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Evaluating the impact of inflammation caused by Norovirus on vitamin A biomarker concentrations

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B.S.
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Abstract

Evaluating the impact of inflammation caused by norovirus on vitamin A biomarker concentrations

By Courtney Poulos Victor

Background: As of 2018, there are still millions of individuals who are vitamin A deficient worldwide, which causes adverse health outcomes such as ocular manifestations and impaired immune system function. While it is known that inflammation has an impact on measurements of vitamin A biomarkers, there is no standardized approach to adjust assessments for inflammation.

Goal: The goal of this research was to evaluate the impact of inflammation caused by norovirus infection on vitamin A biomarkers, retinol and retinol binding protein.

Methods: Using longitudinal data collected from 52 individuals in two separate norovirus challenge studies, we assessed the molar relationship between retinol and retinol binding protein, compared the methodology and utility in the Thurnham and Biomarkers Reflecting Inflammation and Nutritional Determinants of Anemia (BRINDA) inflammation-adjustment approaches on longitudinal data, and assessed the kinetics of serum retinol over the course of an infection using linear-mixed methods.

Results: The average ratio between retinol and RBP was 0.69 (IQR=0.12), which violates popular assumptions but corroborates findings from other studies. While both the BRINDA and Thurnham-adjustment approaches were appropriate using longitudinal data, the mild inflammatory response resulted in 0 or 1 individual in the early convalescence or late period in 7 of the time points. Thus, we were unable to compare the results of the Thurnham correction factor approach. Although the BRINDA-adjustment initially was successful in correcting retinol towards baseline on days 2, 3, and 4 post-exposure, retinol was significantly different (1.02-1.12 μmol/L) from baseline on days 4 ($p=0.03$), 7 ($p<0.001$), 14 ($p<0.001$), and 35 ($p=0.01$). Lastly, we were able to demonstrate a 0.01 μmol/L reduction in retinol as a result of inflammation by day post-exposure ($p=0.03$).

Conclusions: The results of this study demonstrate (1) the presumed molar 1:1 ratio is not upheld even in healthy populations subjected to a norovirus challenge, (2) the BRINDA approach was more appropriate than Thurnham in the face of low-levels of inflammation, and (3) modeling indicated an overall effect of inflammation by day post-exposure on changes in retinol concentration.

Public Health Implications: Because we found that the ratio between retinol and RBP was not equimolar, future assessments that use RBP as a proxy to measure vitamin A levels should consider adjusting the cutoff values for VAD. While both correction methods are appropriate for longitudinal analysis, adjustment for inflammation in populations with low-levels of inflammation should be conducted using the BRINDA approach. Finally, we demonstrated that consideration for inflammation is needed even in populations with low-levels of inflammation as it can still alter serum retinol levels.

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Literature Review

As of 2018, there are still millions of individuals who are vitamin A deficient worldwide. In 2009, the World Health Organization (WHO) synthesized a full report on Global Vitamin A Deficiency (VAD) in at-risk populations using their own global database on VAD (WHO, 2009). They defined populations at-risk of VAD as pregnant women and children, as adequate vitamin A levels are most important for these groups as they have increased physiological requirements for vitamin A. Prevalence of moderate to severe nightblindness, caused by VAD, was deemed a significant public health problem in 66 and 45 countries for pregnant women and preschool children (WHO, 2009), respectively. They also evaluated vitamin A levels biochemically using measures of serum retinol. In this respect, 122 countries and 88 countries were classified as having a significant VAD problem for pre-school children and pregnant women, respectively (WHO, 2009). The report did not address pregnant women and children in countries where the GDP was greater than $15,000 USD. However, a study conducted by Hanson and colleagues in 2016 found that many women of childbearing age in the United States of low socioeconomic status had lower than recommended level of 1.05 μmol/L serum retinol concentrations (Hanson, Lyden, Abresch, & Anderson-Berry, 2016). This study points to the need for further evaluation of adults for VAD in high-income settings.

Most populations evaluated in primary research on VAD are pregnant women and children; little is known about the biological importance for vitamin A in other groups (adult males, adolescents, and teenagers). As mentioned earlier, VAD can cause many problems within populations in addition to ocular manifestations; those who are vitamin A-deficient have an increased suceptibility to infection (Tanumihardjo et al., 2016). This

is of public health significance as, in settings where vitamin A deficiency is most prevalent, so is the risk of infection. These issues can occur in individuals of any age, and are not restricted to only pregnant women and children.

Vitamin A Overview

Vitamin A is an essential nutrient, meaning it cannot be produced by the body, and thus must be obtained from external sources in adequate amounts to maintain several important bodily processes. Vitamin A represents a group of different organic compounds, including retinol, retinal, and retinoic acid which are necessary for many normal homeostatic functions, including vision, maintenance of epithelium, proper immune function, and embryonic growth and development (Tanumihardjo et al., 2016). This nutrient can come from multiple sources in the environment, each of which are important during different life stages. For infants, the majority of their vitamin A intake comes from breast milk, which includes preformed vitamin A and vitamin A carotenoids (Tanumihardjo et al., 2016). From the adolescent stage onward, vitamin A can be consumed from the environment in the form of retinyl esters from animal sources such as dairy products, liver, and fish liver oils (Tanumihardjo et al., 2016). Vitamin A can also be consumed in the form of provitamin A carotenoids through consumption of dark-green leafy vegetables and yellow and orange vegetables and fruit (i.e., corn, citrus fruits, carrots) (Tanumihardjo et al., 2016). Additionally, in instances where there is inadequate intake of vitamin A-rich foods, vitamin A can be provided in the form of capsules (supplementation) or in fortified foods. Food fortification is more cost-efficient, and this method has been employed in high-income settings for decades (Dary & Mora, 2002).

Fortified foods may come in the form of cereals (Dary & Mora, 2002), oils (Dary & Mora, 2002), sugar (Pineda) and noodles (Smitasiri & Solon, 2005).

Since vitamin A is necessary for proper maintenance of many different bodily functions at various life stages, it follows that vitamin A deficiency can lead to a host of problems. The most severe of these is a group of disorders called xeropthalmia. Xeropthalmia is an encompassing term used to describe different types of ocular manifestations including night blindness (Tanumihardjo et al., 2016). Other issues that result from VAD include decreased resistance to infection, which subsequently could lead to further depletion of vitamin A stores in the body, as infections can lead to reduced absorption of nutrients due to intestinal inflammation, diarrhea, and vomiting (Bresnahan & Tanumihardjo, 2014). Additionally, prolonged VAD can result in problems with epithelial surface maintenance, immune competence, reproduction, and growth and development in children (Blomhoff & Blomhoff, 2006).

To monitor the effectiveness of interventions through evaluating the status of VAD prevalence among millions worlwide, research is being done to assess vitamin A status on a population level. A standardized and accurate method to evaluate vitamin A status on a population level is necessary not only to evaluate deficiency, but also to ensure that excessive intake of vitamin A is not occurring. Too much vitamin A could lead to increased risk for developing bone abnormalities, osteoporosis, liver fibrosis, and liver cirrhosis (Tanumihardjo et al., 2016). Further, since vitamin A has a major role in immune function; too much vitamin A could lead to altered immune function (Tanumihardjo et al., 2016). This is especially important in countries like the United States, where food fortification has been ongoing for the past 80 years; fortified foods

includesnack foods, beverages, cereals, margarine and processed dairy foods (Dary & Mora, 2002).

Vitamin A Measurement

There are many different strategies for assessing vitamin A levels among individuals and populations. While liver biopsies are the gold-standard for measuring vitamin A (Tanumihardjo, 2011), this is not practical to use when measuring vitamin A status on a population level. Other methods are presented below, and each has associated advantages and disadvantages. The first category of techniques involves assessing the function of the eye itself, such as dark adaptation testing and electroretinography. Vitamin A deficiency manifests in the form of vision problems. Therefore, evaluating function of the eye is a phenotypic representation of vitamin A status. Within this category of eye function, the earliest method developed for this purpose is called classical dark adaptation testing, and eventually, results led to the establishment of dietary requirements for vitamin A (Medical Research Council (Great Britain), 1949). This method utilizes eye dilation and a series of flash tests to evaluate light intensity detection by the subject (Tanumihardjo et al., 2016). One benefit of this strategy is that it will reveal vitamin A deficiency prior to the subject developing night blindness (Russell, Multack, Smith, Krill, & Rosenberg, 1973). However, this method requires sophisticated equipment and a controlled environment; thus it is not feasible to use in field studies (Russell, Multack, Smith, Krill, & Rosenberg, 1973). Further, since it requires a significant amount of concentration, children often do not perform well with this test (Tanumihardjo et al., 2016). Another disadvantage of this test is that it ignores several potential confounders that also cause eye disorders such as age-related issues (McFarland & Fisher, 1955) and zinc/protein

deficiency (Morrison, Russell, Carney, & Oaks, 1978). This technique was later improved upon and renamed “rapid dark adaptation testing” as it requires less equipment and can be done in a shorter period of time (Tanumihardjo et al., 2016). Although research is being done to develop a field-friendly version of this test (Labrique et. al, 2015), this method currently faces the same disadvantages as classical adaptation testing and is not feasible for use in a population study.

Another physical assessment method of eye function that can be used as a proxy for an individual’s vitamin A stores is electroretinography. This technique is invasive, as it requires physical contact with the eye using a probe to measure the physical response of the retina to light (Tanumihardjo et al., 2016). Although electroretinography has the benefit of being completely objective, it has not been tested extensively and is inappropriate to use in the field and on children (Tanumihardjo et al., 2016). Pupillary threshold testing is also objective with the added advantage that it can be conducted in the field and on children (Tanumihardjo et al., 2016). Like with dark adaptation testing, this test evaluates the pupillary reflex to light exposure to establish a threshold. Additionally, electroretinography has the same issues with confounders as dark adaptation testing and has not been widely tested (Tanumihardjo et al., 2016).

The second category for assessing vitamin A levels is utilization of biochemical indicators to evaluate status of vitamin A levels. While assessment of eye functionality may provide information about individuals or populations who are severely deficient, it is difficult to ascertain levels in those who are only marginally deficient and do not show clinically overt symptoms (Tanumihardjo, 2012). In order to best assess status of a population and target interventions, it is best to understand when there is a problem

before it reaches critical levels. Thus, biochemical indicators provide a more accurate and detailed assessment of vitamin A status compared to physical assessments of eye function.

To understand the concepts behind these biochemical indicator tests, it is important to understand how vitamin A is metabolized and regulated in the body. Since vitamin A metabolites are fat-soluble, they need to be hydrolyzed and mixed with lipids in the stomach in order for them to be absorbed into the intestinal lumen. About 70-90% of ingested preformed vitamin A esters are absorbed and subsequently either used or stored (Blomhoff & Blomhoff, 2006). These mixed compounds are referred to as micelles, and after they are absorbed into the lumen of the intestines, they are cleaved by an enzyme to produce retinol. Retinol is then converted to retinyl esters and enters through the lymph entrance to the bloodstream in a compound called chlyomicra, which is made up of alpha and beta carotene. Whatever is not used after entering the blood stream is subsequently stored in the liver (Tanumihardjo et al., 2016). Afterwards, retinyl esters are hydrolyzed again and subsequently bound to transport protein, retinol binding protein (RBP), and released into the plasma, or re-esterfied and stored (Tanumihardjo et al., 2016). When liver reserves of vitamin A are low, RBP accumulates in the liver (Tanumihardjo et al., 2016). Once adequate levels of vitamin A are consumed, RBP is bound to retinol and released from the liver (Tanumihardjo et al., 2016). Thus, serum concentrations of RBP can be used as a proxy for assessing retinol levels.

One quantitative, biochemical technique to assess vitamin A levels is called a relative-dose-response test. This test operates on the aforementioned principal that RBP is only released from the liver when there is sufficient intake of vitamin A and input of retinyl

esters to the liver (Tanumihardjo, 2012). First, a patient is administered a dose of tagged retinyl ester and then serum samples are collected at baseline and approximately five hours after the dose (Tanumihardjo, 2012). Since this test is invasive and highly dependent on accurately and consistently measuring retinol concentrations in both serum samples, Tanumihardjo and colleagues developed a modified version of this test (Tchum et al., 2006) that does not require a baseline blood draw and tested it in several countries (Tanumihardjo & Olson, 1988). An advantage of the modified relative dose response (MRDR) test is that it can provide more information than just measuring serum retinol concentrations, as serum retinol does not change in response to supplementation of vitamin A unless there is severe deficiency (Tchum et al., 2006). Additionally, measured serum retinol concentrations will be within the range of MRDR test results. Thus, these tests will be in agreement when an individual is vitamin A deficient or has adequate levels of vitamin A. However, although it is useful for defining deficient vs. adequate vitamin A status, the MRDR test is a qualitative, categorical measurement and does not quantitatively define vitamin A status. Further, the test is unable to assess hypervitaminosis A (Tchum et al., 2006). This has important consequences for population assessments in places where VAD is not as prevalent, and toxic liver reserves may be of concern.

Retinol and Retinol Binding Protein

Retinol and retinol binding protein, both biomarkers for vitamin A, are commonly used to assess vitamin A status and offer several advantages over the previously mentioned techniques. These measurements differ from the MRDR test in that actual levels of serum retinol and retinol binding protein are being measured, without administration of a

challenge dose. Measuring serum retinol concentrations to assess vitamin A status is one of the most widely used techniques worldwide. In addition to ease of use compared to other methods previously mentioned, one benefit to measuring serum retinol concentration is the fact that these measures will not decrease unless the individual is significantly deficient, as serum retinol is homeostatically controlled (de Pee & Dary, 2002). When liver vitamin A stores are depleted and an individual is truly vitamin A deficient, then serum retinol levels within this individual also begin to decrease (Stephensen & Gildengorin, 2000). However, the homeostatic control also presents a challenge as serum retinol levels do not reflect when an indivdiual has an excess of vitamin A. Another benefit is that due to the wide use of this technique for measuring vitamin A status in populations, there is an established cutoff for serum retinol concentration of <0.7 umol/L, and when 20% of the children in the population being measured is below this cutoff, vitamin A inadequacy is deemed a public health problem (WHO, 2011). This allows for standardized determination of VAD when utilizing measurement of serum retinol concentrations. However, there are some associated difficulties with measuring serum retinol concentrations. One of these difficulties is related to the tests themselves, one of which is high performance liquid chromatography (HPLC) (de Pee & Dary, 2002). HPLC is a technique used in analytical chemistry which separates, identifies, and quantifies components in a sample (i.e., blood serum). This method may be difficult in field settings, as HPLC analysis requires a venous blood draw and cold-chain transport to a lab for assessment (Tanumihardjo et al., 2016). Further, it is more expensive to use on a population level.

An example of a less invasive technique for measuring biochemical indicators of vitamin A is using dried blood spots as a surrogate to measure serum retinol concentrations (Tanumihardjo et al., 2016). One would not need to draw venous blood, just prick the fingertip of the individual being tested, which is less invasive and less complicated to do in field settings (Tanumihardjo et al., 2016). Additionally, it is also a rapid test that can be conducted quickly on a large number of individuals and does not require constant refrigeration as a venous blood sample would. Although the results may be comparable to that of an HPLC test, there is often a correction factor needed to account for variability in using such a small amount of blood (Tanumihardjo et al., 2016). Further, after a week, retinol concentrations in the dried blood spot tend to decrease and this varies among individuals, which can be much harder to correct for (Tanumihardjo et al., 2016). Breast-milk can also be used as a surrogate for measuring retinol concentrations. However, the amount of retinol varies with the amount of fat in the milk and is only useful in populations where breast-feeding is common (Tanumihardjo et al., 2016). Thus, retinol concentrations derived from breast-milk may not also be an accurate measurement that can be extrapolated when other subsets of the population are included, as using breast milk would only be relevant for breastfeeding mothers and their babies.

Another biochemical indicator that can be used to assess vitamin A status is Retinol Binding Protein (RBP), which is an inflammatory protein biomarker that can be thought of as a proxy for retinol. Under normal conditions, there is assumed to be a 1:1 ratio of retinol to RBP (de Pee & Dary, 2002). Despite this, there is no currently accepted cut-off for RBP levels by WHO to define population-level deficiency. During times of deficiency, RBP can accumulate in the liver and be used as a surrogate measurement

when retinol is limited (Tanumihardjo, 2012). However, this measurement can be confounded by other micronutrient deficiencies. When an individual is zinc-deficient, the liver will not synthesize RBP (Tanumihardjo et al., 2016). One benefit to this measurement is the ability to use immunologic assays to detect RBP, as it is a protein. This is less costly than using chromatography to measure retinol (de Pee & Dary, 2002).

In addition to the previously mentioned hindrances, there are several other difficulties associated with measuring both serum retinol concentrations and retinol binding proteins as assessments of vitamin A status. First, retinol and RBP are static measures of vitamin A status. This means that serum levels may not change immediately in response to increased intake of vitamin A, which is less useful when assessing response to supplementation interventions by measuring these biomarkers (Tanumihardjo, 2012). Secondly, the presumed 1:1 ratio between retinol and retinol binding protein may not be accurate as these biomarkers could be differentially impacted by malnutrition, liver disease, renal failure, and infection (de Pee & Dary, 2002). For example, malnutrition and other micronutrient deficiences can supress serum retinol concentrations and can cause decreased production of retinol binding protein (WHO, 2011). Since both biomarkers are impacted by these factors, one might expect that the 1:1 ratio between retinol and retinol binding protein may not be impacted, and measuring retinol binding protein would still be an accurate assessment of vitamin A status. However, more research needs to be done to assess the relationship between retinol and retinol binding protein under conditions of infection and inflammation.

Vitamin A Measurements and Infection

Vitamin A has historically been called the “anti-infective vitamin” as it plays several key roles in immune function (Semba, 1994). These include enhancement of leukocyte function, adaptive immunity, development of T-helper, T-regulatory, and B-cells, and leukocyte homing and T-regulatory function (Semba, 1994). Unfortunately, populations that have high levels of infection and thus are in most need of a functioning immune system, are also known to be vitamin A deficient (de Pee & Dary, 2002). This creates a vicious cycle in that individuals who are vitamin A deficient are more susceptible to infections, which subesquently deplete their vitamin A levels even more. The important role vitamin A plays in proper immune function highlights the necessity to accurately assess vitamin A status in populations who have high levels of infection.

As mentioned previously, it is known that inflammation caused by infection impacts serum retinol concentrations (Stephensen & Gildengorin, 2000). Inflammation induces the acute phase response to an infection, which increases levels of acute phase proteins such as C-reactive protein (CRP). Stephensen and Gildengorin describe how inflammation, defined by elevated levels of CRP during the acute phase response, can transiently decrease serum retinol concentrations (Stephensen & Gildengorin, 2000). Consequently, serum concentrations of RBP are also impacted (Tanumihardjo, 2012). It is important to note that acute phase response-induced changes of serum retinol and RBP concentrations does not reflect changes in actual liver stores of vitamin A and thus can cause inaccurate measurements of vitamin A status in populations that have high levels of infection. The end result is misclassification of vitamin A deficiency, with a higher likelihood of being classified of vitamin A deficient when one is not actually deficient (Stephensen & Gildengorin, 2000). While the current cut-offs for vitamin A deficiency

are 0.7 and 1.05 μmol/L in children and women respectively, the cut-offs are more likely 0.35 and 0.7 μmol/L in the presence of infection (Tanumihardjo et al., 2016). Larson and colleagues examined 22 nationally representative surveys to assess the relationship between retinol or RBP between CRP and AGP in women of reproductive age and in pre-school children. They found that in women of reproductive age, there was no significant association between either retinol or RBP and CRP or AGP. However, in children, the association between inflammation and retinol or RBP was consistent and statistically significant (Larson et al., 2018).

In order to accurately determine vitamin A status in a population, inflammation should be accounted for in biomarker measurements, as it has been demonstrated that inflammation can alter biomarker measurements for several micronutrients in a way that does not reflect the true micronutrient status of the individual (Tanumihardjo, 2012). One such example is how inflammation alters assesssment of iron status using ferritin. Ferritin, a protein used to measure iron stores, is also an acute phase protein and impacted by infection. In contrast to vitamin A measurements, however, ferritin production is increased during inflammation, subsequently causing overestimation of iron stores (Namaste et al., 2017). In attempt to correct for this, a group of researchers developed the Biomarkers Reflecting Inflammation and Nutritional Determinants of Anemia (BRINDA) project. The goals of this project were to assess a number of questions including whether inflammation adjustment was necessary, the necessity of using both CRP and AGP (α(1)-acid glycoprotein), both acute phase proteins, in adjustment, and the best method for adjusting for inflammation in micronutrient biomarker measurements (BRINDA, 2017). A standardized protocol was developed to correct biomarker measurement based on CRP

and AGP levels; this approach has been adopted by the scientific community in this field in its use for adjustment of ferritin measurements.

While the BRINDA approach has been evaluated and approved by WHO for adjustment of ferritin, more research still needs to be done using other inflammation-adjustment techniques to determine when and how to use CRP and AGP measurements to adjust measures of retinol or retinol binding protein. Although the BRINDA correction has been previously assessed for adjusting retinol and RBP (Larson et al., 2018), other approaches to adjust for inflammation have been proposed. Another method that is used for adjusting for inflammation on both ferritin and vitamin A biomarkers is David Thurnham's approach. This approach allows for the categorization of individuals into different groups based on stage of inflammation: reference (no inflammation), incubation, early convalescense, and late convalescense (Thurnham, Northrop-Clewes, & Knowles, 2015). This method operates on the principle that the effect of infection and inflammation on vitamin A status is affected by severity and duration of the illness (Thurnham, Northrop-Clewes, & Knowles, 2015). By comparing the BRINDA approach and the Thurnham approach on the same data, more evidence will be provided to determine the most accurate method for adjusting for inflammation when measuring biomarkers for vitamin A status.

Due to the compounded effects of malnutrition and infection, it is critical to ensure that assessments of population vitamin A status are accurate. First, more research is needed to determine whether the ratio between retinol and RBP changes over the course of an infection or in response to inflammation. Further, comparing inflammation adjustment methods can provide more evidence to determine whether one method is more

appropriate than another in regards to accurate assessment of vitamin A status. Investigating these questions will address some of the key objectives presented by the BRINDA project and can help to reduce misclassification of vitamin A status in population surveys.

Research Concept and Justification

Despite decades of efforts to alleviate the burden of VAD, it is still a major public health problem. Again, the same populations that have a high prevalence of infection also experience VAD. Further, although it is known that infection impacts vitamin A serum retinol and RBP concentrations, there is no consensus method for correcting for inflammation when measuring vitamin A. Therefore, there is a need to identify the most accurate method for measuring vitamin A micronutrient levels among adults, particularly during enteric inflammation events, in order to best evaluate the need for and performance of vitamin A fortification and supplementation programs worldwide. The goal of this research, then, is to assess how inflammation resulting from norovirus infection impacts concentration of two vitamin A biomarkers (retinol and RBP) that are commonly used in population-based surveys. This study examined norovirus infection, which is one of the most common enteric infections worldwide. Data is derived from participants in norovirus challenge studies in healthy adults from the United States.

Norovirus is one of the most common enteric diseases worldwide, and is the leading cause of vomiting and diarrhea among individuals in the United States (CDC, 2018a). Globally, 1 in 5 cases of gastroenterities and over 680 million cases are caused by norovirus (CDC, 2018b). On average, there are between 19 and 21 million cases of acute gastroenteritis, 56-71,000 hospitalizations, and over 500 deaths, caused by norovirus in

the United States (CDC, 2018b). Norovirus is a single strand, positive sense RNA virus that causes acute gastroenteritis. The virus is transmitted through feces or emesis and is environmentally persistant (Cook, Knight, & Richards, 2016). It can be spread through contaminated water, surfaces, and food (CDC, 2018a). Further, norovirus is highly infectious and can be spread for up to two weeks, sometimes longer, after an infected individual 'recovers' (Seitz et al., 2011). Since the burden of disease is so high worldwide, there have been many research studies conducted on norovirus, including a few unique human experimental studies.

Two of these experimental studies were conducted at Emory University between the years 2006 and 2009. From these studies, 52 individuals (healthy, U.S. adults) were selected to be included in a follow-up study to assess biomarkers of inflammation and micronutrient status before and after a norovirus challenge. Of the 52 individuals included in the study, 26 were infected and 26 were uninfected. Micronutrients measured included iron markers, vitamin A, vitamin D, and B vitamins. The data collected for this study will be utilized to address the research question outlined above.

To determine the most accurate method for assessing vitamin A status on a population level, it is important to first identify the best biomarker for measuring vitamin A concentration in this study population. Although retinol and RBP are thought to be present in equal amounts in serum, this ratio may not be accurate in inflamed populations. Further, the concentrations of each of these biomarkers may differ between individuals who are experiencing inflammation as a result of norovirus infection. Thus, Aim 1 of this paper will be to evaluate the ratio between retinol and RBP over the course of norovirus infection comparing individuals in the study who are experiencing inflammation to those

who are not. The second aim of this research will be to assess the methodology and results of the BRINDA and Thurnham correction methods on longitudinal biomarker concentrations in order to ascertain which method for adjusting for inflammation will be closer to the baseline, or true, vitamin A status of the study population. Aim 3 of this research will be to evaluate changes in vitamin A biomarkers over the course of the infection period.

Research Significance

Results from this research will provide evidence for a more accurate way to adjust for inflammation when assessing population vitamin A status in the context of enteric infections. First, evaluating the 1:1 ratio between retinol and RBP in the face of inflammation (Aim 1) will provide evidence for the usefulness of using RBP as a proxy for retinol. If there is a significant difference in this ratio when an individual is inflamed, RBP may not be the best method for ascertaining vitamin A status. Second, the methodological comparison and results from Aim 2 will contribute more evidence to the most accurate method for utilizing acute phase response proteins, AGP and CRP, when adjusting retinol or retinol binding protein measurements using longitudinal data. Lastly, assessing the changes in vitamin A biomarkers over the course of an infection in a controlled setting will provide a better picture for how vitamin A status changes during infections. Better assessment will lead to information that enables more targeted supplementation and fortification programs that could reduce the burden of vitamin A deficiency worldwide. Further, results from this study may support the need to incorporate biomarkers of inflammation in all nutritional asssessments.

Methods

Study Population and Exposure

Participants were from two norovirus challenge studies conducted at Emory University in 2006 and in 2009 (Leon et al., 2011; Seitz et al., 2011). Enrolled candidates in both studies were apparently healthy adults in the United States that had been screened for the presence of H type 1 histo-blood group antigen carbohydrate. Enrolled participants were positive secretor status (Leon et al., 2011; Seitz et al., 2011). In the first study, participants drank 10 mL of water inoculated with norovirus (final dose of $6.5x10^8$ GEC of virus) (Seitz et al., 2011). Participants in the second study were inoculated with $1.0x10^4$ GEC of norovirus from spiked oysters (Leon et al., 2011). In both studies, patients were hospitalized for 4 days post-inoculation, released, and then returned for follow-up visits.

Selection of Controls to Matched Cases

From these studies, 26 individuals became infected, as determined by a positive reverse transcriptase polymerase chain reaction (RT-PCR) test of stool samples. Infected individuals were matched by age with 26 un-infected individuals from both studies and combined into a single dataset for use in an analysis of cytokine response to norovirus infection conducted by Newman and colleagues in 2016 (Newman et al., 2016). Some infected participants were paired with uninfected individuals from a different challenge group as there were no uninfected individuals from the same study within the three-year age range (Newman et al., 2015).

Inflammation and Nutritional Biomarkers Laboratory Methods

Serum samples were collected from all participants at baseline (pre-inoculation) and on days 1, 2, 3, and 4 post-inoculation. Serum samples were also collected on follow-up visits from week 1 post-exposure up to week 5 (35 days) post-exposures. Individuals who completed all follow-up visits had a total of 10 samples taken. Serum samples from the 52 individuals included in Newman's data set were re-analyzed in 2016 as a result of a nutritional biomarker project led by investigators at Emory University to study the relationship between inflammation and micronutrient concentrations (Williams et. al, in press). Serum samples were stored at -80° C from time of extraction (2006 or 2009) until sent for analysis of CRP, AGP, and nutritional biomarkers in 2016. Retinol was measured using high-performance liquid chromatography with a UV detector and the VitMin Laboratory in Germany used a sandwich ELISA to analyze RBP (Erhardt et. al, 2004). Acute phase proteins were measured in serum extracted from study participants and were also measured by the VitMin Laboratory in Germany using a sandwich ELISA. All subjects from the original challenge studies gave consent for use of samples in future studies (Leon et al., 2011; Seitz et al., 2011). Both studies were approved by the Emory University Institutional Review Board (study identifiers NCT00313404 and NCT00674336). The de-identified database used for this research was deemed non-human subjects research and thus exempt by the Emory University Institutional Review Board (see Appendix).

Data Set

Individuals were defined as infected if they had at least one stool sample from any day post-inoculation test positive by RT-qPCR using the QIAamp Viral RNA Mini Kit (Qiagen, Valencia, CA, USA) (Newman et al., 2016). Symptomatic individuals were

classified as being infected in addition to having at least one more symptom related to gastrointestinal illness (diarrhea alone or emesis plus another symptom), regardless of infection status (Newman et al., 2016). Elevated levels of CRP were defined as greater than 5 mg/L and elevated AGP was defined as greater than 1 g/L (Thurnham, McCabe, Northrop-Clewes, & Nestel, 2003). Individuals were classified as inflamed if they had elevated CRP or AGP at any time days 1-3 post-exposure, given that they were uninflamed at baseline (day 0). A ratio variable was created by dividing retinol (μmol/L) by retinol binding protein (μmol/L) to assess the relationship between the two biomarkers over the course of the study period (Aim 1). Additional select biologic variables that were used in analyses included age and sex (Leon et al., 2011).

Statistical Analysis

All statistical analysis was conducted using SAS 9.4® software. Normality assessment and a primary analysis using Student's t-test or chi-square analysis of the biologic variables, biomarkers, and APPs at baseline was conducted and reported in the results section in order to examine any differences between the study populations (infected vs. uninfected) prior to inoculation with norovirus. Mann-Whitney U tests were used to evaluate to test the significance of any association that may exist between the ratio variable and inflamed variable. A Mann-Whitney U test was used to evaluate any significant differences in the ratio between the inflamed and uninflamed for each day of the study period. There is currently no criterium to evaluate the ratio between retinol and RBP. Therefore, I decided that if it was found that the measure of central tendency was not within 20% of 1(<0.8 or >1.2), retinol would preclude RBP as an outcome for aims 2 and 3; RBP is a proxy for retinol (de Pee & Dary, 2002).

In order to calculate the Thurnham correction factors, individuals were assigned to one of four categories for each day of the study period: reference (CRP less than or equal to 5 mg/L, AGP less than or equal to 1 g/L), incubation (CRP greater than 5 mg/L, AGP less than or equal to 1 g/L), early convalescence (CRP greater than 5 mg/L, AGP greater than 1 g/L), and late convalescence (CRP less than or equal to 5 mg/L, AGP greater than 1 g/L). The geometric means of each category were then divided by the geometric mean of the reference group to calculate the correction factor (Thurnham, Northrop-Clewes & Knowles, 2015). Due to the low levels of inflammation in the population, calculating correction factors for individual days of the study period was not appropriate. All observations across all study time points were pooled in order to calculate the correction factors. These were then applied to the retinol concentrations to calculate adjusted retinol concentration. We considered this approach exploratory, as it ignores the longitudinal nature of the study design. Therefore, pooled arithmetic correction factors were applied to each observation based on the assigned period of infection, but no further analysis was conducted on the retinol data adjusted using the Thurnham approach.

Suchdev and colleagues described the BRINDA correction approach in detail in 2016 (Suchdev et al. 2016). To correct retinol binding protein concentrations using the BRINDA approach, linear regression was used to subtract the influence of CRP and AGP on the vitamin A biomarker. These calculations were done using the SAS 9.4® macro provided on the project's web page (https://brinda-nutrition.org). This macro was then adapted to correct for retinol on each day post-exposure and the adjusted mean retinol concentrations were calculated. Retinol adjusted using the BRINDA macro and unadjusted retinol were pooled by day and plotted linearly for a visual comparison to the

median baseline concentration of retinol. Differences from baseline were assessed using a Wilcoxon ranked-sum test.

To address Aim 3, a mixed-effects regression model was used to evaluate the change in retinol concentrations over the course of an infection, comparing inflamed to uninflamed individuals. A random effects model was used to allow for individual variation in these measurements. Effect modification by covariates of interest (sex, day post-exposure, and symptomatic) was assessed. Confounding by age was addressed through the matching process; the data was assessed for other potential confounders using a priori criteria, followed by a formal analysis. The final model included retinol as the dependent variable and inflamed as the primary independent variable of interest; symptomatic was identified as a confounder and included in the final model. Day post-exposure was identified as an effect modifier on the relationship between inflamed and retinol concentration, and was included in the model. The class statement included the dichotomous variables, inflamed and symptomatic.

$$\textbf{Model: } Retinol = intercept + \theta + \beta_1(inflamed) + \beta_2(symptomatic) + \beta_3(day\_actual) + \beta_4(inflamed*day\_actual) + \varepsilon$$

The interaction term for inflamed and day post-exposure was also included in the final model. The model was repeated using unadjusted retinol and retinol adjusted using the BRINDA correction method.

Results

The objectives of this analysis were (1) to assess for the presence of the 1:1 ratio between retinol and RBP over the course of norovirus infection, (2) compare the methodology and application of two correction techniques to the relevant biomarker(s) to account for the influence of inflammation and evaluate their usefulness on longitudinal data, and (3) evaluate changes in the biomarker(s) due to inflammation from a norovirus challenge, controlling for day post-exposure and symptoms. Important variables defined in the methods section included inflammation, infection, retinol (μmol/L), RBP (μmol/L), symptomatic, day post-infection, AGP (g/L), and CRP (mg/L). There were missing retinol data from one individual at baseline and days 1, 2, 3, and 4 post-exposure. Retinol data was also missing for five other individuals at various time points throughout the study, but not at baseline. Inflammation data was missing for 6 different individuals in the study population.

Baseline Study Population Assessment

To assess baseline differences between the infected and uninfected populations that may impact the study results, the study population was stratified into infected and uninfected groups and analyzed for baseline differences in age, sex, retinol, RBP, CRP, AGP and symptoms. It was found that there were no significant differences in the age between the two groups due to matching. Further, the proportion of males and females in each group were similar. There were also no statistically significant differences in baseline retinol concentrations between the 25 individuals who later became infected and the 26 individuals who remained uninfected ($p=0.35$) [Table 1]. There were also no statistically significant differences in baseline RBP ($p=0.40$). Median baseline CRP concentration

was slightly lower among the infected (0.79 mg/L) than in the uninfected (1.04 mg/L). Similarly, the median baseline AGP concentration was also lower among infected (0.56 g/L) than in the infected (0.64 g/L). However, there were no statistically significant differences for CRP (p=0.96) or AGP (p=0.34) between infected and uninfected individuals at baseline. After norovirus exposure, the majority of the infected individuals displayed symptoms (72%), and approximately 87% were inflamed. In conclusion, it was shown that there were no significant differences in the study population at baseline that would alter the results of the analyses.

Bivariate Analysis

The ratio between retinol and RBP was evaluated across all time points and by inflammation in the study to determine whether the relationship between retinol and RBP differed by inflammation status, and whether the ratio was ever 1:1 in this study population. After the ratio variable was created, the study population was stratified by inflammation and day post-exposure; these results are displayed in Figure 1. There were no statistically significant differences in the ratio between inflamed and uninflamed individuals on any day of the study period. Pooling all study days and subjects, the mean value for the ratio variable was 0.69 (IQR=0.12), indicating that retinol, on average, was lower than RBP. The mean ratio for study participants who were inflamed was 0.68 (IQR=0.13); the mean value for individuals who were not inflamed was 0.70 (IQR=0.11). The mean ratio for subjects who were not infected was 0.70 (IQR=0.11); the mean ratio for infected subjects was 0.68 (IQR=0.13). Retinol was only higher than RBP three times from three different individuals across all time points. Further, the 1:1 ratio was only achieved by one uninfected individual on day 7. As previously mentioned, the cutoff

values for retinol should only be used for RBP when the assumption of the 1:1 ratio holds true. Thus, based on these results, we analyzed data for Aims 2 and 3 below using retinol only as the outcome variable.

Inflammation Adjustment

The second objective of this analysis was to compare the methodology and application of two different inflammation-adjustment methods to repeated measurements of retinol (described in Methods) using the formulas outlined in the Methods section. Using the Thurnham approach (Thurnham, Northrop-Clewes & Knowles, 2015), there were 394, 57, 21, and 20 observations in the reference, incubation, early convalescence, and late convalescence periods. When stratified by day, there were 0 individuals in the early convalescence period on days 0, 1, 14, 21, and 35. Further, there was only one individual in the late convalescence stage on days 1, 2, 21, 28, and 35. Thus, correction factors were calculated and applied using pooled data across all timepoints. Using the Thurnham method (Thurnham, Northrop-Clewes & Knowles, 2015), the geometric means of retinol concentration calculated were 1.21, 1.27, 1.03, and 1.30 μmol/L for the reference, incubation, early convalescence, and late convalescence periods of all individuals throughout the study period. Subsequently, the correction factors were 1.05, 0.85, and 1.07 for the incubation, early convalescence, and late convalescence periods. While the Thurnham-correction methodology was appropriate for a longitudinal design, the application of these correction factors and analysis of the Thurnham-adjusted retinol was not conducted due to low levels of inflammation in the study population. Unlike with the Thurnham-adjustment methods, we were able to make adjustments to retinol at each individual day post-exposure using the BRINDA-approach despite the low levels of

inflammation. The slopes describing the relationship between retinol and CRP or AGP ranged from 0.98-1.06, and 0.84-1.22, respectively. Median BRINDA-adjusted (Suchdev et al. 2016) retinol concentrations were graphed by day alongside unadjusted retinol concentrations and compared to baseline using a Wilcoxon signed rank test [Figure 2]. It is important to note that while the BRINDA adjustment appears to correct towards baseline on the days immediately following exposure (days 2-4), the BRINDA-adjustment corrected retinol concentrations away from baseline on days 7, 14, and 21. As the Thurnham approach was not appropriate for corrections on longitudinal data in this study population, we did not formally compare the two inflammation-adjustment approaches. Thus, the analysis for Aim 3 was conducted with only BRINDA-adjusted serum retinol.

Mixed Effects Analysis

To address the third research objective, a linear mixed effects regression model was used to analyze changes in retinol (unadjusted and adjusted) over the course of an infection. Within the model, inflammation alone was insignificantly associated with changes in unadjusted retinol. However, the changes in unadjusted retinol were statistically significantly associated with day post-exposure ($p=0.01$) and effect of inflammation by day ($p=0.03$) [Table 2]. Similarly, changes in retinol adjusted using the BRINDA approach were statistically significantly associated with day post-exposure ($p<0.01$) and inflammation by day ($p=0.03$) [Table 3]. Again, inflammation alone was not statistically significant in the adjusted model. Although symptomatic status was found to be a confounder in the model selection process, it was not statistically significant within either model. Further, the effect of inflammation by day was significant as a whole, but the

differences in the least-squared means between the inflamed and uninflamed individually by day were not statistically significant in either model.

Discussion

The results of these analyses have demonstrated several important results for assessing VAD on a population level when inflammation is present. Using data from the norovirus infectious challenge study, we were able to ascertain that there was no molar 1:1 ratio of retinol to RBP longitudinally in this population of individuals, regardless of inflammation. Further, we demonstrated reductions in average retinol concentrations post-exposure to norovirus and corrected for these differences using the BRINDA approach. Lastly, through utilizing mixed-effects regression models, we demonstrated a reduction in retinol due to inflammation, and that this effect was different based on day post-exposure.

The assessment of the ratio between retinol and RBP was consistent with other study findings, specifically in that RBP is in excess of retinol (Engle-Stone et. al, 2011; Erikstrup et. al, 2009). Although it is known that inflammation reduces serum retinol concentrations (Stephensen & Gildengorin, 2000), to our knowledge, these findings have never been displayed in a longitudinal analysis from the same population. Further, we were able to demonstrate, for the first time to our knowledge, the utility in the BRINDA approach for inflammation adjustment of retinol on a longitudinal data set.

Retinol: RBP ratio in response to inflammation

Because results from this analysis demonstrated that the molar 1:1 ratio between retinol and RBP concentration does not hold up in a population with low levels of baseline inflammation; extra consideration for analysis must be given to studies that use RBP to estimate VAD prevalence. This result is not unique to our study; investigators have noted recently higher levels of RBP than retinol in several other study populations (Engle-Stone

et. al, 2011; Erikstrup et. al, 2009) which contradicts earlier research that demonstrates a higher correlation between retinol and RBP (Briand et. al, 2001). Consequently, there has been much debate about utilizing retinol cutoffs for RBP measurement (Tanumihardjo et al., 2016). It is important to remember that while RBP and retinol may be highly correlated, RBP measurement is only meant to be a proxy for retinol. However, since serum retinol measurement tends to be higher cost than laboratory methods to assess RBP, RBP is often assessed population-based surveys (Erhardt, Estes, Pfeiffer, Biesalski, & Craft, 2004). Thus, population-specific adjustments to the cutoff for retinol concentration of 0.7 μmol/L should be considered when using RBP to assess vitamin A deficiency on a population level (Engle-Stone et. al, 2011). Further, these results indicate that even in populations with low-levels of inflammation, considerations should be made for adjusting retinol and RBP biomarkers for inflammation.

Evaluation of two methods to adjust for inflammation

Through the application of two inflammation-adjustment approaches to serum retinol, we demonstrated a limitation of the Thurnham approach and the findings from the BRINDA method. To our knowledge, this analysis is the first of its kind to compare inflammation adjustment techniques using longitudinal data. Although both methods are applicable to longitudinal data, the mild immune response to norovirus exposure prevented the accurate calculation of the Thurnham correction factors by day. Although both approaches were constrained by the small sample size of the study population, the Thurnham-adjustment was most affected due to the low-levels of CRP and AGP. While CRP is known to increase up to 1000-fold in response to infections or other inflammatory stimuli (Slaats, Ten Oever, van d Veerdonk, & Netea, 2016), a formal analysis of CRP-

response in this population found the response to be modest with a peak median concentration of 16 mg/L (Williams et. al, in press). AGP-response was also found to be low; the median peak was 0.9 g/L, which is lower than the established cutoff of 1 g/L. Due to the low levels of inflammation, Thurnham correction factors were not applicable on some days post-exposure; thus, we were unable to calculate accurate correction factors for all four different periods on each day. The study population included previously healthy U.S. adults, and the norovirus inoculation did not induce a high acute phase response, particularly in AGP, which resulted in most individuals being classified in the referent and incubation periods. Therefore, the Thurnham correction-factor approach may only be appropriate in larger longitudinal studies of populations with higher levels of inflammation.

In this analysis, the BRINDA approach allowed for more precision and granular application of adjusting retinol concentrations compared to the Thurnham correction factor method. The BRINDA-approach worked well in that corrected retinol concentrations trended toward the baseline median in the days immediately following norovirus exposure. However, the adjustment corrected retinol away from the baseline in the later time points in the study. Since there is no previous literature using these approaches on longitudinal data, I hypothesized that concentrations trended toward baseline on the early days post-exposure due to the immediate inflammatory response. On days 7, 14, and 21 post-exposure, the inflammatory response from norovirus exposure was negligible, the BRINDA approach over-corrected for inflammation, resulting in adjusted concentrations being further from baseline. Additionally, these findings corroborate other studies that have found an inconsistent relationship between

inflammation and vitamin A in women of reproductive age (Larson et. Al, 2018). These results may indicate that correction is not necessary in adult populations where there is no inflammation, but further studies should be done in healthy populations to confirm this hypothesis.

In populations with a high and sustained prevalence of infection, there would be a higher likelihood that APP-response would be more extreme (Bresnahan & Tanumihardjo, 2014). Thus, all periods of infection would be populated at any given time and correction factors would be appropriately calculated using the Thurnham approach. However, when assessing longitudinal data in a smaller cohort with low levels of inflammation to evaluate VAD, the BRINDA approach may be more appropriate.

Changes in retinol over time in response to inflammation

Using the linear mixed effects regression technique, we were able model how retinol changes over the course of an infection. Although inflammation had no statistically significant impact on retinol as a standalone covariate, we still observed significant changes when we modeled the interaction of inflammation over the course of the study period. The observed changes in retinol corroborate other research findings that have found a significant impact of inflammation on serum retinol concentrations (Stephensen & Gildengorin, 2000; Tanumihardjo, 2012). The lack of significance could be explained by the mild APP response to norovirus infection in previously healthy individuals (Williams et. al, in press). Previous studies that have observed these effects involved populations with high levels of inflammation, and have primarily looked at pre-school children and women of reproductive age (Larson et. al, 2018). The low-level inflammatory response could also explain why a statistically significant response between

inflammation was observed only when the effect was pooled for all time points, but not when stratified by each individual day post-exposure. These results highlight the need to evaluate the kinetics of retinol in response to inflammation in a larger study population, particularly pregnant women and children, with higher levels of inflammation.

Strengths and Limitations

A major strength of this research is in utilizing longitudinal data that was collected as part of a controlled challenge experiment. Prior studies have mostly used cross-sectional data, and as a result, were unable to uniquely analyze the causal effect of the inflammatory response on retinol concentrations. With longitudinal data, we were able to assess the response of retinol and RBP following infection. Other causes of inflammation that could have been occurring simultaneously were not measured, but data was collected prior to inoculation in order to account for any baseline levels of inflammation. Further, as the study population were healthy adults in a country with low prevalence of VAD, other factors that could have altered levels of vitamin A in serum were not a concern.

One limitation of this analysis is that other covariates (e.g., BMI, diabetes medications) that could have an impact on the actual mechanism by which inflammation reduces serum RBP concentrations were not accounted for in the analysis as they were not collected in the original study. Another limitation lies in the inability for these results to be extrapolated to the population of most concern: children and pregnant women, particularly in populations where inflammation is common. Further longitudinal analyses should be conducted within these populations to assess the causal relationship between inflammation and altered vitamin A biomarker concentrations.

Conclusions

These results demonstrate that the 1:1 molar ratio between retinol and RBP is not upheld, even in apparently healthy populations where VAD prevalence is low. Thus, adjusting the cutoff for RBP should always be considered when utilizing this as a measurement for estimating population prevalence of VAD. Second, results indicate that although both the Thurnham and BRINDA approaches are appropriate using longitudinal data, the BRINDA approach is more accurate than the Thurnham correction factor approach in populations with low levels of inflammation. Lastly, the results of this analysis demonstrate the necessity to conduct further longitudinal studies which account for inflammation when measuring vitamin A status, even in apparently healthy study populations.

Understanding the relationship between inflammation and serum retinol concentrations is critical to develop a standardized approach for assessment of VAD. Recently, there has been studies that have found that vitamin A supplementation may not be as effective in improving quality of life among populations as previous studies have suggested (Delisle, 2018). As such, there has been talk about directing these funds towards a more effective program. However, prior to drastic changes, an accurate approach to assessing population levels of VAD needs to be implemented to examine the effectiveness of these programs. These results in conjunction with the future studies outlined above can provide insight into the question of the effectiveness of vitamin A supplementation programs.

Public Health Implications

These results have important implications for both clinicians and public health professionals:

- The average ratio between retinol and RBP was less than one regardless of infection status, highlighting the need to revisit the popular assumption that the ratio between retinol and RBP is 1:1 under 'normal' homeostatic conditions.
- Because we found that the ratio between retinol and RBP was not equimolar, we corroborate other findings that retinol is the more accurate assessment of vitamin A status compared to RBP.
- Based on the results described above, any population assessments that use RBP due to lower costs should also collect retinol from a subset of the population in order to estimate population-specific VAD cutoffs for retinol binding protein.
- Both the Thurnham and BRINDA approaches are appropriate for analyzing longitudinal measurements of serum retinol.
- Upon application of the Thurnham correction factor approach in this study, we found that adjustment in populations with low-levels of inflammation should be conducted using the BRINDA approach.
- However, because the BRINDA adjustment significantly corrected retinol measurements away from baseline on days 7, 14, and 21 post-exposure, considerations should be made to prevent over-adjusting for inflammation.
- Finally, we demonstrated that consideration for inflammation should be considered even in populations with low-levels of inflammatory proteins circulating in their serum, as it can still alter serum retinol levels.

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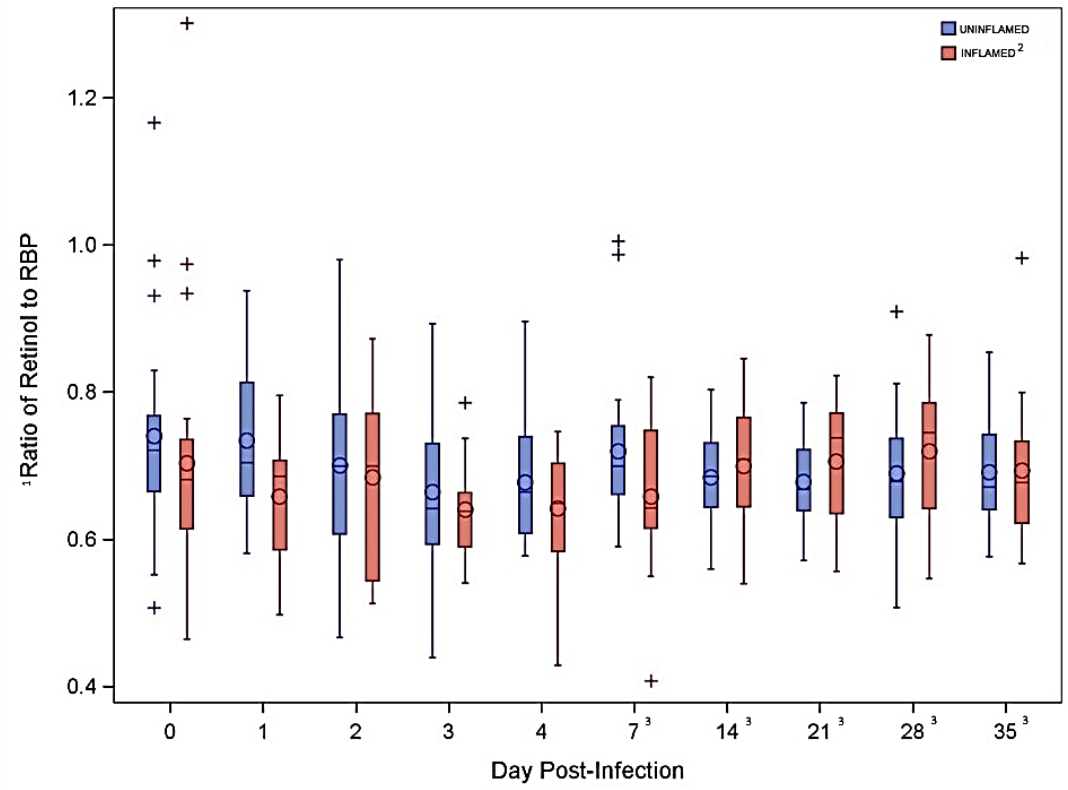
Tables and Figures

Table 1. Study Population Characteristics at Baseline

Study Characteristic[1]	Infected	Uninfected	p-value[2]
Median Age (years) (IQR)	25 (21-28)	26 (21-27)	0.89
Sex (%)	15 Females (60%) 10 Males (40%)	14 Females (54%) 12 Males (46%)	0.78
Median Retinol (µmol/L) (IQR)	1.31 (1.15-1.59)	1.27 (1.09-1.47)	0.40
Median RBP (µmol/L) (IQR)	2.00 (1.77-2.22)	1.74 (1.56-2.00)	0.17
Median CRP (mg/L) (IQR)	0.79 (0.50-1.78)	1.04 (0.53-1.72)	0.96
Median AGP (g/L) (IQR)	0.56 (0.44-0.68)	0.64 (0.47-0.75)	0.34

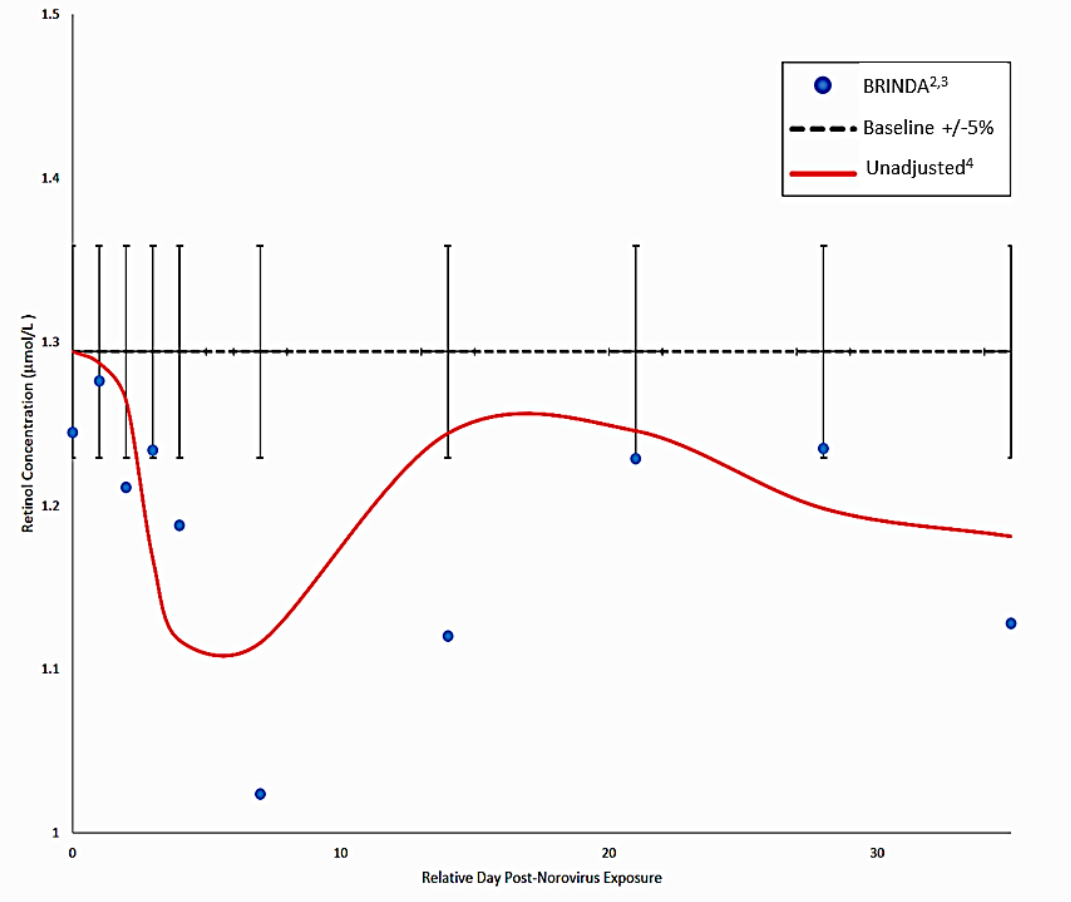
[1]Study characteristics at baseline time point
[2]T-tests and chi-square analysis were run to measure differences in continuous and categorical baseline study characteristics

Figure 1. Ratio of Retinol to RBP in Inflamed vs. Uninflamed Individuals



[1] Ratio variable created by dividing retinol concentration (µmol/L) by RBP concentration (µmol/L) and differences in ratios were assessed using Mann-Whitney U test. There were no significant differences in the ratio between inflamed and uninflamed individuals on any day post-inoculation with Norovirus
[2] Inflamed was defined as elevated levels of CRP or AGP
[3] Days 7, 14, 21, 28, and 35 correspond to weekly follow-up time points and may not be exact

Figure 2. Median Retinol Concentrations throughout Norovirus Infection[1], Unadjusted and Adjusted for Inflammation



[1] Infection (Y/N) defined as having at least one stool sample from any day post-inoculation test positive by RT-qPCR using the QIAamp Viral RNA Mini Kit (Qiagen, Valencia, CA, USA) (Newman et al., 2016)
[2] Biomarkers Reflecting Inflammation and Nutritional Determinants of Anemia
[3] BRINDA-adjusted retinol concentrations significantly different from baseline on days 4 ($p=0.03$), 7 ($p<0.001$), 14 ($p<0.001$), and 35 ($p=0.01$)
[4] Unadjusted retinol concentrations significantly different from baseline on days 3 ($p=0.008$), 4 ($p=0.008$), and 7 ($p=0.002$)

Table 2. Mixed Effects Regression Analysis of Retinol (Unadjusted)

	Bivariate Model	**Multivariate Model**
	Coefficient (p-value)	*Coefficient (p-value)*
Intercept	-	1.19 (<0.01)*
Symptomatic[1]	0.03 (0.69)	-0.07 (0.56)
Inflamed[2]	0.08 (0.36)	0.04 (0.72)
Day Post-Exposure	-0.00 (0.34)	-0.01 (0.01)*
Inflammation by Day[3]	-	0.01 (0.03)*

[1] Symptomatic (Y/N) using asymptomatic as reference
[2] Inflamed (Y/N) using uninflamed as reference
[3] Difference in means by day between inflamed and uninflamed were insignificant and not reported here (see Appendix A)
*Statistically significant at an alpha level of 0.05

Table 3. Mixed Effects Regression Analysis of Retinol (BRINDA-adjusted)

	Bivariate Model	**Multivariate Model**
	Coefficient (p-value)	*Coefficient (p-value)*
Intercept	-	1.17 (<0.01)*
Symptomatic[1]	0.04 (0.63)	-0.07 (0.53)
Inflamed[2]	0.08 (0.32)	0.05 (0.65)
Day Post-Exposure	-0.01 (0.01)*	-0.02 (<0.01)*
Inflammation by Day[3]	-	0.01 (0.03)*

[1] Symptomatic (Y/N) using asymptomatic as reference
[2] Inflamed (Y/N) using uninflamed as reference
[3] Difference in means by day between inflamed and uninflamed were insignificant and not reported here (see Appendix A)
*Statistically significant at an alpha level of 0.05

Appendix A: IRB Approval Letter

EMORY UNIVERSITY
Institutional Review Board

April 15, 2019

Courtney Victor (Poulos), MPH Candidate
Rollins School of Public Health
Global Environmental Health

RE: Determination: No IRB Review Required
Title: *Norovirus infection on Vitamin A biomarkers*
Project Leader: Victor

Dear Courtney:

Thank you for requesting a determination from our office about the above-referenced project. Based on our review of the materials you provided, we have determined that it does not require IRB review because it does not meet the definition of research with "human subjects" or "clinical investigation" as set forth in Emory policies and procedures and federal rules, if applicable. Specifically, in this project, you will use data from a previous Norovirus challenge study as the source material for your thesis. All the data are previously de-identified, no one on the study team was involved in the collection or de-identification of the data, and no one on the study team was involved in analysis of the data while in an identifiable state.

Please note that this determination does not mean that you cannot publish the results. This determination could be affected by substantive changes in the study design, subject populations, or identifiability of data. If the project changes in any substantive way, please contact our office for clarification.

Thank you for consulting the IRB.

Sincerely,

Sam Roberts, BA
Research Protocol Analyst, Sr.

Emory University
1599 Clifton Road, 5th Floor - Atlanta, Georgia 30322
Tel: 404.712.0720 - Fax: 404.727.1358 - Email: irb@emory.edu - Web: http://www.irb.emory.edu

Appendix B: Supplemental Tables

Supplemental Table 1. Median concentrations for unadjusted retinol and BRINDA-adjusted retinol by day.

Day Post-Exposure	Unadjusted Retinol (ug/L)	BRINDA-adjusted Retinol (ug/L)
0	1.29	1.24
1	1.29	1.28
2	1.26	1.21
3	1.17	1.23
4	1.12	1.19
7	1.12	1.02
14	1.24	1.12
21	1.25	1.23
28	1.20	1.24
35	1.18	1.13

Supplemental 2a. Differences of Least Squared Means between Inflamed and Uninflamed by Day (Unadjusted Retinol)

Day Post-Exposure	Estimate	Standard Error	DF	t Value	Pr > |t|	Lower 95% CI	Upper 95% CI
0	0.06	0.12	42.00	0.49	0.63	-0.18	0.30
1	0.07	0.12	42.00	0.62	0.54	-0.16	0.31
2	0.09	0.12	42.00	0.76	0.45	-0.15	0.32
3	0.10	0.11	42.00	0.89	0.38	-0.13	0.33
4	0.12	0.11	42.00	1.02	0.31	-0.11	0.35
7	0.13	0.11	42.00	1.15	0.26	-0.10	0.36
14	0.15	0.11	42.00	1.28	0.21	-0.08	0.38
21	0.16	0.12	42.00	1.40	0.17	-0.07	0.40
28	0.18	0.12	42.00	1.51	0.14	-0.06	0.41
35	0.19	0.12	42.00	1.62	0.11	-0.05	0.43

Supplemental Table 2b. Differences of Least Squared Means between Inflamed and Uninflamed by Day (BRINDA-adjusted Retinol)

Day Post-Exposure	Estimate	Standard Error	DF	t Value	Pr > |t|	Lower	Upper
1	0.06	0.11	42.00	0.58	0.56	-0.16	0.29
2	0.08	0.11	42.00	0.71	0.48	-0.14	0.30
3	0.09	0.11	42.00	0.85	0.40	-0.13	0.31
4	0.11	0.11	42.00	0.98	0.33	-0.11	0.32
5	0.12	0.11	42.00	1.11	0.27	-0.10	0.34
6	0.13	0.11	42.00	1.24	0.22	-0.08	0.35
7	0.15	0.11	42.00	1.36	0.18	-0.07	0.36
8	0.16	0.11	42.00	1.48	0.15	-0.06	0.38
9	0.17	0.11	42.00	1.59	0.12	-0.05	0.40
10	0.19	0.11	42.00	1.69	0.10	-0.04	0.41