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**ASSOCIATION OF ANTIOXIDANT NUTRIENTS AND HUMAN  
PAPILLOMAVIRUS PERSISTENCE, CLEARANCE AND VIRAL LOAD  
AMONG WOMEN IN THE LUDWIG-MCGILL COHORT STUDY**

By

Erin Michelle Siegel

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A Dissertation Submitted to the Faculty of the

COLLEGE OF PUBLIC HEALTH

In Partial Fulfillment of the Requirements  
For the Degree of

DOCTOR OF PHILOSOPHY  
WITH A MAJOR IN EPIDEMIOLOGY

In the Graduate College

THE UNIVERSITY OF ARIZONA

2004

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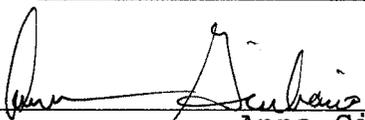
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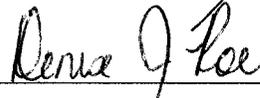
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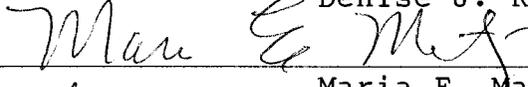
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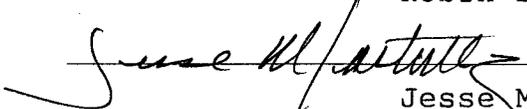
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## ACKNOWLEDGEMENTS

I am deeply indebted to my dissertation director, mentor and friend, Dr. Anna Giuliano. Dr. Giuliano was the Principal Investigator on this study and provided me an amazing opportunity to work with her on this study, which led to my dissertation research. She encouraged me to pursue this degree and has been there for me all the way. Thank you, Anna, for taking me under your wing; providing me guidance, advice, and countless opportunities; and being an amazing role model. I would also like to thank to everyone working in Dr. Giuliano's group for friendship, advice and putting up with me during stressful times.

I wish to thank the co-Investigators on this study. I am very grateful to have worked with Drs. Eduardo Franco and Luisa Villa, the co-Principal Investigators of the Ludwig-McGill Cohort Study. This work would not have been possible without your successful establishment and completion of the Ludwig-McGill Cohort Study. Although Drs. Franco and Villa were in different countries, they encouraged my work, provided valuable opinions with unique prospective and supported me as their own graduate student. Thank you for everything and I am looking forward to years of collaboration. I would like to thank Dr. Neal Craft for overseeing all the serum nutrient analyses and his many insights on retinoic acid analyses.

I would like to thank the members of my doctoral committee, Drs. Robin Harris, Elena Martinez, Denise Roe and Jesse Martinez. You have all supported me during my journey to complete this dissertation and encouraged me to pursue my career goals. Thank you for your advice, patience, and many insights into the world of academic research.

I especially would like to thank everyone in my family who has supported me through the years. Without their support, I would not have been able to accomplish this amazing achievement. I especially thank my beloved husband, Sean for supporting me in his own special way, for putting up with my long nights at the computer, and for being willing to take on new adventures. To Sierra, Karina and Aliya, my daughters, one day I hope you understand the amazing achievement I have made and forget (and forgive) all the times mommy had to work. I love you deeply and hope I have been a role model for you in achieving your future goals. To my family, Arlene, Rich, Gail, Oz, Dean and Mackenzie, thank you for all your love and support over the last decade. Finally, to my mother, Cathie Kieborz, I love you dearly and thank you for raising me with the drive to pursue my goals and strength to overcome obstacles.

## DEDICATION

To Mackenzie, Sierra, Karina and Aliya. May this research prevent you from experiencing the burden of HPV induced cervical dysplasia or cancer.

To all women who have experienced the burden of HPV and cervical cancer. I hope this research contributes to the prevention of cervical cancer worldwide.

All I am,  
All I ever hope to be,  
I owe to my mother.

Abraham Lincoln

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## ABSTRACT

Although an oncogenic human papillomavirus (HPV) infection has been established as the necessary cause of cervical cancer, most HPV infections are transient and rarely progress to significant cervical lesions. Current research has focused on identifying factors associated with viral persistence and clearance. Low nutritional status might influence a woman's risk of having a persistent HPV infection and progression of that infection to cervical dysplasia, or at higher levels increase the probability of clearing infections.

The overall goal of this research was to determine the associations between serum carotenoid, tocopherol, and retinoic acid levels and cervical carcinogenesis among the Ludwig-McGill Cohort Study. A sub-cohort of 846 women, all of whom had HPV results available from four consecutive study visits in the first year of follow-up, had serum samples analyzed for circulating nutrients. We determined the variability of circulating retinoic acid levels over the four months in a sample of 502 women and identified lifestyle, demographic and nutritional factors that were associated with these levels. The relative abundance of retinoic acid isomers was similar for each visit and the within person-variability of total retinoic acid and individual isomers was low.

Using multivariate logistic regression models that serum nutrient levels (retinol,  $\beta$ -carotene, and  $\beta$ -cryptoxanthin), age, race, oral contraceptive use, total number of pregnancies, and season of initial blood draw were significantly associated with endogenous retinoic acid isomer levels. Among the 407 HPV

positive women, endogenous levels of all-*trans* retinoic acid were positively associated with an increased probability of clearing oncogenic and nononcogenic type-specific HPV infections. Furthermore, among the carotenoids and tocopherols examined, we found that higher circulating zeaxanthin concentrations were associated with increased probability of clearing type-specific oncogenic HPV infections. Inverse associations were found between serum tocopherol levels ( $\alpha$ -,  $\delta$ - and  $\gamma$ -tocopherol) and type-specific nononcogenic HPV persistence.

Overall, the findings of this study suggest that women with the highest levels of serum all-*trans* retinoic acid, zeaxanthin, and tocopherol are at a decreased risk for a persistent, type-specific HPV infection of long duration. As both classes of nutrients are modifiable by diet, alterations in diet may be a safe approach to cervical cancer prevention.

## CHAPTER 1

### INTRODUCTION

#### EXPLANATION OF THE PROBLEM

##### Section 1: Background and Significance

Cervical cancer continues to remain a major public health problem as it is the second leading cause of cancer for women worldwide (1). Infection with human papillomavirus (HPV) has been identified as the primary cause of cervical cancer (2). Prospective studies have demonstrated that women with persistent HPV infections are at a significantly greater risk of developing pre-cancerous cervical lesions, or squamous epithelial lesions (SIL), compared with women who are only transiently infected or those not infected at all (3-6).

Implementation of routine “papanicolaou” (Pap) smear screening programs in developed countries has tremendously decreased the rates of invasive cervical cancer over the past two decades (2). Unfortunately, cervical cancer rates remain high in developing countries, such as Mexico and Brazil, which do not have population wide screening programs (1). To decrease health care costs and patient burden worldwide, alternative approaches to prevent cervical lesion development are needed. Currently, there is a tremendous effort being put forth to develop and fully test a vaccine targeting HPV. While preliminary results suggest high efficacy (7), it will be many years before any vaccine is widely available to unscreened populations. Continued research is

needed to identify modifiable factors that could reduce a woman's risk of cervical disease.

Although oncogenic HPV infections have been established as the necessary cause of cervical cancer, most HPV infections are transient and rarely progress to significant cervical lesions (8). The factors that are associated with oncogenic HPV persistence and progression to high-grade SIL are unknown. Potential cofactors, including a lack of antioxidant nutrients (e.g., carotenoids and tocopherols) and retinoic acid, may modulate an oncogenic HPV infection from a transient infection to one that persists and progresses to cancer. Epidemiological evidence suggests that adequate nutrient levels may prevent HPV persistence and progression to cervical dysplasia (9); however, further studies are needed to adequately test the hypothesis that circulating concentrations of antioxidant nutrients are associated with persistent HPV infection and the development of SIL.

## **Section 2: Hypothesis and Specific Aims**

The purpose of this research is to assess the associations between serum carotenoid, tocopherol, and retinoic acid status and cervical carcinogenesis, using prospectively collected HPV data and multiple measures of serum nutrients, among a cohort of high-risk study participants in the Ludwig-McGill Cohort Study (São Paulo, Brazil). The *central hypotheses* for the present study are that high serum concentrations of certain carotenoids, tocopherols, and

retinoic acid will be significantly associated with 1) lower cervical HPV viral load, 2) decreased risk of HPV infection persistence and 3) increased likelihood of HPV clearance among a sub-cohort of women participating in the Ludwig-McGill Cohort study. The following **specific aims** address these hypotheses:

*Specific Aim 1:* To determine the variability of serum retinoic acid (specifically total retinoic acid, 13-*cis* retinoic acid, 9-*cis* retinoic acid and all-*trans* retinoic acid) over a four-month period and to identify nutrient, demographic and lifestyle factors associated with circulating concentrations of retinoic acid among a sample of 502 women (Manuscript 1).

*Specific Aim 2:* To determine the relative risk for type-specific HPV clearance and viral load associated with circulating concentrations of retinoic acid (i.e. total retinoic acid, 13-*cis* retinoic acid, 9-*cis* retinoic acid and all-*trans* retinoic acid) among 407 HPV positive women (Manuscript 2).

*Specific Aim 3:* To determine the relative risk for type-specific HPV persistence and clearance associated with circulating concentrations of carotenoids (i.e. retinol,  $\alpha$ -carotene, *trans*- and *cis*- $\beta$ -carotene, lutein, zeaxanthin,  $\alpha$ - and  $\beta$ -cryptoxanthin, *trans*- and *cis*-lycopene) and tocopherols (i.e.  $\alpha$ -,  $\delta$ -,  $\gamma$ -tocopherol) among 407 HPV positive women (Manuscript 3).

## **BACKGROUND AND LITERATURE REVIEW**

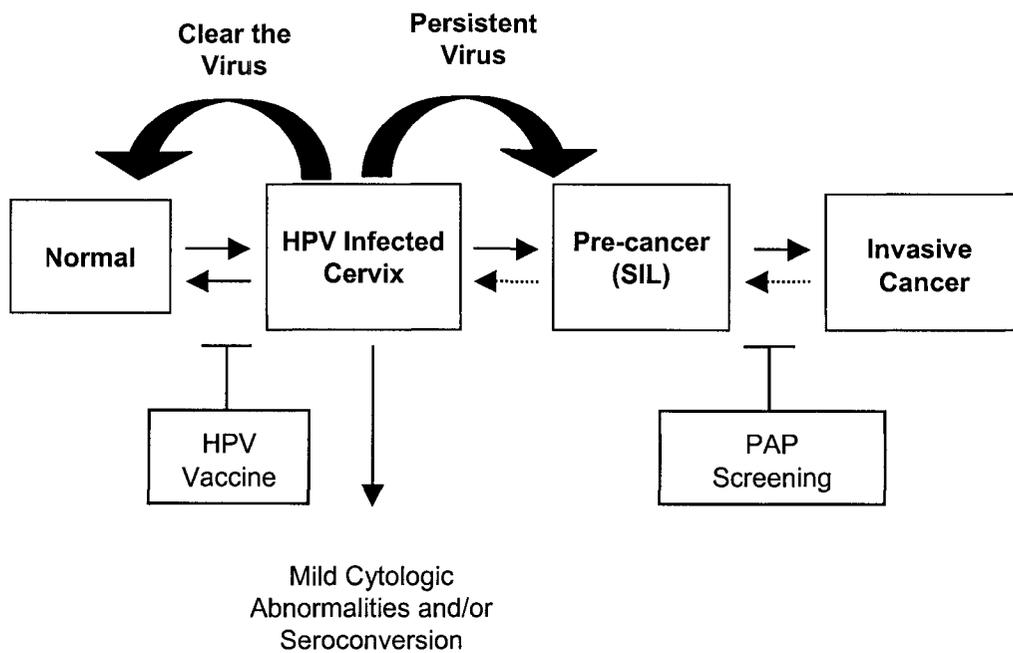
### **Section 1: Overview of Cervical Cancer Worldwide**

Cervical cancer continues to remain a major public health problem as it is the second leading cause of cancer for women worldwide (1). In 2000, the number of cervical cancer cases was estimated to be over 470,000 worldwide with over 230,000 estimated deaths (2). Rates of cervical cancer differ greatly between developed and under-developed countries. For example, age-standardized incidence rates of cervical cancer were reported in 2000 as 7.0/100,000 women and 30.9/100,000 women in the United States (U.S.) and South America, respectively (2). Rates of invasive cervical cancer in the United States and developed countries are lower as a result of the implementation of effective routine "Papanicolaou" (Pap) smear screening programs (2), which currently are not available in developing countries due to high costs.

Pap smear screening identifies cervical cancer and the pre-cursor cervical lesion. Progression from normal to invasive cervical cancer is thought to occur through increasing grades of dysplasia (10) (Figure 1.1, page 20) and the probability of progression is related to the severity of the lesion. The primary mechanism for identification of cervical lesions is through Pap smear screening and cervical cytology diagnosis. In the U.S., cervical cytology is based on the Bethesda System, which identifies two grades of squamous intraepithelial lesions (SIL), low and high-grade (11). Prior to the establishment of the Bethesda system within the United States, cervical histology staging was the primary

Figure 1.1: Progression from HPV Infection to Cervical Cancer

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Most HPV infections are transient with only a small number of infections persisting. These persistent HPV infections are associated with increased risk of developing pre-cancerous lesions. If left untreated, these lesions will progress to invasive cervical cancer.

mechanism for diagnosis of cervical abnormality. Histology continues to be utilized to classify cervical intraepithelial neoplasia (CIN) of biopsy tissues. Within the Bethesda classification system, low-grade SIL (LSIL) represents very mild-to-mild dysplasia (CIN I) and these lesions have a high rate of spontaneous regression (~60%) and a low rate of progression to carcinoma. In contrast, approximately 38% of high grade SIL (HSIL), moderate to severe dysplasia, will spontaneously regress, while 16-36% will progress to invasive cervical cancer (12). HSIL cytology diagnosis includes both CIN II and CIN III lesions defined by histology (11). Therefore, as CIN classifications are embedded within the Bethesda system, the terminology can be used interchangeably understanding that LSIL (CIN I) and HSIL (CIN II and CIN III) are part of the current US cervical cytology classification system (11).

SIL detected through Pap smear screening is effectively treated to prevent development of cancer. While there is a risk of cancer development with SIL, these lesions have a high rate of spontaneous regression with only high-grade lesions progressing to cancer (2). In theory, cytological screening should prevent all cases of cervical cancer. However, due to the logistics of repetitive screening of a large at-risk population, the financial burden of screening and its failure to be comprehensively applied to populations at-risk, cytological screening has not eliminated cervical cancer (13).

To decrease health care costs and patient burden, strategies that can prevent cervical dysplasia are needed. One approach is the development of an

HPV vaccine. Investigators recently reported efficacy of an HPV 16 specific vaccine of 100% (7). However, even with these encouraging early HPV vaccine trials, a primary prevention vaccine is decades away from being available to unscreened populations. In addition, current vaccines only target four HPV types (HPV 16, 18, 6, and 11) and it is unclear to what extent this vaccine will protect against other HPV types. Until a vaccine is fully tested and approved, other approaches to cervical dysplasia and cancer risk reduction are needed. These approaches include identifying relevant risk factors, such as nutritional factors, and modifying these factors to decrease overall cervical dysplasia risk.

## **Section 2: Causal Link between HPV and Cervical Cancer**

The causal role of HPV infections in the development of cervical cancer has been internationally accepted and proposed as the first identified “necessary” cause of human cancer (14). As a “necessary” cause, cervical cancer cannot develop in the absence of an HPV infection. Bosch *et al.* (14), in a systematic review of current evidence, demonstrated that an oncogenic HPV infection continues to meet all the criteria for causality. In brief, anogenital tract cancers and precursor lesions have been strongly linked to infection with HPV among both men and women (15). The International Agency for Research on Cancer (IARC) multicenter case control study provides the strongest evidence with HPV DNA present in over 99% of all cervical cancers and the risk associated with the presence of HPV DNA over 100 fold higher compared to control women (16). In

addition, HPV is present in 85-100% of HSIL (17). These strong associations have been consistently reported in studies conducted worldwide, with different analytical methods for HPV testing and different study designs (14). Only a few specific HPV types cause cervical cancer out of over 100 different HPV types identified. These “oncogenic” types have specific biological activities that are associated with the development of cancer, whereas other types do not have these properties or only manifest them to a weaker extent and are unable to cause cancer (18).

## **2.1 Carcinogenic Mechanisms of HPV**

Papillomaviruses are DNA viruses with virion particles that are small (<55 nm in diameter), non-enveloped, and icosahedral in shape. The papillomavirus family is comprised of over 100 HPV types, which appear to specifically infect epithelial tissues (e.g. skin, cervix, oral cavity, pharyngeal). There are 30-40 HPV types that are mucosatropic and infect the anogenital tract; however, different individual HPV types result in different cervical lesions (19). The association between HPV infection and cervical cancer has been linked to at least 13 HPV types found in the genital tract (19,20), classified as “oncogenic” HPV types. Of these oncogenic HPV types, 16 and 18 are strongly associated with HSIL, carcinoma *in situ* (CIS), and cervical cancer, all of which are cervical lesions unlikely to spontaneously regress (12). HPV types 31, 33, 35, 39, 45, 51, 52, and 58 are associated with an intermediate risk for progression to cervical

cancer. Finally, HPV types 6, 11, 42, 43, and 44 are primarily associated with benign cervical lesions with high spontaneous regression rates (~60%), such as condyloma and LSIL. The HPV types associated with benign lesions are considered “non-oncogenic” and have not been linked to cervical cancer (19,21,22).

## **2.2 Viral Protein Activities**

The papillomavirus genome is circular double-stranded DNA approximately 8 kilo-bases in size (8,000 nucleotides) depending on the individual type (See Figure 1.2, page 25). The HPV genome can be broken down into early and late open reading frames (ORFs) according to the timing of gene expression within the viral lifecycle. Of the eight early genes, E1 encodes for a DNA helicase, allowing the viral genome to unwind during DNA replication. E2 functions as both a transcriptional transactivator and transcriptional repressor of E6 and E7, depending if the full length or a truncated protein is produced. Loss of E2, which frequently occurs at viral integration, results in unrestricted transcription of E6 and E7 viral proteins. E4 is involved in deregulation of host cell mitogenic signaling pathway and allows for release of virus-like particles. E5 is a hydrophobic protein associated with cell membranes (e.g. golgi apparatus, endoplasmic reticulum and cellular membrane) and disrupts the activity of membrane proteins, such as epidermal growth factor receptor (EGFR) and major histocompatibility (MHC) molecules (18,23). E6 and E7 encoded, by oncogenic

HPV types, are considered viral oncoproteins and their functions are discussed below. The functions of E3 and E8 are unknown. The late genes, L1 and L2, are involved in virion production, specifically for the major and minor capsid protein of viral particle. The viral genome also has one known transcriptional regulatory region, between the 3' end of L1 and the 5' end of E6, which contains the transcriptional enhancer region and the E6 promoter (24).

Of the HPV proteins, continued expression of E6 and E7 oncoproteins is required for cervical cancer development. Our understanding of HPV induced carcinogenesis is a direct result of identifying some of the specific viral oncoprotein functions within host cells. The initially identified functions are the binding of E6 and E7 to tumor suppressor proteins p53 and retinoblastoma protein (Rb), respectively, which are classic targets of DNA tumor viruses. Recently, the activities of E6 and E7 have been expanded resulting from continued identification of cellular proteins found to bind HPV E6 and E7, many of which have not yet been fully characterized. The multitude of activities for each oncoprotein is remarkable considering their size, (~150 and 100 amino acids, respectively).

A critical step in the establishment of an HPV infection is the reactivation of DNA synthesis that has been inactivated in differentiating squamous epithelial cells (25) (See Section 2.3). The E7 viral oncoprotein is responsible for the initiation and maintenance of continued DNA synthesis in terminally differentiated cells by interaction with several proteins involved in cell cycle progression. The

binding of E7 to the Rb family of proteins (Rb, p107 and p130) results in phosphorylation of these proteins, enhancement of their degradation and the release of the E2F transcription factors (18). The released E2F transcription factor is constitutively activated and induces expression of genes required for progression from G2 to S phase (DNA synthesis) of the cell cycle. Additional cell cycle proteins are targeted by E7, including cyclins (cyclin A and E) and cyclin-dependent kinase inhibitors (CDKI p21 and p27). High levels of CDKI p21 and p27 in differentiated cells prevent activation of S-phase genes by inhibiting cyclin dependent kinases (e.g. CDK-2). Inhibition of CDKI p21 and p27 by E7 further enhances HPV infected cells to enter S-phase. HPV-induced progression through the cell cycle in terminally differentiated cells is utilized by both oncogenic and nononcogenic HPV types, although the strength of E7 binding is reduced for nononcogenic types (26). E7 has been found to interact with other cellular proteins, although many interactions have not been fully characterized. Known Rb-independent activities of E7 include 1) interaction with histone deacetylation pathway, 2) interaction with components of AP-1 transcription factors, and 3) modulation of M2 pyruvate kinase activity (18).

The uncontrolled cell cycle progression induced by E7 has been shown to induce p53 dependent apoptosis (18). In a coordinated effort to continue HPV replication, viral oncoprotein E6 interferes with p53 induced apoptosis and cell cycle arrest for DNA repair (27). E6 cannot interact with p53 alone and requires interaction with E6-associated protein (E6-AP) (28), which is an ubiquitin-protein

ligase responsible for targeting proteins for degradation, in this case p53. Interaction of E6 with E6-AP is a distinct characteristic of oncogenic HPV types, as non-oncogenic HPV types do not bind E6-AP and, therefore, p53 is not degraded. The E6 induced p53 degradation inhibits the normal cellular response to DNA damage (G1/S cell cycle arrest and initiation of DNA repair) leading to the accumulation of DNA mutations and chromosomal instability (18). E6 p53-independent activities have also been reported, including 1) E6-AP mediated degradation of proapoptotic protein, Bak; 2) activation of telomerase; and 3) reduction of E6-AP mediated degradation of tyrosine kinase Blk (18).

## **2.3 HPV Viral Lifecycle**

### **2.3.1 Productive “Acute” HPV Infection**

To establish an infection, it is likely that HPV infects epithelial stem cells (25,26) or transit-amplifying cells (26) located in the lower layers of the stratified epithelium. Entry is thought to occur through a minor wound or contact of the virus with sites that provide easy access to epithelial stem cells, such as the squamous columnar junction in the transformation zone of the uterine cervix (25). This model of initial HPV infection is somewhat misleading as most studies on the primary target cells for HPV have been conducted in tissue culture systems (26). HPV utilizes host cellular DNA replication machinery to produce approximately 50-100 episomal viral genomes in the nucleus of stem cells, which are passed on to daughter cells during mitosis. During an “acute” HPV infection,

there is very little HPV gene expression occurring in the epithelial stem cells. It is only as infected daughter cells differentiate in the suprabasal layers of the epithelium that the virus begins the productive stage of the infection. This stage is characterized by a high level of viral gene expression, multiplication of the viral genome, synthesis of early and late gene products, encapsulation of the HPV genome, and release of virion particles with exfoliating cells. Viral replicating cells display typical cytopathic changes characteristic of low-grade SIL lesions such as koilocytosis (10). Although a high number of viral genomes are present, this productive viral infection is still regulated by either viral proteins (e.g. E2) or cellular proteins (e.g. cellular interferon factor (CIF) or DNA methylation) and does not represent a viral infection at risk of progressing to cancer (25).

### **2.3.2 “Carcinogenic” HPV Infection**

Within host cells, the response to an “acute” HPV infection is to control expression of viral oncogenes. The loss of controlled viral expression within epithelial stem cells is considered the rate-limiting event that leads to the carcinogenic HPV infection (25), which is characterized by aberrant expression of E6 and E7 within epithelial stem cells and throughout the layers of the epithelium, regardless of differentiation status. There is evidence that loss of both host and viral factors are potentially responsible for aberrant HPV oncogene expression (18). Through the properties of oncogenic HPV type E6 and E7 proteins, their aberrant expression maintains host genome replication, and cell cycle

progression, thereby permitting the accumulation of additional DNA damage until specific clones gain a selective growth advantage and develop into a HSIL. HPV infections that result in HSIL are not productive viral infections because there is an absence of late gene expression, which results in no virion production (29). The severe morphological changes that occur as a result of a “carcinogenic” HPV induced HSIL include changes in overall DNA content (aneuploidy), altered chromatin texture, nuclear membrane changes, large nuclei and increased cell size.

Currently cytological and histological methods cannot distinguish an “acute” oncogenic HPV infection from the early stage of a “carcinogenic” oncogenic HPV infection. The additional detection of HPV DNA in exfoliated cervical cells or cervical lesions (e.g. LSIL) does not provide an adequate distinction either. There are several measures of viral activity, in addition to the detection of HPV DNA and cytological abnormalities, which are currently being investigated as biomarkers that will distinguish these two types of HPV infections, (“acute” vs. “carcinogenic”) including: 1) the continued presence of oncogenic HPV infection, 2) elevated viral load, 3) increased viral oncogene expression throughout the epithelium, 4) presence of integrated virus in LSIL and 5) DNA methylation pattern within viral regulatory regions. Among these biomarkers, type-specific HPV persistence and HPV viral load have been successfully implemented within epidemiological studies.

## **Section 3: Epidemiology of Persistent HPV Infection and Viral Load**

### **3.1 Introduction to HPV Persistence**

Results from several prospective cohort studies of the natural history of HPV infection provide strong evidence that the detection of an oncogenic HPV infection precedes development of a preneoplastic lesions (3-6,30-33). However, HPV infections are relatively common sexually transmitted infections, especially among young women. The majority of these infections are transient (3,8,34-38). The continued presence of an HPV infection has been suggested as a strong biomarker of cervical cancer risk. This section will review the evidence supporting use of persistent HPV infection as a biomarker of SIL and cervical cancer risk. This will be followed by an overview of factors that need to be considered when utilizing HPV persistence as an intermediate biomarker of cervical cancer risk, such as the definition of HPV persistence, the reported rates of HPV persistence, and factors associated with persistence.

### **3.2 Evidence of Increased Risk with Persistent Infection**

HPV persistence increases risk of cervical dysplasia: Prospective studies have demonstrated that women with persistent HPV infections are at a significantly greater risk of developing SIL compared with women who are only transiently infected or those not infected at all (3-6,30-33). Among women with normal cytology or LSIL at enrollment, those with two consecutively positive HPV results are significantly more likely to develop HSIL compared to HPV negative

women (3,5,32,39). These associations are further strengthened when HPV status is assessed at more than two visits, such that the longer a woman remains HPV positive the higher her risk of HSIL development (5,40,41). Results reported from the Ludwig-McGill Cohort Study indicate that women who are persistently HPV positive are four times more likely to have a persistent lesion of the cervix (5). Utilizing the most optimal definition of HPV persistence, that is persistence of incident infection, Woodman *et al.* (42) clearly demonstrate that a majority of HPV infections are transient, HPV infection precedes cervical disease, women with incident oncogenic HPV infections are at increased risk of developing HSIL, and type-specific persistent HPV infection precedes development of dysplasia (42).

Several groups have utilized a more population-based approach to examine the association of persistent HPV infection and SIL risk. These studies typically identify women who test positive for HPV at a routine screening visit as part of an established screening program. HPV positive women are then passively followed until the development of a cervical lesion. A population based follow-up study using national registries in Denmark reported that women (ages 20-29) with type-specific persistent infection were at highly increased risk of SIL development (e.g. OR >100) (43). In the United States, a nested case-control study of incident SIL among Kaiser Permanente patients reported an increased risk of SIL when HPV was detected on average two-years prior to the detection

of the lesion, and that 75% of those that developed HSIL had a type-specific persistent HPV infection (44).

HPV persistence increases risk for Invasive cervical cancer: Countries with socialized medical programs such as Sweden, the Netherlands and Germany, are able to link cases of cervical cancer with archived cervical specimens obtained within population-based cervical cancer screening programs. Nested case-control studies within this setting are an ethical way to study associations of HPV persistence with cervical cancer as an outcome. With these designs, HPV DNA has been detected in cervical specimens years before development of cervical cancer (38,45,46). Furthermore, the same HPV type was detected in 35% of women with cervical cancer prior to the diagnosis, whereas none of the controls have the same HPV type infection detected at both visits (45). Risk of cancer increases for women whose HPV positive test occurred less than six years prior to cancer diagnosis (45). These results provide further evidence that persistent HPV infections precede the development of cervical cancer. Therefore, HPV persistence with the same type is considered a critical intermediate step in the progression to cancer and can be utilized as an intermediate biomarker of cervical cancer risk.

### **3.3 Utilization of HPV Persistence as Intermediate Biomarker**

Definition of HPV persistence: One important aspect for an intermediate biomarker is a clear, consistent definition. Typically, for practical reasons, type-

specific HPV persistence is defined as the detection of the same HPV type two or more times over a certain period (47). Currently, there is no consensus as to how long a time period is required for the definition of an HPV persistent infection (47); however, it has been suggested that it be at least one year or longer (48). There is also no consensus on the time interval between HPV tests within that period of time defining HPV persistence. In prospective epidemiological studies on the natural history of HPV, a majority of the studies utilize intervals between HPV tests of either four or six months, or a combination of the two. There are several benefits of conducting HPV testing every four months, all of which reduce misclassification of the HPV outcome variable. Studies that utilize the four-month interval are less likely to miss infection with a relatively short duration, as it is not biologically feasible for the virus to clear and establish a new infection within four months. HPV testing every four months also provides a better estimate of the time at which an HPV infection is acquired. However, since the median duration of oncogenic infections ranges from 7-10 months (49,50), a four month interval will overestimate persistent infections.

However, the benefits of HPV testing every four months do not outweigh the significant costs of conducting numerous clinical visits and laboratory analyses. Therefore, cost effectiveness analyses recommend HPV testing every six months (51). The addition of HPV variant analyses may help distinguish between persistent infection and new infection. Mayrand *et al.* (52) utilized variant analysis of HPV 16 positive samples taken six months apart and reported

that 8% of what have been labeled as persistent HPV16 infections were actually new infections with different variants and ,therefore, misclassified.

There is a lack of consistency in the literature of reporting type-specific infections. The main reason for not reporting type-specific persistence is the lack of detailed HPV typing data (53). Although continued positivity for different oncogenic HPV types puts a woman at risk for cervical lesions (5), there is added biological significance for identification of the same HPV type. Furthermore, a distinction needs to be made between oncogenic HPV infections and nononcogenic infections as there are known differences in cervical cancer risk (16).

Several approaches are being utilized to better define HPV persistence. One approach is to use data collected over multiple visits in a given time period and to generate a categorical variable (persistent vs. transient). This approach is mainly dependent on the interval at which the longitudinal data will be categorized (e.g. persistence over a 12 month period or 24 month period), however it is also dependent on the time-interval between measurements. Inclusion in the definition of the requirement that type-specific HPV infections occur on consecutive visits enhances the definition. Another approach is to examine visit pairs, such that two consecutive visits define one persistent event (40). The final approach utilizes the longitudinal nature of prospective studies and estimates the duration of HPV infection and the probability of clearing that infection.

Frequency of HPV persistence: Just as there are clear differences in the definition of HPV persistence in the literature, there are also differences in the prevalence of persistent infection (5,43,54-58). Methodological factors that can influence prevalence of persistence include time between study visits, HPV detection methods utilized, ability to report type-specific rates, and population screened (e.g. normal cytology vs. abnormal, adolescent vs. adults). Of the studies that only assessed persistence at two visits, the prevalence ranged from 4.3% (34) to 31.1% (35). Type-specific persistence tends to be lower for types other than HPV 16 (5,42). HPV natural history studies that incorporated more than two assessments have further confirmed these results (6,59).

Prospective studies also provide information of the duration of an HPV infection. The Ludwig-McGill Cohort study reported median duration of oncogenic infections of 8.9 months and nononcogenic infections at 7.0 months (49). These data are supported by data collected within the U.S., with the median duration of oncogenic infection of approximately 7-9 months and nononcogenic infection of 4-6 months (3,39,50). Utilizing slightly different analytical methods, Molano *et al.* (60) reported a median duration of 6 months. Two incident cohorts in Canada (59) and United Kingdom (42) reported a similar median duration of any infections at approximately 13 months, although different durations for type specific infections. Several studies have reported that an HPV infection is maintained longer than LSIL (42,61). Among women with both HPV and LSIL at baseline, clearance of LSIL occurred on average 173 days before loss of HPV

infection (61). HPV 16 infections appear to have longer durations (range 10-14 months) (39,49,50), which corresponds to reported increased prevalence of HPV 16 persistence.

### **3.4 Increased Risk of SIL with High Viral Load**

Recent data have suggested that HPV viral load is related to progression of HPV infection to HSIL (62), and to persistence of these pre-malignant lesions (25). HPV viral load has been assessed utilizing several different assays, either semi-quantitative methods of Hybrid Capture II hybridization signal (63) or low-stringency Polymerase Chain Reaction (PCR) (64), or quantitative measures utilizing real time PCR (65). Overall, results from all methods of viral load assessment have consistently found that high viral load is a significant risk factor for progression to SIL (66-68).

Schlecht and colleagues (67) found a dose-response relationship between viral load and degree of SIL, utilizing the LS-PCR methodology within the Ludwig-McGill Cohort Study. Several independent reports, utilizing different methods, confirmed that women with normal cytology and high viral load have a high probability of progressing to SIL (69-74). Decreases in viral load have also been suggested as a marker for HPV clearance (67,74,75). Women with low viral load were more likely to clear HPV infections compared to women with high viral load infections (74).

As viral load appears to be a biomarker of risk for SIL progression, similar to that of HPV persistence, is it important to identify factors associated with HPV viral load. However, there are few reports of factors associated with HPV viral load. As with HPV persistence, host immunity plays a key role in controlling HPV viral load, as identified in associations found with host HLA class variants (76) and among HIV positive women (77). Recently, Flores-Munguia *et al.* (65) reported that some serum antioxidant levels are inversely associated with oncogenic HPV viral load using real time PCR assay. Viral titer is also shown to be decreased with 13-*cis* retinoic acid treatment using Hybrid Capture II (78). These studies support the potential of nutritional factors influencing viral load; however, larger studies need to be conducted.

### **3.6 Summary**

The relationship between an HPV infection and development of cervical dysplasia and carcinoma is not as straightforward as it may appear from the causal evidence. While it is true that the ultimate endpoint of cervical cancer requires the presence of HPV infection, cervical dysplasia or carcinoma will not develop in the majority of women infected with oncogenic HPV. It is the identification of women at most risk for development of cancer that is the goal of cancer prevention. As discussed above, several factors related to the natural history of the viral infection are being considered as biomarkers of risk for progression to cancer. Future studies need to narrow that focus to clearly

identify these persistent HPV infections that have the highest potential for progression. These studies require a consistently defined measure of HPV persistence that better corresponds to the biology of HPV infection.

## **Section 4: Epidemiological Evidence Supporting Nutrients and Cervical Cancer**

### **4.1 Results from Observational Nutritional Epidemiology**

Although oncogenic HPV infections have been established as the necessary cause of cervical cancer, most HPV infections are transient and rarely progress to significant cervical lesions (8). The factors that are associated with oncogenic HPV persistence and progression to HSIL are unknown. This current research is focused on identifying factors that could potentially alter the lifecycle of HPV during an infection and, therefore, are associated with viral persistence and clearance. Cofactors, such as antioxidant nutrients (e.g., carotenoids and tocopherols) and retinoic acid may modulate an oncogenic HPV infection from a transient infection to one that persists and progresses to cancer.

Antioxidant Nutrients, Cervical Dysplasia and Cancer: Early case-control studies provided encouraging results for an inverse association between antioxidant nutrients, either dietary intake and serum levels, and cervical dysplasia, both carcinoma *in situ* and cervical cancer (9). Of the recent studies that have adequately controlled for HPV status (79-84), several report inverse associations between serum  $\beta$ -carotene and risk of SIL (79,80,83) or invasive

cervical cancer (83). Investigating early cervical disease, Goodman *et al.* (85) found a significant inverse associations between two serum antioxidants (cryptoxanthin and tocopherol) and detection of atypical squamous cells of undetermined significance (ASCUS) during cytological screening. Among recent studies investigating dietary intake and SIL controlling for HPV, one case-control study (86) reported inverse associations with foods containing vitamin C,  $\beta$ -carotene, and folic acid, whereas another case-control study among African American women (87) and a nested case-control study found no dietary associations with SIL (86,88). Another case-control study reported cervical cancer patients were more likely to have lower consumption of vitamin E and vegetables compared to control patients (89).

These significant inverse associations have extended from  $\beta$ -carotene to other carotenoids and cervical dysplasia, including serum lycopene (81,83),  $\alpha$ -carotene (81,84),  $\alpha$ -cryptoxanthin (79),  $\beta$ -cryptoxanthin and lutein/zeaxanthin (84) and SIL. Additional studies suggest that higher serum lycopene (87) concentrations were associated with lower risk for SIL. Among the studies that assessed tocopherol concentrations (57,79-81,83,90), significant inverse associations (57,79,80,83) are observed with risk for SIL or CIN, with a study suggesting an inverse association, although not statistically significant (90). In addition, independent of HPV status,  $\alpha$ -tocopherol is significantly inversely associated with grade of SIL or CIN (57).

A prospective study observed an inverse association between serum retinol level and rate of progression to cervical cancer among women with dysplasia (91). In a population based nested case-control study out of Finland and Sweden, serum levels of retinol were not independent risk factors for cervical cancer, however there was a significant interaction suggesting increased risk of cancer among women with low retinol status and HPV seropositivity (92). After adjusting for HPV, an inverse association of serum retinol has been observed with CIN I (90,93), but was not confirmed by others (79,81). Among HIV positive women, retinol deficiency was significantly associated with increase risk of SIL, after adjustment for HPV (94). Foods with high retinol content were inversely associated with carcinoma *in situ*, but not invasive cancer after adjusting for HPV status (95).

Antioxidant Nutrients and HPV Persistence, Clearance and Viral load To date, there is a limited number of reports that have examined the association between serum concentrations or dietary intake of carotenoids and tocopherols and the natural history of HPV infection, specifically HPV persistence (57,96,97), HPV clearance (98) and viral load (65). Adjusted mean concentrations of serum  $\beta$ -carotene,  $\beta$ -cryptoxanthin, lutein, and  $\alpha$ - and  $\gamma$ -tocopherol were on average 24% ( $p < 0.05$ ) lower among women persistently positive for intermediate and high risk type HPV infection compared with women who were intermittently positive or persistently negative (57). In a small prospective study among women attending a family planning clinic for routine care, Sedjo *et al.* (96) observed significant

inverse associations between dietary intake of lutein, vitamin E, vegetables and serum lycopene levels and HPV persistence (96). In this population of young women, higher levels of vegetable consumption and dietary lutein intake were associated with over a 50% decreased risk of HPV persistence (96). Preliminary findings from a multi-ethnic cohort in Hawaii reported inverse association with oncogenic type-specific HPV persistence and change in serum  $\beta$ -carotene and  $\alpha$ -tocopherol over a 4-month period (99). Giuliano *et al.* (97) examined whether dietary intake of carotenoids was associated with HPV persistence over a 12-month time span within the Ludwig-McGill Cohort Study. Risk of type-specific persistent HPV infection was lower among women reporting intake in the upper two-quartile levels of  $\beta$ -cryptoxanthin, lutein/zeaxanthin and the upper quartile of vitamin C. Consumption of papaya one or more times per week was inversely associated with persistent HPV infection (97).

A few studies have assessed the association between serum antioxidant nutrients and HPV persistence within the context of a  $\beta$ -carotene clinical trial (37,82). These trials reported no association among level of serum retinol,  $\beta$ -carotene, vitamin C and HPV persistence for either the control or supplemented groups (37,82). However, these women were enrolled with pre-existing lesions, 50% LSIL (CIN I) and 49% HSIL (CIN II/III) (37), therefore representing events late in the disease process, in contrast to the analyses of HPV persistence among women with normal or ASCUS cytology at baseline.

Only one study has examined time to clearance of HPV infections in relation to serum nutrient levels. The likelihood of clearing an oncogenic HPV infection was significantly higher with increasing levels of *trans*-lycopene (p for trend, 0.025) and *cis*-lycopene (p for trend, 0.010) among the 84 women with at least one oncogenic infection at baseline (98). Within this same cohort study, Flores-Munguia *et al.* (65) found inverse associations of serum nutrients (*trans*- and *cis*-lycopene,  $\beta$ -cryptoxanthin) and oncogenic HPV viral load determined by real time-PCR. Interestingly,  $\alpha$ -carotene and tocopherols ( $\alpha$ -,  $\delta$ -, or  $\gamma$ -) were positively associated with viral load. These results suggest a possible role of micronutrients in viral burden, which needs to be further characterized in larger prospective studies.

#### **4.2 Limitations of Nutritional Epidemiological Studies**

Since the mid-1980s, numerous observational epidemiological studies have examined the association between cervical cancer risk and nutritional status, with nutritional status assessed as either dietary intake or serological measures of nutrients (100,101). Unfortunately, a majority of these earlier studies had methodological issues in the assessment of nutritional exposures, with only a few assessing the spectrum of carotenoids (102-104). Studies published before 1993 were unable to examine the full range of carotenoids that may be biologically active in serum, due to lacking analytic methods, specifically High-Pressure Liquid chromatography (HPLC) for separating and quantifying the

major carotenoids and their geometric isomers in serum, and due to inadequate nutrient databases for carotenoid content of the dietary intake other than  $\beta$ -carotene (105). Furthermore, a majority of these studies investigating cervical cancer were conducted before a reliable test for HPV status was available. It is now recognized that HPV status, a causal factor for cervical cancer, must be controlled for in the analyses (9,106) and the maintenance of this infection over time needs to be assessed (47). In this regard, another limitation of reported nutritional-cervical cancer studies has been the use of retrospective study designs, which were unable to examine where in the carcinogenesis continuum nutrients were active. Few studies controlled for potential confounders when evaluating cervical cancer risk associated with nutrients (100). Therefore, much of the literature reporting associations between nutrient status and cervical dysplasia or invasive cancer before 1996 had methodological issues and was excluded from this discussion.

### 4.3 Results from Nutrient-based Clinical trials

*$\beta$ -Carotene:* A Phase II study of  $\beta$ -carotene (30 mg/day) supplementation among women with CIN I and II reported response rates over 60% at months 3 and 6, with lower response at month 12 (30%); however, no controls were included to compare these responses to natural regression rates (107). Romney *et al.* (37) conducted a randomized double-blind trial to evaluate the efficacy of oral  $\beta$ -carotene (30 mg/day) for nine months in the regression of predominately

LSIL (CIN I) or HSIL (CIN II) lesions. They found no significant differences in CIN regression rates when comparing the  $\beta$ -carotene group and placebo (37). An additional study reported no benefit of oral  $\beta$ -carotene supplementation for regression of HSIL (CIN II and III) over a two-year period (108). As with other studies of SIL regression, these studies were likely underpowered to detect significant treatment effects above the already high rate of SIL regression (108,109).

Retinoids: Through potent activity as transcriptional regulators, retinoids (natural and synthetic derivatives of vitamin A) have been shown to be cancer chemotherapeutic and chemopreventive [see (110-112) for reviews]. Epidemiological evidence and the success of early phase I/II trials (113-117) of topical *all-trans* retinoic acid in the regression of cervical dysplasia raised enthusiasm for potential potent chemopreventive effects of retinoids. One limitation of these early studies was the lack of a placebo group for accurate comparisons, especially due to the high rate of spontaneous regression of cervical lesions (109,118). Meyskens *et al.* (119) reported a statistically significant histological regression rate of 47% of patients with HSIL (CIN II) randomized to the *all-trans* retinoic acid group (applied via collagen sponge and cervical cap) compared to 27% regression in the placebo group. However, no differences were seen for patients with HSIL (CIN III) (25% *all-trans* RA vs. 31% placebo) (119).

These results have not been replicated with chemopreventive or chemotherapeutic treatment of other retinoids. A randomized double blind clinical trial of oral 9-*cis* RA (aliretinoin) in regression of HSIL (CIN II and III) reported no differences in SIL regression rates between placebo, low-dose or high-dose (N per group 35-40) over a 12 week period (118). Among a small sample of women, oral 13-*cis* retinoic acid over a 6-month period was ineffective at preventing recurrence of SIL following colpoconization (120). Among HIV positive adolescent women with LSIL, treatment with 13-*cis* retinoic acid did not result in improvement to normal cervix, nor did it decrease risk of progression to HSIL (121). In addition to naturally occurring retinoids, chemoprevention trials have been conducted using retinoic acid derivatives. One promising synthetic oral retinoid, N-(4-hydroxyphenyl)retinamide (4-HPR), decreased cell proliferation and induced apoptosis independent of retinoic acid receptors in laboratory models (122,123). Follen *et al.* (124) reported interim analysis of 39 patients enrolled in a double-blind randomized placebo controlled clinical trial of 4-HPR oral dose (200 mg/day) for 12 months in women with HSIL (CIN II and III). At both six and twelve months, the placebo group had a significantly higher regression rate compared to the 4-HPR treatment group, resulting in termination of the trial prior to completion of enrollment (120 patients) (124). Pilot studies of Retinamide II conducted in China have suggested high regression rates using topical treatment (suppositories) in women with all grades of cervical dysplasia;

however, final results from the ongoing randomized clinical trial in this population are not available (125).

Retinoids (*all-trans* retinoic acid, *9-cis* retinoic acid, *13-cis* retinoic acid, or 4-HPR) alone or in combination with inflammatory agents (Interferon- $\alpha$  or - $\gamma$ ) were not effective in the treatment of advanced cervical cancer (126) or recurrent cancer (127-129).

Although retinoid chemoprevention was effective in regression of HSIL (CIN II) lesions, there are toxicities associated with therapy such as varying degrees of teratogenicity and mucocutaneous cytotoxicity (110,130). These toxicities make long-term chemoprevention among healthy populations with naturally occurring retinoic acid unfeasible. An alternative retinoid chemoprevention approach is to develop a method for increasing endogenous concentrations of retinoic acid. Such an approach was demonstrated to be feasible by Sedjo *et al.* (131) who reported the modulation of endogenous retinoic acid following chemopreventive doses of retinyl palmitate. One focus of this dissertation is to examine if the variability of endogenous retinoic acid is associated with variability of dietary intake, implying that diet may modulate levels of retinoic acid safely.

## **SECTION 5: Biological Activity and Anticarcinogenic Mechanisms of Antioxidant Nutrients (Carotenoids, Tocopherols and Retinoic Acid)**

### **5.1 Antioxidant Nutrients: Carotenoids and Tocopherols**

#### **5.1.1 Biological Activity**

Carotenoids, tocopherols and retinoic acid are biologically active and important in maintaining healthy cells through various mechanisms. Both carotenoids and tocopherols are lipid soluble nutrients with potent antioxidant activities. The antioxidant potential of the carotenoids, in order of potency, is lycopene> $\beta$ -carotene= $\beta$ -cryptoxanthin>lutein> $\alpha$ -carotene (132). As antioxidants, these nutrients are part of an antioxidant defense system utilized by cells to control levels of potentially damaging bi-products of normal metabolism, such as reactive oxygen species (ROS) (133). The vitamin E family ( $\alpha$ -, $\delta$ - and  $\gamma$ -tocopherol) are chain-breaking antioxidants that intercept lipid peroxy radicals and terminate lipid peroxidation chain reactions. Vitamin C is not only a free-radical scavenger, but also contributes to the regeneration of tocopherols. Carotenoids quench singlet oxygen and free radicals through their polyene structure (134).

Dietary antioxidants, considered as free-radical scavengers, act to control the levels of ROS within cells to allow normal cellular processes involving ROS to continue without inducing damage. ROS, including superoxide, hydrogen peroxide and hydroxyl radicals, are generated as by-products of reactions occurring within cells, such as electron transport chain, hemoglobin autoxidation,

and Fenton reactions oxidizing metals. Alternatively, ROS are actively produced and utilized by activated immune cells as a host defense against infections. In addition, ROS appear to have a central role in cell signaling by transcription factors, cell proliferation, and apoptosis (133,135). The cellular response to increases in oxidant load is the activation of redox-sensitive transcription factors (Nuclear Factor- $\kappa$ B (NF- $\kappa$ B), Activator Protein-1 (AP-1) and p53) (136). NF- $\kappa$ B is responsible for rapid transcription of defense genes, involved in inflammatory, immune and acute phase responses (136). Continued presence of high oxidant load results in the induction of apoptosis.

The oxidant-antioxidant balance is particularly critical for the immune system, which relies on the production of ROS for host defenses but is also sensitive to oxidative damage (137). ROS can lead to lipid peroxidation, which alters cell membrane integrity and disrupts cell-cell communication via reduction or alteration of membrane-bound receptors. This receptor disruption results in intracellular signaling alterations and decreases in immune cell function (134,138). Immune dysfunction, including decreases in natural killer cell activity and T-cell proliferation, has been observed with high oxidant:antioxidant ratios (139).

As potent antioxidants, carotenoids and tocopherols may strengthen host cellular and humoral immunity (138). Higher serum  $\beta$ -carotene levels are related to increases in delayed-type hypersensitivity (DHT) responses, the number of natural killer cells and cell surface molecules involved in antigen presentation

(137). Influence of serum lycopene and lutein on immune function appears to be lower than that of  $\beta$ -carotene (140), however definitive studies have not confirmed these observations (137). Vitamin E is vital in maintaining the integrity of immune cell membranes and vitamin E deficiencies have been shown to decrease B-cell antibody production and T-cell proliferation (134). These decreases in immune functions with antioxidant deficiency, specifically T-cell activity, provide a host environment that is susceptible to the establishment and persistence of viral infections (141).

#### **5.1.2 Role of Antioxidant Nutrients in HPV Infection and Cervical Cancer**

As indicated above, antioxidant nutrients are required for maintaining health. Elevated levels have been suggested to guard against cervical cancer development and influence HPV infection. The exact biological mechanism by which these nutrients prevent cervical cancer remains unclear. Indirect evidence indicates that during conditions that support oxidative stress, such as decreased intake of antioxidant nutrients or presence of infections, there is a shift in the antioxidant-oxidant balance in favor of high oxidant load. This high oxidant load has been associated with cancer, increased viral activity and decreased immune function. Taken together, the combined presence of HPV infection and decreases in antioxidant nutrients may provide a cellular environment that facilitates HPV persistence and progression to cancer (9,142).

Biological associations of oxidant load, antioxidants and viral activity:

Oxidative stress has been implicated in the pathogenesis of several viral infections, including HIV, influenza, and cytomegalovirus (CMV) (133). Viruses induce oxidative stress not only as the immune system initiates a defense against the virus, but also through specific viral protein activities (143). This viral induced oxidative stress results in activation of redox-sensitive transcription factors that are utilized by viruses to support expression of viral genes and subsequent replication of viral genomes (144). In addition, high oxidant load environments have been shown to influence the virulence of viruses through genomic changes (145).

*In vitro*, oxidative stress increases the replication of HIV (146-148) through the ROS mediated activation of NF- $\kappa$ B, a nuclear transcriptional factor that is obligatory for HIV replication (148). *In vitro* studies have consistently demonstrated that NF- $\kappa$ B activation is inhibited by antioxidants such as  $\alpha$ -tocopherol (149),  $\alpha$ -lipoic acid (150), N-acetylcysteine, and pyrrolidine-dithiocarbamate (PDTC) (149) in HIV infected cells. A recent clinical trial reported a decrease in HIV viral titer among HIV patients supplemented with  $\alpha$ -tocopherol (800 mg/day) compared to non-supplemented HIV patients (151). Another virus, CMV, triggered intracellular increases in ROS *in vitro*, with increases in NF- $\kappa$ B activity. This increase led to NF- $\kappa$ B dependent viral transcription, and subsequent viral replication, and was abrogated in the

presence of antioxidants (152). Using animal models, Peterhans *et al.* (147) demonstrated that influenza viral infection was associated with oxidative stress, and that the resulting ROS increased viral titer (147) and viral infectivity of the virus (153). Administration of antioxidants to animals infected with the influenza virus protected them from the lethal effects of influenza (154). In addition, *in vitro* treatment with PDTC antioxidant inhibited influenza viral synthesis and apoptosis (155). Hepatitis B protein X induces mitochondrial oxidative stress leading to increased activation of two transcription factors (NF- $\kappa$ B and STAT-3) *in vitro*, which could be inhibited by treatment with N-acetylcysteine and PDTC (143).

The findings reported for several viruses, that antioxidant nutrients play a role in down-regulating viral replication and expression, are particularly relevant to cervical carcinogenesis, where viral replication (viral load) and transcriptional activity (expression of HPV 16 E6 and E7 proteins) are critical events. There is evidence to suggest that reactive oxygen species, and their down regulation by antioxidants, may work in a similar manner in HPV infection. Specifically, the effect of antioxidant nutrients on HPV infection can be mediated through the activation of redox-sensitive transcription factors, NF- $\kappa$ B and AP-1 (135,156). AP-1 is a complex of proteins within the c-Jun (c-Jun, c-JunB and c-JunD) and c-Fos (c-Fos, FosB, Fra1 and Fra2) protein families. The specific composition of proteins that form the AP-1 complex, either as homo- or heterodimers, determines the specific activity and binding sites (157). AP-1 has been shown to be a key regulator of HPV transcriptional activity (158,159), and its activity is

altered by HPV E7 in a cell-cycle dependent manner (160). Antioxidant treatment of HPV-16 immortalized human keratinocytes (HKc) with PDTC altered the composition and reduced the activity of the transcriptional AP-1 complex (161) Although not directly shown, the presence of NF- $\kappa$ B binding sites in the HPV upstream regulatory region and evidence of HPV responsiveness to NF- $\kappa$ B activity, suggests that HPV viral replication and expression should be enhanced by the activation of this ROS sensitive transcription factor. Activation of NF- $\kappa$ B occurs in response to cytokines released during an inflammatory response, such as interleukin-1 (IL1) and tumor necrosis factor (TNF- $\alpha$ ). In summary, the indirect evidence indicates that during conditions that support oxidative stress, such as decreases of antioxidants nutrients and/or the presence of a viral infection, there is a shift in the antioxidant-oxidant balance in favor of high oxidant load. This high oxidant load has been associated with increased risk of cancer, increased viral activity and decreased immune function. Taken together, the combined presence of an HPV infection and decreased serum antioxidant nutrients may provide an environment that facilitates HPV persistence and progression to cervical cancer.

Reactive Oxygen Species, Antioxidants and Immunity: The innate immune response towards viral infections includes secretion of interferon (INF- $\gamma$ ) and activation of natural killer cells (9), which is compromised when dietary antioxidants are limiting (137). By preventing oxidant-induced down-regulation of

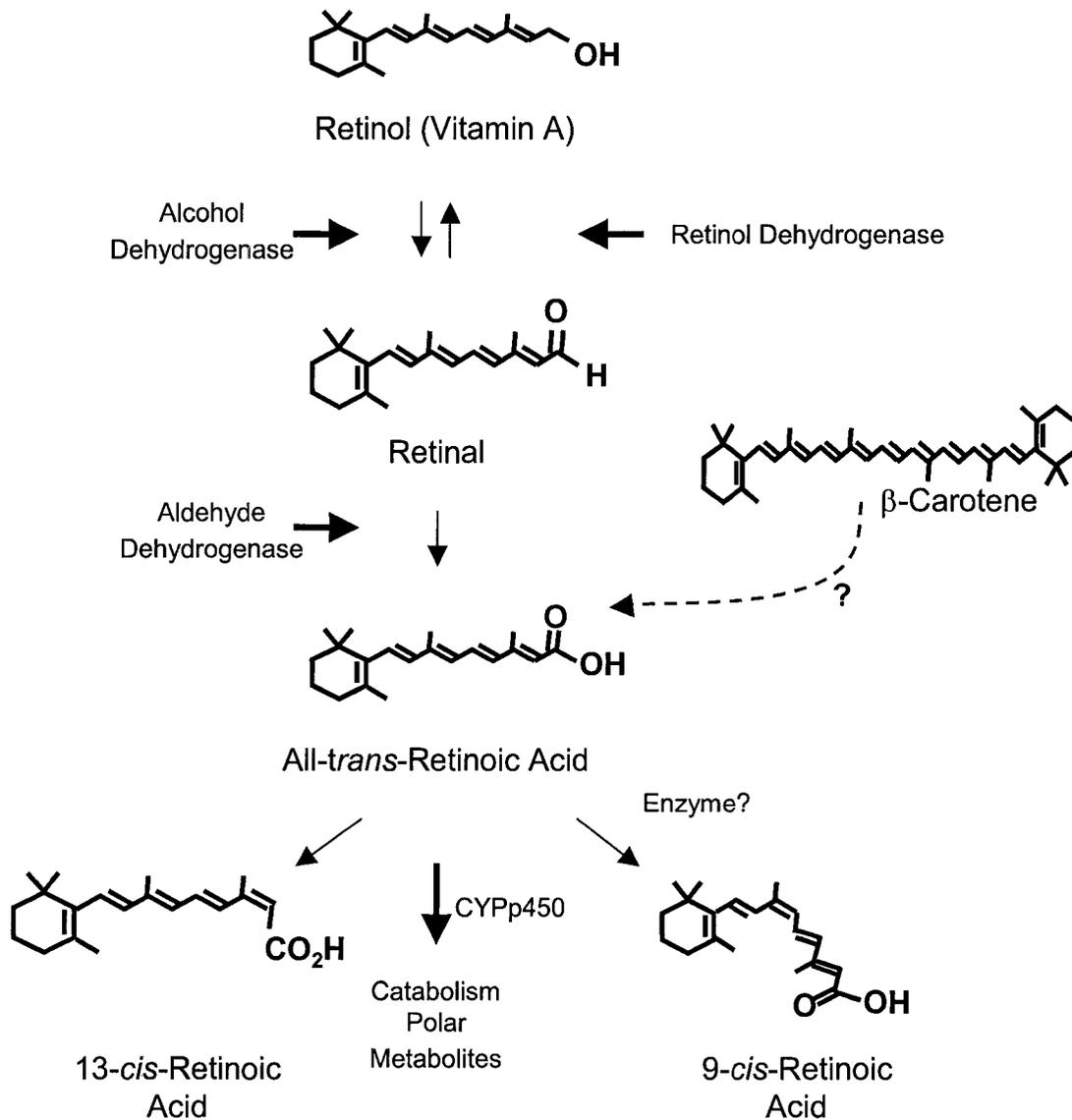
cellular immunity, higher concentrations of carotenoids and tocopherols may decrease risk for persistent HPV infection and lesion progression by modulating T-cell immunity. Recent data indicate that T-cell immunity, and natural killer cell activity in particular, is important to the loss of HPV infection and SIL regression (162). In addition, the importance of acquired immunity (e.g. antibody producing B-cells) has recently been established with the successful prevention of HPV-16 infection persistence following vaccination with HPV-16 specific virus-like particles (7).

## **5.2 Retinoic Acid**

### **5.2.1 Retinoic Acid Metabolism**

Diet provides retinol (preformed Vitamin A) in the form of retinol ester from animal products (liver, eggs, milk, cheese and butter) or as pro-vitamin A carotenoids (e.g.  $\beta$ -carotene,  $\alpha$ -carotene, and  $\beta$ -cryptoxanthin) (163-165). Figure 2.3 (page 55) provides an overview of the metabolism of retinol to retinoic acid. Following absorption, retinol is transferred and stored as esters in the liver. As needed, retinol esters are mobilized from liver stores, transported to specific tissues and are reversibly converted from retinol into retinal. This oxidation to retinal is presumed to be the rate-limiting step in retinoic acid biosynthesis, and potentially involves members of up to three isoenzyme families (medium-chain alcohol dehydrogenases (ADH), short-chain retinol dehydrogenase (RDH), and cytochrome P450 (CYPp450) 1A and 1B)(165,166). Retinal is then irreversibly

Figure 1.3: Metabolism of Retinol (Vitamin A) to Retinoic Acid



Retinol (preformed Vitamin A) is reversibly converted to retinal (rate-limiting step) by medium-chain alcohol dehydrogenases. Retinal is irreversibly converted to all-*trans* retinoic acid via aldehyde dehydrogenases, which can isomerize to 13-*cis* retinoic acid or 9-*cis* retinoic acid. Retinoic acid is catabolized to polar metabolites by CYPp450RA1 or CYP26 enzymes and excreted.

converted to all-*trans* retinoic acid via members of the aldehyde dehydrogenase family (166). All-*trans* retinoic acid can isomerize to 13-*cis* retinoic acid and 9-*cis* retinoic acid; however it is not clear when the majority of the isomerization occurs during metabolism. It has been shown that the conformation of retinol may influence the specific ADH or RDH utilized in metabolism, and that a majority of those identified appear to be selective for all-*trans* retinoic acid (166). 9-*cis* retinoic acid has also been shown to be derived directly from dietary 9-*cis*  $\beta$  carotene (167) or 9-*cis* retinol (168). Recent *in vitro* studies using animal and human tissues have demonstrated a dose-response conversion of  $\beta$ -carotene to retinoic acid (167,169,170). In addition to isomerization, retinoic acid is efficiently catabolized to polar metabolites with little to no biological activity by CYP450RA1 or CYP26 enzymes and excreted (165,166).

### 5.2.2 Biological Activity of Retinoic Acid

Retinoic acid is required for many biological processes including vision, development and reproduction (163-165). Retinoic acid manifests this activity by binding to nuclear retinoid receptors, members of the steroid hormone receptor superfamily (171), and altering transcriptional activity (110,165). To date, two receptors have been identified, retinoic acid receptor (RAR) and retinoid X receptor (RXR) (110). RAR binds to all-*trans* and 9-*cis* retinoic acid with equal affinity, whereas RXR preferentially binds 9-*cis* retinoic acid (172). Each receptor has at least three subtypes ( $\alpha$ ,  $\beta$ , and  $\gamma$ ) and within these subtypes there are

many different isoforms (173). Due to the different receptor isoforms retinoid receptors can interact with a diverse number of receptors forming homodimers and heterodimers not only with retinoid receptors but with other steroid hormone nuclear receptors as well, such as vitamin D receptors and the estrogen receptor (174).

Retinoic acid regulation of epithelial cells: *In vitro*, retinoic acid has been shown to regulate differentiation of normal human ectocervical epithelial cells (175). Low levels of all-*trans* retinoic acid (1-10 nM) stimulated growth of normal human keratinocytes in culture, however high levels (1 $\mu$ M) inhibited growth (176). The reactivity of cells to retinoic acid is dependent on the presence of cellular retinoic acid binding protein (CRABP) and the retinoic acid receptors (RAR/RXR). Using immunohistochemistry, Hillemanns *et al.* demonstrated that CRABP was predominantly present in the basal layer of the normal squamous cervical epithelium biopsy tissue, and the levels of CRABP decreased in more superficial, differentiated cell layers (177). Retinoid receptors are also present in normal ectocervical epithelium. In mouse models, RAR and RXR transcripts were found to be differentially expressed in the two types of cervical cells, stratified squamous and simple columnar epithelium. RAR $\alpha$ -1 and -2 and RXR $\alpha$  and  $\beta$  transcripts were identified in both regions of the cervix, whereas RAR $\beta$ -2 and -3 were only found in columnar epithelium. In the stratified squamous epithelium, RXR $\alpha$  and  $\beta$  was preferentially localized to basal cells and RARs were expressed

in basal and suprabasal layers (178). Similar patterns of expression of RAR $\alpha$ , RAR $\gamma$ , RXR $\alpha$  and RXR $\beta$  were also demonstrated *in vitro* with a LSIL cell line and SiHa cells derived from a high grade cervical tumor (179). High levels of RAR $\beta$  mRNA have been observed in normal cervical cell lines (180) and absent in a dysplastic and carcinoma cell lines (179,180). The differential expression of RAR $\beta$  may be relevant in cervical cancer progression, as RAR $\beta$  is lost in many types of cancers and has been associated with retinoic acid-resistance of tumors (181). RAR $\beta$  is induced by all-*trans* retinoic acid and thought to be responsible for *in vitro* retinoic acid-induced growth inhibition (181).

RARs and RXRs have a multitude of targets and interact with many different nuclear receptors to elicit differentiation and growth inhibition in a cell specific manner. Of relevance to cervical cancer and HPV infection, retinoic acid treatment *in vitro* induces synthesis and secretion of growth inhibitors (TGF- $\beta$ ), and growth factor binding proteins (e.g. insulin-like growth factor binding proteins (IGFBP) and epidermal growth factor binding proteins (EGFBP)). Normal ectocervical cells increase secretion of TGF- $\beta$  and cell proliferation is inhibited in response to retinoic acid treatment; however, normal endocervical cells secrete TGF- $\beta$  and are not inhibited (182). Thus, the heterogeneity of retinoic acid-induced response appears to have cell-type specific activities. In addition, following treatment with  $\beta$ -carotene, there was an increase in TGF- $\beta$  in CIN biopsies (183).

### 5.2.3 Role of Retinoic Acid in HPV Infection and Cervical Cancer

Retinoic acid is essential for differentiation of cervical epithelial cells; however this activity is dependent on the presence of retinoic acid binding protein and, most importantly, the expression of retinoic acid nuclear receptors, which are the biologically active receptors of retinoic acid. Retinoic acid has been demonstrated to inhibit growth and promote differentiation of HPV infected cells and to have a direct influence on HPV oncogene expression *in vitro*. In addition, along the multi-step progression to cervical cancer, it appears that HPV infected cells lose responsiveness to retinoic acid through various mechanisms. Altogether, these data suggest retinoic acid may only be effective early in cervical carcinogenesis, such as by modulating HPV persistence, clearance and viral load.

#### Retinoic acid induces growth inhibition in HPV infected epithelial cells:

Treatment with retinoic acid differentially inhibits growth of HPV-16 immortalized cervical epithelial cells (ECE-16) (175,184) and low-passage human foreskin keratinocytes (HKc/HPV16) (185-187) compared to normal HKc cells treated with retinoids in the absence of an HPV infection. Cervical epithelial cells transfected with HPV 16 (ECE-16) or HPV 18 (ECE-18) and CIN derived cells also demonstrate growth inhibition when treated with *all-trans* retinoic acid, however not to the extent seen in HKc/HPV16 cells. Retinoic acid induced growth inhibition *in vitro* is associated with increases in the synthesis and secretion of TGF- $\beta$ , a growth inhibitor (176) and decreases in HPV induced epidermal growth

factor receptor (EGFR) synthesis (188) and insulin-like growth factor binding proteins (IGFBP) (189,190). It has been demonstrated that these alterations are specifically induced by all-*trans* retinoic acid/RAR interactions and not 9-*cis* retinoic acid/RXR (190,191).

As epithelial cells immortalized with HPV-16 acquire more tumorigenic characteristics, they become resistant to retinoic acid induced growth inhibition (176,192). An *in vitro* system that utilizes continuous passage of HPV-16 infected human foreskin keratinocytes (HKc/HPV16) helps to provide insights into retinoic acid resistance. HKc/HPV16 cells grown for many passages obtain further tumorigenic characteristics at well-characterized points (176). In this model system, HKc/HPV16 cells are initially (e.g. for a low number of passages) more sensitive than normal HKc to growth and differentiation control by all-*trans* retinoic acid (176,185-187), whereas high-passage cells are less sensitive, with only a 20% inhibition of growth with 1mM all-*trans* retinoic acid (176). In this system, differences between low passage and high passage sensitivity to retinoic acid is associated with cellular loss of sensitivity to retinoic acid-induced TGB $\beta$  (a growth inhibitory factor), and not to alteration in RAR or RXR signaling. However, RAR $\beta$ , whose induction has been shown to be lost during progression to cervical cancer (181), has not been examined. Others have reported loss of growth inhibition in cervical carcinoma cell lines (189), with loss of an induction of insulin-like growth factor binding protein. In several *in vitro* model systems, cells in the late stages of HPV-16 induced transformation acquire resistance to retinoic

acid induced differentiation through several different mechanisms, including loss of growth inhibition (176), continued growth stimulation (188-190), and loss of retinoid receptor expression (193).

Retinoic acid induces differentiation in HPV infected epithelial cells:

Retinoic acid is essential for terminal differentiation of cervical epithelial cells by decreasing cellular proliferation and DNA replication. Research examining the activity of retinoic acid on HPV infected epithelial cells, *in vitro*, measures markers of cellular growth and cellular differentiation. Organotypic graft cultures of HPV-16-immortalized human endocervical cells are powerful *in vitro* models of cervical cell differentiation (194). Expression levels of cytokeratin genes are also a marker of epithelial cell differentiation. In addition to inhibiting cellular proliferation, retinoic acid HPV-16 immortalized cervical epithelial cells (175,182,184) are more sensitive to the differentiation, as measured by alterations in cytokeratin gene expression demonstrated cells, induced by retinoids compared to normal cells treated with retinoids in the absence of an HPV infection (175,184). Using an organotypic graft culture of HPV-16-immortalized human endocervical cells, retinoic acid prevents expression of cytokeratin differentiation markers of carcinoma *in situ* and decreases E7 mRNA (195). Further down the carcinogenic pathway, the organotypic graft culture model demonstrates that HPV-16 tumorigenic cells are resistant to retinoic acid treatment by maintaining characteristics of carcinoma *in situ* (192). However, Sarma *et al.* (192) did not investigate possible mechanisms of resistance, such

as loss of retinoid nuclear receptors, retinoic acid binding protein or changes in sensitivity to inhibitory molecules.

Retinoic acid influences viral activity: *In vitro*, physiological concentrations of retinoic acid inhibited the expression of HPV 16 E6 and E7 expression in the low passage HKc/HPV16 cells (185-187) and in CaSki cells (196). Retinoic acid also inhibited late passage HPV-16 mediated immortalization of HKc/HPV16 cells. Decreased levels of viral oncoproteins resulting in increases in p53 and cell cycle arrest in G1 were observed in CaSki cells treated with 1 nM-10  $\mu$ M ( $10^{-9}$  -  $10^{-5}$  M) of all-*trans* or 9-*cis* retinoic acid (196). Other *in vitro* models demonstrated retinoic acid-induced growth suppression in epithelial cervical cells (ECE-16) and CaSki cells without changes in HPV 16 E6 and E7 level (184,197). Both all-*trans* and 9-*cis* retinoic acid inhibited HPV E6 and E7 expression, however all-*trans* retinoic acid was more potent requiring smaller concentrations to observe significant inhibition (196). In addition, retinoic acid treatment resulted in transcriptional repression of HPV 18 with a concomitant down regulation of HPV mRNA levels (193). Retinoic acid may indirectly reduce HPV mRNA levels through influences on AP-1 activity (198) or TGF $\beta$  expression (199). Retinoic acid is a negative regulator of AP-1 (198), the predominate transcription factor utilized by HPV for expression of E6 and E7 (158,159). Alternatively, retinoic acid may reduce HPV mRNA levels through increased expression of TGF $\beta$ , which has been demonstrated to reduce E6 and E7 expression *in vitro* (199).

## **Section 6: Summary of Antioxidant Nutrients, Retinoic Acid and HPV Infection**

Although oncogenic HPV infections have been established as the necessary cause of cervical cancer, most HPV infections are transient and rarely progress to significant cervical lesions (8). The factors that are associated with oncogenic HPV persistence and progression to HSIL are unknown. This current research is focused on identifying factors that can potentially alter the lifecycle of HPV during an infection and, therefore, are associated with viral persistence and clearance. Cofactors, such as antioxidant nutrients (e.g., carotenoids and tocopherols) and retinoic acid may modulate an oncogenic HPV infection from a transient infection to one that persists and progresses to cancer.

Mechanistically, during conditions that support oxidative stress, such as low antioxidant nutrients and/or the presence of a viral infection, there is a shift in the antioxidant-oxidant balance in favor of high oxidant load. The combined presence of an HPV infection and decreased serum antioxidant nutrients has been proposed to provide an environment that facilitates HPV persistence and progression to cervical cancer (9,142). In support of this mechanistic research, epidemiological evidence suggests that antioxidant nutrients may reduce the risk of an HPV persistent infection. Recently published studies (57,96,98) have examined the association between antioxidant nutrient status (dietary intake and serum level) and persistent HPV infection among women in the United States. In a small prospective study among women attending a family planning clinic for

routine care, Sedjo *et al.* (96) observed significant inverse associations between dietary intake of lutein, vitamin E, and vegetables and serum lycopene levels and HPV persistence. These studies have had small sample sizes and did not to assess type-specific HPV persistence (96) and clearance (98). In addition, studies have included only one measure of serum carotenoids and tocopherols which may not have adequately classified nutrient status due to high-within person variability of these measures (200).

In addition to antioxidant activity, pro-vitamin A carotenoids ( $\beta$ -carotene,  $\alpha$ -carotene,  $\alpha$ - and  $\beta$ -cryptoxanthin, and zeaxanthin) are precursors of retinoic acid, a potent regulator of epithelial cell differentiation (112). Retinoic acid has been extensively studied *in vitro* (112,165) and utilized in chemoprevention trials (110); however, assessment of endogenous levels has only recently become available due to advances in HPLC methodology. Recent improvements in HPLC methodologies have facilitated the measurement of endogenous retinoic acid within the context of epidemiological studies. The key improvements include an improved separation of three retinoic acid isomers and implementation of high throughput methods.

The current study, using refined methodology for measuring circulating retinoic acid levels, sought to assess the variability of retinoic acid and the factors associated with endogenous retinoic acid levels. This information is essential to the design of future epidemiological studies focused on assessing retinoic acid-disease associations and intervention studies aimed at modulating retinoic acid

levels. As the activity of HPV in cervical cells is tightly associated with epithelial cell differentiation (25), we hypothesized that factors regulating epithelial cell differentiation (e.g. retinoic acid) may be associated with duration of an HPV infection and viral burden.

The current study sought to address the association of serum antioxidant nutrients (retinoic acid, carotenoids and tocopherols) and type-specific HPV persistence, clearance and viral load over a 12-month period. Data were from Brazilian women participating in a prospective study examining the natural history of HPV infection (Ludwig-McGill Cohort Study) (Specific Aims 2 and 3). Prior to addressing associations between serum retinoic acid and HPV clearance, we determined the variability and factors associated with retinoic acid levels among a sub-sample of 502 women (Specific aim 1).

## **DISSERTATION FORMAT**

This research utilizes data collected from the NIH RO1 grant (CA81310) “Antioxidant Nutrients among a Brazilian Population” awarded to Dr. Anna Giuliano, Professor in the Mel and Enid Zuckerman Arizona College of Public Health and co-Director of the Cancer Prevention and Control Program at the Arizona Cancer Center. This study is nested within the Ludwig-McGill Cohort study being conducted in São Paulo, Brazil. Drs. Eduardo Franco and Luisa Villa are the co-principal investigators for the cohort study and are funded by several international grants, including the National Cancer Institute (CA70269), the Canadian Institutes of Health Research (CIHR) (MA-13647, MOP-49396), and an intramural grant by the Ludwig Institute for Cancer Research. I received an R25 NCI-sponsored Pre-Doctoral Fellowship (R25CA078447) through the Arizona Cancer Center, Cancer Prevention and Control Training Program (Principal Investigator, Anna Giuliano). This fellowship provided a portion of my salary during work on this research.

I began work on this project, when it was funded in August 2000, as the Program Coordinator. My contribution to this project included overall organization, the creation and maintenance of the study database, initiation and tracking of sample shipments from Ludwig Institute for Cancer Research (Brazil) to Craft Technologies (North Carolina) for analysis of retinoic acid, carotenoid and tocopherol analyses, tracking of nutrient results back to Arizona, assessment of missing nutrient data, and compilation of a final data file. I also conducted all

statistical analyses, summarized results and drafted manuscripts for journal submission.

The Epidemiology Graduate Program within the Mel and Enid Zuckerman Arizona College of Public Health at the University of Arizona established that three publishable manuscripts could contribute to the main body of a dissertation. Therefore, three manuscripts from the current study were incorporated into this dissertation. Dr. Anna Giuliano provided assistance on analysis strategies and in the interpretation of the results. Dr. Denise Roe provided statistical consultation.

## **CHAPTER 2**

### **PRESENT STUDY**

#### **METHODS**

##### **Section 1: Overview of the Study**

This research utilizes data collected from the NIH RO1 grant “Antioxidant Nutrients among a Brazilian Population” (P.I Dr. Anna Giuliano), which is nested within the Ludwig-McGill Cohort study in São Paulo, Brazil (P.I. Drs. Eduardo Franco and Luisa Villa). The overall goal of this grant was to determine the association between serum nutrient status (carotenoids, tocopherols and retinoic acid) and cervical carcinogenesis among a cohort of women. A sub-cohort of 846 women was selected for this study. Archived serum samples were available from all four study visits that were scheduled to occur in the first year of the cohort. These samples were analyzed for serum carotenoids and tocopherols by Craft Technologies Inc. (Wilson, NC). Additional HPLC analyses were conducted on serum from the first two study visits (Baseline and visit 2) to determine retinoic acid levels. HPV laboratory data, specifically HPV typing and viral load, were available for all four consecutive visits spanning the first 12-month period from the parent study. Additional questionnaire data were available, including: demographic, cervical cancer risk factor history, and dietary data. All data were merged and analyzed to address the specific aims of this research. I sought to 1) determine the variability and identify factors associated with circulating

concentrations of retinoic acid; 2) estimate the relative risk for type-specific persistent HPV infection and viral load associated with circulating concentrations of retinoic acid, 3) estimate the relative risk for type-specific persistent HPV infection associated with circulating concentrations of carotenoids and tocopherols. The methods, results, and conclusions of this study are presented in the manuscripts appended to this dissertation. The following chapter includes a detailed description of the study methods and a summary of the important findings presented in the manuscripts and any additional analyses conducted that were not included in the manuscripts.

## **Section 2: Study Population**

The Ludwig-McGill Cohort Study is an epidemiological cohort investigation of a systematic sample of women attending a comprehensive maternal and child health maintenance program catering to low-income families in the city of São Paulo, Brazil. The clinic setting where participants are accrued is part of a network of health care institutions maintained by the municipal health department. Study design and methods of the Ludwig-McGill Cohort Study have been reported in detail (201). A summary of Ludwig-McGill Cohort study design and data, which were utilized for this research, is presented in Table 1 (Page 70).

**Table 1: Study Design and Data Utilized from the Ludwig-McGill Cohort Study**

	Participant Visits											
	Months				Years							
	Entry	4	8	12	1.5	2	2.5	3	3.5	4	4.5	5
<b><i>Circulating Nutrient Data:</i></b>												
Carotenoids & Tocopherols	U	U	U	U								
Retinoic Acid	U	U										
<b><i>Laboratory Data:</i></b>												
HPV testing & typing	U	U	U	U	X	X	X	X	X	X	X	X
Viral Load	U	U	U	U	X	X	X	X	X	X	X	X
Molecular Variants					XX (As needed)							
Cytology Rev.	X	X	X	X	X	X	X	X	X	X	X	X
<b><i>Questionnaire Data:</i></b>												
Diet		U									X	
Risk Factors:												
Smoking, Sex Beh.	U	X	X	X		X		X		X		
Full Socio-dem.	U											

X=Data Collected in Parent Study

U=Data Utilized For this Study

Subject Recruitment and Eligibility for Ludwig-McGill Cohort Study:

Women were selected at random from the daily lists of outpatients in the medicine, gynecology, and family planning clinics. The nurse interviewer approached each patient to determine eligibility and to explain the general purpose and nature of the study. The criteria for study eligibility included: (1) age between 18 and 60 years, (2) permanent residence in São Paulo (city), (3) not currently pregnant nor intending to become pregnant in the next 12 months, (4) an intact uterus and not referred for hysterectomy, (5) no vaginal medication use in the 2 days prior to clinic visit, and (6) not treated for cervical disease by

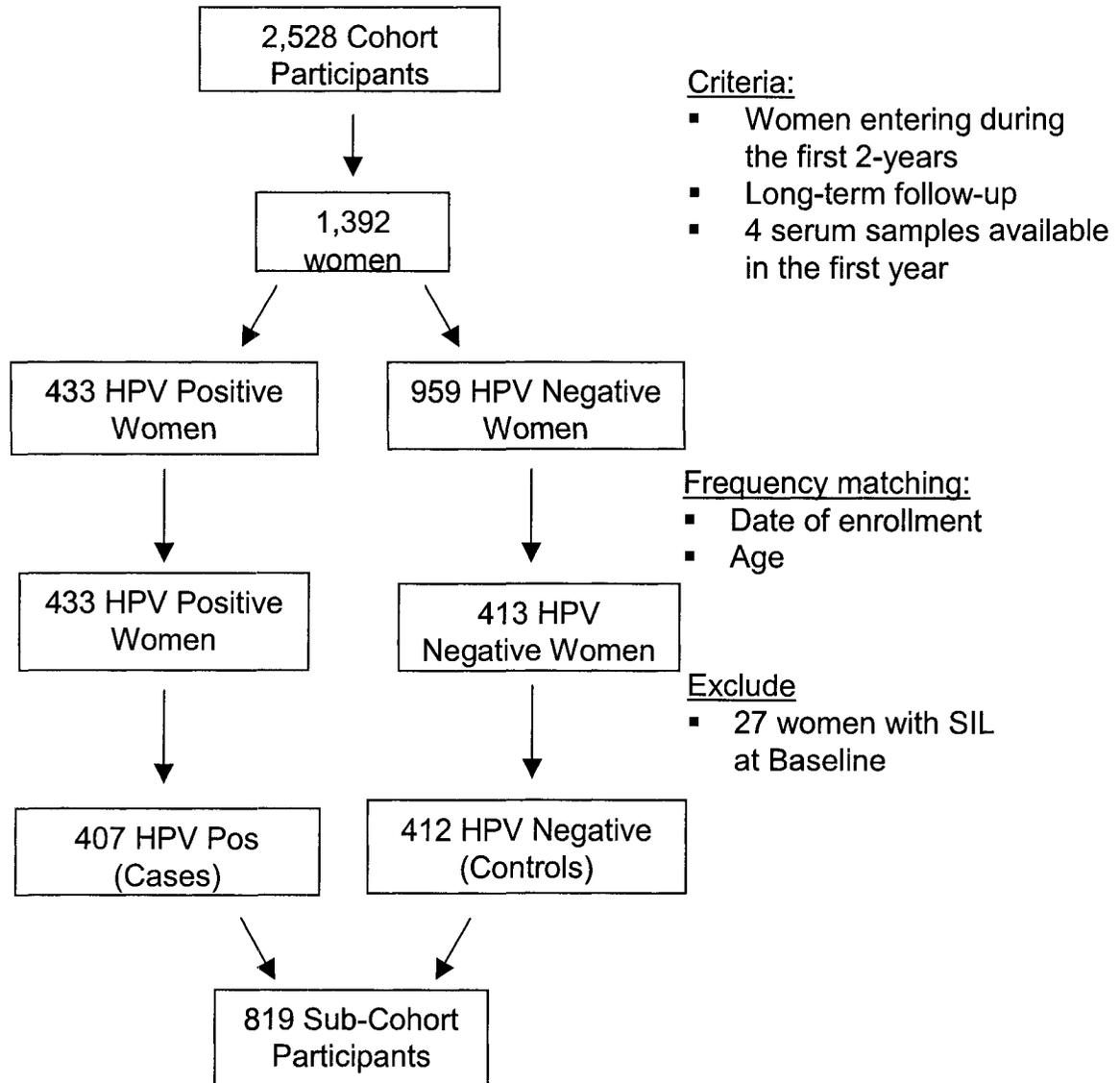
electrocoagulation, cryotherapy, or conization (prevailing methods of treatment) in the 6 months prior to clinic visit. In addition to these criteria, women were considered ineligible if they were not willing to comply with all scheduled visits, over the next 2 years, or if they were unwilling to provide informed consent. All participants signed an informed consent form.

Study Sample: The study sample was a sub-cohort from the Ludwig-McGill Cohort Study for this investigation (See Figure 2.1; Page 72). A sub-cohort of 1,392 women, representing those entering the study during the first 2 years who had long term follow-up and complete HPV data within the first year, was identified. Of these 1,392 women, there were 433 HPV positive women at any one visit during the first study year and 959 women who were consistently negative during the first year. The HPV positive women identified were over 70% of the HPV positive women in the entire cohort at each visit within the first year of follow-up (Table 2, below)

**Table 2. Distribution of HPV Positive Women at Each Study Visit during First Year of Follow-up: Parent vs. Sub-Cohort**

	Total HPV Positive in Parent cohort	HPV Positive in Sub- cohort	Percent HPV Positive within Sub- cohort
HPV Positivity	N	N	%
Visit 1	305	223	73.11
Visit 2	307	228	74.27
Visit 3	304	219	72.04
Visit 4	287	209	72.82

Figure 2.1: Sub-Cohort Selection from Ludwig-McGill Cohort Study



women with HPV infection in the two groups we find no statistically significant differences between the two groups except for income (Appendix D: Table 1). Among HPV negative women, the only variable that differed between groups was age, which was expected since the originally funded study selected HPV negative women matched on age to HPV positive women (Appendix D: Table 2).

### **Section 3: Cohort Study Data Collection**

Participant visits: Participants were followed over at least a five-year period, every four months in year 1 and twice yearly thereafter, for a total of 12 visits. The visit schedule was baseline (0 months), 4, 8, 12, 18, 24, 30, 36, 42, 48, 54, and 60 months. Over the course of this observational study, not all women returned at the designated time points for follow-up, which led to variability of time between study visits. On average, women identified for this present study returned for their month 12 visit  $13.4 \pm 3.4$  months (Range 11 – 50 months) following enrollment. During most of these visits participants were interviewed using a structured questionnaire specific for the current visit and had cervical specimens taken for Pap cytology and HPV testing. A 10 ml blood sample was also drawn by venipuncture in vacutainer tubes without coagulant. Questionnaires and blood specimens were not taken at 18, 30, 42, and 54 months, although HPV typing and PAP cytology was completed.

Study Questionnaires: Seven different color-coded questionnaires were used in this study corresponding to each of the first year visits and subsequent annual follow-up returns. Data from the first four visits were used in this investigation. Questionnaire 1 was administered at enrollment and was the most detailed with 107 questions. Questionnaires 2, 3, and 4 were used for the returns at 4, 8, and 12 months, respectively. The contents of these questionnaires are summarized below.

Questionnaire 1 contained questions on sociodemographic characteristics, tobacco and alcohol consumption, menstrual, sexually transmitted diseased (STD), and gynecologic, sexual, contraceptive, and anal and oral intercourse history. Questionnaire 2 reassessed a selection of socio-demographic variables, as well as sexual history. In addition, this questionnaire assessed sexual activity, menstrual patterns, and gynecologic symptoms since the last interview, as well as frequency of consumption of selected food items and vitamin supplements. Questionnaire 3 repeated selected socio-demographic questions, assesses sexual and contraceptive practices, menstrual patterns, gynecologic symptoms, and tobacco use since the last interview. Questionnaire 4 repeated all questions in questionnaire 3 and had a section on pregnancy history, sexual practices during pregnancy and post-partum, and contraceptive patterns.

Diet Questionnaire: Information on the frequency of consumption of selected food items and the consumption of vitamin and mineral supplements

was obtained at the second visit (month 4). Participants were asked to recall the usual frequency of consumption of the following 15 food items over the past five years: oranges, lemons, carrots, pumpkin, papaya, cauliflower, spinach, broccoli, lettuce, other vegetables, eggs, milk and yogurt, cheese, butter, and liver. These foods were included because there were known to contribute substantially to variation in carotenoid and tocopherol intake among Brazilian women living in São Paulo; however, they cannot be considered a complete index of carotenoid and tocopherol intake. Food consumption frequency categories were as follows: never, less than 1/month, 2-3 times/month, 1-3 times per week, 4-6 times/week, and  $\geq 1$ /day. Use of supplemental vitamin C, E, vitamin B complex, multiple vitamin preparations, and other vitamins was assessed. Participants responded as to whether they never consumed these supplements, occasionally consume supplements (<30 days), or frequently consume supplements (>30 days). Although these data cannot be used to calculate the exact dietary intake of carotenoids and tocopherols, they were utilized to estimate the relative intake of specific carotenoids.

Nutrient Intake Estimation: Nutrient values were calculated from the participants' reported dietary intake using the USDA's Continuing Survey of Food Intake of Individuals (CSFII-86) and Nationwide Food Consumption Survey (NFCS 87-88), unless Brazil specific nutrient values were available. When available, food carotenoid values (e.g.,  $\beta$ -carotene, lutein-zeaxanthin, and  $\beta$ -cryptoxanthin) were derived from published values for foods consumed in São

Paulo, Brazil (202). Dietary intake calculations used age-specific portion sizes for women as previously described (203).

Cervical Cell Specimens: An accelon biosampler was used to collect a sample of ectocervical and endocervical cells at each of the visits. After the smear was placed onto a glass slide and fixed in 95% ethanol, the cytobrush containing exfoliated cells was immersed in a tube containing Tris-EDTA buffer pH 7.4, swirled to release the adhered cells and kept at 4° C at the clinic for at most five days. Once brought to the laboratory at the Ludwig Institute, the tubes containing cell suspensions were frozen until being tested.

The Pap smears were fixed in absolute ethanol, stained, read locally in Brazil for an initial diagnosis, then shipped to McGill University where they were coded and sent to the Jewish General Hospital, Montreal, Canada. The Montreal cytopathology reports were based on the Bethesda System for Cytological Diagnoses (11). Women presenting with HSIL or worse were notified and treated. Women presenting with LSIL or changes consistent with HPV infection on cytology were not recalled for colposcopy and were not treated. Since HSIL was an endpoint both for the parent study and this study, treatment of these lesions did not impact the design or analysis of the study.

## **Section 4: Laboratory Methods**

### **4.1 Human Papillomavirus Analyses**

Polymerase Chain Reaction (PCR) Detection of HPV DNA: Cervical specimen DNA were extracted and purified following standard techniques at the Ludwig Institute for Cancer Research in São Paulo. In brief, cells were digested with 100µg/ml proteinase K for 3 hours at 55 ° C, followed by organic extraction and ethanol precipitation. Specimens were tested for the presence of HPV DNA by a previously described polymerase chain reaction (PCR) protocol amplifying a highly conserved 450 bp segment in the L1 viral gene (flanked by primers MY09/11) (22,36). Typing of the amplified products was performed by hybridization with individual oligonucleotide probes specific for all 27 HPV genital types whose nucleotide sequences for probes within the MY09/11 fragment have been published in the literature. Twenty-seven of these have received a taxonomic entry as HPV types: 6/11, 16, 18, 26, 31, 33, 35, 39, 40, 42, 45, 51-59, 66, 68, 73, 82, 83 and 84 (36). The PCR amplification products were further tested by restriction fragment length polymorphism (RFLP) analysis of the L1 fragment (204) to resolve dubious results from the dot blot hybridization and to distinguish among HPVs that could not be typed by dot blot hybridization with the specific probes (e.g. HPV CP6108, 81). Amplified products that hybridize with the generic probe, but not with any of the type-specific probes, and that additionally do not produce a recognizable band pattern in the RFLP analysis were considered positive for HPV of unknown types. In all, combined use of

specific probing and RFLP analysis effectively increased the number of genital HPV types detected in the study to 40.

HPV PCR Quality Control: Several measures taken to prevent inter-specimen contamination of samples and reagents were as follows: physical isolation of sample processing and reaction areas, use of autoclaved solutions, prealiquoted and pre-mixed reagents, use of highly diluted positive controls, use of disposable gloves, and use of positive displacement pipettes, aerosol-free tips, and “splash-free” tubes. In addition, as standard practice, several negative controls (DNA from C33, an HPV-negative cervical carcinoma derived cell line) were interspersed among test samples as sentinels for possible contamination.

HPV type grouping: The prevalence of any one of the measured HPV types in this population was relatively low (Appendix D: Table 3) (49). Therefore all statistical analyses evaluated type-specific grouped infections; oncogenic HPV types (with and without HPV16) and non-oncogenic HPV types. Table 3 (Appendix D) presents the type-specific HPV pattern data for oncogenic and nononcogenic HPV infections, stratified by incident or prevalent infections. Using the expanded classification system of Bauer *et al.* (20) HPV types were grouped as follows: oncogenic infections included HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, and 68. All other HPV types were considered non-oncogenic. At any given time point the prevalence of multiple infections was approximately 2.0% in this cohort (205) (Appendix D: Table 4), limiting our ability to independently examine associations for women with multiple infections.

Measurement of Viral Load: All cervical specimens found to be positive with the main PCR protocol (MY09/11) were retested by quantitative, low-stringency PCR to measure viral burden in exfoliated cervical cells. This technique was developed and validated by Dr. Villa's group (64) at the São Paulo Branch of the Ludwig Institute. General primers that detect a broad spectrum of HPVs were used (206). This modified system employed low stringency conditions to co-amplify the specific HPV DNA fragment along with sequences from the human genome present in the starting PCR mixture. Standards consisting of mixtures containing varying amounts of reference HPV 16 plasmid added to constant background of normal human DNA were included in duplicate in every assay. In addition, control samples consisting of DNA from two cervical carcinoma cell lines with known quantities of HPV copies were included in duplicate in every assay.

The silver-stained gel bands corresponding to the HPV and to the constant human genome fragments were quantified by densitometry (64). The logarithm of the ratio between these two bands was directly proportional to the logarithm of the amount of HPV DNA in the individual samples. Proper quantification was obtained by linear interpolation in a standard curve constructed with the results from control mixtures. A computer program that automated the calculation of raw densitometric readings had been developed by Dr. Franco to simplify processing of large batches of HPV positive samples. Viral load output was number of HPV copies per cell.

Viral Load Data: As indicated above, viral load data were obtained utilizing a low-stringency PCR method with general HPV primers, which identify a broad spectrum of HPV types (HPV types 1a, 6, 8, 11, 13, 16, 18, 30, 31, 32 and 33) (8); therefore the viral load data represented a combined measure for any HPV type present in the specimen identifiable by the GP5/6 primers. A viral load measure was considered to be low if HPV results were obtained by PCR (MY09/11) but LS-PCR was below the detectable limit. High viral load was detected by PCR and quantified by LS-PCR.

#### **4.2 Serum Nutrient Analyses**

Sample Processing and Storage. All non-fasting blood samples (~10 ml) were collected by venipuncture into vacutainers by a trained nurse at the time of the clinic visit. The samples were centrifuged within 6-8 hours of collection. Aliquots (1 ml) of serum were stored in 1.8 ml Nunc cryovials at  $-20^{\circ}\text{C}$  in a non-frost free freezer until shipped for analyses.

Retrieval and Shipment of Serum Samples: The participant identification numbers and strata identifiers were provided from McGill University, the main data-coordinating center for the parent study. We selected the appropriate identification numbers to be analyzed for each batch and entered them into the tracking form. Careful attention was paid to ensure that each shipment and analysis batch included all four samples for each woman and all women within a matching stratum. The tracking form was then emailed to Ludwig to initiate

sample retrieval. Serum samples were shipped on dry-ice from Brazil to Craft Technologies, Inc in North Carolina, totaling 11 shipments over a two and half year period. After the samples arrived, the integrity of each sample was verified and sample labels were checked. Of the 3,384 serum samples scheduled for shipment, 3,341 were available for nutrient analyses and we obtained carotenoid and tocopherol serum results for 3,332 samples (Appendix D: Table 5).

High-Pressure Liquid Chromatography for Carotenoid and Tocopherols:

Serum retinol, carotenoids and tocopherols were measured in serum samples taken at baseline, months 4, 8, and 12. HPLC analysis was performed for the identification of retinol, nine carotenoids ( $\alpha$ - , *trans*- $\beta$ -, and *cis*- $\beta$ -carotene, lutein, zeaxanthin,  $\alpha$ - and  $\beta$ -cryptoxanthin, *cis*- and *trans*-lycopene) and three tocopherols ( $\alpha$ -,  $\delta$ - and  $\gamma$ -tocopherol) using modified procedures described by Nomura *et al.* (207) by Craft Technologies Inc. (Wilson, NC). Briefly, after thawing stored serum samples, 150  $\mu$ L aliquots of serum were diluted with 150  $\mu$ L of water and deproteinated by vortexing with 300  $\mu$ L of ethanol containing tocol as an internal standard and butylated hydroxytoluene (BHT) as an antioxidant. The samples were extracted twice with 1 mL of hexane and the combined supernatant was evaporated under nitrogen. The residue was dissolved with vortexing in 35  $\mu$ L of ethyl acetate, diluted with 100  $\mu$ L of mobile phase, and ultrasonically agitated for 15 s prior to placement in the autosampler. A 15  $\mu$ L volume was injected.

The HPLC system consisted of a computer data system, solvent degasser, an autosampler maintaining samples at 20°C, a Spherisorb ODS2 column (3  $\mu\text{m}$ , 4.0 x 250 mm with titanium frits), a guard column containing the same stationary phase, a column heater at 31°C, a programmable UV/visible detector to measure retinol at 325 nm and carotenoids at 450 nm; tocol and tocopherols at 296 nm excitation/340 nm emission. The separation was performed isocratically using a mobile phase of 80% acetonitrile: 15% dioxane: 5% methanol/IPA containing 150 mM ammonium acetate: 0.1% triethylamine at a flow rate of 1.2 mL/min.

Linear calibration curves were prepared consisting of multiple concentrations of analytes which span the physiological levels of micronutrients in serum. The calibrants include lutein, zeaxanthin,  $\beta$ -cryptoxanthin, lycopene,  $\alpha$ -carotene,  $\beta$ -carotene, retinol, retinyl palmitate, and  $\delta$ -,  $\gamma$ -, and  $\alpha$ -tocopherols. Serum quantitation was performed by internal standard calibration using peak area ratios. Retinol quantitation was by external standard calibration. In-house quality control (QC) samples were analyzed at the beginning, end, and at 24 sample intervals. The relative standard deviation of analytes in the QC samples usually ranged from 3-10%.

This HPLC system's limit of quantification (LOQ) was 0.004  $\mu\text{g/ml}$  for the carotenoids and 0.15  $\mu\text{g/mL}$  for the tocopherols. Of the total 3,332 samples analyzed over four clinical visits, the following number of samples (percent) were

below the LOQ and assigned a value half way between zero and the lower limits of detection: retinol 3 (0.1%), lutein 16 (0.5%), zeaxanthin 50 (1.5%),  $\alpha$ -cryptoxanthin 1082 (32%),  $\beta$ -cryptoxanthin 330 (10%), *trans*-lycopene 793 (24%), *cis*-lycopene 701 (21%),  $\alpha$ -carotene 811 (24%), *trans*- $\beta$ -carotene 504 (15%), *cis*- $\beta$ -carotene 1037 (31%),  $\alpha$ -tocopherols 46 (1.5%),  $\delta$ -tocopherol 776 (23%) and  $\gamma$ -tocopherol 57 (2%). The distribution of samples below the LOQ by visit is presented in Table 6 (Appendix D). Experience indicated that the benefits of replicate analysis did not justify the effort and expense necessary to perform them. Typically less than 1% of samples yielded significantly different results upon replication within the same batch if appropriate checks were in place. It was more efficient to identify necessary repeats by examining the consistency of the internal standard area, monitoring QC samples, and examining the individual chromatographic profiles. If measurement of reproducibility was the objective of replicates, then QC samples could be used for that purpose.

The recovery of analytes using this method has been determined by both exogenous spikes of standards and exhaustive extraction. In both cases, recovery of all analytes is greater than 90%. Because 100% of the hexane extract cannot be removed from the aqueous phase, a small amount of analyte is not recovered. However, the use of an internal standard accounts for this loss. On-column losses have also been determined by the Flow Injection approach in which standards are injected through the column and peak area is measured,

then standards are injected after the column has been replaced by a meter length of 0.020" Teflon tubing. The ratio of "peak area with column" to "peak area without column" reflects on-column losses due to oxidation, precipitation, and non-specific binding. With the indicated mobile phase modifiers, recovery of all analytes is greater than 94%.

Serum Retinoic Acid HPLC Analytical Procedures: Retinoic acid analyses were conducted on serum samples from the first two visits (months 0 and 4) by Craft Technologies Inc (Wilson, NC). The 9-*cis*- and all-*trans*-retinoic acid were purchased from Sigma Chemical Co. (St. Louis, MO) and 13-*cis*-retinoic acid was obtained from ICN Biomedicals (Aurora, OH). The following reagents were used for sample preparation and analysis: butylated hydroxytoluene (BHT), hexane, ethanol, methanol, acetonitrile, hydrochloric acid, sodium hydroxide, and acetic acid. All solvents were HPLC grade or equivalent and were used without further treatment.

Sample extraction and preparation was performed using a modification of the method reported by Miyagi *et al.* (208). After thawing 500  $\mu$ L aliquots of serum, samples were deproteinated with 500  $\mu$ L of acetonitrile/methanol (19:1) containing 0.01% BHT and made alkaline with 100  $\mu$ L of 2N sodium hydroxide. Samples were extracted by vortex-mixing 45 s with 1.5 mL of hexane containing 0.025% BHT as an antioxidant. The organic phase was discarded. Samples were acidified with 200  $\mu$ L of 2N hydrochloric acid (HCl) and extracted 3x with 1.5 mL of hexane with BHT. The combined supernatant was evaporated under nitrogen.

The residue was dissolved by vortex-mixing with 120  $\mu$ L of mobile phase consisting of 75% acetonitrile, 5% methanol, and 20% of 1% acetic acid. The injection volume was 90  $\mu$ L.

The HPLC separation was performed using a modified method reported by Dimitrova *et al.* (209). HPLC analysis was performed using a ThermoSeparation Products liquid chromatograph with the following components: P4000 solvent delivery system, vacuum degasser, AS3000 autosampler, Spectra FOCUS scanning UV-visible detector, and PC1000 computer-controlled data system (Fremont, CA). On the autosampler, samples were refrigerated at 10° C and the column was maintained at 30° C. Retinoic acid isomers were monitored at 350nm. The analytical column was a Spherisorb ODS2 (3 $\mu$ m, 4.0 $\times$ 250mm) with Javelin guard column containing Keystone ODS2, 3 $\mu$ m (Keystone Scientific, Inc., Bellefonte, PA). The mobile phase had a flow rate of 1 mL/min.

Linear calibration curves were prepared consisting of three concentrations of retinoic acid isomers that spanned the physiological levels in serum. Quantification was performed by external standard calibration using peak area ratios. In-house quality control (QC) samples were analyzed at the beginning and end of each sample queue. The relative standard deviation of analytes in the QC samples ranged from 10-15%. This system had a limit of detection (LOD) of 0.1ng/mL and limit of quantification (LOQ) of 0.3ng/mL (5 pmol/L).

Samples below the LOQ were assigned a value half way between zero and the lower limits of detection: these accounted for 56 (3%) all-*trans*-retinoic acid, 70 (4%) 13-*cis*-retinoic acid, and 254 (15%) 9-*cis*-retinoic acid samples of the 1,658 samples analyzed. The distribution of samples below the LOQ by visit is presented in Table 6 (Appendix D).

Total serum cholesterol: Carotenoids and tocopherols are fat-soluble nutrients, of which uptake, transport and storage is associated with lipids (210). Therefore, the level of serum lipid, as measured by cholesterol, may influence the level of circulating carotenoids and tocopherols. To account for this effect, total serum cholesterol was assessed at visit 3 to enable adjustment for this potential confounding variable. Total cholesterol was determined by coupled enzymatic, colorimetric assay (Sigma kit #401-25P) by Craft Technologies, Inc. (Wilson, NC).

## **Section 5: Data Analyses**

### **5.1 Nutrient Variables**

Retinoic Acid Nutrient variables: Retinoic acid was measured from serum obtained during the first the two clinical visits (visit 1 and 2). Data from individual visits as well as the mean of two retinoic acids measures per individual were included. The study aim (specific aim 1) was to determine factors independently associated with elevated levels of retinoic acid, total and isomer specific. Therefore, we categorized women as having high values if their endogenous

retinoic acid level fell within the upper tertile of the overall distribution and women as having low-to-medium levels if their level was below the upper tertile. There was a difference in chromatographic results across HPLC batches (Appendix D: Table 7 and Figure 1), which were statistically controlled for by including a variable for HPLC batch in all analyses. The retinoic acid category was the same for all samples in two batches that resulted in removal of these data during statistical modeling (N=49 and N=107, respectively). As a result, a smaller number of women were included for these logistic regression models (N: 13-*cis* retinoic acid = 451, 9-*cis* retinoic acid = 393, and all-*trans* retinoic acid = 501). For specific aim 2, tertiles of each retinoic acid isomer were calculated on the basis of the overall nutrient distribution at each visit, creating time-dependent variables. Serum retinoic acid levels were assumed to be constant following the month 4 visit.

Carotenoid and Tocopherol Nutrient variables (Specific aim 1 and 3):

Preliminary studies within the study sample indicated that serum carotenoids and tocopherol values had high within-person variability (Appendix D: Table 8). Therefore, to accurately classify a woman's serum nutrient status and reduce this variability, we calculated the mean of serum carotenoids and tocopherols values at the four time-points for each woman. Using this mean value, we categorized the continuous variable into tertiles based on the distribution of the HPV positive women. To address specific aim 1, *trans*- $\beta$  carotene and *cis*- $\beta$ -carotene were combined for a measure of total serum  $\beta$ -carotene.

## 5.2 HPV Outcome Variables

We examined associations with type-specific HPV persistence using three different approaches; a case-control approach, a prospective approach examining persistent events over time, and a prospective approach examining duration of infection (Figure 2.1, page 90). All outcome variables were classified as oncogenic infections (HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68) or non-oncogenic infections (all other 27 HPV types considered) (20). Women infected with single or multiple oncogenic types and women infected with both oncogenic and non-oncogenic types were categorized as having an oncogenic infection. Only women infected with a single or multiple non-oncogenic types were categorized as having a non-oncogenic infection.

In the case-control approach, HPV status was based on the four HPV evaluations conducted at enrollment and in the first year of follow-up. Two groups of women were identified: those who tested HPV positive in only one of the four assessments or non-consecutively positive for the same type (transient group), and those who tested positive for the same HPV type at two or more consecutive visits in the first year of the study (persistent group). To test the hypothesis that serum nutrient status was associated with persistent HPV infection, women with

Figure 2.2: Hypothetical Example of Different HPV Outcome Variables Considered in Examining the Associations between Serum Nutrients and HPV Persistence and Clearance

Month	0	4	8	12
Visit	1	2	3	4
A. Case-Control	-	+	-	-
	+	+	-	-
B. Longitudinal HPV Events	+	+	-	-
	Persist.		Transient	
			n/a	
C. HPV Clearance	+	+	-	-
	-	+	-	-
	n/a			n/a

(A). HPV persistence which collapses longitudinal data into a dichotomous “case-control” definition. Transient infection (control) is defined as only one out of four visits positive or nonconsecutively positive. Persistent infection (case) is defined as two or more consecutively positive tests for the same HPV type. (B). Approach first described by Ho *et al* (16) utilizing HPV persistence as a repeated event of visit pairs. Women who are HPV positive at a particular measurement are considered persistent if their subsequent measurement is also positive for the same HPV type and transient if their subsequent visit was negative (i.e., if they have cleared the virus) or a different HPV type. (C). Time-dependent cohort analysis for repeated HPV data, with first negative visit being defined as an HPV clearance event. Women with both prevalent and incident infections are included.

transient infections formed the control group and women with persistent infections formed the case group. For this analysis, 230 women had oncogenic HPV infection and 177 women had nononcogenic infection. Of the 230 women with oncogenic infections, 143 had transient infection (controls) and 87 had type-specific persistent HPV infection (cases). Among the women with nononcogenic infections, there were 61 persistent infections (cases) and 116 transient infections (controls).

Our second approach, examining HPV persistence, utilized type-specific persistent events, similar to that used by Ho *et al.* (32) (Specific Aim 3). Women who were HPV positive at a particular measurement were considered persistent if their subsequent measurement was also positive for the same HPV type and transient if their subsequent visit was negative (i.e., if they have cleared the virus) or of a different HPV type. Using HPV measures at 0, 4, 8, and 12 months, we assessed a woman's persistence status over four clinical visits with a maximum of three persistence events possible. Analyses were conducted at the level of individual pairs rather than the individual woman. Of the 407 HPV positive women, 344 women contributed to these analyses with 263 persistent events (+, +) and 277 transient events (+, -).

The third approach was to examine the duration of HPV infections (Specific aims 2 and 3). This analysis was based on 407 women who were either HPV positive at baseline or developed an incident infection during follow-

up. Time to HPV clearance was defined as the number of months to first negative HPV test occurring after previously positive HPV test(s) or until the last visit (month 12) if the participant remained positive throughout the study period. The scheduled visits within this cohort study were based on months following enrollment. However, not all women attended visits at the specific month anticipated. This led to variability in the time between study visits and a total follow-up period for the first four scheduled study visits of 50 months.

The grouping of type-specific clearance by oncogenicity differed from that of the previous two outcome variables. For this analysis, women were considered to have cleared an oncogenic infection, as defined above, if she was no longer positive for the specific type grouping that was originally detected, even if the cervical specimen was positive for a different viral type within the same group or within the nononcogenic grouping.

HPV viral load was included for Specific Aim 2. A viral load measure was defined to be low if HPV results were obtained by PCR (MY09/11) but LS-PCR was below the detectable limit. High viral load was detected by PCR and quantified by LS-PCR. To clarify the time at which serum nutrients were associated with viral load, we conducted separate analyses with increasing time interval ( $n$ ) between exposure (serum retinoic acid level) and outcome assessment (viral load). The time intervals considered were 0 months (simultaneous assessment), 4, 8 or 12 months. In this strategy, women could

contribute multiple time windows in each series of analyses (e.g. enrollment to four months and four months to eight months). Women were only included in these analyses if they were HPV positive at the time of exposure (retinoic acid) assessment with normal/ASCUS cytology, however they could have lost their HPV infection at time viral load assessed.

### **5.3 Statistical Analysis**

Retinoic Acid Variability and Associated Factors (Specific Aim 1): Retinoic acid concentrations were right skewed; therefore we utilized nonparametric statistical methods for most analyses. Differences between month 0 (visit 1) and month 4 (visit 2) were assessed using a Wilcoxon Rank Sum test. Correlations between paired nutrient values were examined by calculating Spearman correlation coefficients. To assess the between- and within-woman variability of retinoic acid, we estimated a linear mixed effects model using logarithmically transformed nutrient values. Only samples that were above the detectable limit of the assay were included in this analysis. The mixed models analyses were performed using PROC MIXED in SAS version 9.0, Cary, North Carolina (211).

We determined if there was a difference in the rank distribution of total retinoic acid and each isomer by lifestyle and demographic characteristics using the Wilcoxon rank-sum test (2 categories) or Kruskal-Wallis test (3 or more categories) without regard to the ordering. For factors that were ordered, a

nonparametric test for trend, which is an extension of the Wilcoxon rank-sum test (212), was used to test whether there was a significant trend across ordered groups. Spearman correlations were calculated between total retinoic acid and each isomer, serum retinol and serum  $\beta$ -carotene for each visit, adjusting for laboratory batch analysis.

Multiple logistic regression was performed to estimate odds ratio, the magnitude of the independent association with retinoic acid levels and 95% confidence interval (CI) were constructed around the odds ratios. Modeling began by using backwards-stepwise logistic regression models to identify the lifestyle and demographics factors that were independently associated with total retinoic acid or individual isomers using a probability of removal set at 0.1. Factors that were considered in the first set of models were race (white vs. non-white), oral contraceptive (OC) use, total number of pregnancies, smoking status, alcohol consumption, season of enrollment, education and income. Age, in intervals of five years, was included in all final models regardless of significance level. A stabilization of the Brazilian economy occurred in July 1994, nine months following initial enrollment in the study, which most likely effected women's socio-economic status and spending patterns. We found this socio-economic phenomenon to be associated with differences in serum carotenoids and controlled for this variable by including an indicator variable (enrollment before July 1994 or after) into the final multivariate models. The retinoic acid category was the same for all samples in two batches, which resulted in removal

of these data during statistical modeling (N=49 and N=107, respectively). To assess the influence of these two batches on our modeling, we repeated the logistic regression analyses removing all samples from the specified batches and found no differences in our conclusions (data not shown).

Using the variables from these prior analyses, as the base model, we examined whether serum retinol and carotenoids (pro-vitamin A:  $\beta$ -carotene,  $\alpha$ -carotene, and  $\beta$ -cryptoxanthin, others: lutein and lycopene) were independently associated with retinoic acid levels using multivariate logistic regression. In addition, we examined if retinoic acid levels were associated with increased vitamin A and pro-vitamin A carotenoid intake and consumption of select foods.

Statistical Modeling of Serum Nutrients and HPV Outcomes: In the case-control approach (Specific Aim 3), logistic regression was performed to estimate the association (odds ratio) and 95% confidence interval (CI) of each serum nutrient with HPV persistence and transient HPV infection.

Since multiple HPV measurements (HPV persistent events or viral load) within a woman were correlated, a generalized estimating equation (GEE) model approach for time-dependent longitudinal data was used for analysis (213,214) (Specific Aims 2 and 3). The GEE model adjusts for the serial correlation within subjects due to the longitudinal nature of the viral load data by modeling the covariance structure within subjects. As the dependent variable was the presence or absence of high viral load the logit link function was used. The

independence working correlation structure with a robust variance estimator to account for within-subject correlation, was selected as the best-fitted covariance pattern, using the Quasilikelihood Information Criterion (QIC) (214). Since viral load may depend on the time since the previous measurement, a time variable was included as a design factor (independent variable) in all GEE models.

The relationship between serum nutrients (retinoic acid, carotenoids and tocopherols) and time to clearance of type-specific HPV infection was also assessed (Specific aims 2 and 3). Probability of maintaining an HPV infection present at baseline or an incident infection during follow-up was examined by estimating the Kaplan Meier survivor function. Missing values during follow-up were treated as censored observations unless there was a subsequent result at a later visit. Multivariate Cox proportional hazards modeling was conducted to obtain the adjusted hazard ratios (AHR) and 95% confidence intervals (95% CI).

Adjustment for Confounding: For all multivariate models (Specific Aims 2 and 3), we considered several variables that could potentially confound the associations including those previously associated with persistence of HPV infection (215) and serum carotenoids and tocopherols. Only those factors that altered the risk estimate by  $\geq 10\%$  were retained in the final multivariate model. Tests for trend were calculated by including the categorical nutrient variables (i.e. tertiles) as an ordinal variable in the multivariate models. All statistical tests were conducted as two-sided.

## RESULTS AND DISCUSSION

### Section 1: Temporal Variation and Identification of Factors Associated with Endogenous Retinoic Acid Isomers in Serum from Brazilian Women

#### 1.1 Results

Overall, total retinoic acid concentrations ranged from 0.81 ng/mL to 8.90 ng/mL (2.70 nmol/L – 29.6 nmol/L) at visit 1 and 0.44 ng/mL to 9.91 ng/mL (1.46 nmol/L- 32.98 nmol/L) at visit 2, with no statically significant difference by visits. Median baseline endogenous concentrations among the 502 women were 1.30 ng/mL (range 0.15-4.90), 1.10 ng/mL (range 0.15 – 3.23) and 0.76 ng/mL (range 0.15 – 3.17) for 13-*cis* retinoic acid, 9-*cis* retinoic acid and all-*trans* retinoic acid, respectively. The relative abundance of the three retinoic acid isomers was similar for each visit, with 13-*cis*-retinoic acid having the highest concentrations, followed by 9-*cis*-retinoic acid and all-*trans*-retinoic acid. Median serum retinol levels were significantly lower at visit 2 compared to visit 1. However, when assessed over four available visits, no significant difference in retinol across visits was observed ( $p=0.08$ ).

The correlation matrix between total retinoic acid, the three retinoic acid isomers, retinol, and  $\beta$ -carotene is presented in Table 9 (Appendix D), adjusted for laboratory analysis batch. Among the individual retinoic acid isomers, correlations were highest between 13-*cis* retinoic acid and 9-*cis* retinoic acid (spearman  $\rho=0.65$ ,  $p < 0.0001$ ). All-*trans* retinoic acid levels were correlated

poorly with 13-*cis* retinoic acid and 9-*cis* retinoic acid (spearman rho =0.11; p-value<0.05, and spearman rho=0.07; p-value= 0.12, respectively). Significant, yet weak, correlations were observed between total retinoic acid and serum retinol (spearman rho=0.20, p<0.0001) and serum  $\beta$ -carotene (rho=0.24, p=0.0001).

Total endogenous retinoic acid level over a four-month time period was highly correlated (Spearman rho=0.67), as were the three isomers, with 9-*cis* retinoic acid having the highest correlation (Spearman rho=0.79). The within person-variability of total retinoic acid and individual isomers was low, with the between- to within-variance ratios ranging from 1.05 to 3.09.

Amount of total retinoic acid and its isomers increased with age (p for trend < 0.001, all-*trans*-retinoic acid p for trend < 0.05), increasing number of pregnancies (p for trend < 0.001, all-*trans* retinoic acid p for trend < 0.05), and increasing level of serum retinol (p for trend < 0.001). Median 13-*cis* retinoic acid and 9-*cis* retinoic acid levels increased with increasing income (p for trend < 0.001), while all-*trans*- retinoic acid decreased with increasing income (p for trend=0.02). For all factors examined, results were similar when retinoic acid concentrations were evaluated for visits 1 and 2 separately, rather than as the mean (data not shown). Median total retinoic acid and two isomers (13-*cis* retinoic acid and 9-*cis* retinoic acid) differed by cigarette smoking status (never, current or former). Median all-*trans* retinoic acid was higher among white women compared to non-white women.

In the multivariate modeling of lifestyle and demographic factors independently associated with retinoic acid levels, age (measured in 5-year intervals) was positively associated with total retinoic acid and 9-*cis* retinoic acid levels (Adjusted OR: 1.34, 95% CI 1.11 – 1.62 and Adjusted OR: 1.62, 95% CI 1.34 – 1.95, respectively). Season of blood draw was significantly associated with 13-*cis* retinoic acid and all-*trans* retinoic acid (Spring vs. Fall: Adjusted OR=0.36, 95% CI 0.15-0.85 and Adjusted OR=0.29, 95% CI 0.12 – 0.72, respectively).

There were strong, positive associations among all retinoids and serum retinol,  $\beta$ -carotene and  $\beta$ -cryptoxanthin. Retinoic acid isomer levels were not associated with serum lutein or lycopene. Increased intake of vitamin A was significantly positively associated with 13-*cis* retinoic acid (adjusted OR=2.65, 95% CI 1.48 – 4.73) and 9-*cis* retinoic acid (adjusted OR=1.85, 95% CI 1.04 – 3.27) and marginally associated with total retinoic acid (Adjusted OR=1.74, 95% CI 0.98 – 3.08). Increased intake of  $\beta$ -carotene was positively associated with 13-*cis* retinoic acid (Adjusted OR=1.78, 95% CI 1.01-3.12).

Increased consumption of liver, pumpkin, and oranges was significantly positively associated with retinoic acid levels (total, 13-*cis* or 9-*cis* retinoic acid) and may be the contributing sources for the positive associations shown with vitamin A and  $\beta$ -carotene intake and serum retinoic acid levels (Appendix D: Table 10). In our study population of Brazilian women, we found that

consumption of pumpkin at least one time per week was associated with higher total serum retinoic acid and 13-*cis* retinoic acid (AOR=2.56, 95% CI 1.26-5.22 and AOR=2.62, 95% CI 1.33-5.18, respectively). In addition, consumption of liver more than once per week was marginally associated with higher 9-*cis* retinoic acid levels (AOR 1.90, 95% CI 0.97 – 3.7).

## 1.2 Discussion

Total serum retinoic acid and three individual retinoic acid isomers (13-*cis*, 9-*cis*, and all-*trans* retinoic acid) were characterized in a large sample of women. The relative abundance of the three retinoic acid isomers was similar for each visit, with 13-*cis* retinoic acid having the highest concentration, followed by 9-*cis* retinoic acid and all-*trans*-retinoic acid. Overall, we observed a wide range of values for this population (total retinoic acid 0.71 – 7.79 ng/mL), which may reflect the heterogeneity of this sample of low-income Brazilian women. While the between-person variability was high, we observed low within-person variability of two measures of serum retinoic acid obtained approximately four months apart.

Furthermore, various socio-demographic factors were related to retinoic acid level. Age, race, oral contraceptive use, total number of pregnancies, and season of initial blood draw were significantly associated with endogenous retinoic acid concentrations; while factors significantly contributing to retinoic acid levels differed for each isomer. All endogenous retinoic acid isomers assessed

in this study were positively associated with serum retinol,  $\beta$ -carotene, and  $\beta$ -cryptoxanthin levels. Associations were confirmed by observing significant associations with pre-formed vitamin A intake and increased consumption of pro-vitamin A containing foods. These associations differed slightly for each retinoic acid isomer suggesting the possibility of independent mechanisms for modulating specific isomer level. Additional laboratory research and population studies would be required to define the mechanisms leading to retinoic acid isomerization and factors that modify this process. At the present time, it would appear that the measurement of multiple isomers of retinoic acid within epidemiological studies provide added information compared to analyses that only assess total endogenous retinoic acid level.

The observed concentrations of 13-*cis* retinoic acid in these women were similar to previously published reports (216-219). Söderlund *et al.* (216) reported an average 13-*cis* retinoic acid concentration of 1.35 ng/mL (range 1.26 – 1.5 ng/mL) in a small sample of Northern European women (mean age 36 years). Others reported higher values (220-222) of 13-*cis* retinoic acid which might reflect differences in study participants (e.g. diet, age, sex, or presence of benign disease) or laboratory methods. Sedjo *et al.* (131) assessed baseline levels of retinoic acid concentrations in a chemoprevention trial and found a relative abundance of each isomer similar to what we observed in this study (13-*cis* retinoic acid > 9-*cis* retinoic acid > all-*trans* retinoic acid).

The variability of retinoic acid measures obtained approximately four months apart was examined. Levels of serum retinoic acid isomers were relatively stable within women, with ratios of between- to within-person variability all above 1.0. This stability was similar to the results published by Tang *et al.* (223) demonstrating no change in 13-*cis* retinoic acid and all-*trans* retinoic acid over a three month period in healthy controls (n=13). Söderlund *et al.* (216) reported within-person coefficients of variability, 13.4% for 13-*cis* retinoic acid and 13.6% for all-*trans* retinoic acid, which were not influenced by period of menstrual cycle. Furthermore, their ratios of between- to within-person variability for 13-*cis* retinoic acid and all-*trans* retinoic acid (1.57 and 1.17, respectively), were similar to the current study (216). Yamakoshi *et al.* (220) also found low between-day variances in serum retinoic acid isomers, all-*trans* retinoic acid (CV=11%) and 13-*cis* retinoic acid (CV=8.4%) in six healthy subjects. Altogether, these results would suggest that a single retinoic acid measurement might be sufficient to accurately classify a woman's retinoic acid level.

Söderlund *et al.* (216) reported individuals' seasonal variation for 13-*cis* retinoic acid, with lower mean values during winter and spring (November-March) compared to summer months. However these differences were small and were interpreted as being of little biological significance. In these Brazilian women, we found lower median values of 13-*cis* retinoic acid in summer, and in the multivariate analyses, women were significantly more likely to have lower 13-*cis* retinoic acid levels in spring and summer compared to fall. In contrast to

Söderlund *et al.* (216), we found significant seasonal differences in all-*trans* retinoic acid, with women more likely to have lower levels in the winter and spring compared to fall. It might be that these seasonal differences might be due to differences in dietary intake; however we did not formally test this question. Future research in this area would need to be conducted.

In this study, serum retinoic acid levels were positively associated with serum retinol and  $\beta$ -carotene levels. Our data were consistent with a previous study in patients with inflammation, which reported lower levels of serum retinol occurring together with low serum levels of retinoic acid (219).  $\beta$ -Carotene supplementation (30 mg/day) was demonstrated to increase serum levels of all-*trans*-retinoic acid after three months in patients with colon polyps (increase from  $2.32 \pm 0.26$  to  $3.00 \pm 0.41$  ng/ml); however, no increase in 13-*cis* retinoic acid, total retinoic acid or retinol with supplementation was observed (223). Our results were also supported by a recent finding that supplementation with retinyl palmitate substantially increased endogenous retinoic acid levels, with all-*trans* retinoic acid having the highest percent increase over time, followed by 13-*cis* retinoic acid (131).

In this study, we reported serum levels of 9-*cis* retinoic acid ranging from below detection (0.3 ng/mL) up to 3.8 ng/ml and contributing a relatively high proportion of total retinoic acid. The most likely explanations for the elevated mean 9-*cis* retinoic acid level are as follows: 1) incomplete resolution of 9-*cis*

retinoic acid from components in the matrix or other isomers, e.g. 9,13-*dicis* retinoic acid; 2) isomerization to 9-*cis* retinoic acid during long term storage at –20°C; and/or 3) unidentified dietary or environmental factors leading to elevated levels. Currently, we do not have a standard for 9,13-*dicis* retinoic acid to compare the retention time to determine if there was co-elution to address the first possible explanation. We have not seen significant isomerization when spiked samples were carried through our extraction and do not believe that artifacts were created during the extraction process. However, since the serum samples utilized in this study were not stored under ideal conditions at -80°C, the second explanation is a possibility.

Lanvers *et al.* (224) reports no degradation of all-*trans* retinoic acid or 13-*cis* retinoic acid in plasma during storage for 3 months at -20°C, whereas others have reported that long-term storage may result in degradation of 13-*cis* retinoic acid (218). To address this possible explanation, we examined mean levels of retinoic acid among women enrolled two years apart and found that the mean retinoic acid levels were similar or elevated among samples taken during the first year of enrollment compared to those taken during the third year (Appendix D: Table 11). Therefore, our retinoic acid concentrations do not appear to be affected by serum storage conditions. In addition, we have comparable overall levels of total retinoic acid to other studies (216-219), and we have compared our study to one whose samples were stored ideally (131) and there are no significant differences.

As with all observational studies, this study does have limitations that need to be recognized. First, similar to other biological markers, the values of retinoic acid presented in this report may not reflect the absolute value of endogenous retinoic acid due to losses that may have occurred during storage or in the extraction process. However, the relative levels of each isomer should not have changed; therefore the associations found in this study are assumed valid with the magnitude of the associations potentially being lower than the true association due to methodological errors. Second, as this study included Brazilian women only, our results cannot be generalized to men. We do believe that the results presented here may be of relevance to other female populations, as we have no reason to believe that this relatively disease free sample of women are different than other groups of women.

The issue of multiple comparisons arises when analyzing nutritional data from large epidemiological studies in that a large number of statistical models are required. For the present study, we did not adjust the significance level but chose to evaluate each serum nutrient as individual associations. By using this approach, there is a chance that single spurious associations will be found based on the nature of statistical probabilities. Therefore, the issue of multiple comparisons needs to be considered when interpreting results. Conclusions should be made in light of consistency with other information and external to the study (225). The present study examined nutritional factors associated with serum retinoic acid utilizing three nutritional measures (i.e. serum nutrient levels,

derived nutrient intake levels, and reported consumption of specific foods). These three measures were utilized to examine the consistency of associations found with serum retinoic acid. Based on these three approaches, serum retinol,  $\beta$ -carotene and  $\beta$ -cryptoxanthin were consistently associated with serum retinoic acid levels. This consistency provides support that our results for this particular nutrient may not be a spurious association found by chance.

The Ludwig-McGill Cohort offered a unique opportunity to assess the association between endogenous retinoic acid levels and lifestyle, demographic and nutritional factors in a sample of over 500 women. There were multiple serum samples available for measurement of retinoic acid allowing for the first assessment of retinoic acid variability over time. Serum retinol and carotenoids were assessed at four time points, which reduced the inherent variability of these measures and, thereby, reduced misclassification of serum nutrient level. Nutrient intake was estimated using Brazil specific carotenoid food content to increase accuracy in estimating carotenoid intake (97).

Supplemental retinoic acid has been prescribed to patient populations to regress pre-neoplastic lesions (110). However, when retinoic acid is taken orally there are significant associated toxicities (130). These toxicities have decreased its utilization in some populations, specifically among reproductive age women. By identifying lifestyle and dietary factors that are associated with increased endogenous levels of retinoic acid, there is potential that modification of these factors will increase levels of retinoic acid, leading to prevention of pre-neoplastic

lesions without the toxicity of supplemental treatment. These modifications could be potentially beneficial to women of reproductive age, especially women at risk for cervical cancer, for whom retinoic acid has been effective at regressing cervical dysplasia (119). Future studies would need to investigate whether increased levels of endogenous retinoic acid were associated with a decreased risk of pre-neoplastic cervical lesions.

## Section 2: Type-Specific HPV Clearance and Viral Load Associated with Endogenous Retinoic Acid Isomers in Brazilian Women: The Ludwig-McGill Cohort Study

### 2.1 Results

Characteristics of study sample. The 407 women included in these analyses attended all four follow-up visits. Among these women, 167 tested positive for the same HPV type at two or more consecutive visits and 240 tested positive only once or at nonconsecutive visits. The median age for this group of women was 31 years, which was not different from the median age of all HPV positive women enrolled in the cohort. Most participants were white (61%), married or in a common law marriage (70%), and not well educated, with 55% of the sample only have attended elementary school or less. At least 80% of these women reported more than two pregnancies and 57% reported using oral contraceptives for less than six years. A majority of these women were ever smokers (53%), with 36% reporting current smoking. Fifty six percent of participants reported age of first sexual intercourse under age 18 and 25% had more than four lifetime sexual partners. The relative abundance of retinoic acid isomers in this sample was highest for 13-*cis* retinoic acid ( $1.32 \pm 0.61$  ng/mL), followed by 9-*cis* retinoic acid ( $1.05 \pm 0.57$  ng/mL) and all-*trans* retinoic acid ( $0.83 \pm 0.38$  ng/mL).

The probability of clearing both oncogenic and nononcogenic HPV infections was positively associated with serum levels of all-*trans* retinoic acid. Specifically, women were 1.82 times more likely to clear an oncogenic HPV

infection if their serum *all-trans* retinoic acid levels were in the middle tertile compared to women with the lowest levels. The probability of clearing a nononcogenic HPV infection was also positively associated with serum *all-trans* retinoic acid levels (Medium: Adjusted Hazard Ratio (AHR) =1.67, 95% CI 1.09-2.57; High: AHR=1.46, 95% CI 0.93-2.28). Higher concentrations of *all-trans* retinoic acid were associated with a shorter duration of non-oncogenic and oncogenic HPV infections ( $p=0.01$  and  $p=0.05$ , respectively).

Over the course of this observational study, not all women returned at the designated time points for follow-up, which led to variability in time between study visits. On average, women identified for this present study returned for their month 12 visit  $13.4 \pm 3.4$  months (Range 11 – 50 months) following enrollment. The median duration of a nononcogenic HPV infection was 12.3, 12.1, and 9.8 months for the lowest, middle and highest tertile of *all-trans* retinoic acid, respectively. Serum *all-trans* retinoic acid levels in the middle tertile were associated with a shorter duration of oncogenic HPV infections compared to the lowest tertile (median time to clearance: 12.1 months vs. 12.9 months, respectively) but this did not appear to be sustained for the highest tertile (median time to clearance 12.4 months).

We examined not only concurrent viral load and retinoic acid measurements, but also the association between serum retinoic acid levels and subsequent viral load measurements at 4, 8 and 12 months. We observed

marginally significant positive associations with high viral load and serum retinoic acid in the highest tertile (all-*trans* retinoic acid: Adjusted OR: 1.79, 95% CI 0.93 – 3.45) measured at the same visit compared to the lowest tertile. Circulating 13-*cis* retinoic acid level measured four months prior was inversely associated with high viral load (middle tertile vs. lowest: Adjusted odds ratios (OR): 0.34, 95% CI 0.13-0.85).

## 2.2 Discussion

This was the first study to examine associations of circulating levels of retinoic acid and clearance of type-specific HPV infections. In this study, higher endogenous all-*trans* retinoic acid concentrations were associated with an increased probability of clearing both oncogenic and non-oncogenic HPV infections. Women with mid to high levels of serum all-*trans* retinoic acid had shorter durations of infection. We found that serum 13-*cis* retinoic acid, but not all-*trans* retinoic acid, was inversely associated with high viral load at the subsequent visit four months later.

The mechanism by which all-*trans* retinoic acid may increase the likelihood of clearing an HPV infection is unknown; however there is indirect evidence for a protective effect of retinoids in cervical carcinogenesis. This indirect evidence stems from epidemiological research involving the precursors of retinoic acid (vitamin A and pro-vitamin A carotenoids), which we have previously shown to be highly associated with serum retinoic acid levels in the

study sample. Inverse associations have been observed for vitamin A and pro-vitamin A carotenoids (dietary intake and circulating levels) and risk of cervical dysplasia (79,80,83,226) and invasive cervical cancer (83,227). Prospective studies have also demonstrated decreased risk of persistent HPV infection with higher dietary intake of pro-vitamin A carotenoids, lutein (96,97),  $\beta$ -cryptoxanthin (97) and vegetables (96).

Retinoids (natural and synthetic derivatives of vitamin A) have been shown to be cancer chemotherapeutic and chemopreventive [see (110-112) for reviews]. In a placebo controlled chemoprevention trial among women with cervical intraepithelial lesions (CIN), Meyskens *et al.* (119) reported a histological regression rate of 47% among patients with a CIN II lesion randomized to all-*trans* retinoic acid treatment group compared to a 27% regression rate in the placebo group. However, no differences were seen for patients with CIN III (25% all-*trans* retinoic acid vs. 31% placebo) (119). Chemoprevention trials with other retinoic acid isomers (9-*cis* and 13-*cis* retinoic acid) (118,120,124) and synthetic retinoids (118,120) have not shown an increase lesion regression compared to spontaneous regression rates of those in the placebo group.

The factors underlying the differential results obtained from cervical chemoprevention trials with retinoids and observational studies are not known. There are at least two possible explanations for these differences. First, there may be differences in the biological activity of each retinoic acid isomer or synthetic derivatives when applied to the cervix compared to those found in

circulation. Alternatively the differences could be attributed to differences in study designs (i.e. different outcome measures, misclassification of lesion grade upon entry, different dosages and treatment periods). Of interest, in this current study, we find that higher levels of endogenous all-*trans* retinoic acid, not 13-*cis* or 9-*cis* retinoic acids, are associated with an increased probability of clearing HPV infections. The protective effect observed in this study combined with those of chemoprevention trials suggests chemopreventive activity for both endogenous and exogenous all-*trans* retinoic acid. To further explore this hypothesis our future studies will examine if endogenous all-*trans* retinoic acid is associated with cervical lesion development.

Retinoic acid is essential for terminal differentiation of cervical epithelial cells by decreasing cellular proliferation and DNA replication. Retinoic acid differentially inhibits growth of HPV-16 immortalized cervical epithelial cells (175,184) and low-passage human foreskin keratinocytes (HKc/HPV16) (185-187) compared to normal HKc cells in the absence of an HPV infection. In addition to decreasing cellular proliferation in the low passage HKc/HPV16 cells, physiological concentrations of retinoic acid inhibited the expression of HPV 16 E6 and E7 (185-187). Retinoic acid may indirectly reduce HPV mRNA levels through influences on AP-1 activity (198) or TGF $\beta$  expression (199). Retinoic acid induced growth suppression, however, appears to be lost in late stages of HPV-16 induced transformation of HKc (176) and cervical carcinoma cells lines. In several *in vitro* model systems, cells in the late stages of HPV-16 induced

transformation acquire resistance to retinoic acid induced differentiation through several different mechanisms, including loss of growth inhibition (176), continued growth stimulation (188-190), and loss of retinoid receptor expression (193). This retinoic acid resistance is consistent with observations that retinoic acid therapy does not reduce recurrence rates of invasive cervical cancer (127-129) nor does it increase regression of high-grade cervical lesions (119). Altogether, these data suggest retinoic acid may only be effective early in cervical carcinogenesis, such as by modulating HPV clearance and viral load. Our results suggest a role of all-trans retinoic acid in the clearance of HPV infections, however further research needs to be conducted to more fully understand the mechanism of clearance.

We observed from the present study that higher all-trans retinoic acid levels are associated with increased probability of clearing both nononcogenic and oncogenic HPV infections; however, the association is strongest for the nononcogenic types. There are several potential explanations for the stronger associations between all-trans retinoic acid levels and clearance of nononcogenic HPV types compared to oncogenic types. First, one of the biological differences between oncogenic and nononcogenic HPV types is the strength of the interaction of viral oncogene E6 with E6-AP, and the resulting p53 degradation. Nononcogenic HPV types do not associate with E6-AP or bins at a much weaker level; therefore, p53 activity remains intact during nononcogenic infections. The ability of all-trans retinoic acid to induce HPV clearance may

require the presence of p53 inducible pathways. Of interest are the findings by Meyskens *et al.* that all-*trans* retinoic acid treatment increased regression of CIN II lesions but not CIN III. The presence of HPV was not determined; however, one could use known frequency of HPV in cervical lesions to predict that CIN III lesions were more likely to be positive for oncogenic HPV types. The lower grade CIN II lesions were probably a combination of oncogenic and nononcogenic types. Synthesizing the results from the current study with those from the chemopreventive trial, one could postulate that lesions, which significantly regressed, were positive for nononcogenic HPV types. This is the first study to suggest such differences by HPV oncogenicity. Alternatively, women with nononcogenic type HPV infections could have had a co-infection with oncogenic types. Therefore, we were unable to rule out the influence of co-infection between oncogenic and nononcogenic and all-*trans* retinoic acid level. Finally, all-*trans* retinoic acid could equally induce clearance of both oncogenic and nononcogenic HPV types. However, this study did not have a large enough sample size to examine the associations limited to single infections. Further research is needed to understand this phenomenon of differential activity of all-*trans* retinoic acid by oncogenic potential.

As with any observational study, this study has limitations that need to be addressed. Our definition of HPV clearance included both prevalent and incident HPV infections. By including prevalent infections, our estimate of duration of infection under represents the true duration. It is impossible to know exactly how

long a woman has been infected when she is found positive at enrollment. In general, women who are HPV positive upon enrollment are more likely to have a persistent infection. The Ludwig-McGill Cohort study did not have enough incident HPV infections to utilize incident HPV persistence as our outcome. Of all the HPV infections detected in the first year, 39% were detected at enrollment and only 22% of the incident infections persisted. We have also assessed HPV clearance over a relatively short period of time and are limited to scheduled clinic visits. Similar to other biological markers, the values of retinoic acid presented in this report may not reflect the absolute value of endogenous retinoic acid due to losses that may have occurred during storage or in the extraction process. However, the relative levels of each isomer are assumed to be relatively stable. Therefore the associations found in this study are valid with the magnitude of the associations potentially being lower than the true association due to methodological errors.

Despite these limitations, the Ludwig-McGill Cohort Study offers a unique opportunity to assess the association between endogenous retinoic acid levels and HPV clearance. This is a large well-characterized cohort with multiple measurements of type-specific HPV infection in a 12-month period. This is the first large population based study to assess endogenous levels of three retinoic acid isomers in multiple serum samples from the same individual.

Our results suggest that circulating retinoic acid may confer protection by decreasing the duration of both oncogenic and nononcogenic HPV infections.

Collectively, this work and *in vitro* studies support the hypothesis that retinoic acids are active during the initial stages of HPV-associated carcinogenesis. Previously, we have demonstrated that serum retinoic acid levels are significantly associated with circulating and dietary levels of vitamin A and pro-vitamin A precursors. Recently, Sedjo *et al.* (131) reported that endogenous retinoic acid is responsive to oral chemopreventive doses of retinyl palmitate (Vitamin A). Collectively, these studies demonstrate that circulating retinoic acid isomers may be modifiable by diet and vitamin A supplementation. Modulating endogenous all-*trans* retinoic acid may be a safe method for decreasing duration of HPV infections and early cervical lesions.

### **Section 3: Associations Between Serum Carotenoids and Tocopherols and Type-Specific HPV Persistence and Clearance: The Ludwig-McGill Cohort Study**

#### **3.1 Results**

These analyses included 407 women participating in the Ludwig-McGill Cohort Study who were enrolled within the first two years of enrollment (1993-1996). The median age for these women was 31 years, which was not different from the median age of all HPV positive women enrolled in the cohort. Most participants were white (61%), married or in a common law marriage (70%), and not well educated, with 55% of the sample having only attended elementary school or less. At least 80% of these women reported more than two pregnancies, and 57% had used oral contraceptives for less than six years. A majority of these women were ever smokers (53%), with 36% reporting current smoking. Fifty-six percent of participants reported age of first sexual intercourse under 18 years and 25% had more than four lifetime sexual partners.

Circulating nutrient concentrations of retinol, carotenoids and tocopherols among HPV positive participants by persistent HPV outcomes were presented. The most abundant circulating carotenoid among the 407 HPV positive women was  $\beta$ -cryptoxanthin (mean, 0.040  $\mu\text{g/mL}$ ), followed by lutein (mean, 0.037  $\mu\text{g/mL}$ ), *cis*-lycopene (mean, 0.026  $\mu\text{g/mL}$ ), *trans*  $\beta$ -carotene (mean, 0.024  $\mu\text{g/mL}$ ), *trans*-lycopene (mean, 0.017  $\mu\text{g/mL}$ ), zeaxanthin (mean, 0.016  $\mu\text{g/mL}$ ),  $\alpha$ -carotene (mean, 0.011  $\mu\text{g/mL}$ ), *cis*- $\beta$ -carotene (mean, 0.007  $\mu\text{g/mL}$ ), and  $\alpha$ -

cryptoxanthin (mean, 0.006  $\mu\text{g/mL}$ ). *Cis*- $\beta$ -carotene and  $\alpha$ -cryptoxanthin were low in this Brazilian study sample with at least 25% of the serum samples having levels below the limit of quantification. The most abundant tocopherol was  $\alpha$ -tocopherol (mean, 5.14  $\mu\text{g/mL}$ ), followed by  $\gamma$ -tocopherol and  $\delta$ -tocopherol (mean, 1.58  $\mu\text{g/mL}$  and 0.083  $\mu\text{g/mL}$ , respectively). Mean serum nutrients were also presented by HPV persistence for both the case-control definition (135 transiently infected (controls) and 165 persistently infected (cases) and the longitudinal HPV events (transient group (+,-) N=277 and persistent group (+,+) N=259).

Case-control analysis of type-specific HPV persistence: For these analyses, we included 230 women with an oncogenic HPV infection detected at some time in the study period and 177 women with a diagnosed nononcogenic infection. Of the 230 women with oncogenic infections, 143 had transient infection (controls) and 87 had type-specific persistent HPV infection (cases). Among the women with nononcogenic infections, there were 61 persistent infections (cases) and 116 transient infections (controls). Higher circulating levels of tocopherols ( $\alpha$ -,  $\delta$ -, and  $\gamma$ -tocopherol) were inversely associated with nononcogenic HPV persistent infection (p for trend, 0.14, 0.04 and 0.05, respectively), but not oncogenic infection. No statistically significant associations were observed between serum retinol, lutein, zeaxanthin,  $\alpha$ -cryptoxanthin,  $\beta$ -cryptoxanthin,  $\alpha$ -carotene, *trans*- and *cis*- $\beta$  carotene and oncogenic or

nononcogenic HPV persistence in this study population. Serum lycopene (*cis*- and *trans*-lycopene) was positively associated with type-specific oncogenic HPV persistence (p for trend 0.03 and 0.03, respectively).

Longitudinal analysis of type-specific HPV persistent events: To fully utilize the longitudinal nature of these data, we examined type-specific HPV persistent events. Of the 407 HPV positive women included in this study, a total of 1,628 visits occurred in the first year of follow-up. These 1,628 visits formed 1,221 pairs of observations with known HPV status at visits  $t$  and  $t+1$ . The distribution of HPV <sub>$t$ ,  $t+1$</sub>  pairs was 277 with (+,-), 263 with (+,+) and 681 with (-,-) or (-,+). The 540 pairs in the (+,-) and (+,+) categories contributed by 344 women and were included in these analyses. Similar to what we observed with the case-control analysis, serum tocopherol levels were inversely associated with persistent HPV events. Circulating levels of  $\alpha$ - and  $\delta$ -tocopherol in the middle or upper tertiles, respectively were marginally inversely associated with type-specific nononcogenic persistent HPV events (Adjusted Odds Ratio (OR)= 0.49, 95% CI 0.23-1.00 and Adjusted OR=0.48, 95% CI 0.21-1.06, respectively) compared to levels in the lowest tertile. Using this analytical approach, serum  $\beta$ -carotene was marginally inversely associated with non-oncogenic persistent events (p for trend=0.07).

Type-specific HPV clearance: The probability of clearing a type-specific oncogenic HPV infection was positively associated with serum levels of zeaxanthin. Specifically, women were 1.59 times more likely to clear an

oncogenic HPV infection if their serum zeaxanthin levels were in the highest tertile compared to women with the lowest serum levels. The probability of clearing nononcogenic HPV infections was not associated with serum zeaxanthin levels, or other serum carotenoid and tocopherol levels. There was a marginally significant increase in probability of clearing type-specific oncogenic HPV infection with higher lutein (Medium: Adjusted Hazards Ratio (HR)=1.54, 95% CI 0.96-2.49) and  $\alpha$ -cryptoxanthin levels (Medium: Adjusted HR=1.52, 95% CI 0.96 - 2.41). There was a marginally significant trend for increased probability of clearing nononcogenic HPV infection by increasing serum retinol level ( $p$  for trend=0.08). Higher concentrations of circulating zeaxanthin were associated with a shorter duration of type-specific oncogenic HPV infections ( $p$ -values = 0.05). The median duration of an oncogenic HPV infection was 13.1, 12.1, and 12.1 months for the lowest, middle and highest tertiles of serum zeaxanthin, respectively. It is important to note that over the course of this observational study, not all women returned at the designated time points for follow-up, which led to variability in time between study visits and longer than 12 months of follow-up in the first year. On average, women identified for this present study returned for their month 12 visit  $13.4 \pm 3.4$  months (Range 11 – 50 months) following enrollment.

### 3.2 Discussion

There is a consensus among the scientific community that HPV is a cause of cervical cancer and that other factors that increase a woman's risk of developing cervical cancer need to be understood within the context of the natural history of HPV infection. To date, most studies examining dietary factors have not been designed to prospectively evaluate nutrients, as most were retrospective studies with either HSIL (CIN II or III) or cancer as the case groups (101). In our earlier studies, we evaluated the role of antioxidants, both dietary intake and serum levels, and risk of persistent HPV infections (57,96) or HPV clearance (98) among women in the U.S. However, we were not able to assess associations with type-specific infections, since we did not have detailed HPV typing to clearly separate oncogenic from non-oncogenic types, and were limited to a relatively short period of follow-up. We also examined the association of dietary intake and type-specific HPV persistence in this sample of Brazilian women (97).

Results of the present study among Brazilian women were consistent with our previous dietary findings in this same Brazilian sample (97) and among women in the U.S. (57,96), where we demonstrated an inverse association with lutein/zeaxanthin consumption and HPV persistence. In addition, inverse associations seen in the earlier studies were consistent for dietary vitamin E intake (57,96) and serum tocopherols (99) and HPV persistence. Preliminary findings from a multi-ethnic cohort in Hawaii also reported inverse associations

with oncogenic type-specific HPV persistence and change in serum  $\beta$ -carotene and  $\alpha$ -tocopherol over a 4-month period (99). Contrary to the earlier findings in U.S. women of inverse associations with HPV persistence (96) and clearance (98), we find in the present study of Brazilian women a positive association with serum lycopene and type-specific oncogenic HPV persistence and no association with HPV clearance. A few prior reports have assessed the association between serum antioxidant nutrients and HPV persistence within the context of a  $\beta$ -carotene clinical trial (37,82). These trials reported no association between serum retinol,  $\beta$ -carotene, vitamin C and HPV persistence in the control or supplemented groups (37,82). However, these women had pre-existing lesions (50% LSIL (CIN I) and 49 % HSIL (CIN II and III)) at enrollment (37), therefore representing relatively late events in disease process. In contrast, women enrolled in the current study had normal or ASCUS cytology at baseline.

In this study we evaluated the nutrient-HPV persistence associations utilizing three different analytical approaches, including a case-control definition, longitudinal analysis of persistence events, and time dependent clearance analyses. To understand the implications of these results, it is important to consider the assumptions of each approach and the validity of the HPV outcome variable utilized in each of the different analyses. Utilizing a case-control approach, our HPV outcome variable collapsed the longitudinal nature of the HPV data into two groups: persistent group (two or more consecutive visits

positive for the same HPV type) and transient group (defined as one of four positive tests or nonconsecutive positive tests of the same type). Among women with persistent infection, a woman with four consecutively positive tests contributes equally to the model as a woman with two positive tests. This logistic regression analysis assumes that an HPV infection lasting four months (i.e. only 2 positive) has the same risk as one that lasts 12 months (i.e. 4 positive visits). Although the utilization of logistic regression is straightforward and easy to interpret, this underlying assumption may not be biologically plausible as it has been demonstrated that risk of cervical disease increases with duration of infection (5). Utilizing this crude definition of HPV persistence, higher circulating levels of tocopherols ( $\alpha$ -,  $\delta$ -, and  $\gamma$ -tocopherol) were inversely associated with nononcogenic HPV persistent infection (p for trend, 0.14, 0.04 and 0.05, respectively).

The logistic regression case control analysis differs from our second approach, which defined HPV persistence as multiple visit pairs and utilizes generalized estimating equations for longitudinal analyses. In this approach, a woman with four consecutively positive visits would contribute three persistence events, compared to a woman with only two consecutively positive events contributing only one event. Therefore, each woman had the potential to contribute up to three events increasing the overall proportion of persistent events. By increasing the number of persistent events, this analysis has more power to detect differences by serum antioxidant level, however the persistent

event spans approximately a four-month period. This approach of defining persistent events in four-month intervals may not be biologically relevant, as it is difficult to determine if an HPV infections lasting four months is truly persistent. Therefore, HPV events defined as persistent based on four month intervals may be misclassified and result in attenuation of the risk estimates to the null. Utilizing this approach, we found that circulating levels of  $\alpha$ - and  $\delta$ -tocopherol in the middle or upper tertiles, respectively were marginally inversely associated with type-specific nononcogenic persistent HPV events (Adjusted Odds Ratio (OR)= 0.49, 95% CI 0.23-1.00 and Adjusted OR=0.48, 95% CI 0.21-1.06, respectively) compared to levels in the lowest tertile. This attenuation is evident in the loss of statistical significance in the GEE modeling compared to the logistic regression analysis.

Although time was included in these generalized estimating equations models as a covariate, the statistical analysis is based on the logit-link function comparing proportion of events and, therefore, the consecutive nature of persistent events is not taken into consideration. Further Cox proportional hazard modeling was performed to utilize the consecutive nature of the HPV infections by estimating the duration of infection from baseline or incident infection. With Cox proportional hazard modeling, the concern about misclassification of the HPV persistent outcome variable is minimal compared to the logistic regression and GEE modeling. Cox proportional hazard modeling is dependent on the clinic visit schedule; therefore, exact timing of clearance is

unknown (60) and these models also assume that the hazard ratio is constant over time. This assumption has been considered a limitation of applying these models to the natural history of HPV infection as risk increased with duration of infections (99). However, this approach estimating HPV clearance is currently the most accurate model with which to examine HPV persistence. Using this approach, we found that the probability of clearing a type-specific oncogenic HPV infection was positively associated with serum levels of zeaxanthin. These results differ from the previous approaches utilized in this current study, which did not find associations with zeaxanthin and oncogenic HPV infections. Oncogenic HPV infections are more likely to be detected over a long period of time (i.e. 12 months), and circulating zeaxanthin appears to reduce the duration of infection (Median duration: 13.1, 12.1, and 12.1 months for the lowest, middle and highest tertiles of serum zeaxanthin). The median duration is much larger than the estimated four-month interval utilized in the GEE analysis, which may explain the lack of association between oncogenic HPV events and zeaxanthin level.

The mechanism by which carotenoids and tocopherols might influence the natural history of HPV infections remains uncertain. However, the potent antioxidant activity of these nutrients may be one mechanism that confers chemopreventive activity. As antioxidants, these nutrients maintain the correct oxidant-antioxidant balance within cells (133) by quenching excess reactive oxygen species (ROS). Although required for normal cellular processes at low

levels, excess ROS can cause a multitude of problems, specifically damage to both cellular DNA and membranes (133) and decreases in immune cell activity (137). Redox-sensitive transcription factors, AP-1 and NF- $\kappa$ B, are activated during conditions of oxidative stress and can potentially lead to alterations in numerous gene expression levels, such as genes involved in cell growth and apoptosis (135).

It has been demonstrated that the activity of some viruses is enhanced under conditions of low antioxidants (146,228-231). This enhanced viral activity may be due to the activation of redox-sensitive transcription factors, AP-1 and NF- $\kappa$ B, which are utilized by several viruses, such as human immunodeficiency virus (HIV), cytomegalovirus (CMV), hepatitis-B virus (HBV) and HPV (143,144,148,152,158,159) to support expression of viral genes and subsequent replication of viral genomes. *In vitro* studies have consistently demonstrated that NF- $\kappa$ B activation is inhibited by antioxidants such as  $\alpha$ -tocopherol (149),  $\alpha$ -lipoic acid (150), N-acetylcysteine, and pyrrolidine-dithiocarbamate (PDTC) (149). The presence of NF- $\kappa$ B binding sites in the HPV upstream regulatory region (24) and evidence that HPV activity is altered by NF- $\kappa$ B activity, suggests the potential for NF- $\kappa$ B to alter HPV viral replication and expression (232). A recent clinical trial reported a decrease in HIV viral titer among HIV patients supplemented with  $\alpha$ -tocopherol (800 mg/day) compared to non-supplemented HIV patients (151). Antioxidant treatment of HPV-16 immortalized human

keratinocytes (HKc) with PDTC altered the composition and reduced the activity of the transcriptional AP-1 complex (161), the key transcription factor for HPV oncogenes (158,159). Although not directly shown, it has been postulated that the combined stress of decreased antioxidant levels and viral infection may allow for a typically transient viral infection to persist over time (9,142).

In addition to their antioxidant activities, it is possible that the observed effects of zeaxanthin and possibly lutein and  $\alpha$ -cryptoxanthin are due to their pro-vitamin A activity. We have recently demonstrated an increased probability of type-specific oncogenic and nononcogenic HPV clearance with elevated serum all-*trans* retinoic acid levels (Manuscript 2). In this present study, we observed a marginal increase in the probability of clearing a type-specific nononcogenic HPV infection with increased serum retinol. However, few studies have found an association between vitamin A consumption and cervical neoplasia risk (9).

Our study among Brazilian woman is the first to demonstrate differences in associations with specific nutrients and type-specific oncogenic versus nononcogenic HPV persistence and clearance. Goodman and colleagues (99) reported associations with serum antioxidants and type-specific oncogenic HPV persistent events, utilizing a similar definition of HPV persistence events. Using generalized logistic models (i.e. GEE logit-link function), they reported a 44% reduction in risk of HPV persistence for women in the highest tertile of plasma  $\beta$ -carotene (positive change between measures) and 57% reduction in risk among women with higher  $\alpha$ -tocopherol (99). In this current study serum zeaxanthin

levels are found to be associated with oncogenic HPV clearance, and not clearance on nononcogenic types.

Both oncogenic and nononcogenic HPV types have the ability to infect cervical epithelial cells and initiate a productive viral infection. Differences in oncogenic and nononcogenic type infections are evident in the strength with which the viral proteins bind to cellular proteins to induce DNA synthesis and cell cycle progression. The continued presence of oncogenic HPV infection is thought to be a result of functional losses within cervical cells that control viral expression. It may be that the protective effect of zeaxanthin becomes evident only after cellular control of oncogenic HPV infections is lost. Antioxidants could be protective at lower levels early in HPV infection for both nononcogenic and oncogenic type infections; however, the associations go undetected until later in the HPV infectious cycle. Another potential target for zeaxanthin is the immune system, as higher antioxidant levels may contribute to increased clearance of HPV infected cells. This mechanism requires further examination.

As with any observational study, there were limitations with this study that need to be addressed. We assessed HPV persistence and clearance over a relatively short period of time. Our definition of HPV persistence and clearance included both prevalent and incident HPV infections. By including prevalent infections our estimate of duration of infection would not represent the true duration because it was impossible to know exactly how long a woman has been infected by the time she was found positive at enrollment. In general, women

found to be HPV positive upon enrollment were more likely to have a persistent infection. Unfortunately the Ludwig-McGill Cohort study did not have sufficient incident HPV infections to utilize incident HPV persistence as our outcome. Of all the HPV infections detected in the first year, 39% were detected at enrollment and only 22% of the incident infections persisted. HPV clearance was defined as the time to the first negative test. Moscicki and colleagues (233) suggested that misclassification might result from using only one time point to determine clearance. However, we utilized PCR based methods, which are highly sensitive and detect low levels of HPV DNA, to assess HPV status. These methods are less likely to result in false negative results compared to other methods (234). Similar to other biological markers, the values of serum nutrients presented in this report might not reflect the absolute value due to losses that might have occurred during storage or in the extraction process. However, the relative levels of each isomer should not have changed; therefore the associations found in this study would be valid with the magnitude of the associations potentially being lower than the true association due to methodological errors.

The issue of multiple comparisons arises when analyzing nutritional data from large epidemiological studies in that a large number of statistical models are required. For the present study, we did not adjust the significance level but chose to evaluate each serum nutrient as individual associations. By using this approach, there is a chance that single spurious associations will be found based on the nature of statistical probabilities. Therefore, the issue of multiple

comparisons needs to be considered when interpreting results. Conclusions should be made in light of consistency with other information and external to the study (225). The findings of an association of serum zeaxanthin levels with HPV clearance consistent with two previously reported studies (96,97). This consistency provides support that our results for this particular nutrient may not be a spurious association found by chance.

This study had many strengths, including the assessment of serum carotenoids and tocopherols at four different time points allowing for an accurate assessment of a woman's nutrient status. The study was nested within a large prospective cohort study that measured HPV at four clinical visits within the first year of follow-up and assessed over 40 HPV types that allowed type-specific HPV infections to be detected.

Overall, results from the current study suggest that higher serum antioxidant nutrients may lead to shorter duration of HPV infections. We found differences in specific nutrient associations between oncogenic and nononcogenic type HPV infections. The consistent associations found for both serum and dietary antioxidants are promising. This research indicates that antioxidant nutrients, modifiable factors, are associated with viral persistence and clearance and suggest that a diet high in antioxidants may confer protection. Future research efforts will examine if serum antioxidant nutrients confer protection against cervical lesion development.

## SUMMARY AND FUTURE DIRECTIONS

Cervical cancer continues to remain a major public health problem as it is the second leading cause of cancer for women worldwide (1). Infection with human papillomavirus (HPV) has been identified as the primary cause of cervical cancer (2). Prospective studies have demonstrated that women with persistent HPV infections are at a significantly greater risk of developing pre-cancerous cervical lesions, squamous epithelial lesion (SIL), compared with women who are only transiently infected or those not infected (3-6).

Although oncogenic HPV infections have been established as the necessary cause of cervical cancer, most HPV infections are transient and rarely progress to significant cervical lesions (8). The factors that are associated with oncogenic HPV persistence and progression to HSIL are unknown. Cofactors, such as antioxidant nutrients (e.g., carotenoids and tocopherols) and retinoic acid may modulate an oncogenic HPV infection from a transient infection to one that persists and progresses to cancer.

Epidemiologic data suggested that carotenoids and tocopherols decrease the risk of persistent HPV infections; however, few studies evaluated these associations and those that did have study limitations. In a small prospective study among women attending a family planning clinic for routine care, Sedjo *et al.* (96) reported significant inverse associations between dietary intake of lutein, vitamin E, vegetables and serum lycopene levels and HPV persistence (96). This cohort had a small sample size and was unable to assess type-specific HPV

persistence (96) and clearance (98). In addition, there was only one measure of serum carotenoids and tocopherols which might not have adequately classified a woman's nutrient status due to the known high-within person variability of these measures (200). Therefore, the present study with its large sample size, multiple HPV measurements, and long follow-up period was needed to adequately test the hypothesis that circulating concentrations of antioxidant nutrients were associated with persistent HPV infection and the development of SIL (Specific Aims 2 and 3).

Overall, the findings of this study suggest that women with the highest levels of serum all-*trans* retinoic acid and tocopherol are at a decreased risk for a persistent type-specific HPV infection. Women with higher circulating zeaxanthin levels are more likely to clear oncogenic HPV infections. From these results and those in the literature, different study populations and different statistical approaches suggest different associations for specific antioxidants (9). For these antioxidant nutrients with similar proposed mechanisms of action, differences in associations for individual nutrients may depend of the study sample being investigated, nutritional status at enrollment, lifestyle factors, and availability and consumption of foods containing specific nutrients. In this study of Brazilian women, women consume an abundant amount of tropical fruits, such as papayas and oranges (97). In a previous study within this population that examined dietary intake and risk of HPV persistence (9), a nutrient database that determined the food carotenoid content specific for the nutritional content from

foods in São Paulo, Brazil (202) were utilized. This Brazilian specific nutrient content may have contributed to the consistency of our findings for both dietary intake and serum nutrients and HPV persistence and clearance. This study within Brazilian women was ideal for the examination of associations with nutritional status, specifically antioxidants, as few women were taking supplements (97), and when supplements were taken they rarely contained carotenoids. Unlike homogeneous populations often studied within the U.S. (235), there was a wide range of serum levels in this population. The mean serum levels were lower than those seen in a study of U.S. women (96), however the overall range of nutrients values was comparable. Future studies are needed in a similar population of women from other developing countries to confirm the results of the present study.

The definitions of HPV persistence, clearance and viral load assessed in these analyses need to be discussed. HPV data were available from four visits within a one-year period, which allowed adequate assessment of HPV persistence by oncogenic type within a one-year period. It has been suggested that a persistent HPV infection should be detectable for at least one full year. Compared to this “gold standard” definition, we defined HPV persistence over a relatively short time period of four-months. Within this large cohort study, there were 18 occurrences of the same oncogenic HPV type detected at all four visits for a woman and 18 occurrences of type-specific nononcogenic infection. Therefore, we lacked statistical power to define our persistence outcome based

on women consistently positive for at least 12-months. By restricting the definition of HPV persistence to 12-months of positivity, women may be more accurately classified as having a persistent type-specific HPV infection, which would reduce misclassification. However, it would require analysis of the entire Ludwig-McGill Cohort study over the entire follow-up period to adequately address differences in risk factors for women who maintain a type-specific HPV infection for at least one year compared to those who do not.

This is the first study to examine association of serum nutrients and HPV persistence and clearance for both oncogenic and nononcogenic types separately. This assessment also allowed an examination of multiple definitions of HPV persistence. However, the rich source of data provided by the Ludwig-McGill Cohort study did not have enough incident HPV infections to utilize incident HPV persistence as our outcome. Of all the HPV infections detected in the first year, 39% were detected at enrollment and only 22% of the incident infections persisted. Other aspects of this complex HPV data that need to be further addressed include analysis of individual HPV types or groups based on phylogenetic classification and analysis of the number of multiple HPV infections present at each visit. Future analyses within this study population will determine if serum carotenoids, tocopherols and retinoic acid are associated with time to SIL development and examination of HPV results for 36-months of follow-up.

As this is the first population-based study assessing serum retinoic acid level, we determined the variability of circulating retinoic acid levels over a four-

months period and lifestyle, demographic and nutritional factors might that explain such variability (Specific Aim 1). Within the sample of 502 women, the relative abundance of retinoic acid isomers was similar at each visit and the within person-variability of total retinoic acid and individual isomers was low. Using multivariate logistic regression models (upper tertile vs. low/middle tertiles of serum retinoic acid), we found that serum retinol,  $\beta$ -carotene, and  $\beta$ -cryptoxanthin levels, age, race, oral contraceptive use, total number of pregnancies, and season of initial blood draw were significantly associated with endogenous retinoic acid isomer levels.

This is the first study to fully characterize serum levels. However, it remains unclear how representative these serum levels are to tissue specific levels. Previous work examined the tissue levels following chemopreventive treatment (236), however these studies occurred prior to newly refined HPLC methodologies. The ultimate goal of this research is for serum retinoic acid levels to serve as a biomarker of overall retinoic acid status or tissue-specific status. Several studies need to be undertaken to determine the utility of serum retinoic acid as such a surrogate biomarker. One recommended study is a Phase II chemoprevention trial be conducted to determine if modulation of diet with increased intake of vitamin A or pro-vitamin A carotenoids will increase serum levels of retinoic acid. Other studies are needed to determine the correlation between serum retinoic acid levels and retinoic acid levels in the cervix or retinoic acid receptor mRNA levels. In these studies, since we are

interested in the effect of retinoic acid on HPV activity, the study population should include both women with HPV infections and those without infection. If the results from this proposed biomarker trial demonstrates that retinoic acid is an adequate biomarker, further large trials would then need to be conducted to examine the efficacy of serum retinoic acid level in the prevention of incident and persistent HPV infection.

The issue of multiple comparisons arises when analyzing nutritional data from large epidemiological studies in that a large number of statistical models are required. For the present study, we did not adjust the significance level but chose to evaluate each serum nutrient as individual associations. By using this approach, there is a chance that single spurious associations will be found based on the nature of statistical probabilities. Therefore, the issue of multiple comparisons needs to be considered when interpreting results. Conclusions should be made in light of consistency with other information and external to the study (225). The present study examined nutritional factors associated with serum retinoic acid utilizing three nutritional measures (i.e. serum nutrient levels, derived nutrient intake levels, and reported consumption of specific foods). These three measures were utilized to examine the consistency of associations found with serum retinoic acid. Bases on these three approaches, serum retinol,  $\beta$ -carotene and  $\beta$ -cryptoxanthin were consistently associated with serum retinoic acid levels. The findings of an association of serum zeaxanthin levels with HPV clearance are consistent with two previously reported studies. This consistency

provides support that our results for this particular nutrient may not be a spurious association found by chance. Results from the present study need to be confirmed in other study population to rule out spurious significance due to multiple comparisons alone.

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**APPENDIX A**

**MANUSCRIPT 1**

**Temporal variation and identification of factors associated with endogenous retinoic acid isomers in serum from Brazilian women<sup>1</sup>**

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**Running Title:** Variability and Factors Associated with Serum Retinoic Acid

**Word Count**=4616

**Target Journal:** Cancer Epidemiology, Biomarkers and Prevention

**KEYWORDS:** retinoic acid, isomers, serum, variability, associations

## CONDENSED ABSTRACT

Retinoids (natural and synthetic derivatives of vitamin A) have cancer chemotherapeutic and chemopreventive activities. Here we assess the variability of circulating retinoic acid (RA) levels and the lifestyle, demographic and nutritional factors that explain such variability. Total RA concentration and the concentrations of RA isomers (*all-trans*, *13-cis*, and *9-cis* RA) were measured by high-pressure liquid chromatography (HPLC) in serum samples obtained 4 months apart from 502 women participating in the Ludwig-McGill Cohort, São Paulo, Brazil. The relative abundance of the three RA isomers was *13-cis*-RA having the highest concentrations, followed by *9-cis*-RA and *all-trans*-RA. The within person-variability of total RA and individual isomers was low. Using multivariate logistic regression models (upper tertile vs. low/middle tertiles of serum RA), we found that age, race, oral contraceptive use, total number of pregnancies, and season of initial blood draw were significantly associated with at least one endogenous retinoic acid isomer level. All endogenous RA isomers were positively associated with serum retinol,  $\beta$ -carotene and  $\beta$ -cryptoxanthin levels. These results have implications for the design of future epidemiological studies focused on assessing RA-disease association and intervention studies aimed at modulating RA levels.

## ABSTRACT

Objective: Retinoids (natural and synthetic derivatives of vitamin A) have cancer chemotherapeutic and chemopreventive activities. Retinoic acid (RA) treatment has been associated with significant regression of preneoplastic lesions. However, serious toxicity associated with some therapies has made long-term chemoprevention in healthy populations unfeasible. Recently serum RA has been shown to increase in response to oral retinol (vitamin A) supplementation. Here we assess the variability of circulating RA levels and the lifestyle, demographic and nutritional factors that explain such variability. Method: Total RA concentration and the concentrations of RA isomers (all-*trans*, 13-*cis*, and 9-*cis* RA) were measured by high-pressure liquid chromatography (HPLC) in serum samples obtained 4 months apart from 502 women participating in the Ludwig-McGill Cohort, São Paulo, Brazil. Results: The relative abundance of the three RA isomers was similar for each visit (baseline and month 4), with 13-*cis*-RA having the highest concentrations, followed by 9-*cis*-RA and all-*trans*-RA. The within person-variability of total RA and individual isomers was low. Using multivariate logistic regression models (upper tertile vs. low/middle tertiles of serum RA), we found that age, race, oral contraceptive use, total number of pregnancies, and season of initial blood draw were significantly associated with at least one endogenous retinoic acid isomer level. All endogenous RA isomers were positively associated with serum retinol,  $\beta$ -carotene and  $\beta$ -cryptoxanthin levels. Conclusion: These results have implications for the design of future epidemiological studies focused on assessing RA-disease association and intervention studies aimed at modulating RA levels.

## INTRODUCTION

Retinoic acid (RA)<sup>3</sup> is required for many biological processes including vision, development and reproduction (1-3). Retinoic acid mediates these activities by binding to nuclear retinoid receptors, members of the steroid hormone receptor superfamily (4), and altering transcriptional activity (1, 5). To date, two families of receptors have been identified, retinoic acid receptor (RAR) and retinoid X receptor (RXR) (5). RAR binds all-*trans* and 9-*cis* RA with equal affinity, whereas RXR preferentially binds 9-*cis* RA (6). Each receptor family has at least three subtypes ( $\alpha$ ,  $\beta$ , and  $\gamma$ ) and within these subtypes there are many different isoforms (7). Due to the different receptor isoforms retinoid receptors can interact with a diverse number of receptors forming homodimers and heterodimers not only with retinoid receptors but with other steroid hormone nuclear receptors as well, such as vitamin D receptors and the estrogen receptor (8).

Through this potent activity as transcriptional regulators, retinoids (natural and synthetic derivatives of vitamin A) have been shown to be cancer chemotherapeutic and chemopreventive [see also (5, 9, 10) for reviews]. RA treatment (all-*trans* RA) has been associated with significant regression of several preneoplastic lesions, including oral leukoplakia (11), cervical dysplasia (12) and actinic keratoses (5). Although retinoid chemoprevention is effective, there are toxicities associated with therapy such as varying degrees of teratogenicity and mucocutaneous cytotoxicity (5, 13). These toxicities make long-term chemoprevention among healthy populations with naturally occurring retinoic acid unfeasible. 13-*cis* RA (isotretinoin) and 4HPR (N-(4-hydroxyphenyl) retinamide, Fenretinide) have shown to have lower toxicities, but it remains unclear if these drugs are

able to induce retinoid receptor mediated changes. An alternative retinoid chemoprevention approach was to develop a method for increasing endogenous concentrations of RA. Such an approach was demonstrated to be feasible by Sedjo *et. al.* (14) who reported the modulation of endogenous RA following chemopreventive doses of retinyl palmitate. The focus of this paper is to examine if the variability of endogenous RA is associated with variability of dietary intake, implying that diet may modulate levels of RA safely.

Prior to developing research and programs to modulate endogenous RA levels, a reliable method for measuring endogenous RA levels is required and an understanding of the factors that influence endogenous RA levels is needed. For over a decade, various methodologies have been reported for the detection of RA in serum (15-25). Recent improvements in high-pressure liquid chromatography (HPLC) methodologies have facilitated the measurement of endogenous RA within the context of epidemiological studies. The key improvements include an improved separation of three RA isomers and implementation of high throughput methods. Using refined methodology for measuring circulating RA levels, it is now possible to assess the variability of RA and the factors associated with endogenous RA levels. This information is essential to the design of future epidemiological studies focused on assessing RA-disease associations and intervention studies aimed at modulating RA levels.

Using blood samples collected four months apart in an ongoing prospective study, the Ludwig-McGill Cohort in São Paulo, Brazil, we investigated the variability of serum RA concentrations and its three major isomers (*all-trans*, *13-cis*, and *9-cis* RA) over a

four-month period. We also examined whether certain lifestyle, demographic and nutritional factors are associated with the variability in such measurements.

## **MATERIALS AND METHODS**

We utilized blood samples previously collected and stored as part of the Ludwig-McGill cohort study of the natural history of human papillomavirus (HPV) infection and cervical neoplasia (26). Briefly, from 1993 to 1997, the Ludwig-McGill Cohort enrolled a systematic sample of 2,528 women attending a comprehensive maternal and child health maintenance program catering to low income families in the city of São Paulo, Brazil. The clinic setting where participants were being accrued is part of a network of primary, secondary, and tertiary health care institutions maintained by the municipal health department. Cohort participants were examined every four months in the first year and twice yearly, thereafter, for a total of five years. At each study visit, participants were interviewed based on a structured questionnaire specific for the current visit (26). The mean age at enrollment was 31 years (median age 33 years) (26). The institutional review boards and ethical committees of all institutions with which the authors are affiliated approved these protocols. Each study participant signed an approved informed consent document.

Study Sample: Of the 2,528 women in the cohort, we identified a sub-cohort of women (n=1,392), representing mostly those entering the study during the first two years (1993-1995) and who had provided long-term follow-up. We then selected the 846 women who provided data and serum for all four visits within the first year. Of these

women, 810 had sufficient serum for RA analysis at visits 1 (month 0) and 2 (month 4). We then limited the study sample to those women that were HPV negative and had normal cervical cytology (n=502).

Diet questionnaire: Information on the frequency of consumption of selected food items and the consumption of vitamin and mineral supplements was obtained at the second visit (month 4). Participants were asked to recall the usual frequency of consumption, during the past 5 years, of the following 15 food items: oranges, lemons, carrots, pumpkin, papaya, cauliflower, spinach, broccoli, lettuce, other vegetables, eggs, milk and yogurt, cheese, butter, and liver. These foods contributed substantially to variation in intake of carotenoids and tocopherols, among Brazilian women living in São Paulo. The food consumption-frequency categories were as follows: never, <1 time/month, 2-3 times/month, 1-3 times/week, 4-6 times/week, and  $\geq 1$  time/day. Women in this study did not report a substantial consumption of vitamin and mineral supplements (27).

Nutrient Intake Values: Diet provides retinol (preformed Vitamin A) in the form of retinol ester from animal products (liver, eggs, milk, cheese and butter) or as pro-vitamin A carotenoids (e.g.  $\beta$ -carotene,  $\alpha$ -carotene, and  $\beta$ -cryptoxanthin) (1-3). Through a series of controlled metabolic reactions, retinol and pro-vitamin A carotenoids are converted to retinoic acid, *in vivo*. Therefore, nutrient intake of vitamin A and carotenoid rich foods are the focus of this report. The determination of nutrient values for this population have been reported previously (27). In brief, nutrient values were calculated from the participants' reported dietary intake by use of the United States Drug

Administration's Continuing Survey of Food Intake of Individuals (CSFII-86) and Nationwide Food Consumption Survey (NFCS 87-88), unless Brazil-specific nutrient values were available. When available, food carotenoid values (e.g.,  $\beta$ -carotene, lutein/zeaxanthin, and  $\beta$ -cryptoxanthin) were derived from published values for foods consumed in São Paulo, Brazil (28). Dietary-intake calculations used age-specific portion sizes for women, as described elsewhere (29). From these databases, nutrient values were obtained for vitamin A, carotenoids ( $\alpha$ - and  $\beta$ -carotene,  $\beta$ -cryptoxanthin, and lutein/zeaxanthin), and vitamin C.

Since the questionnaire used in this study was short, we examined the association between consumption of certain fruits and vegetables and corresponding serum concentrations of carotenoids. Serum concentrations of certain carotenoids were correlated with consumption of citrus fruits and carrots (27) in a group of 100 women. In addition, we evaluated the crude and energy-adjusted (30) associations between intake of carotenoids and serum concentrations of carotenoids and found stronger associations when the energy-adjusted intake values for carotenoids were used (data not shown). The primary purpose of energy-adjustment of nutrient values was to adjust for the tendency of individuals to consistently under- or over-report intake values when a frequency checklist is used (30). Therefore, all dietary intake analyses used energy-adjusted nutrient values.

Sample Processing and Storage. All non-fasting blood samples (~10 ml) were collected by venipuncture into green top vacutainers by a trained nurse at the time of the clinic visit. The samples were centrifuged within 6-8 hours of collection. Aliquots (1 ml) of serum were stored in 1.8 ml Nunc cryovials at  $-20^{\circ}\text{C}$  in a non-frost free freezer

until shipped for analyses. Serum from the first two clinical visits (months 0 and 4) was utilized to measure retinoic acid. To address the known variability of serum carotenoids (31), we determined serum retinol and carotenoids in four serum samples obtained within the first year (months 0, 4, 8, and 12).

Total serum cholesterol: Carotenoids and tocopherols are fat-soluble nutrients, of which uptake, transport and storage is associated with lipids (32). Therefore, the level of serum lipid, as measured by cholesterol, may influence the level of circulating carotenoids and tocopherols. To account for this effect, total serum cholesterol was assessed at the Visit 3 to enable adjustment for this potential confounding variable. Total cholesterol was determined by coupled enzymatic, colorimetric assay (Sigma kit #401-25P) by Craft Technologies Inc (Wilson, NC).

Serum Retinoic Acid HPLC Analytical Procedures: Retinoic acid analyses were conducted on serum samples from the first two visits (months 0 and 4). The 9-*cis*- and all-*trans*-RA were purchased from Sigma Chemical Co. (St. Louis, MO) and 13-*cis*-RA was obtained from ICN Biomedicals (Aurora, OH). The following reagents were used for sample preparation and analysis: butylated hydroxytoluene (BHT), hexane, ethanol, methanol, acetonitrile, hydrochloric acid, sodium hydroxide, and acetic acid. All solvents were HPLC grade or equivalent and were used without further treatment.

Sample extraction and preparation was performed using a modification of the method reported by Miyagi *et al.* (15). After thawing 500  $\mu\text{L}$  aliquots of serum, samples were deproteinated with 500  $\mu\text{L}$  of acetonitrile/methanol (19:1) containing 0.01% BHT and made alkaline with 100  $\mu\text{L}$  of 2N sodium hydroxide. Samples were extracted by

vortex-mixing 45 s with 1.5 mL of hexane containing 0.025% BHT as an antioxidant. The organic phase was discarded. Samples were acidified with 200  $\mu$ L of 2N hydrochloric acid (HCl) and extracted 3x with 1.5 mL of hexane with BHT. The combined supernatant was evaporated under nitrogen. The residue was dissolved by vortex-mixing with 120  $\mu$ L of mobile phase consisting of 75% acetonitrile, 5% methanol, and 20% of 1% acetic acid. The injection volume was 90  $\mu$ L.

The HPLC separation was performed using a modified method reported by Dimitrova *et al.* (16). HPLC analysis was performed using a ThermoSeparation Products liquid chromatograph with the following components: P4000 solvent delivery system, vacuum degasser, AS3000 autosampler, Spectra FOCUS scanning UV-visible detector, and PC1000 computer-controlled data system (Fremont, CA). On the autosampler, samples were refrigerated at 10° C and the column was maintained at 30° C. RA isomers were monitored at 350nm. The analytical column was a Spherisorb ODS2 (3 $\mu$ m, 4.0 $\times$ 250mm) with Javelin guard column containing Keystone ODS2, 3 $\mu$ m (Keystone Scientific, Inc., Bellefonte, PA). The mobile phase had a flow rate of 1 mL/min.

Linear calibration curves were prepared consisting of three concentrations of retinoic acid isomers that spanned the physiological levels in serum. Quantification was performed by external standard calibration using peak area ratios. In-house quality control (QC) samples were analyzed at the beginning and end of each sample queue. The relative standard deviation of analytes in the QC samples ranged from 10-15%. This system had a limit of detection (LOD) of 0.1ng/mL and limit of quantification (LOQ) of 0.3ng/mL (5 pmol/L). Samples below the LOQ were assigned a value half way between

zero and the lower limits of detection: these accounted for 10 (1%) all-*trans*-RA, 20 (2%) 13-*cis*-RA, and 85 (8.5%) 9-*cis*-RA samples of the 1004 samples analyzed.

Serum Retinol and Carotenoids HPLC Analytical Procedures: Archival serum from all four visits within the first year (months 0, 4, 8, and 12) on study were analyzed to detect retinol and carotenoids in serum using a modification of the procedures described by Nomura et al. (33). The percent coefficient of variation was 8% or less for retinol and all carotenoids measured. This HPLC system's LOQ was 0.004 µg/ml for the carotenoids. Of the total 2,008 samples analyzed over four clinical visits, the following number of samples (percent) were below the LOQ and assigned a value half way between zero and the lower limits of detection: retinol 2 (<0.1%), α-cryptoxanthin 654 (33%), β-cryptoxanthin 187 (9%), α-carotene 514 (26%), *trans*-β-carotene 296 (15%), *cis*-β-carotene 621 (30%), lutein 10 (0.5%), and lycopene 425 (21%). Due to many samples being below the detectable limit for α-cryptoxanthin and α-carotene, these results will not be utilized in the analyses. *Trans*-β carotene and *cis*-β-carotene were combined for a measure of total serum β-carotene.

Statistical Analysis: Since retinoic acid concentrations were right skewed, we utilized nonparametric statistical methods for most analyses. Differences between month 0 (visit 1) and month 4 (visit 2) were assessed using a Wilcoxon Rank Sum test and correlation between paired nutrient values was examined by calculating Spearman correlation coefficient. To assess the between- and within-woman variability of RA, we estimated a linear mixed effects model using logarithmically transformed nutrient values. Only samples that were above the detectable limit of the assay were included in this

analysis. The mixed models analyses were performed using PROC MIXED in SAS version 9.0, Cary, North Carolina (34).

As the differences in RA concentrations were low between visits (Table 1), we calculated the mean of two retinoic acids measures per individual. We determined if there was a difference in the rank distribution of total RA and each isomer by lifestyle and demographic characteristics using the Wilcoxon rank-sum test (2 categories) or Kruskal-Wallis test (3 or more categories) without regard to the ordering. For factors that were ordered, a nonparametric test for trend, which is an extension of the Wilcoxon rank-sum test (35), was used to test whether there was a significant trend across ordered groups. Spearman correlations were calculated between total RA and each isomer, serum retinol and serum  $\beta$ -carotene for each visit, adjusting for laboratory batch analysis.

Multiple logistic regression was performed to estimate the magnitude of associations (odds ratio) and 95% confidence interval (CI) with RA levels and various socio-demographic and lifestyle factors. The study aim was to determine factors independently associated with elevated levels of RA, total and isomer specific. Therefore, we categorized women as having high values if their endogenous RA level fell within the upper tertile of the overall distribution and women as having low-to-medium levels if their level was below the upper tertile. Modeling began by using backwards-stepwise logistic regression models to identify the lifestyle and demographics factors that were independently associated with total retinoic acid or individual isomers with a probability of removal set at 0.1. Factors that were considered in the first set of models were race (white vs. non-white), oral contraceptive (OC) use, total number of

pregnancies, smoking status, alcohol consumption, season of enrollment, education and income. Age, in intervals of five years, was included in all final models regardless of significance level. A stabilization of the Brazilian economy occurred in July 1994, nine months following initial enrollment in the study, which most likely effected women's social economic status and spending patterns. We found this to be associated with differences in serum carotenoids and controlled for this confounding variable by inputting an indicator variable (enrollment before July 1994 or after) into the final multivariate models. Finally, a difference in chromatographic results across HPLC run batches was observed. This was controlled statistically by including a variable for HPLC batch in all analyses. However, since the RA category was the same for all samples in two batches these data were dropped during statistical modeling (N=49 and N=107, respectively). As a result, a smaller number of women was included in these logistic regression models (N: 13-*cis* RA = 451, 9-*cis* RA = 393, and all-*trans* RA = 501). To assess the influence of these two batches on our modeling, we repeated the logistic regression analyses removing all samples from the specified batches and found no differences in our conclusions (data not shown).

Using the above as a base model, we examined whether serum retinol and carotenoids (pro-vitamin A:  $\beta$ -carotene,  $\alpha$ -carotene, and  $\beta$ -cryptoxanthin, others: lutein and lycopene) were independently associated with RA levels using multivariate logistic regression. In addition, we examined if RA levels were associated with increased vitamin A and pro-vitamin A carotenoid intake and consumption of select foods. To test for linear trends, we treated all nutrient variables as continuous in the multivariate logistic

regression models. Statistical significance was set at the 0.05 level and all analyses were two-sided. Univariate and logistic regression analyses were performed in Stata 7.0 (College Station, TX) and mixed models analyses and adjusted spearman correlations were performed in SAS 9.0 (Cary, NC (36)).

## RESULTS

The mean, median, and range of the concentrations of endogenous RA, retinol and  $\beta$ -carotene at each visit are presented in **Table 1**. Overall, total RA concentrations ranged from 0.81 ng/mL to 8.90 ng/mL (2.70 nmol/L – 29.6 nmol/L) at visit 1 and 0.44 ng/mL to 9.91 ng/mL (1.46 nmol/L- 32.98 nmol/L) at visit 2 and did not differ by visit. Median endogenous concentrations at visit 1 for the 502 women were 1.30 ng/mL (range 0.15-4.90), 1.10 ng/mL (range 0.15 – 3.23) and 0.76 ng/mL (range 0.15 – 3.17) for 13-*cis* RA, 9-*cis* RA and all-*trans* RA, respectively. The relative abundance of the three retinoic acid isomers was similar for each visit, with 13-*cis*-RA having the highest concentrations, followed by 9-*cis*-RA and all-*trans*-RA. Median serum retinol levels were significantly lower at visit 2 compared to visit 1. However, when assessed over four available visits, no significant difference in retinol across visits was observed ( $p=0.08$ ) (data not shown).

Spearman correlation coefficients for total RA, RA isomers, retinol and  $\beta$ -carotene between visits 1 and 2 are shown in **Table 2**. Total endogenous RA level over a four-month time period was highly correlated (Spearman  $\rho=0.67$ ), as were the three isomers, with 9-*cis* RA having the highest correlation (Spearman  $\rho=0.79$ ). The within

person-variability of total RA and individual isomers was low, with the between- to within-variance ratios ranging from 1.05 for 3.09 (**Table 2**).

We also examined the association between RA isomer concentrations and lifestyle and demographic factors using the mean concentration of visits 1 and 2 for each woman (**Table 3**). Total RA and each of the RA isomers increased with age (p for trend < 0.001, all-*trans*-RA p for trend < 0.05), increasing number of pregnancies (p for trend < 0.001, all-*trans*-RA p for trend < 0.05), and increasing level of serum retinol (p for trend < 0.001). Median 13-*cis*-RA and 9-*cis*-RA levels increased with increasing income (p for trend < 0.001), while all-*trans*-RA decreased with increasing income (p for trend=0.02). For all factors examined, results were similar when RA concentrations were evaluated for visits 1 and 2 separately, rather than as the mean (data not shown). Median total RA and two isomers (13-*cis*-RA and 9-*cis*-RA) differed by cigarette smoking status (never, current or former). Median all-*trans*-RA was higher among white women compared to non-white women.

**Table 4** presents results of multivariate modeling of lifestyle and demographic factors independently associated with RA levels. Age, measured in 5-year intervals, was positively associated with total RA and 9-*cis* RA levels (Adjusted OR: 1.34, 95% CI 1.11 – 1.62 and Adjusted OR: 1.62, 95% CI 1.34 – 1.95, respectively). Season of blood draw was significantly associated with 13-*cis* RA and all-*trans* RA (Spring vs. Fall: Adjusted OR=0.36, 95% CI 0.15-0.85 and Adjusted OR=0.29, 95% CI 0.12 – 0.72, respectively).

**Table 5** presents independent associations between nutritional factors and serum RA. There were strong, positive associations among all retinoids and serum retinol,  $\beta$ -

carotene and  $\beta$ -cryptoxanthin. RA isomer levels were not associated with serum lutein or lycopene. The associations between intake of specific nutrients and circulating concentrations of RA isomers are also reported in **Table 5**. Increased intake of vitamin A was significantly positively associated with 13-*cis* RA (adjusted OR=2.65, 95% CI 1.48 – 4.73) and 9-*cis* RA (adjusted OR=1.85, 95% CI 1.04 – 3.27) and marginally associated with total RA (Adjusted OR=1.74, 95% CI 0.98 – 3.08). Increased intake of  $\beta$ -carotene was positively associated with 13-*cis* RA (Adjusted OR=1.78, 95% CI 1.01-3.12). Increased consumption of liver, pumpkin, and oranges was significantly positively associated with RA levels (Total, 13-*cis* or 9-*cis* RA) and may be the contributing sources for the positive associations shown with vitamin A and  $\beta$ -carotene intake and serum RA levels (data not shown).

## DISCUSSION

We characterized total serum RA and three individual RA isomers (13-*cis*, 9-*cis*, and all-*trans* RA) in a large sample of women and determined factors associated with these levels. The relative abundance of the three RA isomers was similar for each visit, with 13-*cis*-RA having the highest concentrations, followed by 9-*cis*-RA and all-*trans*-RA. Overall, we observed a wide range of values for this population (total RA 0.71 – 7.79 ng/mL), which may reflect the heterogeneity of this sample of low-income Brazilian women. While the between-person variability was high, we observed low within-person variability of two measures of serum RA obtained approximately four months apart.

Furthermore, various socio-demographic factors were related to retinoic acid level. Age, race, oral contraceptive use, total number of pregnancies, and season of initial blood draw were significantly associated with endogenous RA concentrations; however, factors significantly contributing to RA levels differed for each isomer. All endogenous RA isomers assessed in this study were positively associated with serum retinol,  $\beta$ -carotene, and  $\beta$ -cryptoxanthin levels. Associations were confirmed by observing significant associations with pre-formed vitamin A intake and increased consumption of pro-vitamin A containing foods. These associations differed slightly for each RA isomer suggesting the possibility of independent mechanisms for modulating specific isomer level. Additional laboratory research and population studies are required to define the mechanisms leading to RA isomerization and factors that modify this process. At the present time, it would appear that the measurement of multiple isomers

of retinoic acid within epidemiological studies provide added information compared to analyses that only assess total endogenous retinoic acid level.

The observed concentrations of 13-*cis* RA in these women were similar to previously published reports (17-20). Söderlund et al (17) reported an average 13-*cis* RA concentration of 1.35 ng/mL (range 1.26 – 1.5 ng/mL) in a small sample of Northern European women (mean age 36 years). Others reported higher values (21-23) of 13-*cis* RA which might reflect differences in study participants (e.g. diet, age, sex, or presence of benign disease) or laboratory methods. Sedjo *et al.* (14) assessed baseline levels of RA concentrations in a chemoprevention trial and found a relative abundance of each isomer similar to what we observed in this study (13-*cis* RA > 9-*cis* RA > all-*trans* RA).

The variability of retinoic acid measures obtained approximately four months apart was examined. Levels of serum RA isomers were relatively stable within women, with ratios of between- to within-person variability all above 1.0. This stability was similar to the results published by Tang *et al.* (24) demonstrating no change in 13-*cis* RA and all-*trans* RA over a three month period in 13 healthy controls. Overall, Söderlund *et al* (17) reported within-person coefficients of variability of 13.4% for 13-*cis* RA and 13.6% for all-*trans* RA, which were not influenced by period of menstrual cycle among 17 women. The ratios of between- to within-person variability for 13-*cis* RA and all-*trans* RA (1.57 and 1.17, respectively), were similar to those reported in this study (17). Yamakoshi *et al* (21) also found low between-day variances in serum RA isomers, all-*trans* RA (CV=11% ) and 13-*cis* RA (CV=8.4%) in six healthy subjects. Altogether,

these results would suggest that a single retinoic acid measurement might be sufficient to accurately classify a woman's retinoic acid level.

Söderlund *et al.* (17) have reported seasonal variation for 13-*cis* RA, with lower mean values during winter and spring (November-March) compared to summer months; however these differences were small and were interpreted as being of little biological significance. In these Brazilian women, we found lower median values of 13-*cis* RA in summer and, in the multivariate analyses, women were significantly more likely to have lower 13-*cis* RA levels in spring and summer compared to fall. In contrast to Söderlund *et al.* (17), we found significant seasonal differences in all-*trans* RA, with women more likely to have lower levels in the winter and spring, compared to fall. It might be that these seasonal differences might be due to differences in dietary intake, however we did not formally test this question. Future research in this area would need to be conducted.

In this study, serum RA levels were positively associated with serum retinol and  $\beta$ -carotene levels. Our data were consistent with a previous study in patients with inflammation, which reported lower levels of serum retinol occurring together with low serum levels of RA (20).  $\beta$ -Carotene supplementation (30 mg/day) was demonstrated to increase serum levels of all-*trans*-RA after three months in patients with colon polyps (increase from  $2.32 \pm 0.26$  to  $3.00 \pm 0.41$  ng/ml); however, no increase in 13-*cis* RA, total RA or retinol with supplementation were observed (24). Our results are further supported by a recent finding that supplementation with retinyl palmitate substantially increased endogenous RA levels, with all-*trans* RA having the highest percent increase over time, followed by 13-*cis* RA (14).

In this study, we are reporting serum levels of *9-cis* RA ranging from below detection (0.3 ng/mL) up to 3.8 ng/ml and contributing to relatively high proportion of total RA. The most likely explanations for the elevated *9-cis* RA levels include: 1) incomplete resolution of *9-cis* retinoic acid from components in the matrix or other isomers, e.g. *9,13-dicis* retinoic acid; 2) isomerization to *9-cis* retinoic acid during long term storage at  $-20^{\circ}\text{C}$ ; and/or 3) unidentified dietary or environmental factors leading to elevated levels. Currently, we do not have a standard for *9,13-dicis* retinoic acid to compare the retention time to determine if there was co-elution to address the first possible explanation. We have not seen significant isomerization when spiked samples were carried through our extraction and do not believe that artifacts were created during the extraction process. However, since the serum samples utilized in this study were not stored under ideal conditions at  $-80^{\circ}\text{C}$ , the second explanation is a possibility.

Lanvers *et al.* (25) report no degradation of *all-trans* RA or *13-cis* RA in plasma after 3 months storage at  $-20^{\circ}\text{C}$ , whereas others have reported degradation of *13-cis*-RA with long-term storage (19). To address this issue, we compared mean levels of RA in women among women enrolled two years apart and found that the mean RA levels were similar or elevated among samples obtained during the first year of enrollment compared to those obtained during the third year (data not shown). Therefore, our RA concentrations do not appear to be affected by serum storage conditions. In addition, we have comparable overall levels of total RA to other studies (17-20), and we have compared our study to one whose samples were stored ideally (14) and there were no

significant differences (data not shown). Further research in this area needs to be conducted to clarify these results.

As with all observational studies, this study does have limitations that need to be recognized. Similar to other biological markers, the values of RA presented in this report may not reflect the absolute value of endogenous RA due to losses that may have occurred during storage or in the extraction process. However, the relative levels of each isomer should not have changed; therefore the associations found in this study are assumed valid. The magnitude of the associations will potentially be lower than the true association due to any methodological errors. Another potential limitation is that this study included Brazilian women only. Our results cannot be generalized to men but since we have no reason to believe that this relatively disease free sample of women is different from other groups of women, the results presented here may be of relevance to other female populations.

The issue of multiple comparisons arises when analyzing nutritional data from large epidemiological studies in that a large number of statistical models are required. For the present study, we did not adjust the significance level but chose to evaluate each serum nutrient as individual associations. By using this approach, there is a chance that single spurious associations will be found based on the nature of statistical probabilities. Therefore, the issue of multiple comparisons needs to be considered when interpreting results. Conclusions should be made in light of consistency with other information and external to the study (37). The present study examined nutritional factors associated with serum retinoic acid utilizing three nutritional measures (i.e. serum nutrient levels, derived

nutrient intake levels, and reported consumption of specific foods). These three measures were utilized to examine the consistency of associations found with serum retinoic acid. Based on these three approaches, serum retinol,  $\beta$ -carotene and  $\beta$ -cryptoxanthin were consistently associated with serum retinoic acid levels. This consistency provides support that our results for this particular nutrient may not be a spurious association found by chance.

The Ludwig-McGill Cohort offers a unique opportunity to assess the association between endogenous RA levels and lifestyle, demographic and nutritional factors in a sample of over 500 women. There are multiple serum samples available for measurement of RA allowing for the first assessment of RA variability over time. In addition, serum retinol and carotenoids are available from four time points, a design that reduces the inherent variability of these measures and misclassification of serum nutrient level. Nutrient intake is estimated using Brazil specific carotenoid food content to increase accuracy in estimating carotenoid intake (27).

Previous studies have administered RA to patient populations for possible regression of preneoplastic lesions. However, when RA is given orally, significant toxicities have been reported. These toxicities have decreased utilization of RA in some populations, specifically among reproductive age women. Through identification of factors that are associated with increased endogenous levels of RA, there is potential that modification of these factors will increase levels of RA, and this leads to prevention of pre-neoplastic lesions without the toxicity of supplemental treatment. This potential modification would be potentially beneficial to women of reproductive age, especially

women at risk for cervical cancer, for whom retinoic acid has been effective at regressing cervical dysplasia (12). Futures studies need to investigate whether increased levels of endogenous RA are associated with a decreased risk of pre-neoplastic cervical lesions.

#### **ACKNOWLEDGMENTS**

We are indebted to Maria L. Baggio and Lenice Galan for management of the patients and specimen collections and to Silvaneide Ferreira for data entry, sample retrieval and shipment and laboratory analysis. We appreciated the thoughtful comments on this manuscript by Drs. Robin Harris, Elena Martinez and Jesse Martinez.

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**Table 1: Endogenous Levels of Retinoic Acid, Retinol and  $\beta$ -Carotene in Brazilian Women<sup>a</sup>**

	Visit 1			Visit 2			P-value <sup>b</sup>
	Mean $\pm$ SD	Median	Range	Mean $\pm$ SD	Median	Range	
Total Retinoic Acid (ng/mL)	3.21 $\pm$ 1.23	3.07	0.81 - 8.90	3.19 $\pm$ 1.33	3.12	0.44 - 9.91	0.958
<i>Cis</i> -isomer							
13- <i>cis</i> RA (ng/mL)	1.35 $\pm$ 0.74	1.21	0.15 - 5.60	1.30 $\pm$ 0.74	1.25	0.15 - 7.00	0.604
9- <i>cis</i> RA (ng/mL)	1.02 $\pm$ 0.58	1.06	0.15 - 3.90	1.05 $\pm$ 0.59	1.08	0.15 - 3.83	0.445
All- <i>trans</i> -isomer (ng/mL)	0.83 $\pm$ 0.39	0.76	0.15 - 3.50	0.83 $\pm$ 0.45	0.76	0.15 - 5.14	0.810
Retinol ( $\mu$ g/mL)	0.465 $\pm$ 0.169	0.437	0.007 - 2.083	0.446 $\pm$ 0.172	0.416	0.075 - 1.998	0.012
Total $\beta$ -Carotene ( $\mu$ g/mL)	0.044 $\pm$ 0.079	0.020	0.004 - 0.650	0.032 $\pm$ 0.039	0.021	0.004 - 0.609	0.762

a. N=502

b. P-values testing the null hypothesis that the difference between visit 1 and visit 2 is zero using the Wilcoxon Rank Sum test

**Table 2: Spearman Correlation Coefficients, Between and Within-person Variances, Between-to-Within Ratio of Serum Retinoids**

	Spearman Correlation <sup>a</sup>	N <sup>b</sup>	Between- person Variance <sup>c</sup>	Within-person Variance	B/W Ratio
Total Retinoic Acid (ng/mL)	0.67	502	0.122	0.068	1.796
<i>cis</i> -isomer					
13- <i>cis</i> RA (ng/mL)	0.57	480	0.139	0.132	1.053
9- <i>cis</i> RA (ng/mL)	0.79	424	0.165	0.053	3.092
All- <i>trans</i> -isomer (ng/mL)	0.56	485	0.100	0.086	1.161

a: All  $P < 0.001$

b: Random Effects models were restricted to data above limit of quantitation, N varies by nutrient. Spearman Correlation coefficient includes 502 women

c: Log transformed nutrient values used in Random Effects analysis.

**Table 3: Serum Total Retinoic Acid and Retinoic Acid isomer Concentrations in Women Stratified by Lifestyle and Demographic Characteristics<sup>a</sup>**

	N	Median Concentration (ng/mL)			
		Total RA	13- <i>cis</i> -RA	9- <i>cis</i> RA	All- <i>trans</i> RA
<b>Age</b>					
< 20 yrs	21	2.75	1.22	1.04	0.61
20-29 yrs	198	2.84	1.11	0.91	0.74
30-39 yrs	176	3.46	1.45	1.22	0.78
≥ 40 yrs	106	3.39	1.32	1.21	0.79
<i>p-trend</i> <sup>o</sup>		< 0.001	< 0.001	< 0.001	0.04
<b>Ethnicity</b>					
White	328	3.19	1.30	1.12	0.79 <sup>d</sup>
Non-white	173	3.07	1.28	1.07	0.71
<b>Cigarette Smoking</b>					
Never	277	3.09 <sup>c</sup>	1.22 <sup>d</sup>	1.09 <sup>d</sup>	0.74
Current	147	3.20	1.33	1.13	0.79
Former	77	3.24	1.33	1.11	0.76
<b>Alcohol</b>					
Never	174	3.20	1.37	1.10	0.74
≤ 1 glass/week	262	3.10	1.25	1.07	0.79
> 1 glass/week	66	3.25	1.33	1.22	0.74
<b>Years Consuming Alcohol</b>					
Never	174	3.20	1.37	1.10	0.74
≤ 5 years	113	3.18	1.35	1.10	0.70
6-10 years	84	2.88	1.06	1.00	0.85
11-15 year	38	3.25	1.25	1.14	0.90
>15 yrs	73	3.36	1.41	1.20	0.78
<i>p-trend</i>		0.78	0.81	0.80	0.06
<b>Oral contraceptive use</b>					
Never	78	3.16	1.41	1.07	0.73
< 6 years	276	3.06	1.20	1.06	0.74
≥ 6 years	147	3.40	1.39	1.20	0.80
<i>p-trend</i>		0.10	0.53	0.18	0.07
<b>Total # of Pregnancies</b>					
0-1	87	2.87	1.11	1.01	0.72
2-3	214	3.02	1.20	1.06	0.73
≥ 4	199	3.47	1.41	1.18	0.80
<i>p-trend</i>		< 0.001	< 0.001	< 0.001	0.05
<b>Education</b>					
Illiterate	35	3.19	1.33	1.23	0.74
< Elementary	77	3.24	1.30	1.08	0.76
Elementary	283	3.10	1.29	1.10	0.75
< High School	43	3.11	1.35	1.07	0.70
≥ High School	63	3.26	1.38	1.13	0.84
<i>p-trend</i>		0.76	0.94	0.28	0.61

Table 3 - Continued

<i>Income (US\$)</i>					
< 250	126	2.81	1.05	1.00	0.82
250-450	123	3.13	1.20	1.07	0.75
451-725	118	3.19	1.33	1.15	0.79
≥ 725	118	3.30	1.41	1.17	0.69
<i>p-trend</i>		< 0.001	< 0.001	< 0.001	0.02
<i>Total # Household members</i>					
1-3	108	3.15	1.25	1.10	0.87
4	148	3.10	1.30	1.07	0.73
5	119	3.28	1.30	1.13	0.73
≥ 6	126	3.11	1.32	1.10	0.73
<i>p-trend</i>		0.99	0.34	0.96	0.04
<i>Season (visit 1)<sup>f</sup></i>					
Fall	198	3.20 <sup>b</sup>	1.38 <sup>b</sup>	1.10 <sup>b</sup>	0.82 <sup>b</sup>
Winter	95	3.19	1.21	1.20	0.72
Spring	99	3.10	1.31	1.05	0.62
Summer	110	2.55	0.85	0.81	0.83
<i>Total Serum Cholesterol (mg/dL)</i>					
44 - 134	126	2.83	1.19	0.99	0.73
135 - 160	118	3.28	1.38	1.11	0.73
161 - 188	127	3.13	1.25	1.13	0.73
189 - 386	129	3.37	1.34	1.20	0.84
<i>p-trend</i>		< 0.001	0.01	< 0.001	0.08
<i>Tertiles of Serum Retinol<sup>g</sup></i>					
Low (0.165 - 0.392 µg/ml)	165	2.88	1.19	1.00	0.71
Medium (0.393 - 0.478 µg/ml)	177	3.19	1.33	1.12	0.73
High (0.479 - 1.550 µg/ml)	160	3.46	1.40	1.22	0.88
<i>p-trend</i>		< 0.001	< 0.001	< 0.001	< 0.001
<i>Tertiles of Serum β Carotene<sup>g</sup></i>					
Low (0.004 - 0.017 µg/ml)	165	2.98	1.27	1.05	0.67
Medium (0.018 - 0.031 µg/ml)	159	3.22	1.32	1.15	0.82
High (0.032 - 0.287 µg/ml)	178	3.33	1.30	1.17	0.83
<i>p-trend</i>		0.26	0.69	0.21	< 0.001

a: Mean retinoic acid concentration at visits 1 and 2 per individual.

b-d: Differences in retinoic acid concentration using Wilcoxon rank-sum test (2 categories) or the Kruskal-Wallis test (3 or more categories): b) p-value < 0.001; c) p < 0.01; d) p < 0.05

e: Nonparametric test for linear trend across ordered groups

f: For Season of blood sampling, only visit 1 concentrations are shown.

g: Tertiles distribution of mean of 4 measurements per women within 12 months

**Table 4: Associations of Total Retinoic Acid and Retinoic Acid Isomers with Lifestyle and Demographic Factors among Brazilian Women**

	<i>Total RA (n=451)<sup>a</sup></i>				<i>13-cis RA (n=451)<sup>a</sup></i>				
	Low - Med RA (N=281)	High RA (N=170)	Crude OR <sup>b</sup>	Adjusted OR <sup>c</sup> (95% CI)	Low-Med RA (N=282)	High RA (N=169)	Crude OR <sup>b</sup>	Adjusted OR <sup>c</sup> (95% CI)	
<i>Age, 5yr (Mean ± SD)</i>	29 ± 8.3	35 ± 8.5	1.42	1.34 (1.11 - 1.62)	30 ± 8.6	33 ± 8.5	1.07	1.05 (0.89 - 1.23)	
<i>Race</i>									
White	180	111	1.00		177	114	1.00		
Not-White	101	59	0.98		105	55	0.81		
<i>Season (visit 1)</i>									
Fall	105	77	1.00		97	80	1.00	1.00	Referent
Winter	61	34	1.03		61	34	0.67	0.68	(0.32 - 1.44)
Spring	60	32	0.58		65	27	0.34	0.36	(0.15 - 0.85)
Summer	55	18	0.65		60	23	0.42	0.45	(0.22 - 0.92)
<i>OC Use</i>									
Never	41	31	1.00	1.00	43	29	1.00		
< 6 yrs	172	74	0.50	0.47 (0.24 - 0.91)	162	84	0.64		
≥ 6 yrs	68	65	1.17	0.89 (0.43 - 1.83)	77	56	0.93		
<i>Total # of Pregnancies</i>									
0-1	61	16	1.00	1.00	55	22	1.00		
2-3	125	66	2.26	1.90 (0.91 - 3.97)	127	64	1.31		
≥ 4	95	88	3.48	2.42 (1.13 - 5.18)	100	83	1.75		

a: Cutpoint between low-medium RA level and High: Total RA 3.65 ng/μl; 13-cis RA 1.53 ng/μl ; 9-cis RA 1.30 ng/μl; all-trans RA 0.92 ng/μl

b: Crude models are adjusted for laboratory analysis batch

c: Adjusted simultaneously for all variables in the table for each specific RA isomer, laboratory analysis batch and economy

**Table 4 - Continued**

<i>9-cis RA (n=393)<sup>a</sup></i>					<i>all-trans RA (N=501)<sup>a</sup></i>				
Low-Med RA (N=233)	High RA (N=160)	Crude OR <sup>b</sup>	Adjusted OR <sup>c</sup> (95% CI)		Low-Med RA (N=334)	High RA (N=168)	Crude OR <sup>b</sup>	Adjusted OR <sup>c</sup> (95% CI)	
29 ± 8.5	34 ± 8.3	1.57	1.62	(1.34 - 1.95)	31 ± 8.6	32 ± 8.6	1.00	0.95	(0.81 - 1.12)
140	115	1.00	1.00	Referent	211	117	1.00		
93	45	0.58	0.63	(0.39 - 1.02)	123	50	0.65		
89	65	1.00			112	86	1.00	1.00	Referent
43	39	1.18			74	21	0.43	0.44	(0.21 - 0.91)
60	32	0.64			82	17	0.35	0.29	(0.12 - 0.72)
42	24	0.74			66	44	0.66	0.86	(0.47 - 1.58)
38	27	1.00			55	23	1.00		
135	76	0.72			187	89	1.17		
60	57	1.31			92	55	1.43		
47	22	1.00			64	23	1.00	1.00	Referent
100	61	1.28			147	67	1.16	1.19	(0.61 - 2.33)
86	77	1.84			123	76	1.67	1.89	(0.93 - 3.85)

**Table 5: Association of Total Retinoic Acid and Retinoic Acid Isomers and Nutritional Factors**

	Total RA (ng/mL) (n=451)				13-cis RA (ng/mL) (n=451)				9-cis RA (ng/mL) (n=393)				All-trans RA (ng/mL) (N=501)							
	Low - High	Crude	Adjusted <sup>b</sup>		Low - High	Crude	Adjusted <sup>b</sup>		Low - High	Crude	Adjusted <sup>b</sup>		Low - High	Crude	Adjusted <sup>b</sup>					
	Med RA	OR <sup>a</sup>	OR (95% CI)		Med RA	OR <sup>a</sup>	OR (95% CI)		Med RA	OR <sup>a</sup>	OR (95% CI)		Med RA	OR <sup>a</sup>	OR (95% CI)					
<b>A. Circulating Nutrients:</b>																				
<i>Serum Retinol</i>																				
Low (0.165 - 0.392 μg/mL)	103	37	1.00	1.00	-	95	45	1.00	1.00	-	83	31	1.00	1.00	-	119	45	1.00	1.00	-
Medium (0.393 - 0.478 μg/mL)	98	66	2.30	2.18 (1.20-3.95)		104	60	1.27	1.34 (0.76 - 2.36)		80	63	2.73	2.55 (1.43 - 4.54)		122	55	1.54	1.65 (0.93 - 2.95)	
High (0.479 - 1.550 μg/mL)	78	68	3.38	3.32 (1.74-6.32)		83	63	2.04	2.30 (1.26 - 4.19)		69	66	4.21	3.84 (2.08 - 7.09)		92	67	3.34	4.04 (2.16 - 7.54)	
<i>p-trend</i>				0.001					0.006					0.001					0.001	
<i>Serum β Carotene</i>																				
Low (0.165 - 0.392 μg/mL)	93	51	1.00	1.00	-	93	51	1.00	1.00	-	101	40	1.00	1.00	-	110	40	1.00	1.00	-
Medium (0.393 - 0.478 μg/mL)	97	46	1.07	0.99 (0.53-1.84)		95	48	0.89	0.84 (0.45 - 1.56)		74	46	1.50	1.31 (0.72 - 2.38)		97	57	1.83	1.82 (0.98 - 3.39)	
High (0.479 - 1.550 μg/mL)	62	68	4.23	4.58 (2.12-9.89)		69	61	3.04	3.17 (1.48 - 6.77)		36	67	4.98	4.86 (2.22 - 10.63)		100	60	2.08	3.04 (1.42 - 6.55)	
<i>p-trend</i>				0.001					0.008					0.001					0.004	
<i>Serum β Cryptoxanthin</i>																				
Low (0.002 - 0.012 μg/mL)	116	44	1.00	1.00	-	107	53	1.00	1.00	-	111	40	1.00	1.00	-	124	44	1.00	1.00	-
Medium (0.013 - 0.029 μg/mL)	90	61	2.14	1.97 (1.09-3.56)		95	56	1.21	1.20 (0.68 - 2.14)		81	51	1.56	1.22 (0.69 - 2.16)		107	59	1.63	1.82 (1.03 - 3.23)	
High (0.03 - 1.47 μg/mL)	73	66	4.19	3.76 (1.89-7.48)		80	59	2.04	2.24 (1.14 - 4.42)		40	69	4.32	3.21 (1.65 - 6.26)		102	64	1.82	2.39 (1.23 - 4.64)	
<i>p-trend</i>				0.001					0.022					0.001					0.009	
<i>Serum Lutien</i>																				
Low (0.009 - 0.027 μg/mL)	109	46	1.00	1.00	-	102	53	1.00	1.00	-	92	45	1.00	1.00	-	119	49	1.00	1.00	-
Medium (0.028 - 0.040 μg/mL)	90	63	1.45	1.38 (0.78-2.45)		89	63	1.38	1.21 (0.69 - 2.11)		78	56	1.27	1.07 (0.61 - 1.89)		109	58	1.1	1.18 (0.68 - 2.07)	
High (0.041 - 0.219 μg/mL)	80	63	2.22	2.12 (1.17-3.83)		91	52	1.41	1.35 (0.76 - 2.41)		62	59	1.85	1.55 (0.86 - 2.79)		105	60	1.23	1.57 (0.89 - 2.76)	
<i>p-trend</i>				0.013					0.36					0.147					0.120	
<i>Serum Lycopene</i>																				
Low (0.002 - 0.0093 μg/mL)	109	52	1.00	1.00	-	108	53	1.00	1.00	-	104	45	1.00	1.00	-	120	52	1.00	1.00	-
Medium (0.0094 - 0.018 μg/mL)	92	60	1.29	1.15 (0.65-2.04)		93	59	1.05	1.06 (0.60 - 1.87)		82	54	1.44	1.37 (0.78 - 2.42)		102	60	1.35	1.31 (0.75 - 2.27)	
High (0.0181 - 0.1203 μg/mL)	78	59	1.72	1.56 (0.77-3.16)		81	56	1.53	1.55 (0.77 - 3.11)		46	61	2.00	1.70 (0.83 - 3.49)		111	55	0.93	1.15 (0.59 - 2.25)	
<i>p-trend</i>				0.230					0.253					0.129					0.580	

**Table 5 - Continued**

**B. Energy Adjusted Nutrient Intake Value:**

<i>Vitamin A (RE)</i>																				
Low	109	41	1.00	1.00	-	110	40	1.00	1.00	-	97	40	1.00	1.00	-	122	46.00	1.00	1.00	-
Medium	86	65	1.99	1.90 (1.07-3.38)		89	62	2.45	2.33 (1.31 - 4.17)		71	58	1.78	1.70 (0.96 - 3.02)		105	62.00	1.18	1.17 (0.68 - 2.02)	
High	86	65	1.90	1.74 (0.98-3.08)		84	67	2.75	2.65 (1.48 - 4.73)		66	62	2.00	1.85 (1.04 - 3.27)		107	60.00	1.07	1.04 (0.60 - 1.81)	
<i>p-trend</i>				0.063					0.001					0.038					0.917	
<i>α-carotene (ug/mg)</i>																				
Low	91	57	1.00	1.00	-	96	52	1.00	1.00	-	80	52	1.00	1.00	-	111	57	1.00	1.00	-
Medium	95	58	0.95	0.89 (0.51-1.57)		100	53	1.02	0.97 (0.56 - 1.69)		75	56	1.13	0.94 (0.54 - 1.66)		106	61	1.23	1.19 (0.70 - 2.03)	
High	95	56	0.80	0.70 (0.40-1.24)		87	64	1.25	1.25 (0.72 - 2.17)		79	52	0.89	0.78 (0.44 - 1.38)		117	50	1.10	0.72 (0.42 - 1.25)	
<i>p-trend</i>				0.227					0.419					0.391					0.245	
<i>β-carotene (ug/mg)</i>																				
Low	95	54	1.00	1.00	-	101	48	1.00	1.00	-	77	58	1.00	1.00	-	119	49	1.00	1.00	-
Medium	91	59	1.26	1.21 (0.68-2.13)		93	57	1.56	1.57 (0.88 - 2.78)		77	51	0.86	0.73 (0.41 - 1.29)		107	60	1.12	1.23 (0.71 - 2.13)	
High	95	58	1.11	1.07 (0.61-1.90)		89	64	1.90	1.78 (1.01 - 3.12)		80	51	0.84	0.71 (0.41 - 1.26)		108	59	0.72	1.08 (0.63 - 1.85)	
<i>p-trend</i>				0.826					0.049					0.252					0.820	
<i>β-cryptoxanthin (ug/mg)</i>																				
Low	98	51	1.00	1.00	-	94	55	1.00	1.00	-	76	53	1.00	1.00	-	118	50	1.00	1.00	-
Medium	94	58	1.11	1.02 (0.57-1.81)		100	51	0.97	0.97 (0.55 - 1.70)		76	58	1.13	0.96 (0.55 - 1.69)		112	55	1.10	1.08 (0.63 - 1.87)	
High	89	63	1.49	1.39 (0.79-2.48)		89	63	1.41	1.39 (0.80 - 2.41)		82	50	0.91	0.73 (0.41 - 1.31)		104	63	1.56	1.60 (0.93 - 2.77)	
<i>p-trend</i>				0.249					0.232					0.291					0.088	
<i>Lutein (ug/mg)</i>																				
Low	97	52	1.00	1.00	-	94	55	1.00	1.00	-	75	54	1.00	1.00	-	119	49	1.00	1.00	-
Medium	97	55	1.06	1.00 (0.56-1.78)		104	48	0.92	0.93 (0.53 - 1.63)		78	54	1.04	0.89 (0.51 - 1.57)		109	58	1.22	1.17 (0.68 - 2.01)	
High	87	64	1.51	1.44 (0.81-2.54)		85	66	1.56	1.57 (0.90 - 2.72)		81	52	0.95	0.80 (0.45 - 1.41)		106	61	1.57	1.63 (0.94 - 2.83)	
<i>p-trend</i>				0.206					0.101					0.434					0.080	

a Crude model adjusted for analysis batch

b Logistic regression model adjusted simultaneously for variable in the table 5 for total RA and each isomer, laboratory analysis batch and economy. Cholesterol is included in model for serum retinol and β-carotene

**APPENDIX B**

**MANUSCRIPT 2**

**Type-specific HPV clearance and viral load associated with endogenous retinoic acid isomers in Brazilian women: the Ludwig-McGill Cohort Study<sup>1</sup>**

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**Target Journal:** Cancer Epidemiology, Biomarkers and Prevention

## FOOTNOTES:

1. Support for this study was provided by the National Cancer Institute (CA70269, CA81310), Canadian Institutes of Health Research (CIHR) (MA-13647, MOP-49396), and by an intramural grant by the Ludwig Institute for Cancer Research. E.L.F. is recipient of a Distinguished Scientist Award from the CIHR. E.M.S. is recipient of a NCI Cancer Prevention and Control Pre-Doctoral Fellowship (R25CA078447).
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**ABSTRACT:**

Background: Although oncogenic HPV infections have been established as the necessary cause of cervical cancer, most HPV infections are transient and rarely progress to significant cervical lesions. Current research is focused on identifying factors associated with viral persistence and clearance. The purpose of this study is to determine the association of circulating retinoic acid (RA), a potent regulator of epithelial cell differentiation and growth, and early events in cervical carcinogenesis, specifically HPV clearance and viral load. Methods: Using blood samples archived from 407 women participating in the Ludwig-McGill Cohort in São Paulo, Brazil, we investigated the association between concentrations of three RA isomers (*all-trans*, *13-cis*-, and *9-cis*-RA) and time to type-specific HPV clearance within a 12-month period. RA levels were determined at months 0 and 4 by high-pressure liquid chromatography (HPLC). Probability of clearing a type-specific HPV infection was estimated using Cox proportional hazard models. Results: Endogenous levels of *all-trans* RA were positively associated with an increased probability of clearing a type-specific HPV infection. Women with *all-trans* RA in the highest tertile had a shorter duration of type-specific HPV infection. Reduction of HPV viral load was associated with *13-cis* retinoic acid levels taken four months prior to viral load measure. Conclusions: Higher endogenous retinoic acid concentrations may increase the probability of clearing both oncogenic and nononcogenic HPV infection thereby decreasing risk of cervical disease in high-risk women.

## INTRODUCTION

Human papillomavirus (HPV) infection has been shown to cause virtually all cases of cervical cancer, the second leading cancer among women worldwide (1). Prospective studies have shown that women with persistent HPV infections are more likely to develop cervical lesions compared to women with transient HPV infections (2). Although oncogenic HPV infections have been established as the necessary cause of cervical cancer, most HPV infections are transient and rarely progress to significant cervical lesions (3). Current research is focused on identifying factors that could potentially alter the lifecycle of HPV during an infection and, therefore, are associated with viral persistence and clearance. Activity of HPV in cervical cells is tightly associated with epithelial cell differentiation (4) and factors that regulate differentiation may be related to the maintenance of an HPV infection. We sought to determine if the duration and viral burden of cervical HPV infections are associated with endogenous levels of retinoic acid (RA), a potent regulator of epithelial cell differentiation and growth (5).

Retinoic acid is the biologically active form of vitamin A, which is obtained from the diet as retinol esters or as pro-vitamin A carotenoids (6). There is evidence that vitamin A and pro-vitamin A carotenoids are associated with decreased risk of cervical neoplasia (7); however, the most compelling results have been reported from chemoprevention trials (8). Several clinical trials with retinoic acid or retinoic acid derivatives have been conducted to examine the

ability of this compound to induce regression of cervical dysplasia (8-12). Meyskens *et al.* (8) reported a histological regression rate of 47% of patients with CIN II randomized to the topical all-*trans* retinoic acid group compared to 27% regression in the placebo group. The protective activity of retinoic acid treatment is not sustained for high-grade lesion regression (e.g. CIN III) (8) or in reduction of cancer recurrence (13-15). This differential activity of retinoic acid between early and late stages of cervical disease suggests that retinoic acid may be most active during early stages of cervical carcinogenesis (16), at which point HPV is establishing itself as a persistent infection. This hypothesis is supported by *in vitro* observations during early passage of HPV infected foreskin keratinocyte (HKc); there is a reduction in HPV associated cellular proliferation and HPV oncogenic expression with retinoic acid treatment, followed by retinoic acid resistance in late-passage HPV-16 immortalized HKc (16).

The purpose of this study was to determine the association of circulating retinoic acid and early events in cervical carcinogenesis, specifically HPV clearance and viral load among women participation in the Ludwig-McGill Cohort study in São Paulo, Brazil. We measured endogenous levels of RA and three isomers (13-*cis* RA, 9-*cis* RA, and all-*trans* RA) at the baseline and month four visit and assessed association with type specific oncogenic and nononcogenic HPV clearance over a 12-month period.

## METHODS

The Ludwig-McGill Cohort Study is an epidemiologic cohort investigation focused on the natural history of HPV infections among women attending a comprehensive maternal and child health maintenance program catering to low-income families, in São Paulo, Brazil between 1993 to 1997 (17). The clinic setting where participants were accrued is part of a network of primary, secondary, and tertiary health care institutions maintained by the municipal health department. Cohort participants were examined every four months in the first year, and twice yearly thereafter for a total of five years. Over the course of this observational study, not all women returned at the designated time points for follow-up, which led variability in time between study visits. On average, women identified for this present study returned for their month 12 visit  $13.4 \pm 3.4$  months (Range 11 – 50 months) following enrollment. At each study visit, participants were interviewed based on a structured questionnaire specific for the current visit (17). The institutional review boards and ethical committees of all institutions, with which the authors were affiliated, approved these protocols. Each study participant signed an approved informed consent document.

*Study Sample:* Of the 2,528 women in the cohort, we identified a sub-cohort of women (n=1,392), representing mostly those entering the study during the first two years (1993-1995) and who provided long-term follow-up. From this sub-cohort (n=1,392), we selected women who had complete HPV data and serum available from all four visits within the first year (n=846), of which 818 had

normal/ASCUS cytology at enrollment and complete serum RA analysis at baseline and month 4. Of these 819 women, 407 tested positive for HPV at least once out of the first four visits, and 412 women were HPV negative at all four visits. To study the association between HPV clearance and serum retinoic acid levels, only the 407 women who tested positive for HPV in any one of the four evaluations were included in this analysis.

*Cervical cell specimens.* At each of the visits, an accelon biosampler was used to collect a sample of ectocervical and endocervical cells. After the smear was prepared on a glass slide and fixed in 95% ethanol, the sampler containing exfoliated cells was immersed in a tube containing Tris-EDTA buffer (pH 7.4), was swirled to release the adhered cells, and was maintained at the clinic for, at most, 5 days at 4°C. Once they were brought to the laboratory at the Ludwig Institute, the tubes containing cell suspensions were frozen until testing.

*HPV DNA detection methods-polymerase chain reaction (PCR) methods.* All HPV analyses were performed at the Ludwig Institute for Cancer Research, São Paulo, Brazil. Cervical-specimen DNA was extracted and purified in accordance with standard techniques. In brief, cells were digested with 100 µg/mL proteinase K for 3 hours at 55°C, followed by organic extraction and ethanol precipitation. Specimens were tested for the presence of HPV DNA by a previously described PCR protocol amplifying a highly conserved 450-bp segment in the L1 viral gene (flanked by primers MY09/11) (18, 19). Typing of the

amplified products was performed by hybridization with individual oligonucleotide probes specific for all 27 HPV genital types whose nucleotide sequences for probes within the MY09/11 fragment have been published elsewhere (19), namely types 6/11, 16, 18, 26, 31, 33, 35, 39, 40, 42, 45, 51-59, 66, 68, 73, 82, 83, and 84 . The PCR amplification products were further tested by restriction fragment-length polymorphism (RFLP) analysis of the L1 fragment (20), to resolve dubious results from the dot-blot hybridization and to distinguish among HPVs that could not be typed by dot-blot hybridization with the specific probes. This allowed us to extend the detection range to >40 genital HPV types, including HPV types 32, 34, 44, 62, 64, 67, 69-72, CP6108, and IS39. Amplified products that hybridized with the generic probe but not with any of the type-specific probes and that also did not produce a recognizable band pattern in the RFLP analysis were considered to be positive for HPV of unknown types. Using the expanded classification system of Bauer *et al.* (21), HPV types are grouped as follows: oncogenic infections (HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68) and non-oncogenic infections (all other 27 HPV types considered).

Measurement of Viral Load: All cervical specimens found to be positive with the main PCR protocol (MY09/11) were retested by quantitative, low-stringency PCR (LS-PCR) to measure viral burden in exfoliated cervical cells. General primers that detect a broad spectrum of HPVs were used (22). This modified system employed low stringency conditions to co-amplify the specific HPV DNA fragment along with sequences from the human genome present in

the starting PCR mixture. Standards consisting of mixtures containing varying amounts of reference HPV 16 plasmid added to constant background of normal human DNA were included in duplicate in every assay. In addition, control samples consisting of DNA from two cervical carcinoma cell lines with known quantities of HPV copies were included in duplicate in every assay. The silver-stained gel bands corresponding to the HPV and to the constant human genome fragments were quantified by densitometry (23). The logarithm of the ratio between these two bands was directly proportional to the logarithm of the amount of HPV DNA in the individual samples. Proper quantification was obtained by linear interpolation in a standard curve constructed with the results from control mixtures. Viral load output was number of HPV copies per cell.

*Serum Sample Processing and Storage.* All non-fasting blood samples (~10 ml) were collected by venipuncture into vacutainers by a trained nurse at the time of the clinic visit. The samples were centrifuged within 6-8 hours of collection. Aliquots (1 ml) of serum were stored in 1.8 ml Nunc cryovials at –20°C in a non-frost free freezer until shipped for analyses.

*High-pressure liquid chromatography (HPLC) analysis of serum retinoic acid:* Retinoic acid analyses were conducted for serum samples from the first two visits (months 0 and 4) using a modification of a method initially reported Horst *et al.* (24). The relative standard deviation of analytes in the QC samples ranged from 10-15%. Samples below the detectable limit of the assay (0.3 ng/ml or 5 pmol/L) were assigned a value half way between zero and the lower limits of

detection, which consisted of 31 (4%) all-*trans*-RA, 38 (5%) 13-*cis*-RA, and 120 (15%) 9-*cis*-RA samples of the 814 samples analyzed.

Statistical Analysis: Tertiles of each RA isomer were calculated on the basis of the overall nutrient distribution at each visit, creating time-dependent variables. Serum RA levels were assumed to be constant following the month 4 visit. There was a difference in chromatographic results across HPLC batches, which was statistically controlled for by including a variable for HPLC batch in all analyses.

The relationship between the serum retinoic acid and time to clearance of type-specific HPV infection was assessed. This analysis was based on 407 women who were HPV positive at baseline and those who developed an incident infection during follow-up. Time to HPV clearance was defined as the number of months to first negative HPV test occurring after previously positive HPV test(s) or until the last visit (month 12) if the participant remained positive throughout the study period. Type-specific clearance was grouped by oncogenicity, with women considered to have cleared an oncogenic infection if she was no longer positive for the specific type grouping that was originally detected, even if the cervical specimen was positive for a different viral type within the same group or within the nononcogenic grouping. Probability of maintaining an HPV infection present at baseline or an incident infection during follow-up was examined by estimating the Kaplan Meier survivor function. Missing values during follow-up were treated as censored observations unless there was a subsequent result at a later visit.

Multivariate Cox proportional hazards modeling was conducted to obtain the adjusted hazard ratios (AHR) and 95% confidence interval (95% CI). Several variables that could potentially confound the associations were considered; including those previously associated with persistence of HPV infection (25) and serum retinoic acid levels. Only those factors that altered the risk estimate by  $\geq 10\%$  were retained in the final multivariate model (income, education, age, marital status, race and age at first intercourse). Tests for trends were performed by treating all categorical nutrient variables as continuous, in the multivariate models

We examined the association of serum retinoic acid and HPV viral load at each visit. A viral load measure was considered to be low if HPV results were obtained by PCR (MY09/11) but LS-PCR was below the detectable limit. High viral load was detected by PCR and quantified by LS-PCR. To identify the time at which serum retinoic acid was associated with viral load, we conducted separate analyses with increasing time interval (n) between exposure (serum retinoic acid level) and outcome assessment (viral load). The time intervals considered were 0 months (simultaneous assessment), 4, 8 or 12 months. In this strategy, women could contribute multiple time windows in each series of analyses (e.g. enrollment to four months and four months to eight months). Women were only included in these analyses if they were HPV positive at the time of exposure (retinoic acid) assessment with normal/ASCUS cytology, however they could have lost their HPV infection at time of viral load. Since

multiple viral load measurements within a woman were correlated, a generalized estimating equation (GEE) model approach for time-dependent longitudinal data was used for analysis (26, 27). The GEE model adjusts for the serial correlation within subjects due to the longitudinal nature of the viral load data by modeling the covariance structure within subjects. As the dependent variable was the presence or absence of high viral load the logit link function was used. The independence working correlation structure with a robust variance estimator was selected as the best-fitted covariance pattern, using the Quasilikelihood Information Criterion (QIC) (27). Since viral load may depend on the time since the previous measurement, a time variable was included as a design factor (independent variable) in all GEE models. All statistical tests performed were 2-sided. Statistical analyses were performed by use of Intercooled STATA (Version 7.0; Stata).

## RESULTS

Characteristics of study sample. The 407 women in these analyses attended all four follow-up visits. Among these women, 167 tested positive for the same HPV type at two or more consecutive visits and 240 tested positive only once or at nonconsecutive visits. **Table 1** presents the demographic characteristics of this study sample. The median age for this group of women was 31 years, which was not different from the median age of all HPV positive women enrolled in the cohort. Most participants were white (61%), married or in

a common law marriage (70%), and not well educated, with 55% of the sample only have attended elementary school or less. At least 80% of these women reported more than two pregnancies and 57% reported using oral contraceptives for less than six years. A majority of these women were ever smokers (53%), with 36% reporting current smoking. Fifty six percent of participants reported age of first sexual intercourse under age 18 and 25% had more than four lifetime sexual partners. The relative abundance of retinoic isomers in this sample was highest for 13-*cis* retinoic acid ( $1.32 \pm 0.61$  ng/mL), followed by 9-*cis* retinoic acid ( $1.05 \pm 0.57$  ng/mL) and all-*trans* retinoic acid ( $0.83 \pm 0.38$  ng/mL) (**Table 2**).

**Table 3** presents the risk of type-specific HPV clearance by circulating retinoic acid levels, stratified by oncogenic and nononcogenic type infections. The probability of clearing both oncogenic and nononcogenic HPV infections was positively associated with serum levels of all-*trans* retinoic acid. Specifically, women were 1.82 times more likely to clear an oncogenic HPV infection if their serum all-*trans* retinoic acid levels were in the middle tertile compared to women with the lowest levels. The probability of clearing nononcogenic HPV infections was also positively associated with serum retinoic acid levels (Medium: AHR=1.67, 95% CI 1.09-2.57; High: AHR=1.46, 95% CI 0.93-2.28). The cumulative probability of clearing a type-specific oncogenic or nononcogenic infection by all-*trans* retinoic acid levels adjusted for laboratory analysis batch is presented in **Figure 1**. Higher concentrations of all-*trans* retinoic acid were associated with a shorter duration of non-oncogenic and oncogenic HPV

infections ( $p=0.01$  and  $p=0.05$ , respectively). The median duration of a nononcogenic HPV infection was 12.3, 12.1, and 9.8 months for the lowest, middle and highest tertile of all-*trans* retinoic acid, respectively. Serum all-*trans* retinoic acid level in the middle tertile were associated with shorter duration of oncogenic HPV infections compared to the lowest level (median time to clearance: 12.1 months vs 12.9 months, respectively) but this did not appear to be sustained for the highest level (median time to clearance 12.4 months). Over the course of this observational study, not all women returned at the designated time points for follow-up, which led variability in time between study visits. On average, women identified for this present study returned for their month 12 visit  $13.4 \pm 3.4$  months (Range 11 – 50 months) following enrollment.

**Table 4** presents the association of HPV viral load and serum retinoic acid levels. We examined not only concurrent viral load and retinoic acid measure (0 months apart), but the association between serum retinoic acid levels and subsequent viral load measures at 4, 8 or 12 months. We observed marginally significant positive associations with high viral load and serum retinoic acid in the highest tertile, (all-*trans* RA: Adjusted OR: 1.79, 95% CI 0.93 – 3.45) measured at the same visit compare to the lowest tertile. Circulating 13-*cis* RA level measured four months prior was inversely associated with high viral load (middle tertile vs lowest: Adjusted Odds ratios: 0.34, 95% CI 0.13-0.85).

## DISCUSSION

This is the first study to examine associations of circulating levels of retinoic acid and clearance of type-specific HPV infections. In this study, higher endogenous all-*trans* retinoic acid concentrations were associated with increased probability of clearing both oncogenic and non-oncogenic HPV infections. Women with mid to high levels of serum all-*trans* retinoic acid had shorter durations of infection. We found that serum 13-*cis* retinoic acid, but not all-*trans* retinoic acid, was inversely associated with high viral load at the subsequent four month visit.

The mechanism by which all-*trans* retinoic acid may increase the likelihood of clearing an HPV infection is unknown; however there is indirect evidence for a protective effect of retinoids in cervical carcinogenesis. This indirect evidence stems from epidemiological research involving the precursors of retinoic acid (vitamin A and pro-vitamin A carotenoids). We previously have shown these precursors to be highly associated with serum retinoic acid levels in this study sample. Inverse association have also been observed for vitamin A and pro-vitamin A carotenoids (dietary intake and circulating levels) and risk of cervical dysplasia (28-31) and invasive cervical cancer (30, 32). Prospective studies have demonstrated decreased risk of persistent HPV infection with higher dietary intake of pro-vitamin A carotenoids lutein (33, 34),  $\beta$ -cryptoxanthin (33) and vegetables (34).

Retinoids (natural and synthetic derivatives of vitamin A) have been shown to be both cancer chemotherapeutic and chemopreventive [see (5, 35, 36) for reviews]. In a placebo controlled chemoprevention trial among women with cervical intraepithelial lesions (CIN), Meyskens *et al.* (8) report a histological regression rate of 47% among patients with CIN II lesion randomized to the all-*trans* retinoic acid treatment group compared to 27% regression rate in the placebo group. However, no differences are seen for patients with CIN III (25% all-*trans* RA vs. 31% placebo) (8). Chemoprevention trials with other retinoic acid isomers (9-*cis* and 13-*cis* retinoic acid) (11, 12, 37) and synthetic retinoids (11, 37) have not shown an increase of lesion regression compared to spontaneous regression rates in the placebo group.

The factors underlying the differential results obtained from cervical chemoprevention trials with retinoids and observational studies are not known. There are at least two possible explanations for these differences. First, there may be differences in the biological activity of each retinoic acid isomer or synthetic derivatives when applied to the cervix compared to those found in circulation. Alternatively the differences could be attributed to differences in study designs (i.e. different outcome measures, misclassification of lesion grade upon entry, different dosages and treatment periods). Of interest, in this current study, we find that higher levels of endogenous all-*trans* retinoic acid, not 13-*cis* or 9-*cis* retinoic acids, are associated with an increased probability of clearing HPV infections. The protective effect observed in this study combined with those of

chemoprevention trials suggests chemopreventive activity for both endogenous and exogenous all-*trans* retinoic acid. To further explore this hypothesis our future studies will examine if endogenous all-*trans* retinoic acid is associated with cervical lesion development.

Retinoic acid is essential for terminal differentiation of cervical epithelial cells by decreasing cellular proliferation and DNA replication. Retinoic acid differentially inhibits growth of HPV-16 immortalized cervical epithelial cells (38, 39) and low-passage human foreskin keratinocytes (HKc/HPV16) (40-42) compared to normal HKc cells in the absence of an HPV infection. In addition to decreasing cellular proliferation in the low passage HKc/HPV16 cells, physiological concentrations of retinoic acid inhibits the expression of HPV 16 E6 and E7 (40-42). Retinoic acid may indirectly reduce HPV mRNA levels through influences on Activator protein-1 (AP-1) activity (43) or TGF $\beta$  expression (44). The retinoic acid induces growth suppression; however, this suppression appears to be lost in late stages of HPV-16 induced transformation of HKc (16) and cervical carcinoma cells lines. In several *in vitro* model systems, cells in the late stages of HPV-16 induced transformation acquire resistance to retinoic acid induced differentiation through several different mechanisms, including loss of growth inhibition (16), continued growth stimulation (45-47), and loss of retinoid receptor expression (48). This retinoic acid resistance is consistent with observations that retinoic acid therapy does not reduce recurrence rates of invasive cervical cancer (13-15) nor does it increase regression of high-grade

cervical lesions (8). Altogether, these data suggest retinoic acid may only be effective early in cervical carcinogenesis, such as by modulating HPV clearance and viral load.

We observed in the present study that higher *all-trans* retinoic acid levels are associated with increased probability of clearing both nononcogenic and oncogenic HPV infections; however, the association is strongest for the nononcogenic types. There are several potential explanations for the stronger associations between *all-trans* retinoic acid levels and clearance of nononcogenic HPV types compared to oncogenic types. First, one of the biological differences between oncogenic and nononcogenic HPV types is the strength of the interaction of viral oncogene E6 with E6-AP, and the resulting p53 degradation. Nononcogenic HPV types do not associate with E6-AP or bins at a much weaker level; therefore, p53 activity remains intact during nononcogenic infections. The ability of *all-trans* retinoic acid to induce HPV clearance may require the presence of p53 inducible pathways. Of interest are the findings by Meyskens *et al.* that *all-trans* retinoic acid treatment increased regression of CIN II lesions but not CIN III. The presence of HPV was not determined; however, one could use known frequency of HPV in cervical lesions to predict that CIN III lesions were more likely to be positive for oncogenic HPV types. The lower grade CIN II lesions were probably a combination of oncogenic and nononcogenic types. Synthesizing the results from the current study with the chemopreventive trial, one could propose that the lesions that significantly

regressed were positive for nononcogenic HPV types. This is the first study to suggest such differences by HPV oncogenicity. Alternatively, women with nononcogenic type HPV infections could have had a co-infection with oncogenic types. Therefore, we were unable to rule out the influence of co-infection between oncogenic and nononcogenic and all-*trans* retinoic acid level. Finally, all-*trans* retinoic acid could equally induce clearance of both oncogenic and nononcogenic HPV types. However, this study did not have a large enough sample size to examine the associations limited to single infections. Further research is needed to understand this phenomenon of differential activity of all-*trans* retinoic acid by oncogenic potential.

As with any observational study, this study has limitations. Our definition of HPV clearance includes both prevalent and incident HPV infections. By including prevalent infections in the definition, the estimate of duration of infection does not represent the true duration. It is impossible to know exactly how long a woman has been infected by the time she is found positive at enrollment. In general, women who are HPV positive upon enrollment are more likely to have a persistent infection. The Ludwig-McGill cohort study did not have enough incident HPV infections to utilize incident HPV persistence as our outcome. Of all the HPV infections detected in the first year, 39% were detected at enrollment and only 22% of the incident infections persisted. In this study, we assess HPV clearance over a relatively short period of time and have been limited to screening only at scheduled clinic visits. Similar to other biological markers, the

values of RA presented in this report may not reflect the absolute value of endogenous RA due to losses that may have occurred during storage or in the extraction process. However, the relative levels of each isomer should be stable; therefore the associations found in this study are assumed valid, with the magnitude of the associations potentially being lower than the true association.

The issue of multiple comparisons arises when analyzing nutritional data from large epidemiological studies in that a large number of statistical models are required. For the present study, we did not adjust the significance level but chose to evaluate each serum nutrient as individual associations. By using this approach, there is a chance that single spurious associations will be found based on the nature of statistical probabilities. Therefore, the issue of multiple comparisons needs to be considered when interpreting results. Conclusions should be made in light of consistency with other information and external to the study (49).

Despite these limitations, the Ludwig-McGill Cohort Study offers a unique opportunity to assess the association between endogenous retinoic acid levels and HPV clearance. This is a large well-characterized cohort with multiple measurements of type-specific HPV infection in a 12-month period. This is the first large population based study to assess endogenous levels of three retinoic acid isomers in multiple serum samples from the same individual.

Our results suggest that circulating retinoic acid may confer protection by decreasing the duration of both oncogenic and nononcogenic HPV infections.

Collectively, this work and *in vitro* studies support the hypothesis that retinoic acids are active during the initial stages of HPV-associated carcinogenesis. Previously, we have demonstrated that serum retinoic acid levels are significantly associated with circulating and dietary levels of vitamin A and pro-vitamin A precursors. Recently, Sedjo *et al.* (50) reported that endogenous retinoic acid is responsive to oral chemopreventive doses of retinyl palmitate (Vitamin A). Collectively, these studies demonstrate that circulating retinoic acid isomers may be modifiable by diet and vitamin A supplementation. Modulating endogenous all-*trans* retinoic acid may be a safe method for decreasing duration of HPV infections and early cervical lesions.

## **ACKNOWLEDGMENTS**

We are indebted to Maria L. Baggio and Lenice Galan for management of the patients and specimen collections and to Silvaneide Ferreira for data entry, sample retrieval and shipment and laboratory analysis. We appreciated the thoughtful comments on this manuscript by Drs. Denise Roe, Robin Harris, Elena Martinez and Jesse Martinez.

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**FIGURE LEGENDS**

Figure 1: Probability of type specific HPV clearance by tertile of all-*trans* retinoic acid concentrations for oncogenic HPV infections (A) and nononcogenic HPV infections (B)

**Table 1: Demographic Characteristics of HPV Positive Women Enrolled in the Ludwig-McGill Cohort Study (N=407)**

	N	%
<i>Age</i>		
Mean $\pm$ SD, yrs	31.5	8.9
<i>Ethnicity</i>		
White	246	60.6
Non-white	160	39.4
<i>Cigarette Smoking</i>		
Never	190	46.8
Current	149	36.7
Former	67	16.5
<i>Oral contraceptive use</i>		
Never	66	16.3
< 6 years	231	56.9
$\geq$ 6 years	109	26.9
<i>Total # of Pregnancies</i>		
0-1	78	19.4
2-3	169	42.0
4-5	115	28.6
$\geq$ 6	40	10.0
<i>Age @ first intercourse</i>		
$\leq$ 15	121	29.8
16-17	106	26.1
$\geq$ 18	179	44.1
<i>Lifetime # sexual partners</i>		
0 - 1	147	36.2
2-3	157	38.7
$\geq$ 4	102	25.1
<i>Total # sex partners past 5 yrs</i>		
0-1	266	65.5
$\geq$ 2	140	34.5

Table 1-Continued

<i>Total # sexual partners past year</i>		
0-1	362	90.3
≥ 2	39	9.7
<i>Marital Status</i>		
Single	77	19.0
Married	154	37.9
Widowed / Divorced	46	11.3
Common Law	129	31.8
<i>Education</i>		
Illiterate	22	5.4
< Elementary	64	15.8
Elementary	223	54.9
< High School	50	12.3
≥ High School	47	11.6
<i>Income (US\$)</i>		
< 250	103	26.1
250-450	98	24.9
451-725	90	22.8
≥ 725	103	26.1

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**Table 2: Circulating Retinoic Acid Concentrations Among HPV Positive Women**

	Mean	SD	Median	Min <sup>1</sup>	Max
Total Retinoic Acid (ng/mL)	3.21	1.15	3.15	0.56	7.69
<i>cis</i> -isomer					
13- <i>cis</i> RA (ng/mL)	1.32	0.61	1.34	0.15	3.91
9- <i>cis</i> RA (ng/mL)	1.05	0.57	1.10	0.15	3.20
<i>All-trans</i> -isomer (ng/mL)	0.83	0.38	0.77	0.15	3.17

1. Limit of quantification (LOQ) was 0.30 ng/mL, values below LOQ were given value of 0.15 ng/mL

**Table 3: Association Between Type-specific Oncogenic and Nononcogenic HPV Clearance and Serum Retinoic Acid Concentrations**

Nutrients (ng/mL)	Oncogenic HPV				Non-Oncogenic HPV			
	No. Cleared / women-months <sup>1</sup>	Crude HR	Adjusted <sup>2</sup> HR	Adjusted <sup>2</sup> (95% CI)	No. Cleared / women-months <sup>1</sup>	Crude HR	Adjusted <sup>3</sup> HR	Adjusted <sup>3</sup> (95% CI)
<i>Total Retinoic Acid</i>								
Low (< 2.65)	40 / 704.13	1.00	1.00	Referent	59 / 721.77	1.00	1.00	Referent
Medium (2.66 - 3.65)	49 / 714.23	1.11	1.25	(0.70 - 2.24)	47 / 872.37	0.71	0.62	(0.36 - 1.05)
High (> 3.65)	38 / 676.53	0.92	1.05	(0.54 - 2.04)	55 / 782.70	0.97	0.91	(0.53 - 1.58)
<i>13-cis RA</i>								
Low (< 1.02)	45 / 764.07	1.00	1.00	Referent	58 / 696.27	1.00	1.00	Referent
Medium (1.03 - 1.52)	39 / 585.47	1.13	1.07	(0.62 - 1.87)	45 / 731.07	0.67	0.64	(0.38 - 1.07)
High (> 1.53)	43 / 745.37	0.93	0.96	(0.52 - 1.76)	58 / 949.50	0.78	0.77	(0.44 - 1.34)
<i>9-cis RA</i>								
Low (< 0.89)	47 / 809.30	1.00	1.00	Referent	61 / 838.47	1.00	1.00	Referent
Medium (0.90 - 1.29)	35 / 575.33	1.10	1.20	(0.67 - 2.14)	48 / 700.13	1.21	1.16	(0.67 - 2.01)
High (> 1.30)	45 / 710.27	1.28	1.28	(0.69 - 2.34)	52 / 838.32	1.15	1.17	(0.67 - 2.05)
<i>all-trans RA</i>								
Low (< 0.64)	42 / 776.27	1.00	1.00	Referent	55 / 990.63	1.00	1.00	Referent
Medium (0.65 - 0.93)	48 / 583.53	1.54	1.82	(1.14 - 2.91)	48 / 605.10	1.66	1.67	(1.09 - 2.57)
High (> 0.94)	37 / 735.10	1.00	1.18	(0.68 - 2.06)	58 / 781.10	1.34	1.46	(0.93 - 2.28)

<sup>1</sup> Numbers do not correspond to the crude measures since the Cox proportional hazard model accounts for the change in the RA over time while the numbers provided assume the HR does not vary over time.

<sup>2</sup> Oncogenic models adjusted for laboratory analysis batch, income, education, race and age

<sup>3</sup> Nononcogenic models adjusted for laboratory analysis batch, income, education, age, marital status, and age at first intercourse

**Table 4: Association of Serum Retinoic Acid Concentration and Viral Load According to Months Between Measures<sup>1</sup>**

Time interval (n) between RA and VL <sup>2</sup>	RA Level	<i>Total Retinoic Acid</i>					<i>13-cis Retinoic Acid</i>				
		Viral Load <sup>3</sup>		Adjusted <sup>4</sup>			Viral Load		Adjusted <sup>4</sup>		
		Low (N)	High (N)	Crude OR	OR	95% CI	Low (N)	High (N)	Crude OR	OR	95% CI
<b>0</b>	Low	78	47	1.00	1.00	Referent	78	52	1.00	1.00	Referent
	Medium	90	38	1.23	1.30	(0.56 - 3.02)	84	30	0.79	0.78	(0.35 - 1.75)
	High	77	39	1.78	2.10	(0.87 - 5.10)	84	42	1.10	1.20	(0.54 - 2.67)
<b>4</b>	Low	96	31	1.00	1.00	Referent	85	34	1.00	1.00	Referent
	Medium	99	31	0.80	0.82	(0.31 - 2.15)	83	26	0.63	0.34	(0.13 - 0.85)
	High	90	31	0.89	1.02	(0.40 - 2.65)	97	33	0.73	0.54	(0.21 - 1.34)
<b>8</b>	Low	105	23	1.00	1.00	Referent	101	27	1.00	1.00	Referent
	Medium	106	27	1.38	1.00	(0.36 - 2.83)	97	24	0.81	0.59	(0.23 - 1.50)
	High	87	32	2.08	1.73	(0.51 - 5.86)	98	31	1.02	0.72	(0.24 - 2.14)
<b>12</b>	Low	78	15	1.00	1.00	Referent	87	13	1.00	1.00	Referent
	High	81	17	0.98	0.89	(0.25 - 3.18)	72	19	2.35	1.53	(0.28 - 8.36)

\* RA, retinoic acid; VL, viral load; HPV, human papillomavirus; OR, odds ratio; CI, confidence interval

1. Results obtained from a time-dependent generalized estimating equation approach with a logit link for binary outcomes
2. Separate analyses conducted with increasing time interval (n) between exposure (serum retinoic acid level) and outcome assessment (viral load). The time intervals considered were 0 months (simultaneous assessment), 4, 8 or 12 months.
3. Total number of observations are not the same due to missing data
4. All models adjusted for time, age, oncogenicity, and oral contraceptive use. Additional confounders for each time intervals: (n=0) lifetime number of partners and number in household; (n=4/8) smoking, number of pregnancies, income; (n=12) marital status.
5. 12-month interval only utilized baseline RA measure, therefore RA is dichotomized at median

**Table 4 - Continued**

<i>9-cis Retinoic Acid</i>					<i>all-trans Retinoic Acid</i>				
Viral Load		Crude OR	Adjusted <sup>4</sup>		Viral Load		Crude OR	Adjusted <sup>4</sup>	
Low (N)	High (N)		OR	95% CI	Low (N)	High (N)		OR	95% CI
85	49	1.00	1.00	Referent	92	44	1.00	1.00	Referent
75	36	1.57	1.92	(0.80 - 4.58)	78	28	1.04	0.81	(0.43 - 1.52)
86	39	1.20	1.97	(0.76 - 5.14)	76	52	1.79	1.79	(0.93 - 3.45)
101	35	1.00	1.00	Referent	100	37	1.00	1.00	Referent
90	24	0.58	0.81	(0.30 - 2.17)	88	20	0.65	0.67	(0.27 - 1.67)
94	34	0.80	1.11	(0.37 - 3.38)	97	36	1.09	1.66	(0.76 - 3.64)
105	30	1.00	1.00	Referent	105	35	1.00	1.00	Referent
97	19	0.51	0.60	(0.20 - 1.77)	90	15	0.51	0.53	(0.22 - 1.27)
94	33	0.88	0.84	(0.26 - 2.70)	101	32	0.96	1.02	(0.39 - 2.67)
83	19	1.00	1.00	Referent	98	19	1.00	1.00	Referent
76	13	0.58	0.21	(0.04 - 1.02)	91	13	0.48	0.39	(0.11 - 1.46)

Figure 1A

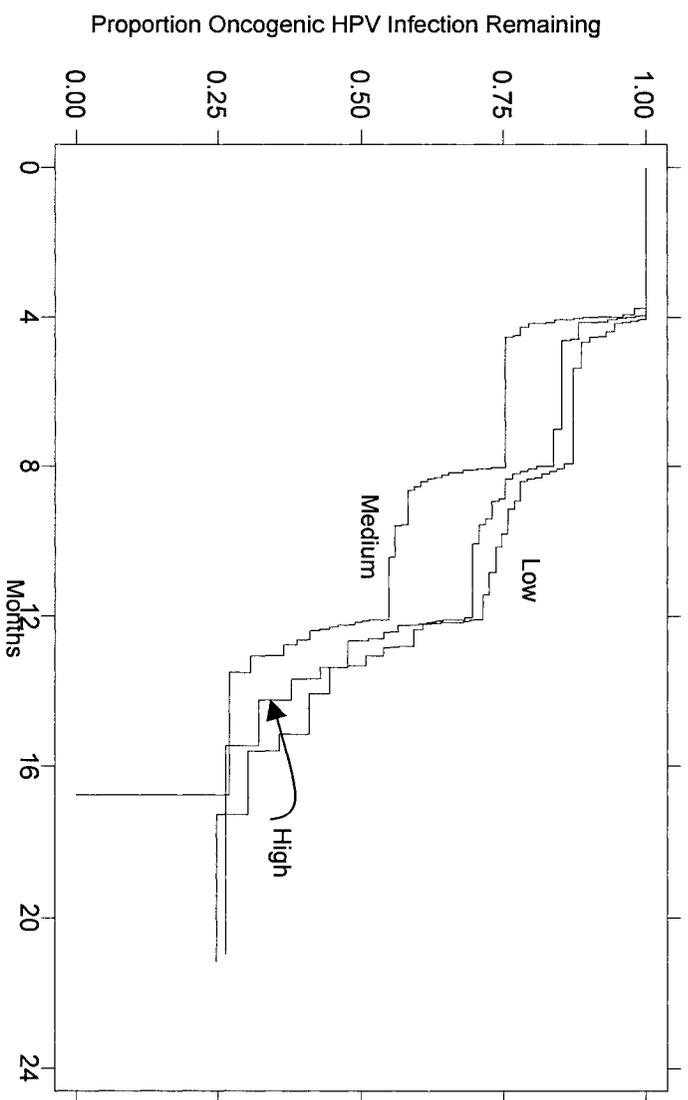
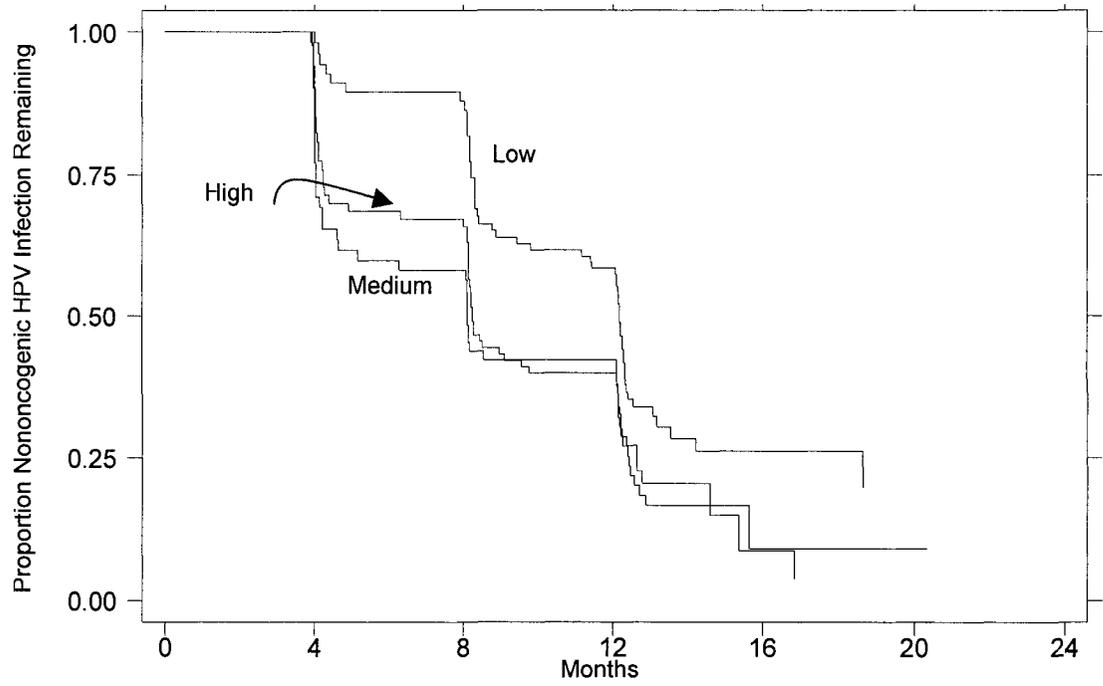


Figure 1B



**APPENDIX C**

**MANUSCRIPT 3**

**Associations between serum carotenoids and tocopherols and type-specific HPV persistence and clearance: the Ludwig-McGill Cohort Study<sup>1</sup>**

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**Target Journal:** International Journal of Cancer

**FOOTNOTES**

1. Support for this study was provided by the National Cancer Institute (CA70269, CA81310), Canadian Institutes of Health Research (CIHR) (MA-13647, MOP-49396), and by an intramural grant by the Ludwig Institute for Cancer Research. E.L.F. is recipient of a Distinguished Scientist Award from the CIHR. E.M.S. is recipient of a NCI Cancer Prevention and Control Pre-Doctoral Fellowship (R25CA078447).
2. Presented in part at the 21<sup>st</sup> International Papillomavirus Conference, Mexico City 2004 (Abstract Number 70)
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**ABSTRACT**

Although oncogenic HPV infections have been established as the necessary cause of cervical cancer, most HPV infections are transient and rarely progress to significant cervical lesions. Current research is focused on identifying factors that can potentially alter the lifecycle of HPV and therefore modulate viral persistence and clearance, such as nutritional status. This current study evaluates the association between serum antioxidant nutrients (carotenoids and tocopherols) and type-specific HPV persistence and clearance over a 12-month period among 407 Brazilian women participating in a prospective study examining the natural history of HPV infection (Ludwig-McGill Cohort Study). We measured circulating antioxidants (carotenoids and tocopherol) at four different clinical visits for each woman to accurately classify their nutrient status. In addition, we utilized several statistical approaches to examine the association between carotenoids and tocopherols and the natural history of HPV infections. In a case-control analysis, higher circulating levels of tocopherols ( $\alpha$ -,  $\delta$ -, and  $\gamma$ -tocopherol) were inversely associated with nononcogenic HPV persistent infection (p for trend, 0.14, 0.04 and 0.05, respectively). Similarly, in prospective GEE models, circulating levels of  $\alpha$ - and  $\delta$ -tocopherol in the middle or upper tertiles were marginally inversely associated with type-specific nononcogenic persistent HPV events (Adjusted Odds Ratio (AOR) = 0.49, 95% CI 0.23-1.00 and AOR=0.48, 95% CI 0.21-1.06, respectively) compared to levels in the lowest tertile. The probability of clearing a type-specific oncogenic HPV infection was positively associated with serum levels of zeaxanthin (p for trend= 0.05). Higher concentrations of circulating zeaxanthin were associated with a shorter duration of type-

specific oncogenic HPV infections ( $p=0.05$ ). This research suggests that antioxidant nutrients are modifiable factors that are associated with viral persistence and clearance, and that diets high in antioxidant may confer protection.

## INTRODUCTION

Human papillomavirus (HPV) infection has been shown to be the cause of cervical cancer, the second leading cancer among women worldwide<sup>1</sup>. Prospective studies have shown that women with persistent HPV infections are more likely to develop cervical lesions compared to women with transient HPV infections<sup>2</sup>. Although oncogenic HPV infections have been established as the necessary cause of cervical cancer, most HPV infections are transient and rarely progress to significant cervical lesions<sup>3</sup>. Current research is focused on identifying factors that could potentially alter the lifecycle of HPV and, therefore, modulate viral persistence and clearance. Nutritional status may be an important co-factor affecting both HPV persistence and progression of persistent HPV infections to CIN<sup>4,5</sup>.

Although the association between nutrient status and cervical carcinogenesis has been extensively investigated<sup>6</sup>, several important questions remain. First, do nutrients act as co-factors in HPV-related carcinogenesis and where in this continuum are different classes of nutrients active? Epidemiological evidence suggests that antioxidant nutrients may reduce the risk of persistent HPV infection. In a small prospective study among women attending a family planning clinic for routine care, we observed significant inverse associations between dietary intake of lutein, vitamin E, vegetables and serum

lycopene levels and HPV persistence<sup>7</sup>. This study did not assess type-specific HPV persistence<sup>7</sup> or clearance<sup>8</sup>. In addition, serum carotenoids and tocopherols were assessed only once, which may not have adequately classified a woman's nutrient status due to within-person variability of these measures<sup>9</sup>.

This current study was conducted to further evaluate the association between serum antioxidant nutrients (carotenoids and tocopherols) and type-specific HPV persistence and clearance over a 12-month period among Brazilian women participating in a prospective study examining the natural history of HPV infection (Ludwig-McGill Cohort Study). We measured circulating carotenoids and tocopherol at four different clinical visits for each woman to accurately classify their nutrient status. In addition, we utilized several analytic approaches to examine the association between carotenoids and tocopherols and HPV persistence and clearance (i.e. case-control and longitudinal events).

## **METHODS**

The Ludwig-McGill Cohort Study is an epidemiological cohort investigation of women attending a comprehensive maternal and child health maintenance program catering to low-income families, in São Paulo, Brazil between 1993 to 1997<sup>10</sup>. The clinic setting where participants were accrued is part of a network of primary, secondary, and tertiary health care institutions maintained by the municipal health department. Cohort participants were examined every four months in the first year, and twice yearly thereafter for a total of five years. At each study visit, participants were interviewed based on a structured questionnaire specific for the current visit<sup>10</sup>. The institutional

review boards and ethical committees of all institutions with which the authors are affiliated approved these protocols. Each study participant signed an approved informed consent document.

*Study Sample:* Of the 2,528 women in the cohort, we identified a sub-cohort of women (n=1,392), representing mostly those entering the study during the first two years (1993-1995) and who had long-term follow-up. From this sub-cohort, we selected women who had complete HPV data and serum available from all four visits within the first year (n=846). Among the 846 women, 819 had normal or ASCUS cytology at baseline. Of these 819 women, 407 tested positive for HPV at least once during the first four visits and 412 women were HPV negative at all four visits. To study the association between HPV persistence and serum carotenoids and tocopherol levels, only the 407 women who tested positive for HPV at any one of the four visits were included in this analysis.

*Cervical cell specimens.* At each of the visits, an accelon biosampler was used to collect a sample of ectocervical and endocervical cells. After the smear was prepared on a glass slide and fixed in 95% ethanol, the sampler containing exfoliated cells was immersed in a tube containing Tris-EDTA buffer (pH 7.4), was swirled to release the adhered cells, and was maintained at the clinic for, at most, 5 days at 4°C. Once they were brought to the laboratory at the Ludwig Institute, the tubes containing cell suspensions were frozen until testing.

HPV DNA detection methods-polymerase chain reaction (PCR) methods. All HPV analyses were performed at the Ludwig Institute for Cancer Research, São Paulo, Brazil. Cervical-specimen DNA was extracted and purified in accordance with standard techniques. In brief, cells were digested with 100 µg/mL proteinase K for 3 hours at 55°C, followed by organic extraction and ethanol precipitation. Specimens were tested for the presence of HPV DNA by a previously described PCR protocol amplifying a highly conserved 450-bp segment in the L1 viral gene (flanked by primers MY09/11)<sup>11, 12</sup>. Typing of the amplified products was performed by hybridization with individual oligonucleotide probes specific for all 27 HPV genital types, whose nucleotide sequences for probes within the MY09/11 fragment have been published elsewhere<sup>12</sup> namely types 6/11, 16, 18, 26, 31, 33, 35, 39, 40, 42, 45, 51-59, 66, 68, 73, 82, 83, and 84 . The PCR amplification products were further tested by restriction fragment-length polymorphism (RFLP) analysis of the L1 fragment<sup>13</sup>, to resolve dubious results from the dot-blot hybridization and to distinguish among HPVs that could not be typed by dot-blot hybridization with the specific probes. This allowed us to extend the detection range to >40 genital HPV types, including HPV types 32, 34, 44, 62, 64, 67, 69-72, CP6108, and IS39. Amplified products that hybridized with the generic probe but not with any of the type-specific probes and that also did not produce a recognizable band pattern in the RFLP analysis were considered to be positive for HPV of unknown types.

Serum Sample Processing and Storage. All non-fasting blood samples (~10 ml) were collected by venipuncture into vacutainers by a trained nurse at the time of the clinic visit. The samples were centrifuged within 6-8 hours of collection. Aliquots (1

ml) of serum were stored in 1.8 ml Nunc cryovials at  $-20^{\circ}\text{C}$  in a non-frost free freezer until shipped for analyses.

Serum Retinol and Carotenoids HPLC Analytical Procedures. Archival serum from all four visits within the first year (months 0, 4, 8, and 12) on study were analyzed to detect retinol, carotenoids and tocopherols in serum using a modification of the procedures described by Nomura *et al.*<sup>14</sup>. The percent coefficient of variation was 8% or less for retinol, carotenoids and tocopherols measured. This HPLC system's limit of quantification (LOQ) was 0.004  $\mu\text{g/ml}$  for the carotenoids and 0.015  $\mu\text{g/mL}$  for tocopherols. Of the total 3,332 samples analyzed over four clinical visits, the following number of samples (percent) were below the LOQ and assigned a value half way between zero and the lower limits of detection: retinol 3 (0.1%), lutein 16 (0.5%), zeaxanthin 50 (1.5%),  $\alpha$ -cryptoxanthin 1082 (32%),  $\beta$ -cryptoxanthin 330 (10%), *trans*-lycopene 793 (24%), *cis*-lycopene 701 (21%),  $\alpha$ -carotene 811 (24%), *trans*- $\beta$ -carotene 504 (15%), *cis*- $\beta$ -carotene 1037 (31%),  $\alpha$ -tocopherols 46 (1.5%),  $\delta$ -tocopherol 776 (23%) and  $\gamma$ -tocopherol 57 (2%). Some serum nutrients, specifically  $\alpha$ -carotene,  $\alpha$ -cryptoxanthin, *cis*- $\beta$ -carotene, had a large number of samples that were below the LOQ. Therefore when categorized into tertiles, as discussed in the Statistical Analysis section below, the lowest tertile is comprised entirely of those samples that were below the limit of quantification (e.g. 33% of the samples below detection and considered the lowest group).

Total serum cholesterol: Carotenoids and tocopherols are fat-soluble nutrients, of which uptake, transport and storage is associated with lipids<sup>15</sup>. Therefore, the level of serum lipid, as measured by cholesterol, may influence the level of circulating

carotenoids and tocopherols. To account for this effect, total serum cholesterol was assessed at visit 3 to enable adjustment for this potential confounding variable. Total cholesterol was determined by coupled enzymatic, colorimetric assay (Sigma kit #401-25P) by Craft Technologies Inc (Wilson, NC).

## STATISTICAL ANALYSIS

Nutrient variables: Preliminary studies within the study sample suggested a high within-person variability for serum carotenoids and tocopherol values (data not shown). Therefore, to accurately classify a woman's serum nutrient status and reduce this variability, we calculated the mean of serum carotenoids and tocopherols values at the four time-points for each woman. Using this mean value, we categorized the continuous variable into tertiles based on the distribution of each nutrient among HPV positive women.

HPV Outcome Variables: We examined associations with type-specific HPV persistence using three different approaches: a case-control approach, a prospective approach examining persistent events over time and a prospective approach examining duration of infection (**Figure 1**). All outcome variables were grouped as oncogenic infections (HPV types 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, and 68) or non-oncogenic infections (all other 27 HPV types considered)<sup>16</sup>. Women infected with single or multiple oncogenic types and women infected with both oncogenic and non-oncogenic types were categorized as having an oncogenic infection. Only women infected with a

single or multiple non-oncogenic types were categorized as having a non-oncogenic infection.

In the case-control approach, HPV status was based on the four HPV evaluations conducted at enrollment and in the first year of follow-up. Two groups of women were identified: those who tested HPV positive in only one of the four assessments or were non-consecutively positive for the same type (transient group), and those who tested positive for the same HPV type at two or more consecutive visits in the first year of the study (persistent group). To test the hypothesis that serum nutrient status was associated with persistent HPV infection, women with transient infections formed the control group and women with persistent infections formed the case group. For this analysis, 230 women had an oncogenic HPV infection and 177 women had nononcogenic infections. Of the 230 women with oncogenic infections, 143 were classified as transient infections (controls) and 87 had type-specific persistent HPV infections (cases). Among the women with nononcogenic infections, there were 61 persistent infections (cases) and 116 transient infections (controls).

The second approach examining HPV persistence defined type-specific persistent events similar to those used by Ho *et al.*<sup>17</sup>. Women who were HPV positive at a specific visit were considered persistent if their subsequent assessment was also positive for the same HPV type and transient if the subsequent visit was negative (i.e., if they have cleared the virus) or a different HPV type infection. Using HPV assessments at 0, 4, 8, and 12 months, we defined persistence status over four clinical visits with a maximum total of three persistence events possible per woman. Analyses were conducted at the

level of individual pairs rather than the individual woman. Of the 407 HPV positive women, 344 women contributed to these analyses with 263 persistent events (+, +) and 277 transient events (+,-).

The third approach was to examine the duration of HPV infections. This analysis was based on 407 women who were HPV positive at baseline or those who developed an incident infection during follow-up. Time to HPV clearance was defined as the number of months to first negative HPV test occurring after previously positive HPV test(s) or until the last visit (month 12) if the participant remained positive throughout the study period. The scheduled visits within this cohort study were based on months following enrollment. However, not all women attended visits at the specific month anticipated. This led to variability in the time between study visits and a total follow-up period for the first four scheduled study visits of 50 months. The grouping of type-specific clearance by oncogenicity differed from that of the previous two outcome variables. For this analysis, a woman was considered to have cleared an oncogenic infection if she was no longer positive for the specific oncogenic type grouping originally detected, even if the cervical specimen was positive for a different oncogenic viral type or within the nononcogenic grouping.

*Statistical Modeling:* In the case-control approach, logistic regression was performed to estimate the association (odds ratio) and 95% confidence interval (CI) of each serum nutrient with HPV persistent and transient HPV infection. In the prospective approach of HPV persistent events, the multiple HPV measures over time within a woman were correlated. Therefore, a generalized estimating equation (GEE) model

approach for time-dependent longitudinal data was employed<sup>18, 19</sup>. The GEE adjusts for the serial correlation within subjects due to the longitudinal nature of the data by modeling the covariance structure within subjects. The dependent variable was the presence or absence of persistent infection; therefore the logit link function was used. The independent working correlation structure with a robust variance estimator to account for within-subject correlation was selected as the best-fitted covariance pattern, using the Quasilikelihood Information Criterion (QIC)<sup>19</sup>. Since HPV persistence may depend on the time since the previous measurement, a time variable was included as a design factor (independent variable) in all GEE models.

The relationship between serum carotenoids and tocopherols and time to clearance of type-specific HPV infection was also assessed. Probability of maintaining an HPV infection present at baseline or an incident infection during follow-up was examined by estimating the Kaplan Meier survivor function. Missing values during follow-up were treated as censored observations unless there was a subsequent result at a later visit. Multivariate Cox proportional hazards modeling was conducted to obtain the adjusted hazard ratios (AHR) and 95% confidence interval (95% CI).

*Adjustment for Confounding:* For all multivariate models, we considered several variables that could potentially confound the associations; including those previously associated with persistence of HPV infection<sup>20</sup> and serum carotenoids and tocopherols. Only those factors that altered the risk estimate by  $\geq 10\%$  were retained in the final multivariate model. Tests for trend were performed by treating all categorical nutrient variables as continuous in the multivariate models. All statistical tests performed were

two-sided. Statistical analyses were performed by use of Intercooled STATA (version 7.0; Stata).

## RESULTS:

These analyses include 407 women participating in the Ludwig-McGill cohort study, mainly women enrolled within the first two years of enrollment (1993-1996). The median age for these women was 31 years, which was not different from the median age of all HPV positive women enrolled in the cohort. Most participants were white (61%), married or in a common law marriage (70%), and not well educated, with 55% of the sample having only attended elementary school or less. At least 80% of these women reported more than two pregnancies, and 57% had used oral contraceptives for less than six years. A majority of these women were ever smokers (53%), with 36% reporting current smoking. Fifty-six percent of participants reported age of first sexual intercourse under 18 years and 25% had more than four lifetime sexual partners.

Circulating nutrient concentrations of retinol, carotenoids and tocopherols among HPV positive participants by persistent HPV outcomes are presented in **Table 1**. The most abundant circulating carotenoid was  $\beta$ -cryptoxanthin (mean, 0.040  $\mu\text{g/mL}$ ), followed by lutein (mean, 0.037  $\mu\text{g/mL}$ ), *cis*-lycopene (mean, 0.026  $\mu\text{g/mL}$ ), *trans*  $\beta$ -carotene (mean, 0.024  $\mu\text{g/mL}$ ), *trans*-lycopene (mean, 0.017  $\mu\text{g/mL}$ ), zeaxanthin (mean, 0.016  $\mu\text{g/mL}$ ),  $\alpha$ -carotene (mean, 0.011  $\mu\text{g/mL}$ ), *cis*- $\beta$ -carotene (mean, 0.007  $\mu\text{g/mL}$ ), and  $\alpha$ -cryptoxanthin (mean, 0.006  $\mu\text{g/mL}$ ). *Cis*- $\beta$ -carotene and  $\alpha$ -cryptoxanthin were low in this Brazilian study sample with at least 25% of the serum samples having levels

below the limit of quantification. The most abundant tocopherol was  $\alpha$ -tocopherol (mean, 5.14  $\mu\text{g/mL}$ ), followed by  $\gamma$ -tocopherol and  $\delta$ -tocopherol (mean, 1.58  $\mu\text{g/mL}$  and 0.083  $\mu\text{g/mL}$ , respectively). Mean serum nutrients are also presented in **Table 1** by HPV persistence for both the case-control definition (135 transiently infected (controls) and 165 persistently infected (cases) and the longitudinal HPV events (transient group (+,-) N=277 and persistent group (+,+) N=259).

*Case-control analysis of type-specific HPV persistence:* For these analyses, we included 230 women with an oncogenic HPV infection and 177 women with nononcogenic infection. Of the 230 women with oncogenic infections, 143 had transient infection (controls) and 87 had type-specific persistent HPV infection (cases). Among the women with nononcogenic infections, there were 61 persistent infections (cases) and 116 transient infections (controls). The unadjusted and multivariate adjusted associations between individual serum carotenoids and tocopherols and type-specific HPV infections that persisted are reported in **Table 2**. No statistically significant associations were observed between serum retinol, lutein, zeaxanthin,  $\alpha$ -cryptoxanthin,  $\beta$ -cryptoxanthin,  $\alpha$ -carotene, *trans*- and *cis*- $\beta$  carotene and oncogenic or nononcogenic HPV persistence in this study population. Serum lycopene (*cis*- and *trans*-lycopene) was positively associated with type-specific oncogenic HPV persistence (p for trend 0.03 and 0.03, respectively). Higher circulating levels of tocopherols ( $\alpha$ -,  $\delta$ -, and  $\gamma$ -tocopherol) were inversely associated with nononcogenic HPV persistent infection (p for trend, 0.14, 0.04 and 0.05, respectively), but not oncogenic infection.

Longitudinal analysis of type-specific HPV persistent events: To fully utilize the longitudinal nature of these data, we examined type-specific HPV persistent events (**Figure 1**). Of the 407 HPV positive women included in this study, a total of 1,628 visits occurred in the first year of follow-up. These 1,628 visits formed 1,221 pairs of observations with known HPV status at visits  $t$  and  $t+1$ . The distribution of HPV <sub>$t$ ,  $t+1$</sub>  pairs was 277 with (+,-), 263 with (+,+) and 681 with (-,-) or (-,+). The 540 pairs in the (+,-) and (+,+) categories were contributed by 344 women and included in these analyses. The associations between type-specific persistent HPV events and serum carotenoids and tocopherols are presented in **Table 3**. Similar to what we observed with the case-control analysis, serum tocopherol levels were inversely associated with persistent HPV events. Circulating levels of  $\alpha$ - and  $\delta$ -tocopherol in the middle or upper tertiles, respectively were marginally inversely associated with type-specific nononcogenic persistent HPV events (Adjusted Odds Ratio (OR)= 0.49, 95% CI 0.23-1.00 and Adjusted OR=0.48, 95% CI 0.21-1.06, respectively) compared to levels in the lowest tertile. Using this analytical approach, serum  $\beta$ -carotene was marginally inversely associated with non-oncogenic persistent events ( $p$  for trend=0.07).

Type-specific HPV clearance: **Table 4** presents the risk of type-specific HPV clearance by circulating carotenoid and tocopherol levels, stratified by oncogenic and nononcogenic type infections. The probability of clearing a type-specific oncogenic HPV infection was positively associated with serum levels of zeaxanthin. Specifically, women were 1.59 times more likely to clear an oncogenic HPV infection if their serum zeaxanthin levels were in the highest tertile compared to women with the lowest serum

levels. The probability of clearing nononcogenic HPV infections was not associated with serum zeaxanthin levels, or other serum carotenoid and tocopherol levels. There was a marginally significant increase in probability of clearing type-specific oncogenic HPV infection with higher lutein (Medium: Adjusted Hazard Ratio (HR)=1.54, 95% CI 0.96-2.49) and  $\alpha$ -cryptoxanthin levels (Medium: Adjusted HR=1.52, 95% CI 0.96 - 2.41). There was a marginally significant trend for increased probability of clearing nononcogenic HPV infection by increasing serum retinol level (p for trend=0.08). The cumulative probability of clearing a type-specific oncogenic or nononcogenic infection by zeaxanthin levels is presented in **Figure 2**. Higher concentrations of circulating zeaxanthin were associated with a shorter duration of type-specific oncogenic HPV infections (p=0.05). The median duration of an oncogenic HPV infection was 13.1, 12.1, and 12.1 months for the lowest, middle and highest tertile of serum zeaxanthin, respectively. These results extend past 12-months of follow-up due to the variability in time between study visits. On average, women identified for this present study returned for their month 12 visit  $13.4 \pm 3.4$  months (Range 11 – 50 months) following enrollment.

## **DISCUSSION**

There is a consensus among the scientific community that HPV is a cause of cervical cancer and that other factors that increase a woman's risk of developing cervical cancer need to be understood within the context of the natural history of HPV infection. To date, most studies examining dietary factors have not been designed to prospectively evaluate nutrients, as most were retrospective studies with either HSIL (CIN II or III) or

cancer as the case groups<sup>5</sup>. In our earlier studies, we evaluated the role of antioxidants, both dietary intake and serum levels, and risk of persistent HPV infections<sup>4, 7</sup> or HPV clearance<sup>8</sup> among women in the U.S.. However, we were not able to assess associations with type-specific infections, since we did not have detailed HPV typing to clearly separate oncogenic from non-oncogenic types, and were limited to a relatively short period of follow-up. We also examined the association of dietary intake and type-specific HPV persistence in this sample of Brazilian women<sup>21</sup>.

Results of the present study among Brazilian women were consistent with our previous dietary findings in this same Brazilian sample<sup>21</sup> and among women in the U.S.<sup>4, 7</sup>, where we demonstrated an inverse association with lutein/zeaxanthin consumption and HPV persistence. In addition, inverse associations seen in the earlier studies were consistent for dietary vitamin E intake<sup>4, 7</sup> and serum tocopherols<sup>22</sup> and HPV persistence. Preliminary findings from a multi-ethnic cohort in Hawaii also reported inverse associations with oncogenic type-specific HPV persistence and change in serum  $\beta$ -carotene and  $\alpha$ -tocopherol over a 4-month period<sup>22</sup>. Contrary to the earlier findings in U.S. women of inverse associations with HPV persistence<sup>7</sup> and clearance<sup>8</sup>, we find in the present study of Brazilian women a positive association with serum lycopene and type-specific oncogenic HPV persistence and no association with HPV clearance. A few prior reports have assessed the association between serum antioxidant nutrients and HPV persistence within the context of a  $\beta$ -carotene clinical trial<sup>23, 24</sup>. These trials reported no association between serum retinol,  $\beta$ -carotene, vitamin C and HPV persistence in the control or supplemented groups<sup>23, 24</sup>. However, these women had pre-existing lesions

(50% LSIL (CIN I) and 49 % HSIL (CIN II and III)) at enrollment<sup>24</sup>, therefore representing relatively late events in disease process. In contrast, women enrolled in the current study had normal or ASCUS cytology at baseline.

In this study we evaluated the nutrient-HPV persistence associations utilizing three different analytical approaches, including a case-control definition, longitudinal analysis of persistence events, and time dependent clearance analyses. To understand the implications of these results, it is important to consider the assumptions of each approach and the validity of the HPV outcome variable utilized in each of the different analyses. Utilizing a case-control approach, our HPV outcome variable collapsed the longitudinal nature of the HPV data into two groups: persistent group (two or more consecutive visits positive for the same HPV type) and transient group (defined as one of four positive tests or nonconsecutive positive tests of the same type). Among women with persistent infection, a woman with four consecutively positive tests contributes equally to the model as a woman with two positive tests. This logistic regression analysis assumes that an HPV infection lasting four months (i.e. only 2 positive) has the same risk as one that lasts 12 months (i.e. 4 positive visits). Although the utilization of logistic regression is straightforward and easy to interpret, this underlying assumption may not be biologically plausible as it has been demonstrated that risk of cervical disease increases with duration of infection<sup>2</sup>. Utilizing this crude definition of HPV persistence, higher circulating levels of tocopherols ( $\alpha$ -,  $\delta$ -, and  $\gamma$ -tocopherol) were inversely associated with nononcogenic HPV persistent infection (p for trend, 0.14, 0.04 and 0.05, respectively).

The logistic regression case control analysis differs from our second approach, which defined HPV persistence as multiple visit pairs and utilizes generalized estimating equations for longitudinal analyses. In this approach, a woman with four consecutively positive visits would contribute three persistence events, compared to a woman with only two consecutively positive events contributing only one event. Therefore, each woman had the potential to contribute up to three events increasing the overall proportion of persistent events. By increasing the number of persistent events, this analysis has more power to detect differences by serum antioxidant level, however the persistent event spans approximately a four-month period. This approach of defining persistent events in four-month intervals may not be biologically relevant, as it is difficult to determine if an HPV infections lasting four months is truly persistent. Therefore, HPV events defined as persistent based on four month intervals may be misclassified and result in attenuation of the risk estimates to the null. Utilizing this approach, we found that circulating levels of  $\alpha$ - and  $\delta$ -tocopherol in the middle or upper tertiles, respectively were marginally inversely associated with type-specific nononcogenic persistent HPV events (Adjusted Odds Ratio (OR)= 0.49, 95% CI 0.23-1.00 and Adjusted OR=0.48, 95% CI 0.21-1.06, respectively) compared to levels in the lowest tertile. This attenuation is evident in the loss of statistical significance in the GEE modeling compared to the logistic regression analysis.

Although time was included in these generalized estimating equations models as a covariate, the statistical analysis is based on the logit-link function comparing proportion of events and, therefore, the consecutive nature of persistent events is not taken into

consideration. Further Cox proportional hazard modeling was performed to utilize the consecutive nature of the HPV infections by estimating the duration of infection from baseline or incident infection. With Cox proportional hazard modeling, the concern about misclassification of the HPV persistent outcome variable is minimal compared to the logistic regression and GEE modeling. Cox proportional hazard modeling is dependent on the clinic visit schedule; therefore, exact timing of clearance is unknown<sup>25</sup> and these models also assume that the hazard ratio is constant over time. This assumption has been considered a limitation of applying these models to the natural history of HPV infection as risk increased with duration of infections<sup>22</sup>. However, this approach estimating HPV clearance is currently the most accurate model with which to examine HPV persistence. Using this approach, we found that the probability of clearing a type-specific oncogenic HPV infection was positively associated with serum levels of zeaxanthin. These results differ from the previous approaches utilized in this current study, which did not find associations with zeaxanthin and oncogenic HPV infections. Oncogenic HPV infections are more likely to be detected over a long period of time (i.e. 12 months), and circulating zeaxanthin appears to reduce the duration of infection (Median duration: 13.1, 12.1, and 12.1 months for the lowest, middle and highest tertiles of serum zeaxanthin). The median duration is much larger than the estimated four-month interval utilized in the GEE analysis, which may explain the lack of association between oncogenic HPV events and zeaxanthin level.

The mechanism by which carotenoids and tocopherols might influence the natural history of HPV infections remains uncertain. However, the potent antioxidant activity of

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these nutrients may be one mechanism that confers chemopreventive activity. As antioxidants, these nutrients maintain the correct oxidant-antioxidant balance within cells<sup>26</sup> by quenching excess reactive oxygen species (ROS). Although required for normal cellular processes at low levels, excess ROS can cause a multitude of problems, specifically damage to both cellular DNA and membranes<sup>26</sup> and decreases in immune cell activity<sup>27</sup>. Redox-sensitive transcription factors, Activator Protein (AP)-1 and Nuclear Factor (NF)- $\kappa$ B, are activated during conditions of oxidative stress and can potentially lead to alterations in numerous gene expression levels, such as genes involved in cell growth and apoptosis<sup>28</sup>.

It has been demonstrated that the activity of some viruses is enhanced under conditions of low antioxidants<sup>29-33</sup>. This enhanced viral activity may be due to the activation of redox-sensitive transcription factors, AP-1 and NF- $\kappa$ B, which are utilized by several viruses, such as human immunodeficiency virus (HIV), cytomegalovirus (CMV), hepatitis-B virus (HBV) and HPV<sup>34-39</sup> to support expression of viral genes and subsequent replication of viral genomes. *In vitro* studies have consistently demonstrated that NF- $\kappa$ B activation is inhibited by antioxidants such as  $\alpha$ -tocopherol<sup>40</sup>,  $\alpha$ -lipoic acid<sup>41</sup>, N-acetylcysteine, and pyrrolidine-dithiocarbamate (PDTC)<sup>40</sup>. The presence of NF- $\kappa$ B binding sites in the HPV upstream regulatory region<sup>42</sup> and evidence that HPV activity is altered by NF- $\kappa$ B activity, suggests the potential for NF- $\kappa$ B to alter HPV viral replication and expression<sup>43</sup>. A recent clinical trial reported a decrease in HIV viral titer among HIV patients supplemented with  $\alpha$ -tocopherol (800 mg/day) compared to non-supplemented HIV patients<sup>44</sup>. Antioxidant treatment of HPV-16 immortalized human

keratinocytes (HKc) with PDTC altered the composition and reduced the activity of the transcriptional AP-1 complex<sup>45</sup>, the key transcription factor for HPV oncogenes<sup>37, 38</sup>. Although not directly shown, it has been postulated that the combined stress of decreased antioxidant levels and viral infection may allow for a typically transient viral infection to persist over time<sup>6, 46</sup>.

In addition to their antioxidant activities, it is possible that the observed effects of zeaxanthin and possibly lutein and  $\alpha$ -cryptoxanthin are due to their pro-vitamin A activity. We have recently demonstrated an increased probability of type-specific oncogenic and nononcogenic HPV clearance with elevated serum all-*trans* retinoic acid levels<sup>47</sup>. In this present study, we observed a marginal increase in the probability of clearing a type-specific nononcogenic HPV infection with increased serum retinol. However, few studies have found an association between vitamin A consumption and cervical neoplasia risk<sup>6</sup>.

Our study among Brazilian woman is the first to demonstrate differences in associations with specific nutrients and type-specific oncogenic versus nononcogenic HPV persistence and clearance. Goodman and colleagues<sup>22</sup> reported associations with serum antioxidants and type-specific oncogenic HPV persistent events, utilizing a similar definition of HPV persistence events. Using generalized logistic models (i.e. GEE logit-link function), they reported a 44% reduction in risk of HPV persistence for women in the highest tertile of plasma  $\beta$ -carotene (positive change between measures) and 57% reduction in risk among women with higher  $\alpha$ -tocopherol<sup>22</sup>. In this current study serum

zeaxanthin levels are found to be associated with oncogenic HPV clearance, and not clearance on nononcogenic types.

Both oncogenic and nononcogenic HPV types have the ability to infect cervical epithelial cells and initiate a productive viral infection. Differences in oncogenic and nononcogenic type infections are evident in the strength with which the viral proteins bind to cellular proteins to induce DNA synthesis and cell cycle progression<sup>48</sup>. The continued presence of oncogenic HPV infection is thought to be a result of functional losses within cervical cells that control viral expression<sup>49</sup>. It may be that the protective effect of zeaxanthin becomes evident only after cellular control of oncogenic HPV infections is lost. Antioxidants could be protective at lower levels early in HPV infection for both nononcogenic and oncogenic type infections; however, the associations go undetected until later in the HPV infectious cycle. Another potential target for zeaxanthin is the immune system, as higher antioxidant levels may contribute to increased clearance of HPV infected cells. This mechanism requires further examination.

As with any observational study, there were limitations with this study that need to be addressed. We assessed HPV persistence and clearance over a relatively short period of time. Our definition of HPV persistence and clearance included both prevalent and incident HPV infections. By including prevalent infections our estimate of duration of infection would not represent the true duration because it was impossible to know exactly how long a woman has been infected by the time she was found positive at enrollment. In general, women found to be HPV positive upon enrollment were more likely to have a persistent infection. Unfortunately the Ludwig-McGill Cohort study did

not have sufficient incident HPV infections to utilize incident HPV persistence as our outcome. Of all the HPV infections detected in the first year, 39% were detected at enrollment and only 22% of the incident infections persisted. HPV clearance was defined as the time to the first negative test. Moscicki and colleagues<sup>50</sup> suggested that misclassification might result from using only one time point to determine clearance. However, we utilized PCR based methods, which are highly sensitive and detect low levels of HPV DNA, to assess HPV status. These methods are less likely to result in false negative results compared to other methods<sup>51</sup>. Similar to other biological markers, the values of serum nutrients presented in this report might not reflect the absolute value due to losses that might have occurred during storage or in the extraction process. However, the relative levels of each isomer should not have changed; therefore the associations found in this study would be valid with the magnitude of the associations potentially being lower than the true association due to methodological errors.

The issue of multiple comparisons arises when analyzing nutritional data from large epidemiological studies in that a large number of statistical models are required. For the present study, we did not adjust the significance level but chose to evaluate each serum nutrient as individual associations. By using this approach, there is a chance that single spurious associations will be found based on the nature of statistical probabilities. Therefore, the issue of multiple comparisons needs to be considered when interpreting results. Conclusions should be made in light of consistency with other information and external to the study<sup>52</sup>. The findings of an association of serum zeaxanthin levels with HPV clearance consistent with two previously reported studies<sup>7, 21</sup>. This consistency

provides support that our results for this particular nutrient may not be a spurious association found by chance.

This study had several strengths, including the assessment of serum carotenoids and tocopherols at four different time points allowing for an accurate assessment of a woman's nutrient status. The study was nested within a large prospective cohort study that measured HPV at four clinical visits within the first year of follow-up and assessed over 40 HPV types that allowed type-specific HPV infections to be detected.

Overall, results from the current study suggest that higher serum antioxidant nutrients may lead to shorter duration of HPV infections. We found differences in specific nutrient associations between oncogenic and nononcogenic type HPV infections. The consistent associations found for both serum and dietary antioxidants are promising. This research indicates that antioxidant nutrients, modifiable factors, are associated with viral persistence and clearance and suggest that a diet high in antioxidants may confer protection. Future research efforts will examine if serum antioxidant nutrients confer protection against cervical lesion development.

#### **ACKNOWLEDGEMENTS**

We are indebted to Maria L. Baggio and Lenice Galan for management of the patients and specimen collections and to Silvaneide Ferreira for data entry, sample retrieval and shipment and laboratory analysis. We appreciated the thoughtful comments on this manuscript by Drs. Robin Harris, Elena Martinez, Denise Roe and Jesse Martinez.

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## FIGURE LEGENDS

**Figure 1:** Graphic representation of the different approaches utilized to examine HPV persistence. All approaches imply the same HPV type at each positive visit (+). (A). HPV persistence which collapses longitudinal data into a dichotomous “case-control” definition. Transient infection (control) is defined as only one out of four visits positive or nonconsecutive visits positive. Persistent infection (case) is defined as two or more consecutively positive tests for the same HPV type. (B). Approach first described by Ho *et al.*<sup>17</sup> utilizing HPV persistence as a repeated event of visit pairs. Women who are HPV positive at a particular measurement are considered persistent if their subsequent measurement is also positive for the same HPV type and transient if their subsequent visit was negative (i.e., if they have cleared the virus) or a different HPV type. (C). Time-dependent cohort analysis for repeated HPV data, with first negative visit being defined as an HPV clearance event. Women with both prevalent and incident infections are included.

**Figure 2:** Probability of type specific HPV clearance by tertile of zeaxanthin concentrations for oncogenic HPV infections (A) and nononcogenic HPV infections (B)

**Figure 1.** Hypothetical Examples of Different HPV Outcome Variables Considered in Examining the Associations between Serum Nutrients and HPV Persistence and Clearance

Month	0	4	8	12
Time	1	2	3	4
<b>A. Case-Control</b>				
HPV <sub>t</sub>	-	+	-	-
Outcome	Transient			
HPV <sub>t</sub>	+	+	-	-
Outcome	Persistent			
<b>B. Longitudinal Events</b>				
HPV <sub>t</sub>	+	+	-	-
HPV <sub>(t, t+1)</sub>	(+,+)	(+,-)	(-, -)	
Outcome	Pers.	Trans.	n/a	
<b>C. Clearance</b>				
HPV <sub>t</sub>	+	+	+	-
Outcome				Clearance Event
HPV <sub>t</sub>	-	+	-	-
Outcome	n/a	Enter Follow-up	Clearance Event	n/a

**Table 1: Mean (SD) of Serum Carotenoids and Tocopherols Among HPV Positive Woman and Stratified by HPV Persistence**

<i>Nutrients (μg/mL)</i>	<b>HPV Positive</b>		<b>HPV Persistence</b>							
			<b>Case-Control Analysis</b>				<b>Longitudinal Events</b>			
	(N=407)		Transient (N=135)		Persistent (N=165)		+,- (N=277)		+ ,+ (N=259)	
	Mean	(SD)	Mean	(SD)	Mean	(SD)	Mean	(SD)	Mean	(SD)
Retinol	0.458	0.142	0.460	0.153	0.456	0.124	0.452	0.143	0.455	0.125
Lutein	0.037	0.043	0.035	0.020	0.040	0.062	0.035	0.019	0.044	0.084
Zeaxanthin	0.016	0.007	0.015	0.007	0.016	0.007	0.015	0.006	0.016	0.007
α Cryptoxanthin	0.006	0.006	0.006	0.004	0.006	0.008	0.006	0.004	0.007	0.010
β Cryptoxanthin	0.040	0.171	0.035	0.143	0.049	0.205	0.033	0.133	0.063	0.257
trans-Lycopene	0.017	0.015	0.016	0.015	0.018	0.016	0.018	0.016	0.017	0.015
cis-Lycopene	0.026	0.018	0.025	0.018	0.027	0.017	0.026	0.018	0.026	0.017
α Carotene	0.011	0.009	0.011	0.009	0.012	0.009	0.011	0.009	0.012	0.010
trans-β Carotene	0.024	0.027	0.024	0.027	0.025	0.028	0.025	0.028	0.025	0.029
cis-β Carotene	0.007	0.005	0.007	0.004	0.007	0.006	0.007	0.005	0.007	0.006
a Tocopherol	5.135	1.959	5.113	1.973	5.167	1.943	5.060	1.813	5.111	1.848
δ Tocopherol	0.083	0.034	0.083	0.036	0.082	0.032	0.082	0.036	0.082	0.031
γ Tocopherol	1.578	0.577	1.578	0.563	1.578	0.598	1.579	0.557	1.549	0.593

**Table 2: Odd Ratio estimates for the associations between nononcogenic and oncogenic HPV persistence and serum antioxidant nutrient level: Case-Control Analysis<sup>1</sup>**

	Non Oncogenic HPV Types					Oncogenic HPV Types				
	Trans. (N) <sup>2</sup>	Pers. (N)	Crude OR	Adjusted <sup>3</sup> OR	Adjusted <sup>3</sup> 95% CI	Trans. (N)	Pers. (N)	Crude OR	Adjusted <sup>3</sup> OR	Adjusted <sup>3</sup> 95% CI
<b>Carotenoids (µg/mL)</b>										
<b>Retinol</b>										
≤ 0.380	40	19	1.00	1.00	Referent	47	26	1.00	1.00	Referent
0.381 - 0.453	41	21	1.08	0.89	(0.39 - 2.00)	45	29	1.16	1.23	(0.61 - 2.50)
0.434 - 1.900	35	20	1.20	0.89	(0.36 - 2.19)	51	31	1.10	1.11	(0.54 - 2.27)
<i>p-trend</i>					0.80					0.79
<b>Lutein</b>										
≤ 0.0292	38	16	1.00	1.00	Referent	48	30	1.00	1.00	Referent
0.0293-0.0415	38	23	1.44	1.58	(0.68 - 3.66)	49	23	0.75	0.65	(0.31 - 1.35)
0.042-0.807	40	21	1.25	1.27	(0.53 - 3.05)	42	33	1.15	1.06	(0.53 - 2.14)
<i>p-trend</i>					0.61					0.86
<b>Zeaxanthin</b>										
≤ 0.0125	37	17	1.00	1.00	Referent	49	29	1.00	1.00	Referent
0.0126-0.0202	40	17	0.93	1.00	(0.42 - 2.40)	48	25	0.88	0.89	(0.44 - 1.83)
0.0203-0.054	39	26	1.45	1.56	(0.67 - 3.61)	46	32	1.18	1.12	(0.56 - 2.25)
<i>p-trend</i>					0.28					0.75
<b>α Cryptoxanthin</b>										
≤ 0.005	39	26	1.00	1.00	Referent	53	34	1.00	1.00	Referent
0.006-0.0075	36	16	0.67	0.65	(0.28 - 1.48)	47	23	0.76	0.75	(0.37 - 1.52)
0.0076-0.088	41	18	0.66	0.62	(0.28 - 1.37)	43	29	1.05	0.87	(0.42 - 1.80)
<i>p-trend</i>					0.22					0.69
<b>β Cryptoxanthin</b>										
≤ 0.0207	38	19	1.00	1.00	Referent	52	33	1.00	1.00	Referent
0.0208-0.0395	39	18	0.92	0.99	(0.43 - 2.32)	44	17	0.61	0.62	(0.29 - 1.34)
0.040-2.20	39	23	1.18	0.93	(0.40 - 2.16)	47	36	1.21	1.04	(0.51 - 2.09)
<i>p-trend</i>					0.86					0.95
<b>trans-Lycopene</b>										
≤ 0.013	31	21	1.00	1.00	Referent	58	22	1.00	1.00	Referent
0.0135-0.0271	41	20	0.72	0.65	(0.28 - 1.49)	42	28	1.76	1.85	(0.88 - 3.88)
0.0272-0.120	44	19	0.64	0.64	(0.27 - 1.53)	43	36	2.21	2.33	(1.09 - 5.02)
<i>p-trend</i>					0.32					0.03
<b>cis-Lycopene</b>										
≤ 0.0210	34	17	1.00	1.00	Referent	52	23	1.00	1.00	Referent
0.0211-0.0397	42	29	1.38	1.23	(0.53 - 2.83)	45	22	1.11	1.31	(0.61 - 2.84)
0.0398-0.1633	40	14	0.70	0.59	(0.22 - 1.56)	46	41	2.02	2.21	(1.05 - 4.62)
<i>p-trend</i>					0.27					0.03
<b>α Carotene</b>										
≤ 0.0087	43	19	1.00	1.00	Referent	44	27	1.00	1.00	Referent
0.0088-0.016	30	23	1.74	1.66	(0.70 - 3.95)	55	23	0.68	0.75	(0.36 - 1.55)
0.0165-0.065	43	18	0.95	0.97	(0.42 - 2.28)	44	36	1.33	1.31	(0.63 - 2.72)
<i>p-trend</i>					0.87					0.44
<b>trans-β Carotene</b>										
≤ 0.0184	37	23	1.00	1.00	Referent	50	28	1.00	1.00	Referent
0.0185-0.036	35	20	0.92	0.99	(0.43 - 2.27)	52	25	0.86	1.01	(0.49 - 2.07)
0.037-0.238	44	17	0.62	0.65	(0.28 - 1.53)	41	33	1.44	1.35	(0.64 - 2.82)
<i>p-trend</i>					0.33					0.42
<b>cis-β Carotene</b>										
≤ 0.005	42	20	1.00	1.00	Referent	53	30	1.00	1.00	Referent
0.006-0.008	39	26	1.40	1.10	(0.50 - 2.45)	43	25	1.03	0.88	(0.43 - 1.78)
0.008-0.165	35	14	0.84	0.74	(0.30 - 1.82)	47	31	1.17	1.02	(0.51 - 2.03)
<i>p-trend</i>					0.55					0.97

Table 2 - Continued

Tocopherols ( $\mu\text{g/mL}$ )										
<b><math>\alpha</math> Tocopherol</b>										
$\leq 4.27$	32	23	1.00	1.00	Referent	57	27	1.00	1.00	Referent
4.28-6.03	48	14	0.41	0.20	(0.08 - 0.54)	35	27	1.63	1.89	(0.90 - 3.95)
6.04-17.81	36	23	0.89	0.43	(0.16 - 1.15)	51	32	1.32	1.31	(0.63 - 2.73)
<i>p-trend</i>				0.14					0.45	
<b><math>\delta</math> Tocopherol</b>										
$\leq 1.36$	37	23	1.00	1.00	Referent	53	28	1.00	1.00	Referent
1.37-1.77	37	22	0.96	0.75	(0.34 - 1.69)	45	28	1.18	1.06	(0.52 - 2.16)
1.78-4.85	42	15	0.57	0.39	(0.16 - 0.95)	45	30	1.26	1.44	(0.71 - 2.92)
<i>p-trend</i>				0.04					0.31	
<b><math>\gamma</math> Tocopherol</b>										
$\leq 1.36$	33	20	1.00	1.00	Referent	53	25	1.00	1.00	Referent
1.37-1.77	42	20	0.79	0.54	(0.23 - 1.27)	46	32	1.47	1.72	(0.85 - 3.47)
1.78-4.85	41	20	0.80	0.39	(0.15 - 1.00)	44	29	1.40	1.68	(0.81 - 3.47)
<i>p-trend</i>				0.05					0.158	

\* HPV, human papillomavirus; Trans, Transient HPV group; Pers., Persistent HPV group; OR, odds ratio; CI, confidence interval

1. Results obtained from logistic regression with binary outcome of transient or persistent HPV infection ( Figure 1)
2. Total number of observations did not add up to 135 among transient and 165 among persistent due to missing data
3. Adjusted for serum cholesterol, age, race, number of sexual partners (1 year), income and number in household

**Table 3: Odd Ratio estimates for the associations between nononcogenic and oncogenic HPV events and serum antioxidant nutrient level: GEE Analysis<sup>1</sup>**

	Non Oncogenic HPV Types					Oncogenic HPV Types				
	+, <sup>-2</sup> (N) <sup>3</sup>	+,+	Crude OR	Adjusted <sup>4</sup> OR 95% CI		+, <sup>-</sup> (N)	+,+	Crude OR	Adjusted <sup>5</sup> OR 95% CI	
<b>Carotenoids (µg/mL)</b>										
<b>Retinol</b>										
≤0.380	43	38	1.00	1.00	Referent	51	47	1.00	1.00	Referent
0.381 - 0.453	50	34	0.77	0.58	(0.29 - 1.14)	46	50	1.18	1.43	(0.77 - 2.65)
0.434 - 1.900	35	37	1.20	1.07	(0.50 - 2.30)	52	53	1.11	1.22	(0.65 - 2.30)
<i>p-trend</i>				0.95					0.53	
<b>Lutein</b>										
≤0.0292	41	33	1.00	1.00	Referent	49	55	1.00	1.00	Referent
0.0293-0.0415	44	35	0.99	1.01	(0.50 - 2.01)	48	39	0.72	0.84	(0.42 - 1.65)
0.042-0.807	42	41	1.18	0.95	(0.44 - 2.04)	52	56	0.96	1.03	(0.55 - 1.93)
<i>p-trend</i>				0.90					0.89	
<b>Zeaxanthin</b>										
≤0.0125	43	31	1.00	1.00	Referent	45	55	1.00	1.00	Referent
0.0126-0.0202	43	28	0.90	0.97	(0.47 - 2.02)	48	43	0.73	0.77	(0.40 - 1.50)
0.0203-0.054	42	50	1.65	1.47	(0.72 - 3.01)	56	52	0.76	0.81	(0.44 - 1.46)
<i>p-trend</i>				0.29					0.49	
<b>α Cryptoxanthin</b>										
≤0.005	45	47	1.00	1.00	Referent	50	62	1.00	1.00	Referent
0.006-0.0075	39	27	0.66	0.80	(0.40 - 1.61)	50	42	0.68	0.69	(0.36 - 1.33)
0.0076-0.088	44	35	0.76	0.68	(0.33 - 1.41)	49	46	0.76	0.81	(0.45 - 1.49)
<i>p-trend</i>				0.29					0.51	
<b>β Cryptoxanthin</b>										
≤0.0207	40	36	1.00	1.00	Referent	51	56	1.00	1.00	Referent
0.0208-0.0395	49	28	0.63	0.70	(0.34 - 1.44)	45	33	0.67	0.74	(0.37 - 1.49)
0.040-2.20	39	45	1.28	1.11	(0.52 - 2.36)	53	61	1.05	1.11	(0.61 - 2.01)
<i>p-trend</i>				0.80					0.73	
<b>trans-Lycopene</b>										
≤0.013	38	38	1.00	1.00	Referent	51	43	1.00	1.00	Referent
0.0135-0.0271	42	36	0.86	0.73	(0.37 - 1.46)	39	50	1.52	2.07	(1.03 - 4.15)
0.0272-0.120	48	35	0.73	0.53	(0.26 - 1.09)	59	57	1.15	1.29	(0.71 - 2.37)
<i>p-trend</i>				0.09					0.47	
<b>cis-Lycopene</b>										
≤0.0210	40	29	1.00	1.00	Referent	47	43	1.00	1.00	Referent
0.0211-0.0397	50	52	1.43	1.27	(0.63 - 2.58)	40	44	1.20	1.50	(0.73 - 3.07)
0.0398-0.1633	38	28	1.02	0.66	(0.28 - 1.55)	62	63	1.11	1.29	(0.68 - 2.43)
<i>p-trend</i>				0.33					0.47	
<b>α Carotene</b>										
≤0.0087	49	35	1.00	1.00	Referent	47	50	1.00	1.00	Referent
0.0088-0.016	33	43	1.82	1.66	(0.82 - 3.37)	46	40	0.82	0.81	(0.41 - 1.57)
0.0165-0.065	46	31	0.94	0.79	(0.37 - 1.67)	56	60	1.01	1.04	(0.56 - 1.93)
<i>p-trend</i>				0.51					0.85	
<b>trans-β Carotene</b>										
≤0.0184	46	41	1.00	1.00	Referent	51	50	1.00	1.00	Referent
0.0185-0.036	37	41	1.24	1.42	(0.70 - 2.88)	45	47	1.07	1.13	(0.60 - 2.15)
0.037-0.238	45	27	0.67	0.50	(0.25 - 1.02)	53	53	1.02	1.03	(0.57 - 1.86)
<i>p-trend</i>				0.07					0.93	
<b>cis-β Carotene</b>										
≤0.005	48	33	1.00	1.00	Referent	55	52	1.00	1.00	Referent
0.006-0.008	47	53	1.64	1.55	(0.79 - 3.04)	48	41	0.90	0.88	(0.47 - 1.67)
0.008-0.165	33	23	1.01	0.75	(0.34 - 1.66)	46	57	1.31	1.33	(0.72 - 2.46)
<i>p-trend</i>				0.59					0.70	

**Table 3 - Continued**

Tocopherols ( $\mu\text{g/mL}$ )										
<b><math>\alpha</math> Tocopherol</b>										
$\leq 4.27$	43	39	1.00	1.00	Referent	56	51	1.00	1.00	Referent
4.28-6.03	47	28	0.66	0.49	(0.23 - 1.00)	38	49	1.42	1.40	(0.71 - 2.73)
6.04-17.81	38	42	1.22	0.96	(0.43 - 2.14)	55	50	1.00	1.05	(0.56 - 1.97)
<i>p-trend</i>				0.93					0.82	
<b><math>\delta</math> Tocopherol</b>										
$\leq 1.36$	42	39	1.00	1.00	Referent	56	46	1.00	1.00	Referent
1.37-1.77	44	41	1.00	0.88	(0.44 - 1.79)	45	54	1.46	1.53	(0.82 - 2.87)
1.78-4.85	42	29	0.74	0.48	(0.21 - 1.06)	48	50	1.27	1.40	(0.75 - 2.60)
<i>p-trend</i>				0.07					0.29	
<b><math>\gamma</math> Tocopherol</b>										
$\leq 1.36$	37	34	1.00	1.00	Referent	51	44	1.00	1.00	Referent
1.37-1.77	50	37	0.81	0.65	(0.31 - 1.33)	52	60	1.34	1.36	(0.72 - 2.56)
1.78-4.85	41	38	1.01	0.56	(0.25 - 1.24)	46	46	1.16	1.22	(0.63 - 2.39)
<i>p-trend</i>				0.16					0.57	

\* HPV, human papillomavirus; OR, odds ratio; CI, confidence interval

1. Results obtained from a time-dependent generalized estimating equation approach with a logit link for binary outcomes
2. HPV results of two consecutive visits were grouped as a pair, see Figure 1
3. Total number of observations did not add up to 259 among (+,+) and 277 among (+, -) due to missing data
4. Adjusted for serum cholesterol, time, age, race, total number of pregnancies, lifetime number of sexual partners and marital status.
5. Adjusted for serum cholesterol, time, age, smoking and lifetime number of sexual partners

**Table 4: Hazard ratios for the association between nononcogenic and oncogenic HPV clearance and serum antioxidant nutrient level**

	Non Oncogenic HPV Types				Oncogenic HPV Types				
	No Cleared / Woman-	Crude HR	Adjusted <sup>1</sup> HR	95% CI	No Cleared / Woman-	Crude HR	Adjusted <sup>2</sup> HR	95% CI	
<b>Carotenoids (µg/mL)</b>									
<b>Retinol</b>									
≤ 0.380	52 / 792.6	1.00	1.00	Referent	77 / 774.6	1.00	1.00	Referent	
0.381 - 0.453	52 / 829.6	1.03	1.19	(0.80-1.79)	75 / 691.4	1.00	1.04	(0.67-1.58)	
0.434 - 1.900	56 / 709.0	1.26	1.45	(0.96-2.19)	76 / 637.4	1.07	1.09	(0.69-1.71)	
<i>p-trend</i>			0.08				0.71		
<b>Lutein</b>									
≤ 0.0292	60 / 747.3	1.00	1.00	Referent	40 / 755.1	1.00	1.00	Referent	
0.0293-0.0415	47 / 753.5	0.79	0.79	(0.54-1.17)	39 / 602.8	1.36	1.54	(0.96-2.49)	
0.042-0.807	53 / 830.2	0.88	0.93	(0.63-1.36)	49 / 745.6	1.32	1.32	(0.85-2.60)	
<i>p-trend</i>			0.69				0.24		
<b>Zeaxanthin</b>									
≤ 0.0125	61 / 784.3	1.00	1.00	Referent	35 / 737.4	1.00	1.00	Referent	
0.0126-0.0202	51 / 716.3	0.94	1.02	(0.69-1.49)	44 / 678.2	1.54	1.44	(0.92-2.26)	
0.0203-0.054	48 / 830.6	0.81	0.86	(0.57-1.27)	49 / 687.9	1.53	1.59	(1.01-2.52)	
<i>p-trend</i>			0.46				0.05		
<b>α Cryptoxanthin</b>									
≤ 0.005	54 / 869.9	1.00	1.00	Referent	43 / 810.6	1.00	1.00	Referent	
0.006-0.0075	57 / 755.1	1.27	1.29	(0.87 - 1.89)	41 / 600.0	1.39	1.52	(0.96 - 2.41)	
0.0076-0.088	49 / 706.1	1.30	1.34	(0.89 - 2.02)	44 / 692.9	1.25	1.44	(0.92 - 2.26)	
<i>p-trend</i>			0.15				0.11		
<b>β Cryptoxanthin</b>									
≤ 0.0207	54 / 791.6	1.00	1.00	Referent	40 / 673.6	1.00	1.00	Referent	
0.0208-0.0395	54 / 778.3	1.11	1.18	(0.80-1.74)	41 / 744.4	1.05	1.21	(0.76-1.93)	
0.040-2.20	52 / 761.2	1.10	1.12	(0.75-1.67)	47 / 685.4	1.22	1.39	(0.88-2.19)	
<i>p-trend</i>			0.57				0.15		
<b>trans-Lycopene</b>									
≤ 0.013	56 / 876.0	1.00	1.00	Referent	41 / 716.8	1.00	1.00	Referent	
0.0135-0.0271	48 / 691.0	1.10	1.10	(0.74-1.63)	38 / 629.2	1.05	1.04	(0.65-1.66)	
0.0272-0.120	56 / 764.1	1.29	1.22	(0.81-1.82)	49 / 757.5	1.13	1.26	(0.81-1.96)	
<i>p-trend</i>			0.34				0.29		
<b>cis-Lycopene</b>									
≤ 0.0210	52 / 794.3	1.00	1.00	Referent	41 / 698.4	1.00	1.00	Referent	
0.0211-0.0397	55 / 758.1	1.12	1.07	(0.72-1.60)	41 / 664.1	1.04	1.08	(0.68-1.72)	
0.0398-0.1633	53 / 778.6	1.62	1.13	(0.74-1.72)	46 / 740.9	1.08	1.22	(0.77-1.93)	
<i>p-trend</i>			0.57				0.39		
<b>α Carotene</b>									
≤ 0.0087	59 / 795.4	1.00	1.00	Referent	41 / 683.5	1.00	1.00	Referent	
0.0088-0.016	48 / 814.8	0.84	0.81	(0.55-1.20)	42 / 686.4	0.96	1.15	(0.72-1.82)	
0.0165-0.065	53 / 720.9	0.14	1.01	(0.69-1.49)	45 / 733.6	1.04	1.29	(0.81-2.04)	
<i>p-trend</i>			0.98				0.28		
<b>trans-β Carotene</b>									
≤ 0.0184	58 / 819.4	1.00	1.00	Referent	41 / 726.0	1.00	1.00	Referent	
0.0185-0.036	50 / 789.3	0.86	0.83	(0.56-1.22)	39 / 643.7	1.08	1.09	(0.69-1.71)	
0.037-0.238	52 / 722.4	1.06	1.03	(0.69-1.53)	48 / 733.8	1.15	1.33	(0.86-2.08)	
<i>p-trend</i>			0.94				0.20		
<b>cis-β Carotene</b>									
≤ 0.005	56 / 777.9	1.00	1.00	Referent	46 / 735.7	1.00	1.00	Referent	
0.006-0.008	49 / 840.4	0.78	0.76	(0.51 - 1.12)	40 / 631.8	0.96	0.92	(0.59 - 1.42)	
0.008-0.165	55 / 712.8	1.14	1.09	(0.74 - 1.61)	42 / 736.0	0.91	0.97	(0.62 - 1.52)	
<i>p-trend</i>			0.70				0.89		

**Table 4 - Continued**Tocopherols ( $\mu\text{g/mL}$ ):

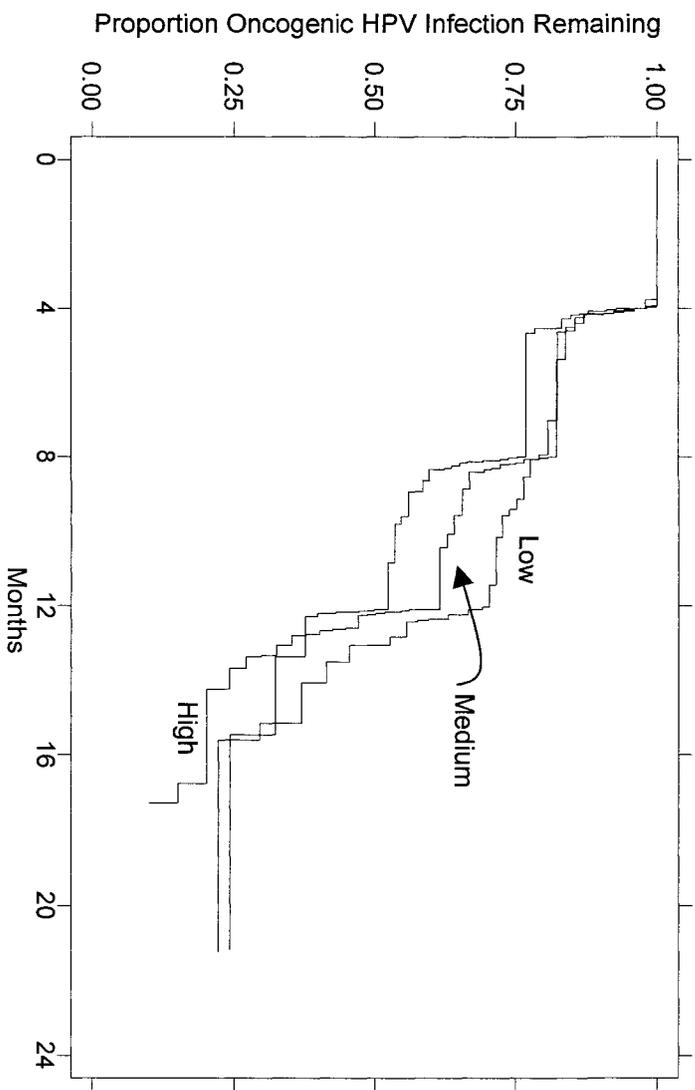
<b><math>\alpha</math> Tocopherol</b>									
$\leq 4.27$	57 / 782.7	1.00	1.00	Referent	46 / 696.8	1.00	1.00	Referent	
4.28-6.03	52 / 768.0	0.92	1.03	(0.68-1.54)	41 / 665.9	1.00	1.05	(0.66-1.66)	
6.04-17.81	51 / 780.4	0.91	1.02	(0.67-1.58)	41 / 740.8	0.84	0.93	(0.58-1.51)	
<i>p-trend</i>			0.91				0.77		
<b><math>\delta</math> Tocopherol</b>									
$\leq 1.36$	54 / 768.1	1.00	1.00	Referent	47 / 718.2	1.00	1.00	Referent	
1.37-1.77	60 / 826.9	1.1	1.12	(0.77-1.63)	40 / 686.6	0.92	0.91	(0.59-1.47)	
1.78-4.85	46 / 736.1	0.85	0.91	(0.61-1.37)	41 / 698.7	0.88	0.78	(0.50-1.22)	
<i>p-trend</i>			0.69				0.27		
<b><math>\gamma</math> Tocopherol</b>									
$\leq 1.36$	54 / 757.1	1.00	1.00	Referent	46 / 667.0	1.00	1.00	Referent	
1.37-1.77	60 / 798.4	1.12	1.09	(0.75-1.60)	46 / 739.8	0.98	0.97	(0.63-1.50)	
1.78-4.85	46 / 775.6	1.25	0.9	(0.59-1.38)	36 / 696.6	0.75	0.69	(0.43-1.12)	
<i>p-trend</i>			0.96				0.14		

\* HPV, human papillomavirus; HR, hazard ratio; CI, confidence interval

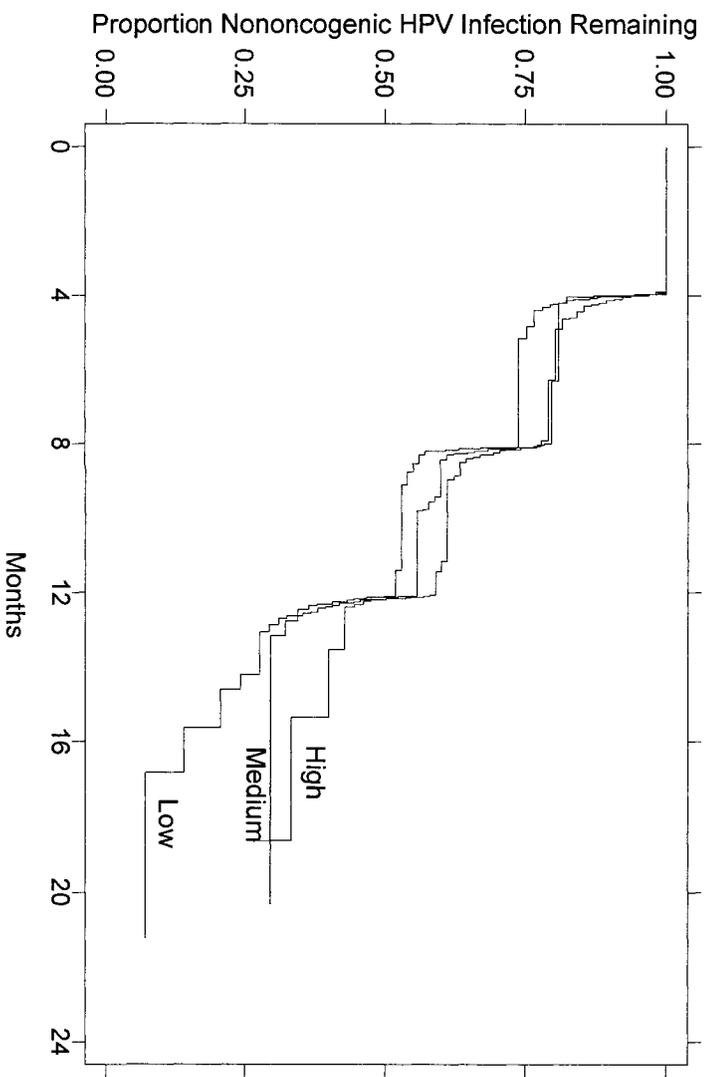
1. Adjusted for serum cholesterol, age, number sexual partners (5 years), and income

2. Adjusted for serum cholesterol, age, race, number sexual partners (5 years), education, total number of pregnancies

**Figure 2A**



**Figure 2B**



**APPENDIX D**

**ADDITIONAL TABLES**

Table 1: Comparison of Baseline Demographic and Lifestyle factors among HPV positive women: Parent Cohort vs. Sub-cohort

	Parent cohort <sup>a</sup>		Subcohort		P-value <sup>b</sup>
	N	%	N	%	
<i>HPV Status</i>					
Ever Positive <sup>c</sup>	191		433	100	-
<i>Age (mean ± SD)</i>					
	30	9	31	9	0.214
<i>Ethnicity</i>					
White	111	58.1	268	61.9	0.373
Non-white	80	41.9	165	38.1	
<i>Education</i>					
< Elementary	31	16.2	89	20.6	0.18
Elementary	126	66.0	245	56.6	
High School	30	15.7	86	19.9	
> High School	4	2.1	13	3.0	
<i>Marital Status</i>					
Single	33	17.4	81	18.7	0.54
Married	69	36.3	164	37.9	
Widow	6	3.2	8	1.9	
Divorced	12	6.3	39	9.0	
Common Law	70	36.8	141	32.6	
<i>Number living in household</i>					
1-2	10	5.3	31	7.2	0.709
3	34	17.9	68	15.8	
4	51	26.8	103	24.0	
5	41	21.6	84	19.5	
6	24	12.6	61	14.2	
≥ 7	30	15.8	83	19.3	
<i>Monthly Income (US\$)</i>					
≤ 250	31	16.3	108	25.7	0.01
251 - 450	44	23.2	107	25.4	
451 - 725	43	22.6	96	22.8	
≥ 725	72	37.9	110	26.1	

a Parent cohort restricted to women with similar follow-up as subcohort, n=1028

b p-value for difference in observed vs expected Chi2 statistic

c Women with a HPV positive test at any visit during the first 12 months

Table 1 Continued:	Parent cohort		Subcohort		P-value <sup>b</sup>
	N	%	N	%	
<i>Cigarette Smoking</i>					
Never	79	41.4	197	45.5	0.211
Current	69	36.1	164	37.9	
Former	43	22.5	72	16.6	
<i>Age @ first intercourse</i>					
≤15	31	16.2	93	21.5	0.231
16-17	37	19.4	92	21.3	
18-19	65	34.0	117	27.0	
20-50	58	30.4	131	30.3	
<i>Lifetime # sexual partners</i>					
0 - 1	65	34.0	154	35.6	0.934
2 - 3	76	39.8	168	38.8	
≥ 4	50	26.2	111	25.6	
<i>Total # sex partners past 5 yrs</i>					
0-1	128	67.0	283	65.4	0.687
≥ 2	63	33.0	150	34.6	
<i>Total # sexual partners past year</i>					
0-1	177	93.2	386	90.2	0.231
≥ 2	13	6.8	42	9.8	
<i>Oral contraceptive use</i>					
Never	30	15.7	71	16.4	0.629
<6 years	116	60.7	246	56.8	
≥ 6 years	45	23.6	116	26.8	
<i>Total # of Pregnancies</i>					
0-1	38	19.9	83	19.4	0.974
2-3	76	39.8	177	41.3	
4-6	58	30.4	124	28.9	
≥ 7	19	10.0	45	10.5	
<i>Age of Menarche</i>					
0-11	48	25.3	101	23.3	0.602
12-19	142	74.7	332	76.7	

a Parent cohort restricted to women with similar follow-up as subcohort, n=1028

b p-value for difference in observed vs expected Chi2 statistic

c Women with a HPV positive test at any visit during the first 12 months

**Table 2: Comparison of Baseline Demographic and Lifestyle factors among HPV Negative Women: Parent Cohort vs. Sub-cohort**

	Parent cohort <sup>a</sup>		Subcohort		P-value <sup>b</sup>
	N	%	N	%	
<i>HPV Status</i>					
Never HPV positive <sup>c</sup>	829		412		-
<i>Age (mean ± SD)</i>					
	35	8	32	9	0.001
<i>Ethnicity</i>					
White	553	66.7	271	65.8	0.744
Non-white	276	33.3	141	34.2	
<i>Education</i>					
< Elementary	187	22.6	93	22.6	0.515
Elementary	501	60.6	237	57.5	
High School	121	14.6	69	16.8	
> High School	18	2.2	13	3.2	
<i>Marital Status</i>					
Single	51	6.2	20	4.9	0.121
Married	462	55.7	235	57.0	
Widow	20	2.4	5	1.2	
Divorced	46	5.6	13	3.2	
Common Law	250	30.2	139	33.7	
<i>Number living in household</i>					
1-2	32	3.9	20	4.9	0.497
3	170	20.5	73	17.7	
4	239	28.9	132	32.0	
5	169	20.4	92	22.3	
6	105	12.7	47	11.4	
≥ 7	113	13.7	48	11.7	
<i>Monthly Income (US\$)</i>					
≤ 250	194	23.7	103	25.8	0.244
251 - 450	208	25.4	103	25.8	
451 - 725	178	21.8	98	24.6	
≥ 725	238	29.1	95	23.8	

a Parent cohort restricted to women with similar follow-up as subcohort, n=1028

b p-value for difference in observed vs expected Chi2 statistic

c Based on the first 12 months on study

Table 2 - Continued

	Parent cohort <sup>a</sup>		Subcohort		P-value <sup>b</sup>
	N	%	N	%	
<i>Cigarette Smoking</i>					
Never	404	48.7	232	56.3	0.042
Current	274	33.1	117	28.4	
Former	151	18.2	63	15.3	
<i>Age @ first intercourse</i>					
≤15	266	32.1	103	25.0	0.069
16-17	165	19.9	97	23.5	
18-19	193	23.3	101	24.5	
20-50	205	24.7	111	26.9	
<i>Lifetime # sexual partners</i>					
0 - 1	410	49.5	205	49.9	0.269
2 - 3	257	31.0	140	34.1	
≥ 4	162	19.5	66	16.1	
<i>Total # sex partners past 5 yrs</i>					
0-1	698	84.3	346	84.2	0.958
≥ 2	130	15.7	65	15.8	
<i>Total # sexual partners past year</i>					
0-1	809	98.2	399	97.6	0.467
≥ 2	15	1.8	10	2.4	
<i>Oral contraceptive use</i>					
Never	116	14.0	64	15.5	0.43
<6 years	447	53.9	230	55.8	
≥ 6 years	266	32.1	118	28.6	
<i>Total # of Pregnancies</i>					
0-1	117	14.2	67	16.3	0.372
2-3	367	44.4	188	45.9	
4-6	253	30.6	122	29.8	
≥ 7	89	10.8	33	8.1	
<i>Age of Menarche</i>					
0-11	173	21.0	96	23.4	0.327
12-19	652	79.0	314	76.6	

a Parent cohort restricted to women with similar follow-up as subcohort, n=1028

b p-value for difference in observed vs expected Chi2 statistic

c Based on the first 12 months on study

Table 3a. Patterns of Oncogenic Type-Specific HPV Infections, Stratified by Incident or Prevalent Infections (n=818)

HPV Type	HPV -    HPV +		Incident HPV infection				Prevalent HPV infection						
			Transient <sup>1</sup>		Persistent <sup>2</sup>		Transient <sup>1</sup>		Persistent <sup>2</sup>				
			1 +	2 NC +	2 +	3 +	1 +	2 NC +	2 +	3 NC +	2	Total	3 +
16	763	55	28	0	2	6	6	0	2	0	2	5	6
18	803	15	4	0	2	0	6	0	0	0	0	2	1
31	789	29	13	0	4	0	2	0	2	1	3	5	2
33	805	13	1	0	6	0	3	0	2	1	3	0	0
35	805	13	6	2	1	1	1	0	2	0	2	0	0
39	813	5	3	0	1	1	0	0	0	0	0	0	0
45	802	16	6	0	1	0	6	1	1	0	1	0	1
51	777	41	22	1	3	2	7	0	4	0	4	0	2
52	793	25	9	1	5	0	6	0	2	0	2	1	1
56	805	13	6	0	2	2	2	0	0	1	1	0	0
58	792	26	10	0	3	0	3	1	4	0	4	2	3
59	802	16	5	0	2	0	7	0	0	1	1	0	1
68	804	14	8	0	0	0	5	0	0	0	0	0	1
Total		281	121	4	32	12	54	2	19	4	23	15	18

<sup>1</sup> Transient HPV infection defined as only one visit positive for specific type (1+) or two non-consecutive visits positive (2 NC+)

<sup>2</sup> Persistent HPV infections defined as two (2+ or 3 NC+) or more (3+ or 4+) consecutively positive results for the same HPV type

Table 3b. Patterns of Nononcogenic Type-Specific HPV Infections, Stratified by Incident or Prevalent Infections (n=818)

HPV Type	HPV -    HPV +		Incident HPV infection				Prevalent HPV infection						
			Transient <sup>1</sup>		Persistent <sup>2</sup>		Transient <sup>1</sup>		Persistent <sup>2</sup>				
			1 +	2 NC +	2 +	3 +	1 +	2 NC +	2 +	3 NC +	Total	3 +	4 +
6/11	794	24	10	0	2	0	8	0	1	1	2	1	1
26	814	4	2	0	0	0	2	0	0	0	0	0	0
32	817	1	1	0	0	0	0	0	0	0	0	0	0
34	816	2	0	0	0	0	2	0	0	0	0	0	0
40	806	12	4	0	3	0	2	1	0	0	0	0	2
42	817	1	1	0	0	0	0	0	0	0	0	0	0
44	814	4	2	0	0	0	2	0	0	0	0	0	0
53	762	56	27	0	7	2	10	0	6	2	8	1	1
54	803	15	8	1	2	0	2	0	0	0	0	0	2
55	804	14	10	0	0	1	2	0	0	0	0	1	0
57	818	0	0	0	0	0	0	0	0	0	0	0	0
61	793	25	5	0	5	1	6	1	2	0	2	1	4
62	808	10	6	0	3	0	1	0	0	0	0	0	0
64	818	0	0	0	0	0	0	0	0	0	0	0	0
66	806	12	4	1	1	0	2	0	1	1	2	1	1
67	816	2	1	0	0	1	0	0	0	0	0	0	0
69	817	1	1	0	0	0	0	0	0	0	0	0	0
70	801	17	5	0	1	0	5	1	3	0	3	0	2
71	812	6	4	0	0	1	1	0	0	0	0	0	0
72	810	8	1	0	2	0	0	1	2	1	3	1	0
73	805	13	8	0	2	0	3	0	0	0	0	0	0
81	804	14	6	0	0	1	1	0	2	2	4	2	0
82	814	4	3	0	1	0	0	0	0	0	0	0	0
83	808	10	8	0	0	0	0	0	0	0	0	0	2
84	796	22	10	0	1	1	4	1	2	0	2	1	2
is39	813	5	3	0	0	0	2	0	0	0	0	0	0
c6108	816	2	2	0	0	0	0	0	0	0	0	0	0
Unknown		28	20	1	0	0	5	0	1	0	1	0	1
Total		284	152	3	30	8	60	5	20	7	27	9	18

<sup>1</sup> Transient HPV infection defined as only one visit positive for specific type (1+) or two non-consecutive visits positive (2 NC+)

<sup>2</sup> Persistent HPV infections defined as two (2+ or 3 NC+) or more (3+ or 4+) consecutively positive results for the same HPV type

**Table 4: Summary and Distribution of HPV Co-Infections**

	Transient Infection (N)	Persistent Infection (N)
<b>Infection Summary</b>		
Number of Single Infection		
Oncogenic	100	69
Nononcogenic	98	79
Total	198	148
Number of Co-infections		
2	56	15
3	16	4
4	3	0
5	1	0
Total	76	19
<b>Distribution of Co-Infections</b>		
Oncogenic Only		
2	9	7
3	2	2
4	0	0
Non oncogenic Only		
2	15	1
3	3	0
4	0	0
Oncogenic : Nononcogenic		
1 : 1	32	7
1 : 2	5	0
1 : 3	0	0
1 : 4	1	0
2 : 1	6	2
2 : 2	2	0
3 : 1	1	0
3 : 2	0	0

**Table 5. Summary of Missing Serum Nutrient Analyses, N=3,384**

	Visit 1	Visit 2	Visit 3	Visit 4	Total Missing
<b>Carotenoids and Tocopherols</b>					
Sample not available (n/s) <sup>a</sup>	12	11	9	15	47
Vial Empty upon arrival (e/v) <sup>b</sup>	1	0	0	0	1
No sample Remaining Following RA analyses (i/s)	2	2	0	0	4
<b>Total Missing</b>	<b>15</b>	<b>13</b>	<b>9</b>	<b>15</b>	<b>52</b>
<b>Retinoic Acid</b>					
Sample not available (n/s)	12	11			23
Vial Empty upon arrival (e/v)	1	0			1
No sample Remaining Following CT/TP analyses (i/s)	4	6			10
<b>Total Missing</b>	<b>17</b>	<b>17</b>			<b>34</b>

a: Duplicate samples sent to replace missing visit:

- Missing Visit 1: Duplicate V-2 (ID=916)
- Missing Visit 1: Duplicate V-3 (ID=3338)
- Missing Visit 2: Duplicate V-4 (ID=2432)
- Missing Visit 3: Duplicate V-4 (ID=2440)
- Missing Visit 4: Duplicate V-2 (ID=1957)

b: ID = 412

**Table 6. Frequency (%) of Serum Nutrient Samples Below the Limit of Quantification<sup>1</sup>, by Visit**

	Visit 1		Visit 2		Visit 3		Visit 4		Total	
	N	%	N	%	N	%	N	%	N	%
<b>Carotenoids (µg/mL)</b>										
Lutein	7	1%	1	0%	3	0%	5	1%	16	0.5%
Zeaxanthin	11	1%	12	1%	14	2%	13	2%	50	1.5%
α-Cryptoxanthin	264	32%	280	34%	287	34%	251	30%	1082	32.5%
β-Cryptoxanthin	83	10%	88	11%	76	9%	83	10%	330	9.9%
<i>trans</i> -Lycopene	194	23%	212	25%	198	24%	189	23%	793	23.8%
<i>cis</i> -Lycopene	170	20%	184	22%	169	20%	178	21%	701	21.0%
α-Carotene	205	25%	204	24%	198	24%	204	25%	811	24.3%
<i>trans</i> -β-Carotene	140	17%	122	15%	111	13%	131	16%	504	15.1%
<i>cis</i> -β-Carotene	264	32%	257	31%	264	32%	252	30%	1037	31.1%
<b>Tocopherols (µg/mL)</b>										
α-Tocopherol	13	2%	11	1%	7	1%	15	2%	46	1.4%
δ-Tocopherol	190	23%	193	23%	200	24%	193	23%	776	23.3%
γ-Tocopherol	14	2%	14	2%	14	2%	15	2%	57	1.7%
<b>Retinoids</b>										
Retinol (µg/mL)	1	0%	0	0%	1	0%	1	0%	3	0.1%
13- <i>cis</i> RA (ng/ml)	24	3%	46	6%	-	-	-	-	70	4.2%
9- <i>cis</i> RA (ng/ml)	130	16%	124	15%	-	-	-	-	254	15.3%
<i>all-trans</i> RA (ng/ml)	22	3%	34	4%	-	-	-	-	56	3.4%

<sup>1</sup>Limit of Quantification: Carotenoids=0.004 µg/mL; Tocopherols = 0.015 µg/mL; Retinoic Acid = 0.03 ng/mL

Figure 1. Distribution of Retinoic Acid Concentrations by HPLC Batch Number

Figure 1a.

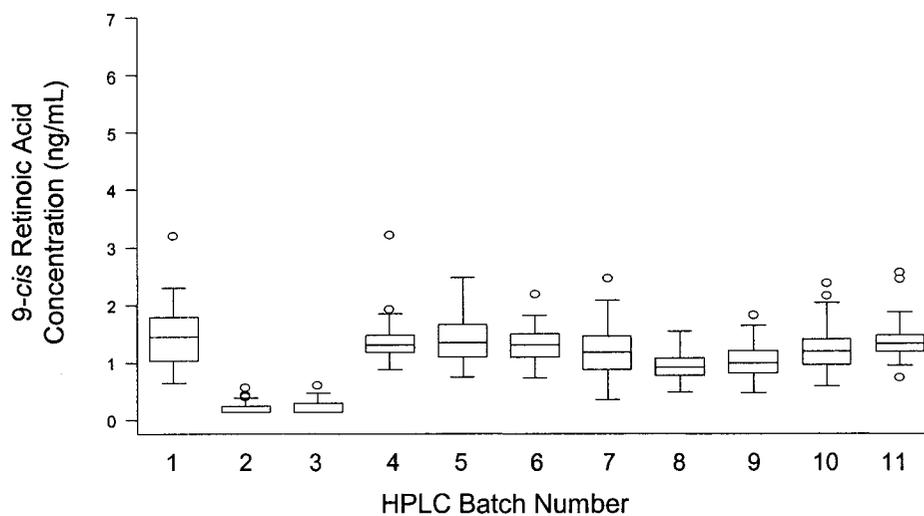
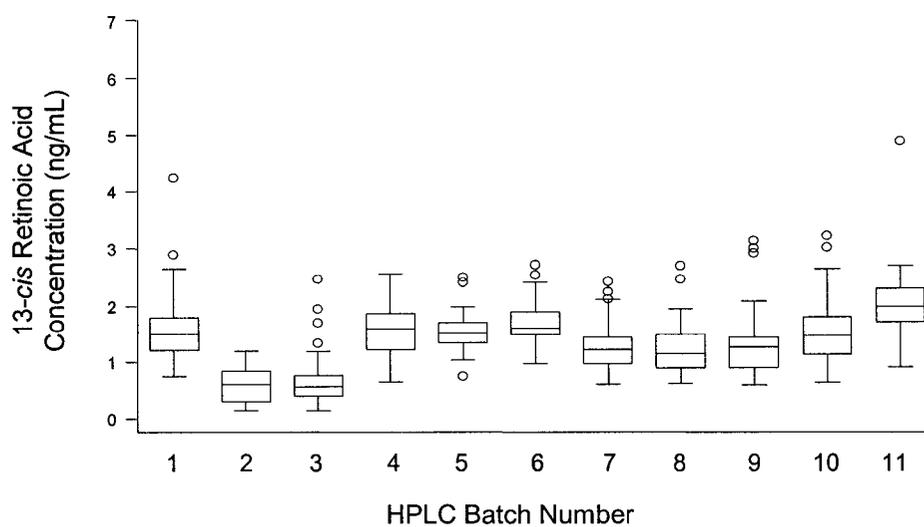
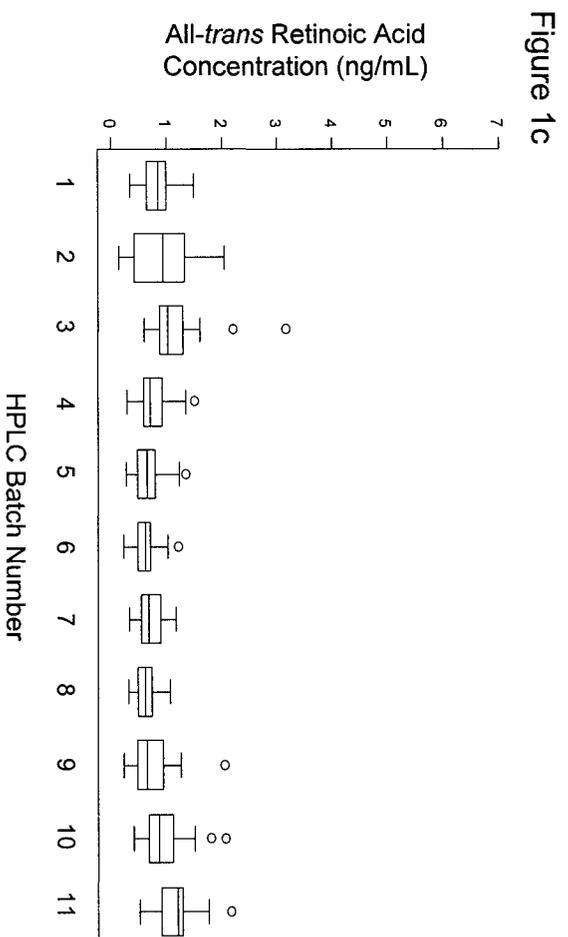


Figure 1b





**Table 7: Mean (SD) Retinoic Acid Concentrations by HPLC Laboratory Batch number<sup>1</sup>**

Laboratory Batch	N	Total Retinoic Acid (ng/mL)		13- <i>cis</i> Retinoic Acid (ng/mL)		9- <i>cis</i> Retinoic Acid (ng/mL)		all- <i>trans</i> Retinoic Acid (ng/mL)	
		Mean	SD	Mean	SD	Mean	SD	Mean	SD
1	122	3.921	1.205	1.549	0.758	1.512	0.613	0.853	0.305
2	158	1.779	0.699	0.588	0.387	0.217	0.118	0.938	0.541
3	190	1.961	0.862	0.622	0.468	0.220	0.136	1.093	0.443
4	168	3.783	0.953	1.622	0.509	1.381	0.388	0.780	0.329
5	136	3.644	0.972	1.560	0.450	1.419	0.405	0.660	0.318
6	152	3.638	0.879	1.680	0.496	1.332	0.350	0.621	0.271
7	152	3.109	0.943	1.221	0.528	1.181	0.437	0.705	0.273
8	138	2.949	0.950	1.295	0.724	0.977	0.278	0.676	0.266
9	152	3.058	1.129	1.348	0.801	1.012	0.316	0.695	0.370
10	180	3.704	1.004	1.504	0.636	1.256	0.409	0.944	0.423
11	72	4.820	1.448	2.105	0.853	1.468	0.508	1.246	0.512
Total	1620	3.194	1.268	1.316	0.727	1.042	0.588	0.828	0.415

<sup>1</sup> Oneway ANOVA, p-value < 0.001 for total retinoic acid and all isomers areAll retinoic acid, by batch

Table 8: Partial correlation coefficients, between and within-person variances, between/within ratio, and number of measurements for 80% reliability for serum carotenoids and tocopherols

	Partial Correlation <sup>1</sup>	Between- woman Variance <sup>2</sup>	Within- woman Variance <sup>2</sup>	Between / Within Ratio	Number for 80% Reliability <sup>3</sup>
Retinol	0.60 ***	0.0422	0.0279	1.51	3
Lutein	0.61 ***	0.1316	0.0802	1.64	3
Zeaxanthin	0.59 ***	0.1476	0.1023	1.44	3
cis-Zeaxanthin	0.58 ***	0.1726	0.1146	1.51	3
$\alpha$ -Cryptoxanthin	0.52 ***	0.1499	0.1409	1.06	4
$\beta$ -Cryptoxanthin	0.71 ***	0.5975	0.2562	2.33	2
Lycopene	0.42 **	0.2595	0.3051	0.85	5
cis-Lycopene	0.48 ***	0.2881	0.3089	0.93	5
$\alpha$ -Carotene	0.66 ***	0.3485	0.1767	1.97	2
$\beta$ -Carotene	0.72 ***	0.5376	0.2064	2.6	2
cis- $\beta$ -Carotene	0.35 *	0.0809	0.1491	0.54	8
$\alpha$ -Tocopherol	0.59 ***	0.0396	0.0261	1.51	3
$\gamma$ -Tocopherol	0.43 **	0.0547	0.0711	0.77	6

<sup>1</sup> Taken from Roe D, Siegel EM, Franco E, Papenfuss M, Marshall JR, Craft N, Villa L, Giuliano AR. Multiple measures of serum carotenoids in the Brazilian HPV Natural History Cohort: Implications for cohort analysis

<sup>2</sup> Partial Pearson's correlation coefficient adjusted for the time period in which the first measurement occurred.

<sup>3</sup> Linear mixed effects model was used to estimate the between- versus within-woman variability, adjusted for period.

<sup>4</sup> Number of replicates required to insure 80% reliability

\*  $p < 0.05$ , \*\*  $p < 0.01$ , \*\*\*  $p < 0.001$

**Table 9: Correlation matrix for total retinoic acid, retinoic acid isomers, retinol and  $\beta$ -carotene in seum samples take 4-months apart**

<b>Spearman Correlation Coefficient (time1, time2)</b>				
	Total Retinoic Acid (ng/mL)	13- <i>cis</i> RA (ng/mL)	9- <i>cis</i> RA (ng/mL)	All- <i>trans</i> -isomer (ng/mL)
Total Retinoic Acid (ng/mL)	----			
13- <i>cis</i> RA (ng/mL)	0.87 , 0.87 <.0001 , <.0001			
9- <i>cis</i> RA (ng/mL)	0.77 , 0.83 <.0001 , <.0001	0.54 , 0.63 <.0001 , <.0001		
All- <i>trans</i> - isomer (ng/mL)	0.36 , 0.43 <.0001 , <.0001	0.10 , 0.17 0.0245 , 0.0001	0.06 , 0.13 0.1734 , 0.0027	
Retinol ( $\mu$ g/mL)	0.21 , 0.17 <.0001 , 0.0001	0.13 , 0.11 0.004 , 0.0132	0.18 , 0.18 <.0001 , <.0001	0.14 , 0.14 0.0014 , 0.0017
Total $\beta$ - Carotene ( $\mu$ g/mL)	0.19 , 0.22 <.0001 , <.0001	0.12 , 0.15 0.0057 , 0.0009	0.16 , 0.22 0.0002 , <.0001	0.17 , 0.19 0.0002 , <.0001

**Table 10: Association between consumption of specific food and Serum Retinoic Acid levels**

	Total Retinoic Acid (ng/mL) (N=451)				13- <i>cis</i> Retinoic Acid (ng/mL) (N=451)					
	Low	High	Crude <sup>a</sup>	Adjusted <sup>b</sup>	Low	High	Crude <sup>a</sup>	Adjusted <sup>c</sup>		
	N	N	OR	OR (95% CI)	N	N	OR	OR (95% CI)		
<b>Liver</b>										
Never or < 1 time/year	70	29	1.00	1.00	Referent	64	35	1.00	1.00	Referent
≥ 1 time/year, < 1 time/mo.	57	26	0.88	0.72	(0.33 - 1.56)	59	24	0.63	0.60	(0.28 - 1.28)
1-3 times/month	103	74	1.72	1.67	(0.90 - 3.11)	110	67	1.24	1.19	(0.65 - 2.16)
≥ 1 time/week	50	42	1.74	1.59	(0.78 - 3.26)	49	43	1.59	1.63	(0.81 - 3.26)
<i>p for trend</i>				0.042				0.065		
<b>Orange</b>										
Never or < 1 time/month	29	14	1.00	1.00	Referent	25	18	1.00	1.00	Referent
1-3 times/month	57	26	1.34	1.57	(0.59 - 4.14)	54	29	1.01	1.05	(0.42 - 2.60)
1-3 times/week	117	71	1.61	1.86	(0.78 - 4.48)	121	67	1.00	1.05	(0.46 - 2.39)
≥ 4 time/week	78	60	2.49	2.64	(1.07 - 6.48)	83	55	1.32	1.29	(0.55 - 3.01)
<i>p for trend</i>				0.022				0.475		
<b>Lemon</b>										
Never or < 1 time/year	26	17	1.00	1.00	Referent	25	18	1.00	1.00	Referent
≥ 1 time/year, < 1 time/mo.	65	37	0.89	0.86	(0.34 - 2.19)	68	34	0.82	0.82	(0.33 - 2.02)
1-3 times/month	72	39	0.94	1.02	(0.40 - 2.56)	66	45	1.26	1.40	(0.58 - 3.37)
≥ 1 time/week	118	78	0.94	0.82	(0.35 - 1.96)	124	72	0.88	0.92	(0.40 - 2.09)
<i>p for trend</i>				0.701				0.935		
<b>Carrot</b>										
Never or < 1 time/month	48	24	1.00	1.00	Referent	45	27	1.00	1.00	Referent
1-3 times/month	54	42	1.22	1.07	(0.49 - 2.30)	62	34	0.76	0.77	(0.36 - 1.63)
1-3 times/week	135	75	0.92	0.78	(0.39 - 1.56)	139	71	0.75	0.77	(0.40 - 1.48)
≥ 4 time/week	44	30	0.98	0.79	(0.34 - 1.81)	37	37	1.50	1.42	(0.65 - 3.11)
<i>p for trend</i>				0.346				0.490		
<b>Pumpkin</b>										
Never or < 1 time/year	77	31	1.00	1.00	Referent	76	32	1.00	1.00	Referent
≥ 1 time/year, < 1 time/mo.	92	54	1.44	1.24	(0.65 - 2.36)	92	54	1.15	1.16	(0.62 - 2.18)
1-3 times/month	63	40	1.86	1.61	(0.81 - 3.20)	65	38	1.66	1.68	(0.86 - 3.29)
≥ 1 time/week	49	45	2.61	2.42	(1.18 - 4.96)	50	44	2.46	2.51	(1.27 - 4.97)
<i>p for trend</i>				0.011				0.004		

a Crude adjusted for Laboratory Batch

b Adjusted for Laboratory Batch, age (5 years), total number of pregnancies, OC use and economy

c Adjusted for Laboratory batch, age (5 years), season and economy

Table 10 - Continued

	Total Retinoic Acid (ng/mL) (N=451)				13- <i>cis</i> Retinoic Acid (ng/mL) (N=451)					
	Low N	High N	Crude <sup>a</sup> OR	Adjusted <sup>b</sup> OR (95% CI)	Low N	High N	Crude <sup>a</sup> OR	Adjusted <sup>c</sup> OR (95% CI)		
<b>Papaya</b>										
Never or < 1 time/year	33	17	1.00	1.00	Referent	30	20	1.00	1.00	Referent
≥ 1 time/year, < 1 time/mo.	49	27	1.05	0.86	(0.34 - 2.13)	49	27	0.85	0.84	(0.34 - 2.08)
1-3 times/month	70	34	0.69	0.56	(0.23 - 1.34)	73	31	0.61	0.63	(0.27 - 1.51)
1-3 times/week	93	63	1.26	1.08	(0.48 - 2.44)	96	60	1.06	1.05	(0.46 - 2.35)
≥ 4 time/week	36	30	1.49	1.31	(0.51 - 3.34)	35	31	1.53	1.59	(0.64 - 3.98)
<i>p for trend</i>				0.273				0.156		
<b>Spinach</b>										
Never or < 1 time/year	162	85	1.00	1.00	Referent	160	87	1.00	1.00	Referent
≥ 1 time/year, < 1 time/mo.	40	34	1.40	1.37	(0.73 - 2.58)	41	33	1.26	1.12	(0.61 - 2.06)
1-3 times/month	53	31	0.91	0.81	(0.43 - 1.53)	53	31	0.86	0.80	(0.43 - 1.48)
≥ 1 time/week	26	21	1.26	1.32	(0.62 - 2.82)	29	18	0.82	0.76	(0.36 - 1.62)
<i>p for trend</i>				0.821				0.379		
<b>Collard Greens</b>										
Never or < 1 time/year	43	24	1.00	1.00	Referent	43	24	1.00	1.00	Referent
≥ 1 time/year, < 1 time/mo.	68	37	0.70	0.74	(0.34 - 1.58)	67	38	0.79	0.87	(0.41 - 1.83)
1-3 times/month	75	64	1.46	1.68	(0.81 - 3.49)	82	57	1.21	1.38	(0.68 - 2.81)
≥ 1 time/week	95	46	0.71	0.81	(0.39 - 1.69)	91	50	0.91	0.93	(0.46 - 1.87)
<i>p for trend</i>				0.930				0.900		
<b>Broccoli</b>										
Never or < 1 time/year	122	71	1.00	1.00	Referent	121	72	1.00	1.00	Referent
≥ 1 time/year, < 1 time/mo.	49	35	1.02	1.04	(0.54 - 2.00)	57	27	0.68	0.67	(0.36 - 1.27)
1-3 times/month	67	42	0.89	0.79	(0.43 - 1.43)	65	44	1.05	1.01	(0.57 - 1.79)
≥ 1 time/week	43	23	0.84	0.77	(0.38 - 1.56)	40	26	0.92	0.86	(0.44 - 1.70)
<i>p for trend</i>				0.344				0.812		

a Crude adjusted for Laboratory Batch

b Adjusted for Laboratory Batch, age (5 years), total number of pregnancies, OC use and economy

c Adjusted for Laboratory batch, age (5 years), season and economy

Table 10 - Continued

	9- <i>cis</i> Retinoic Acid (ng/mL) (N=393)				All- <i>trans</i> Retinoic Acid (ng/mL) (N=502)					
	Low N	High N	Crude <sup>a</sup> OR	Adjusted <sup>d</sup> OR (95% CI)	Low N	High N	Crude <sup>a</sup> OR	Adjusted <sup>e</sup> OR (95% CI)		
<b>Liver</b>										
Never or < 1 time/year	59	29	1.00	1.00	Referent	84	28	1.00	1.00	Referent
≥ 1 time/year, < 1 time/mo.	46	27	1.00	0.76	(0.36 - 1.61)	59	29	1.32	1.10	(0.53 - 2.30)
1-3 times/month	92	62	1.20	1.09	(0.59 - 2.02)	130	71	1.47	1.34	(0.74 - 2.44)
≥ 1 time/week	36	42	1.90	1.73	(0.85 - 3.52)	61	39	1.63	1.50	(0.75 - 2.99)
<i>p for trend</i>				0.096				0.197		
<b>Orange</b>										
Never or < 1 time/month	20	17	1.00	1.00	Referent	33	14	1.00	1.00	Referent
1-3 times/month	51	23	0.50	0.58	(0.23 - 1.48)	65	28	1.65	1.99	(0.79 - 5.02)
1-3 times/week	98	68	0.78	0.89	(0.39 - 2.04)	139	68	1.51	1.63	(0.71 - 3.71)
≥ 4 time/week	65	52	1.03	1.00	(0.43 - 2.31)	97	58	1.65	1.68	(0.73 - 3.89)
<i>p for trend</i>				0.441				0.541		
<b>Lemon</b>										
Never or < 1 time/year	22	18	1.00	1.00	Referent	32	14	1.00	1.00	Referent
≥ 1 time/year, < 1 time/mo.	50	37	0.75	0.59	(0.24 - 1.45)	73	37	0.86	0.92	(0.38 - 2.24)
1-3 times/month	61	36	0.66	0.64	(0.26 - 1.54)	85	42	0.93	1.04	(0.43 - 2.48)
≥ 1 time/week	101	69	0.65	0.55	(0.24 - 1.25)	144	75	0.89	0.90	(0.39 - 2.04)
<i>p for trend</i>				0.265				0.823		
<b>Carrot</b>										
Never or < 1 time/month	44	20	1.00	1.00	Referent	54	27	1.00	1.00	Referent
1-3 times/month	47	39	1.59	1.54	(0.71 - 3.33)	70	37	0.79	0.83	(0.40 - 1.71)
1-3 times/week	107	72	1.28	1.13	(0.56 - 2.29)	151	77	0.81	0.90	(0.47 - 1.71)
≥ 4 time/week	36	29	1.49	1.31	(0.57 - 3.01)	59	27	0.60	0.60	(0.28 - 1.29)
<i>p for trend</i>				0.878				0.285		
<b>Pumpkin</b>										
Never or < 1 time/year	58	39	1.00	1.00	Referent	84	33	1.00	1.00	Referent
≥ 1 time/year, < 1 time/mo.	73	52	0.87	0.71	(0.38 - 1.32)	107	55	1.20	1.03	(0.56 - 1.91)
1-3 times/month	59	32	0.75	0.56	(0.28 - 1.09)	81	39	1.09	1.09	(0.57 - 2.08)
≥ 1 time/week	44	36	1.09	0.86	(0.43 - 1.74)	62	40	1.39	1.44	(0.74 - 2.80)
<i>p for trend</i>				0.485				0.283		

a Crude adjusted for Laboratory Batch

d Adjusted for Laboratory batch, age (5 years), race, and economy

e Adjusted for Laboratory batch, age (5 years), season, total number of pregnancies, and economy

Table 10 - Continued

	9- <i>cis</i> Retinoic Acid (ng/mL) (N=393)					All- <i>trans</i> Retinoic Acid (ng/mL) (N=502)				
	Low N	High N	Crude <sup>a</sup> OR	Adjusted <sup>d</sup> OR (95% CI)		Low N	High N	Crude <sup>a</sup> OR	Adjusted <sup>e</sup> OR (95% CI)	
<b>Papaya</b>										
Never or < 1 time/year	23	18	1.00	1.00	Referent	36	19	1.00	1.00	Referent
≥ 1 time/year, < 1 time/mo.	40	26	0.82	0.53	(0.21 - 1.33)	58	25	0.77	0.78	(0.32 - 1.90)
1-3 times/month	57	37	0.82	0.63	(0.27 - 1.48)	79	38	0.77	0.76	(0.33 - 1.73)
1-3 times/week	76	57	0.90	0.62	(0.27 - 1.40)	118	57	0.81	0.82	(0.37 - 1.80)
≥ 4 time/week	38	22	0.79	0.48	(0.19 - 1.25)	43	29	1.31	1.51	(0.61 - 3.70)
<i>p for trend</i>				0.312					0.373	
<b>Spinach</b>										
Never or < 1 time/year	128	85	1.00	1.00	Referent	178	101	1.00	1.00	Referent
≥ 1 time/year, < 1 time/mo.	42	26	0.83	0.70	(0.37 - 1.33)	56	26	0.85	0.87	(0.46 - 1.64)
1-3 times/month	41	30	0.91	0.63	(0.33 - 1.19)	67	24	0.47	0.50	(0.27 - 0.94)
≥ 1 time/week	23	19	1.04	0.86	(0.41 - 1.83)	33	17	0.94	1.03	(0.49 - 2.15)
<i>p for trend</i>				0.295					0.258	
<b>Collard Greens</b>										
Never or < 1 time/year	37	25	1.00	1.00	Referent	60	18	1.00	1.00	Referent
≥ 1 time/year, < 1 time/mo.	56	34	0.63	0.65	(0.30 - 1.39)	76	40	1.41	1.49	(0.70 - 3.18)
1-3 times/month	66	57	0.98	0.89	(0.43 - 1.86)	93	57	1.94	2.28	(1.09 - 4.76)
≥ 1 time/week	75	44	0.67	0.65	(0.31 - 1.34)	105	53	1.30	1.35	(0.66 - 2.77)
<i>p for trend</i>				0.435					0.472	
<b>Broccoli</b>										
Never or < 1 time/year	96	70	1.00	1.00	Referent	143	72	1.00	1.00	Referent
≥ 1 time/year, < 1 time/mo.	44	28	0.79	0.73	(0.38 - 1.41)	59	34	0.94	0.94	(0.52 - 1.72)
1-3 times/month	53	43	1.07	0.87	(0.48 - 1.58)	89	30	0.55	0.56	(0.31 - 1.00)
≥ 1 time/week	41	19	0.69	0.55	(0.27 - 1.12)	43	32	1.82	1.76	(0.91 - 3.38)
<i>p for trend</i>				0.156					0.734	

a Crude adjusted for Laboratory Batch

d Adjusted for Laboratory batch, age (5 years), race, and economy

e Adjusted for Laboratory batch, age (5 years), season, total number of pregnancies, and economy

**Table 11: Comparison of Serum Retinoic Acid Values by Visit Date<sup>1</sup>**

	July-August 1994 (N=29)			July-August 1995 (N=24)			P-value <sup>2</sup>
	Mean $\pm$ SD	Median	Range	Mean $\pm$ SD	Median	Range	
Total Retinoic Acid (ng/mL)	3.81 $\pm$ 0.84	3.81	2.12 - 5.31	3.00 $\pm$ 0.69	2.85	2.03 - 4.26	0.050
<i>Cis</i> -isomer							
13- <i>cis</i> RA (ng/mL)	1.71 $\pm$ 0.50	1.61	0.76 - 2.57	1.18 $\pm$ 0.40	1.13	0.69 - 2.48	0.009
9- <i>cis</i> RA (ng/mL)	1.37 $\pm$ 0.27	1.40	0.76 - 1.84	1.07 $\pm$ 0.33	1.02	0.50 - 1.67	0.029
All- <i>trans</i> -isomer (ng/mL)	0.71 $\pm$ 0.29	0.67	0.28 - 1.36	0.74 $\pm$ 0.23	0.67	0.33 - 1.18	0.270
Retinol ( $\mu$ g/mL)	0.44 $\pm$ 0.06	0.43	0.29 - 0.54	0.45 $\pm$ 0.09	0.45	0.28 - 0.65	0.100
Total $\beta$ -Carotene ( $\mu$ g/mL)	0.05 $\pm$ 0.03	0.04	0.02 - 0.13	0.03 $\pm$ 0.01	0.03	0.02 - 0.06	0.570

	May-June 1995 (N=35)			May-June 1996 (N=46)			P-value <sup>2</sup>
	Mean $\pm$ SD	Median	Range	Mean $\pm$ SD	Median	Range	
Total Retinoic Acid (ng/mL)	3.51 $\pm$ 0.68	3.26	2.56 - 5.15	3.75 $\pm$ 1.08	3.77	1.96 - 6.66	0.640
<i>Cis</i> -isomer							
13- <i>cis</i> RA (ng/mL)	1.52 $\pm$ 0.34	1.49	0.90 - 2.43	1.65 $\pm$ 0.64	1.57	0.79 - 3.23	0.962
9- <i>cis</i> RA (ng/mL)	1.32 $\pm$ 0.37	1.25	0.74 - 2.46	1.16 $\pm$ 0.30	1.18	0.60 - 2.00	0.057
All- <i>trans</i> -isomer (ng/mL)	0.68 $\pm$ 0.18	0.66	0.41 - 1.11	0.93 $\pm$ 0.33	0.96	0.41 - 1.80	0.002
Retinol ( $\mu$ g/mL)	0.44 $\pm$ 0.13	0.45	0.24 - 0.95	0.46 $\pm$ 0.12	0.44	0.27 - 0.93	0.960
Total $\beta$ -Carotene ( $\mu$ g/mL)	0.03 $\pm$ 0.02	0.03	0.01 - 0.06	0.02 $\pm$ 0.01	0.02	0.01 - 0.05	0.001

1. Analysis does not include Batched 2 and 3

2: P-values testing differences in Median Rank between periods (Ranksum)