited related to small sample size and sample variability, a number of interesting, if not significant conclusions can be reached. The data collected in this study will provide a useful foundation for future work and enable a more refined approach to future studies. Although findings in this study did not reach the level of significance, some speculations are possible from the study results. For example, the greatest mean difference (not significant) between VTC and PLC groups appeared in sICAM-1 nasal lavage from study day 1 to day 4. This suggests that future study designs could be shortened while still capturing the physiological response to vitamin C supplementation.

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APPENDIX A

INFORMED CONSENT WITH IRB APPROVAL

To:	Carol Johnston ABC 139
From:	Carol Johnston, Chair Biosci IRB
Date:	04/05/2013
Committee Action:	**Expedited Approval**
Approval Date:	04/05/2013
Review Type:	Expedited F2 F4 F7
IRB Protocol #:	1303008998
Study Title:	Vitamin C supplementation and sICAM-1Concentrations in Asthmatic Adults
Expiration Date:	04/04/2014

The above-referenced protocol was approved following expedited review by the Institutional Review Board.

It is the Principal Investigator's responsibility to obtain review and continued approval before the expiration date. You may not continue any research activity beyond the expiration date without approval by the Institutional Review Board.

Adverse Reactions: If any untoward incidents or severe reactions should develop as a result of this study, you are required to notify the Biosci IRB immediately. If necessary a member of the IRB will be assigned to look into the matter. If the problem is serious, approval may be withdrawn pending IRB review.

Amendments: If you wish to change any aspect of this study, such as the procedures, the consent forms, or the investigators, please communicate your requested changes to the Biosci IRB. The new procedure is not to be initiated until the IRB approval has been given.

Please retain a copy of this letter with your approved protocol.

CONSENT FORM

Impact of a Nutritional Supplement on sICAM-1 Expression in Nasal Secretions of Asthmatics

INTRODUCTION

The purposes of this form are to provide you (as a prospective research study participant) information that may affect your decision as to whether or not to participate in this research and to record the consent of those who agree to be involved in the study.

RESEARCHERS

Dr. Carol Johnston, Associate Director and Professor, School of Nutrition and Health Promotion, and Lindsay Gnant, a graduate student, have invited your participation in a research study that will last 18 days.

STUDY PURPOSE

The purpose of this research is to evaluate the effect of a dietary supplement on markers of inflammation and asthma symptoms.

DESCRIPTION OF RESEARCH STUDY

You have informed us that you have been diagnosed by a doctor with asthma and that you currently suffer from asthma. If you decide to participate in this 18-day study you will join a study examining the effect of a dietary supplement on markers of inflammation in the respiratory tract and in the blood as well as on asthma symptoms. You will be randomly assigned (by a coin toss) to receive either a nutritional supplement or a placebo. Neither you nor the researcher you are working with will know if you were assigned to the supplement group or the placebo group. You will be asked to take a capsule twice a day during the study and to keep a record of respiratory symptoms each day during the study. You will be asked to not change your typical diet or physical activity patterns during the study and to not drink fruit/vegetable juice during the study. The study involves four visits to the test site at the ASU Nutrition Labs on the Phoenix Downtown Campus. To measure markers of inflammation, you will be asked to provide a fasting blood sample on three occasions and a nasal wash sample on three occasions. A trained phlebotomist or registered nurse will draw all blood samples; each sample of blood is about 2 tablespoons. The nasal wash procedure will be conducted under sterile conditions with distilled water. This procedure involves tilting your head back and allowing a small amount of saline solution (about 1 teaspoon for each nostril) to be placed inside your nose with a dropper. After saline is placed into each nostril, you will be asked to lift your head forward and allow the liquid to drain into a collection cup.

If you say YES, then your participation will last for 18 days at the Arizona State University Downtown Phoenix Campus. Approximately 20 of subjects will be participating in this study.

RISKS

Potential risks of participating in this study involve general risks associated with giving a blood sample such as bruising or irritation at the site of venipuncture and faintness. The nasal washing procedure may tickle your nose or make you sneeze. Side effects with nasal washing (which include death) have been related to the use of contaminated water; in this study, only distilled water will be used and aseptic procedures will be followed for preparing the saline solution. Ingestion of the dietary supplement may be associated with GI tract disturbances, which are usually alleviated when the supplement is ingested with food. As with any research, there is some possibility that you may be subject to risks that have not yet been identified.

BENEFITS

Although there are no direct benefits to you, the possible benefits of your participation in the research are that you are contributing to scientific knowledge regarding the efficacy of a dietary supplement on symptoms of asthma.

NEW INFORMATION

If the researchers find new information during the study that would reasonably change your decision about participating, then they will provide this information to you.

CONFIDENTIALITY
All information obtained in this study is strictly confidential unless disclosure is required by law. The results of this research study may be used in reports, presentations, and publications, but the researchers will not identify you. In order to maintain confidentiality of your records, Dr. Carol Johnston will assign you a participant number. Your name will not appear on any records aside from this consent form. This form will be kept in Dr. Carol Johnston's locked office to maintain your confidentiality.

WITHDRAWAL PRIVILEGE
It is OK for you to say no. Even if you say yes now, you are free to say no later, and withdraw from the study at any time. Your decision will not affect your relationship with Arizona State University or otherwise cause a loss of benefits to which you might otherwise be entitled.

COSTS AND PAYMENTS
The researchers want your decision about participating in the study to be absolutely voluntary. Yet they recognize that your participation may pose some costs related to time and travel. Participants will receive a $10 gift card to Target at visit #4.

COMPENSATION FOR ILLNESS AND INJURY
If you agree to participate in the study, then your consent does not waive any of your legal rights. However, no funds have been set aside to compensate you in the event of injury.

VOLUNTARY CONSENT
Any questions you have concerning the research study or your participation in the study, before or after your consent, will be answered by Carol Johnston, carol.johnston@asu.edu or (602)827-2265 and Lindsay Gnant, lstubbs@asu.edu.

If you have questions about your rights as a subject/participant in this research, or if you feel you have been placed at risk; you can contact the Chair of the Human Subjects Institutional Review Board, through the ASU Office of Research Integrity and Assurance, at 480-965 6788. This form explains the nature, demands, benefits and any risk of the project. By signing this form you agree knowingly to assume any risks involved. Remember, your participation is voluntary. You may choose not to participate or to withdraw your consent and discontinue participation at any time without penalty or loss of benefit. In signing this consent form, you are not waiving any legal claims, rights, or remedies. A copy of this consent form will be offered to you.

Your signature below indicates that you consent to participate in the above study.

ASU IRB Approved
Sign SM
Date 4/5/13 - 5/14/14

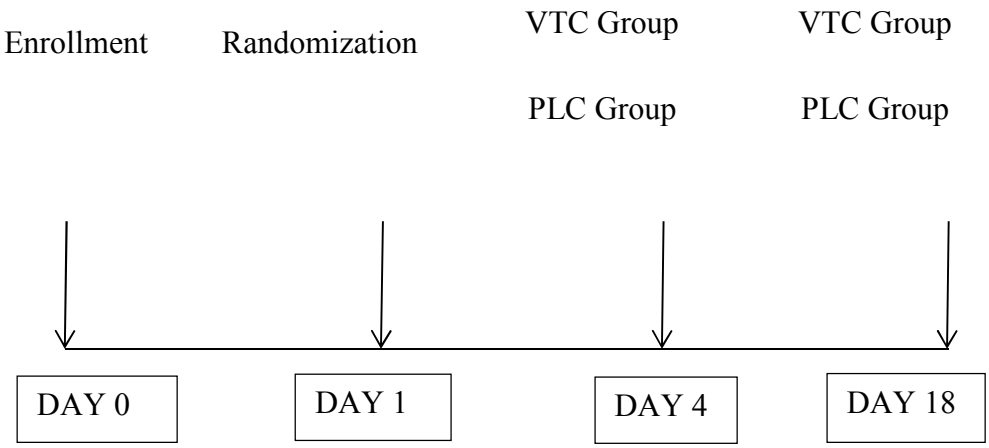
Subject's Signature _____ Printed Name _____ email _____ Date

INVESTIGATOR'S STATEMENT
"I certify that I have explained to the above individual the nature and purpose, the potential benefits and possible risks associated with participation in this research study, have answered any questions that have been raised, and have witnessed the above signature. These elements of Informed Consent conform to the Assurance given by Arizona State University to the Office for Human Research Protections to protect the rights of human subjects. I have provided (offered) the subject/participant a copy of this signed consent document."

Signature of Investigator_____ Date_____

APPENDIX B

METHODOLOGY TIMELINE



Study Visit 1	Study Visit 2	Study Visit 3	Study Visit 4
• Informed consent • NL • Blood Collection • Anthropometric Data • DSDS REAPS • WURSS-21 • Medical history • Vitamin C FFQ	• Blood Collection • NL • Study booklet distributed • Supplement or Placebo distributed	• NL	• Blood collection • NL • Study booklet collected • Vitamin C FFQ • Remaining supplement or Placebo Collected

VTC	Vitamin C Group, experimental arm
PLC	Placebo Group, control arm
NL	Nasal Lavage
DSDS	Daytime Symptom Diary Scale, asthma symptom questionnaire
REAPS	Rapid Eating Assessment for Participants short form, diet quality assessment
WURSS-21	Wisconsin Upper-Respiratory Symptom Survey-21, assessment for cold symptoms
FFQ	Food Frequency Questionnaire, assessment of dietary vitamin C intake

APPENDIX C

SUBJECT RECRUITMENT AND SCREENING SURVEY

Adults with Asthma Needed for ASU Asthma Trial

The ASU Nutrition Program is recruiting adults with doctor-diagnosed asthma (18-65 years of age).

This 18-day trial will examine whether a dietary supplement may improve inflammation and symptoms of asthma. If you are willing to provide 3 blood samples and 3 nasal washings, and to record respiratory tract symptoms daily for 18 days, you may be interested in this trial.

To apply for the study, please visit our recruitment site:
https://www.surveymonkey.com/s/ASUAsthmaStudy

For more information, contact Lindsay Gnant; lstubbs@asu.edu or Carol Johnston; Carol.Johnston@asu.esu.

ASU Asthma Study Recruitment Survey

*1. Please provide your e-mail address.

*2. Select your gender.
Male
Female

*3. Has your doctor diagnosed you with asthma?
Yes
No

*4. Do you currently experience symptoms of asthma?
Yes
No

*5. Do you currently smoke cigarettes?
Yes
No

*6. Do you take prescription medications every day to control your asthma?
Yes
No

*7. Do you use prescription medications (such as an inhaler) to treat your asthma symptoms when you have an asthma attack?
Yes
No

*8. Are you between 18 and 65 years old?
Yes
No

*9. Do you exercise vigorously over 4 times weekly and/or consider yourself a competitive athlete?
Yes
No
Not sure

*10. Do you weigh at least 110 pounds?
Yes
No
Not sure

*11. Are you willing to have a small amount of blood taken from an arm vein (<1 Tablespoon) on 3 occasions during the trial?

Yes
No
Not sure

*12. Are you willing to provide a nasal wash on 3 occasions during the trial? (This entails dripping sterile saline solution into both nostrils while your head is tilted back, waiting several moments, then tilting the head down and allowing the nasal wash to run into a cup.)

Yes
No
Not sure

*13. Are you currently being treated by a physician for a chronic disease or condition other than asthma (e.g., cancer, diabetes, arthritis, inflammatory bowel disease, heartdisease, hepatitis, etc.)?

Yes
No

*14. Do you regularly take vitamin/mineral supplements?

Yes
No

If yes, please list these supplements.

*15. Are you willing to (1) not drink any fruit or vegetable juice for 18 days and (2) not change your diet or activity level for 18 days?

Yes
No
Not sure

*16. Are you willing to record respiratory tract symptoms daily for 18 days?

Yes
No
Not sure

*17. Are you able to come to the ASU Downtown Phoenix Campus on 4 occasions in the next several months? (The ASU nutrition laboratories are located at 5th Street and Van Buren.)

Yes
No
Not sure

APPENDIX D

SUBJECT QUESTIONNAIRES

REAPS (Rapid Eating Assessment for Participants - Shortened Version)

CJSegal-Isaacson, EdD RD, Judy-Wylie-Rosett, EdD RD, Kim Gans, PhD, MPH

In an average week, how often do you:	Usually/ Often	Sometimes	Rarely/ Never	Does not apply to me
1. Skip breakfast?	O	O	O	
2. Eat 4 or more meals from sit-down or take out restaurants?	O	O	O	
3. Eat less than 2 servings of whole grain products or high fiber starches a day? **Serving** = 1 slice of 100% whole grain bread; 1 cup whole grain cereal like Shredded Wheat, Wheaties, Grape Nuts, high fiber cereals, oatmeal, 3-4 whole grain crackers, ½ cup brown rice or whole wheat pasta, boiled or baked potatoes, yuca, yams or plantain.	O	O	O	
4. Eat less than 2 servings of fruit a day? **Serving** = ½ cup or 1 med. fruit or ¾ cup 100% fruit juice.	O	O	O	
5. Eat less than 2 servings of vegetables a day? **Serving** = ½ cup vegetables, or 1 cup leafy raw vegetables.	O	O	O	
6. Eat or drink less than 2 servings of milk, yogurt, or cheese a day? **Serving** = 1 cup milk or yogurt; 1½ - 2 ounces cheese.	O	O	O	
7. Eat more than 8 ounces (see sizes below) of meat, chicken, turkey or fish per day? ***Note**: 3 ounces of meat or chicken is the size of a deck of cards or ONE of the following: 1 regular hamburger, 1 chicken breast or leg (thigh and drumstick), or 1 pork chop.*	O	O	O	Rarely eat meat, chicken, turkey or fish O
8. Use regular processed meats (like bologna, salami, corned beef, hotdogs, sausage or bacon) instead of low fat processed meats (like roast beef, turkey, lean ham; low-fat cold cuts/hotdogs)?	O	O	O	Rarely eat processed meats O
9. Eat fried foods such as fried chicken, fried fish, French fries, fried plantains, tostones or fried yuca?	O	O	O	
10. Eat regular potato chips, nacho chips, corn chips, crackers, regular popcorn, nuts instead of pretzels, low-fat chips or low-fat crackers, air-popped popcorn?	O	O	O	Rarely eat these snack foods O
11. Add butter, margarine or oil to bread, potatoes, rice or vegetables at the table?	O	O	O	
12. Eat sweets like cake, cookies, pastries, donuts, muffins, chocolate and candies more than 2 times per day.	O	O	O	
13. Drink 16 ounces or more of non-diet soda, fruit drink/punch or Kool-Aid a day? ***Note:** 1 can of soda = 12 ounces*	O	O	O	
	YES			**NO**
14. You or a member of your family usually shops and cooks rather than eating sit-down or take-out restaurant food?	O			O
15. Usually feel well enough to shop or cook.	O			O

16. How willing are you to make changes in your eating habits in order to be healthier?	1 Very willing	2	3	4	5 Not at all willing

HEALTH HISTORY QUESTIONNAIRE ID#_____

Height _____ Weight _____
to be completed by investigator

1. Gender: M F

2. Age: _____

3. What year were you diagnosed by a physician for asthma? _____
Have you experienced asthma-related symptoms in the recent months? _____ Yes _____ No
Describe your asthma status:

_____
_____
_____
_____

4. Ethnicity: (please circle one) Native American African-American Caucasian Hispanic Asian Other

5. Education (please circle): HS diploma Current college student BS degree MS degree PhD degree

6. Do you smoke? _____ No, never
_____ Yes # Cigarettes per day = _____
_____ I used to, but I quit _____ months ago [or _____ years ago]

7. Do you take steroidal drugs for asthma (such as……..) _____ Yes _____ No

8. Do you take any medications regularly? _____ Yes ____No *If yes, list type and frequency:*

Medication	month/year initiated	Dosage	Frequency

9. Do you currently take supplements (vitamins, minerals, herbs, etc.)? _____ Yes _____ No
If yes, list type and frequency:

Supplement	month/year initiated	Dosage	Frequency

10. Are you currently being treated by a physician for any condition other than asthma? ____ Yes ____ No
If yes, please elaborate _____
_____

11. How would you rate your lifestyle?
Not active _____ Active _____ Somewhat active _____ Very Active _____

-----Please turn over-----

12. During the previous **7-day period** (one week), how many times on the average did you do the following kinds of exercise for **more than 15 minutes** during your free time?

Light activities:
(e.g., yoga, archery, fishing from river bank, bowling, horseshoes, golf, snow-mobiling, easy walking)
Times per week: 0 1 2 3 4 5 6 7 8 9 10+

Moderate activities:
(e.g., fast walking, baseball, tennis, easy bicycling, volleyball, badminton, easy wimming, alpine skiing, popular and folk dancing)
Times per week: 0 1 2 3 4 5 6 7 8 9 10+

Vigorous activities:
(e.g., running, jogging, hockey, football, soccer, squash, basketball, cross country skiing, judo, roller skating, vigorous swimming, vigorous long distance bicycling)
Times per week: 0 1 2 3 4 5 6 7 8 9 10+

13. Do you consider yourself a competitive athlete? _____ Yes _____ No

14. How would you rate your lifestyle?
Not active _____ Active _____
Somewhat active _____ Very Active _____

15. Do you have any food allergies? Yes No If yes, explain: _____

16. How many times per week do you drink fruit or vegetable juice? _____

17. Circle how often you eat these foods (circle appropriate frequency):

- Cruciferous vegetables (broccoli, kale, Brussels sprouts, cauliflower, cabbage, collard greens, turnip greens, asparagus) daily.....3-6x/wk.....1-2x/wk.....3x/month.....2x/month.....1x/month.....6-12x/yr.....less
- Melons (cantaloupe, honeydew, watermelon, etc) daily.....3-6x/wk.....1-2x/wk.....3x/month.....2x/month.....1x/month.....6-12x/yr.....less
- Citrus fruits (oranges, grapefruit, lemons, etc) daily.....3-6x/wk.....1-2x/wk.....3x/month.....2x/month.....1x/month.....6-12x/yr.....less
- Citrus juices daily.....3-6x/wk.....1-2x/wk.....3x/month.....2x/month.....1x/month.....6-12x/yr.....less
- Strawberries daily.....3-6x/wk.....1-2x/wk.....3x/month.....2x/month.....1x/month.....6-12x/yr.....less
- Papaya, mangos, kiwi (including juices) daily.....3-6x/wk.....1-2x/wk.....3x/month.....2x/month.....1x/month.....6-12x/yr.....less
- Peppers (sweet green, red, yellow, hot green chili, hot red chili, jalapeno) daily.....3-6x/wk.....1-2x/wk.....3x/month.....2x/month.....1x/month.....6-12x/yr.....less
- Highly fortified breakfast cereals (total, all bran, 100% bran, honey buckwheat crisp, bran buds, product 19, ovaltine, maypo, instant breakfast, etc) daily.....3-6x/wk.....1-2x/wk.....3x/month.....2x/month.....1x/month.....6-12x/yr.....less
- Fortified energy/fitness bars/drinks (power bars, Powerade, etc) daily.....3-6x/wk.....1-2x/wk.....3x/month.....2x/month.....1x/month.....6-12x/yr.....less

Wisconsin Upper Respiratory Symptom Survey – 21 --- *Daily Symptom Report*

Day:	*Date:*	*Time:*	*ID:*

Please fill in one circle for each of the following items:

	Not sick	Very mildly		Mildly		Moderately		Severely
	0	1	2	3	4	5	6	7
How sick do you feel today?	O	O	O	O	O	O	O	O

Please rate the **average severity of your cold symptoms over the last 24 hours** for each symptom:

	Do not have this symptom	Very mild		Mild		Moderate		Severe
	0	1	2	3	4	5	6	7
Runny nose	O	O	O	O	O	O	O	O
Plugged nose	O	O	O	O	O	O	O	O
Sneezing	O	O	O	O	O	O	O	O
Sore throat	O	O	O	O	O	O	O	O
Scratchy throat	O	O	O	O	O	O	O	O
Cough	O	O	O	O	O	O	O	O
Hoarseness	O	O	O	O	O	O	O	O
Head congestion	O	O	O	O	O	O	O	O
Chest congestion	O	O	O	O	O	O	O	O
Feeling tired	O	O	O	O	O	O	O	O

Over the **last 24 hours**, how much has your cold interfered with your ability to:

	Not at all	Very mildly		Mildly		Moderately		Severely
	0	1	2	3	4	5	6	7
Think clearly	O	O	O	O	O	O	O	O
Sleep well	O	O	O	O	O	O	O	O
Breathe easily	O	O	O	O	O	O	O	O
Walk, climb stairs, exercise	O	O	O	O	O	O	O	O
Accomplish daily activities	O	O	O	O	O	O	O	O
Work outside the home	O	O	O	O	O	O	O	O
Work inside the home	O	O	O	O	O	O	O	O
Interact with others	O	O	O	O	O	O	O	O
Live your personal life	O	O	O	O	O	O	O	O

Compared to yesterday, I feel that my cold is…

Very much better	Somewhat better	A little better	The same	A little worse	Somewhat worse	Very much worse
O	O	O	O	O	O	O

Please list any products (including prescription or over-the-counter medicines, herbal preparations or supplements, and/or lozenges) taken to relieve respiratory symptoms.

Name	Dosage	Time(s) taken

Daytime Symptom Diary Scale questions

1) How often did you experience asthma symptoms today?

0	1	2	3	4	5	6
None of the time						All of the time

2) How much did your asthma symptoms bother you today?

0	1	2	3	4	5	6
Not at all bothered						Severely bothered

3) How much activity could you do today?

0	1	2	3	4	5	6
More than usual activity						Less than usual activity

4) How often did your asthma affect your activities today?

0	1	2	3	4	5	6
None of the time						All of the time

Nocturnal Diary Scale question

1) Did you wake up with asthma symptoms. (This can be awakening in the middle of the night or on awakening in the morning)?

❏ No ❏ Once ❏ More than once ❏ Awake "all night"

Daytime Symptom Diary Scale and Nocturnal Diary Scale (developed by N.C. Santanello et al.)

APPENDIX E

LABORATORY PROTOCOLS FOR PLASMA VITAMIN C

Reagent Preparation:

Store all reagents under refrigeration prior to use.

1. Prepare 10% trichloroacetic acid (TCA) solution

   Mass 10 g TCA, add deionized water to a volume of 100 mL

2. Prepare 5% TCA

   Mass 5 g TCA, add deionized water to a volume of 100 mL

3. Prepare 9N H_2SO_4

   Measure 300 mL deionized water, slowly add to a volume of 100 mL 36N H_2SO_4

4. Prepare 2,4-dinitrophenlylhydrazine, tiourea, copper sulfate (DTC) reagent

   Mass: 0.9 g 2,4-dinitrophenlylhydrazine

   0.12 g thiourea

   0.015 g copper sulfate pentahydrate

   Combine in a 50 ml beaker with stir bar, slowly add 30 mL 9N H_2SO_4

   Stir until dissolved

5. Prepare ascorbic acid stock solution

   Mass 5 mg ascorbate, add 5% TCA to a volume of 25 mL

   Store 1 mL aliquots at -80°C

6. Prepare working ascorbic acid solution immediately prior to running assay

   Pipette 500 µL acorbic acid stock solution, add 5% TCA to a volume of 25 mL

Specimen Collection: 7 mL lavender top tube

Whole blood may be maintained for a short period of time at room temperature on a rocker prior to processing.

Specimen Processing:

1. Spin down whole blood in a refrigerated centrifuge at 4° C at 2800 RPM for 10 minutes
2. Pipette 2.5 mL 10% TCA into a glass tube, place the tube in ice.
3. Add 2.5 mL plasma to the tube containing 2.5 mL 10% TCA
4. Vortex for 10 seconds to mix
5. Spin down sample in a refrigerated centrifuge at 4° C at 4000 RPM for 20 minutes.
6. Divide supernatants into 1.5 mL aliquots and freeze at -80°C immediately

Assay Procedure:

1. Prepare 6 ascorbic acid standards in duplicate in glass test tubes according to the following table:

Standard	Ascorbic Acid Working Solution (µL)	5% TCA (µL)	DTC (µL)
1 (0 mg/dL)	0	500	100
2 (0.4 mg/dL)	100	400	100
3 (0.8 mg/dL)	200	300	100
4 (1.2 mg/dL)	300	200	100
5 (1.6 mg/dL)	400	100	100
6 (2 mg/dL)	500	0	100

2. Prepare samples in duplicate:

   Pipette 500 µL sample and 100 µL DTC reagent into a glass test tube

3. Cover test tubes to prevent evaporation. Incubate samples and standards together at 37°C for 3 hours.

4. Add 750 μL ice cold 65% H_2SO_4 to each tube. Vortex for 5 seconds after each addition.
5. Incubate at room temperature for 30 minutes
6. Read absorbance at 520 nm in spectrophotometer, using standard #1 as a blank
7. Produce a standard curve from using standards 1-6. Plot ascorbic acid concentration (mg/dL) on the horizontal axis and absorbance on the vertical axis. Use a linear model to produce a line of best fit. Calculate sample ascorbic acid concentration in mg/dL from the standard curve.