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Validating Resonance Raman Spectroscopy: a Non-invasive Assessment of Skin Carotenoids as a Biomarker of Fruit and Vegetable Intake in Children

by

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A thesis submitted in partial fulfillment of the requirements for the

Degree of

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in

Health and Human Movement

Approved:

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Abstract

Background: Adult studies have found a strong correlation between serum carotenoids and skin carotenoids measured by resonance Raman spectroscopy (RRS). No published studies have examined correlations between skin and serum carotenoids among children.

Objectives: (1) To validate skin RRS methodology against serum carotenoid measurements by high-performance liquid chromatography and (2) to determine if RRS skin carotenoids can be used as a valid biomarker of total fruit and vegetable (FV) intake among children.

Design: Participants were 45 healthy children age 5-17 who provided 3 blood samples used to assess serum carotenoid concentrations and 3 RRS skin measurements (using a Biophotonic Scanner™) within a 4 week period. Dietary intake of FV was assessed 3 times within 4 weeks using a 27 item food frequency questionnaires (FFQ) and the ASA24™-Kids, an automated multiple-pass 24-hour recall (24HDR). Estimates of intake from three FFQ, completed at least 7 days apart, were averaged. Estimates of intake from 24HDR were collected on 2 weekdays and a weekend day and averaged.

Results: Levels of skin and serum carotenoids were highly correlated (R^2=.63, p<.001). A linear regression model predicted that for every unit increase of total FV from FFQ and total FV as assessed by 24HDR, scanner score was predicted to increase by 3,798 (p=.001) and 3,504 (p=.001), respectively. Similar results were observed for reported high carotenoid vegetables intake. For each milligram of consumed beta-carotene, total carotenoids, and alpha-carotene, there was an increase in scanner score of 3,354 (p=.011), 4,556 (p=.008), and 12,299 (p=.002), respectively.

Conclusions: Skin carotenoids measured by RRS were strongly correlated with serum carotenoid levels and were positively associated with estimates of intake from FFQ and ASA24™-Kids among children age 5-17. Skin carotenoids may be used as biomarker of FV intake among children.

Introduction

Accurately measuring fruit and vegetable (FV) intake in children is imperative to assess changes in consumption behavior. Collecting accurate food recalls in young children has historically been difficult due to their immature cognitive ability and reporting skills (1). Current tools for collecting food recalls in children are time and labor intensive. Food frequency questionnaires (FFQ) are the easiest to implement. However, recalls in children younger than 10 years have poor correlation to actual intake (2). A more accurate method, 24-hour recall (24HDR), is more time and labor intensive and not valid in children age 9 and younger (3).

Carotenoids are pigments found in fruits and vegetables that are important bioactive nutrients for humans (4). Concentration of serum carotenoids is correlated with fruit and vegetable intake in adults (5), but measuring serum carotenoids requires blood and measures are sensitive to day-to-day variation in carotenoid consumption. A non-invasive method to measure skin carotenoids utilizes resonance Raman spectroscopy (RRS). Past research has validated this measure against serum/plasma carotenoids in adults (5, 6) and against FV consumption in adults (5) and it has been used as a biomarker of change in FV intake in adults.

Others have found concentration of carotenoids from RRS positively correlated with FV consumption in children (7, 8). However, currently there are no published studies looking at the comparison of carotenoids in blood to RRS in children which is needed to validate this tool as a biomarker of FV intake in children.

The purpose of this study was to measure the correlation between skin and serum carotenoids and the correlation of these biomarkers to reported FV intake from 24HDR

and FFQ in children.

Subjects and Methods

Power calculations were computed using a correlation of 0.35 between the FFQ and the serum carotenoid concentrations. This is the approximate correlation identified in studies of adults (9) and is the lowest expected correlation of the comparisons we are proposing. A sample of 44 was estimated to give us approximately 90% power (alpha = 0.01, two-tail) to detect a correlation of at least 0.35.

Subjects

A total of 47 healthy Cache County, Utah school children ages 5-17 were recruited through local elementary and secondary schools. The Institutional Review Board at Utah State University reviewed and approved the research protocol. A letter of information was sent via email to parents of two local elementary schools (see Appendix B). Older children were recruited by word of mouth and from siblings of elementary school children. Researchers recruited a purposeful sample of approximately 9% (5) children from each grade k-12, 45% of which were male. The ethnicity of the participant population reflected the ethnicity of the local school-age population (16% Hispanic, 6% Asian, 2% Pacific Islander, and 76% Caucasian). Parents of children completed a qualifying online survey (Appendix C). If the child met the study inclusion/exclusion criteria, the parents were mailed/emailed and asked to complete a Center for Human Nutrition Studies (CHNS) health history questionnaire (Appendix D) which includes past and present medical history, past and current medication, and nutritional supplement use. Children were excluded if they had a health history or habits that were known to affect

carotenoid levels (10). These exclusions included major illness in the two weeks before the study began, use of topical self-tanning lotion, chronic disease such as asthma or type 1 diabetes, and sun exposure for more than two hours per day without use of sun screen. Three children were excluded from the study due to a reported history of asthma.

Researchers obtained parent consent and child assent from participants in person. Participants were asked to maintain their normal lifestyle including activity, supplement use, and dietary habits for the duration of the study. Participants received $20 at each of the three clinic visit and a $25 bonus for completing the study for a total of $85.

Protocol

Participants completed three clinic visits scheduled in the morning seven days apart. Prior to the visit, the children completed a 10 hour overnight fast from eating or drinking anything except water. During the first clinic visit, height was measured using a Seca 223 digital stadiometer (Hangzhou, China) and weight was measured using a Detecto 758C digital scale (Webb City, MO). Height and weight were used to calculate BMI (weight in kg/height in meters^2). BMI was categorized based on age and gender percentiles. At each clinic visit the following occurred. An 8 ml blood sample was collected from an arm vein into untreated glass vacutubes. Tubes were protected from the light to minimize light-induced degradation of serum carotenoids. Within 15 minutes of blood sampling, skin carotenoid concentrations were measured by trained researchers using the BioPhotonic Scanner^{TM} (Pharmanex, LLC), a portable RRS devise. The child placed his/her palm against the light window of the scanner and held it there for 90 seconds. The scanner emitted a light and displayed a score in Raman counts of 0-

70,000+. Each child was scanned twice using the same scanner at all three clinic visits. At each visit, if there was more than 2,000 Raman counts difference between scanner scores, they were scanned a third time. This was done to minimize the individual variation in scanner score. The two scanner scores that were within 2,000 Raman counts were averaged for the true score. Children and parents were blinded to the results of their scanner score.

Participants completed a computer assisted 24HDR (ASA24™-Kids automated multiple-pass method), a 27-item one week look-back FFQ (11) (Appendix E), and health check list which reported lifestyle questions regarding the prior 7 days including hours of sun exposure, smoke exposure, supplement use, illness, and use of any new medications since prior clinic visit (See Appendix G). Parents assisted participants under the age of 10 to complete these forms.

Dietary Assessment

For our FFQ, we used a modified form of a beverage and snack questionnaire (BSQ) developed and validated by Neuhouser et al. (11). The BSQ asks children to report how often they consumed a list of fruits, vegetables, snacks, and beverages over the past week. For purposes of this study, we modified this BSQ by adding additional questions about HCV and condensing snack items into more general categories. Our modified BSQ tool contained 27 items compared to the 19 item tool used by Neuhouser.

We collected multiple 24HDR to assess total habitual dietary intake, including two weekdays and a weekend day, at least 7 days apart, within our four week study. We used the National Cancer Institute ASA24™-Kids (12). The ASA24™ is based upon the

USDA Automated Multiple-Pass Method (AMPM) system involving five requests for intake data across the same 24HDR. Participants age 10 and older and parents of younger children completed automated multiple-pass method recalls online. The first recall was completed during a clinic visit. Subsequent recalls were completed at home and the completion verified during clinic visits.

Resonance Raman Spectroscopy

The RRS unit used in this research was the Biophotonic Scanner™, NuSkin, LLC. The carotenoid level was determined by calculating the average height of the peak Raman absorbance signal obtained and quantified from excitation of skin carotenoids using a low-intensity blue (λ=473 nm) LED light with green light (510 nm) detection (13). Skin carotenoids were reported as Raman intensity counts. The higher the count, the higher the concentration of carotenoid molecules detected at the site of measurement.

Serum Carotenoids by HPLC

Serum carotenoids were measured in the laboratory using high-performance liquid chromatography (HPLC) (14). They were extracted with hexane after the addition of α-tocopheral acetate as an internal standard. Extracts were dried under N_2 and resuspended in mobile phase. Carotenoids were quantified using a Shimadzu AT-20A HPLC equipped with an autosampler and diode array detector. Separation of the carotenoids was achieved by employing a Kromasil C18 5 μM 25 x 4.6 cm column. The resulting chromatograph allows for the quatification of β-carotene, lycopene and lutein.

The samples were prepared in a room with reduced light to minimize photodegradation of the carotenoids. The samples were measured in duplicate. All

chromatographic runs included quality control material to ensure comparability between analytical runs.

Statistical Analysis

Descriptive statistics including *t* tests and ANOVA were used to examine differences to describe the distribution of assessments and characteristics of the study population and to compare means across subgroups (age, sex, ethnicity, and BMI). We examined the association of potential confounding factors including smoke exposure, illness, and sun exposure to both skin and serum carotenoids. We tested for linearity by visual inspection of the scatter plots. To determine the normality of the data, we conducted Levene's statistics for all of our descriptive statistic calculations. For the linear regression analysis, we confirmed that skewness and kurtosis statistics were $\leq \pm 1$. Outliers within independent variables were top-coded to the next closest value. This was done by converting the scores to Z values (15). Z values that were above the range of 1.96, or one standard deviation, were changed to be the next value closest to 1.96. This was done to be able to retain the outliers in the data set. Two to four results were top-coded for each independent variable (averaged total FV from FFQ and 24HDR; averaged total HCV from FFQ and 24HDR; beta-carotene, alpha-carotene, lycopene, and total carotenoids from 24HDR; averaged total serum carotenoids, serum lycopene, and serum beta-carotene; weekly and total averaged scanner scores and serum carotenoids). Intraclass correlation coefficients (ICC) were used to assess how well the scanner scores were correlated over multiple scannings obtained at one visit.

Linear regression models were used to control for the variation in scanner scores between scanner units. We explored other potential factors to refine this model including age, sex, ethnicity, BMI, and weight. Weight, but not BMI, was significant so we included weight in the model along with scanner unit when exploring associations between our independent and dependent variables. Average scanner score or serum carotenoids served as the dependent variables. This linear regression model was created to examine associations between assessments including the following: Weekly counts of RRS and the concentration of weekly serum carotenoids; total averaged counts of RRS intensity and total averaged concentration of serum carotenoids, RRS intensity and serum carotenoids and diet assessment of FV consumption and serum carotenoids. Statistics were conducted using SPSS software 20.0 with a P value of 0.05 or below to be considered statistically significant.

Results

Of the 47 children, 2 were missing one or more assessments and were excluded. One child was unable to complete the study due to time conflicts and one was unable to complete 2 of the 3 blood draws. The average age for participants was 10.5 years.

There were no differences in mean scanner scores and serum carotenoids by gender, age groups, or BMI groups, report of illness or sun exposure. (**See Table 1**). There was a difference in mean serum carotenoids and HCV consumption from 24HDR between ethnicity groups. In addition, children from grades 6-8 reported significantly more HCV intake on FFQ than children in grades K-5. No child reported having smoke exposure in the home.

TABLE 1

Resonance Raman spectroscopy (RRS), serum carotenoid (SC), fruit and vegetable (FV) and high carotenoid vegetable (HCV) intake by baseline characteristics of the study population (n=45).

	N (%)	**RRS score[1]**	**SC value[2]**	**FFQ FV[3]**	**FFQ HCV[4]**	**24HDR FV[5]**	**24HDR HCV[6]**
Grade							
K-5th	25 (55)	33162	.59	1.5	.5	1.9	.41
6-8th	12 (25)	29161	.66	1.75	.65*	2.3	.42
9-12th	8 (19)	28676	.49	1.9	.75	2.7	.61
Sex							
Male	20 (45)	30909	.53	1.7	.65	2.2	.43
Female	25 (55)	31743	.67	1.5	.55	2	.47
Ethnicity							
Caucasian	34 (76)	30575	.55	1.5	.55	2	.34
Hispanic	7 (16)	31547	.67	1.9	.75	2.5	.73
Asian	3 (6)	43283	.95*	2.1	.90	2.4	1.1**
Pacific Is.	1 (2)	18866	.31	2.7	.95	4.6	.35
BMI							
>85%ile	7 (16)	29180	.52	1.75	.70	2.6	.36
<5%ile	4 (8)	29015	.55	1.45	.48	1.7	.18
5-85%	34 (76)	31797	.61	1.65	.60	2.1	.49
Mean Sun Exposure/day							
0-29 Min	5 (11)	35973	.57				
30-60 Min	24 (54)	31891	.58				
1-2 Hours	15 (33)	25580	.49				
>2 Hours	1 (2)	25032	.73				
Illness							
Yes	13 (29)	33598	.60				
No	32 (71)	30299	.59				

[1] Mean RRS palm measures of skin carotenoids at 3 time points.
[2] Mean SC (µg/ml) measures at 3 time points.
[3] Mean FV servings from 3 food frequency questionnaires
[4] Mean HCV servings from 3 food frequency questionnaires
[5] Mean FV servings from 3, 24-hour recalls
[6] Mean HCV servings from 3, 24-hour recalls
[*] $P \leq .05$, ** $P \leq .01$

Skin carotenoids compared to serum carotenoids

The duplicate or triplicate (if scores differed by more than 2000 units) scanner scores from each clinic visit were highly correlated (Week 1 ICC = 0.99; $p<0.001$; Week 2 ICC=.98, $p<.001$; Week 3 ICC=.99, $p<.001$) and so these scanner scores were averaged. In addition, averaged scanner scores from visit 1, 2, and 3 were highly correlated (ICC=.98, $p<.001$) so these scores were averaged to create the variable average scanner score. Serum carotenoids were also highly correlated between visits (ICC=.88, $p<.001$) so these scores were averaged to create the average serum level. Using linear regression models we observed skin carotenoid levels to be highly correlated with serum carotenoids at each clinic visit (Visit 1: R^2=.49, $p<.001$; Visit 2: R^2=.56, $p<.001$; Visit 3: R^2=.56, $p<.001$). The averaged skin and averaged serum carotenoid levels were also highly correlated (R^2=.63, $p<.001$).

Skin carotenoids compared to diet

Using multivariable linear regression models that controlled for scanner unit and weight of child, we observed that reported FV and HCV consumption for the FFQ was correlated with skin carotenoid levels. The results are listed in **Table 2**. Each serving of averaged total FV and HCV reported from the FFQ was associated with a 3,798 (p=.001) and 6,355 (p= .03) unit increase in scanner score, respectively. The results from the FV intake estimated from the 24HDR were similar. From the 24HDR averages, for each cup of averaged total FV and HCV, scanner score increased 3,504 (p=.001) and 10,820 (p=.027) respectively. Carotenoids consumption was also estimated from the 24HDR. For every milligram of consumed beta-carotene, total carotenoids, and alpha-carotene

there was an increase in scanner score of 3,354 (p=.01), 4,556 (p<=.008) and 12,299 (p=.002) respectively. Lutein and zeaxanthin results were reported combined and did not correlate with averaged scanner score. Lycopene from 24HDR was not significantly associated with scanner score.

Table 2

Multivariable Linear regression analysis of the R^2 and Beta representing the relationship between total carotenoids measured by resonance Raman spectroscopy (RRS) and total carotenoids in serum (SC) and FV and carotenoids intake estimated from FFQ and 24HDR after controlling for weight and scanner unit.

	RRS Model R^2	RRS β	SC Model R^2	SC β
FFQ FV	.32***	.49	.18*	.39
FFQ HCV	.21*	.39	.14	.33
24HDR FV	.31***	.48	.08	.24
24HDR HCV	.21*	.33	.13	.30
24HDR TC	.25**	.40	.23**	.46
24HDR lycopene	.19	.29	.09	.37
24HDR α carotene	.30**	.45	.18	.38
24HDR β carotene	.24**	.39	.23*	.28
24HDR LZ	.13	.14	.09	.23

FFQ, food frequency questionnaire; 24HDR, 24-hour daily recall; HCV, high-carotenoid vegetables; LZ, lutein + zeaxanthin; β, standard coefficient.

*p<.05; **p<.01; ***p<.001

Serum carotenoids compared to diet

Averaged total serum carotenoids were significantly correlated with the averaged total FV but not HCV from FFQ. Averaged total serum carotenoids did not significantly

correlate with total FV or HCV from 24HDR.

Total carotenoid and beta-carotene levels from the 24HDR correlated significantly to total serum carotenoids levels. However, serum lycopene did not. Since lutein was combined with zeaxanthin in the 24HDR, correlations with serum lutein were not included in the model. Serum beta carotene significantly correlated to beta carotene intake from the 24HDR (R^2= .27, $p<.05$); serum lycopene did not correlate significantly to lycopene from 24HDR. Correlations of serum lutein and lutein + zeaxanthin from 24HDR were not included in the model.

Discussion

Our findings indicate that the RRS skin carotenoid concentrations may be a valid and reliable indicator of serum concentrations of carotenoids and total FV intake among children. Skin carotenoid levels were positively correlated (R^2=.63, $p<.001$) to serum carotenoid concentrations measured by HPLC among children. Similarly, skin carotenoid levels were positively correlated to total and carotenoid containing FV intake as assessed from both a FFQ and multiple 24HDR (Table 2). Skin carotenoids may provide a useful indicator of usual FV intake among children, a group for which it is difficult to obtain accurate information about usual dietary intake.

This magnitude of correlation observed for skin carotenoids measured from RRS and serum carotenoids measured with HPLC is similar to that observed among adults. (5, 6). This is the first report of these correlations among children. We found skin carotenoid levels were approximately normally distributed in contrast to serum carotenoid concentrations, which are known to be skewed due to their relatively short half-life and

the large influenced from carotenoids consumed in the previous meal (16). Skin carotenoids are less influenced by daily changes in dietary FV intake (13). Using RRS to assess skin carotenoids is fast, inexpensive, compact, and non-invasive, making it possible to use this technology outside of the laboratory setting. Thus, RRS may be a preferred method of assessing carotenoid levels and may better represent average FV intake among children.

We also observed high correlations between skin carotenoids and total FV and HCV from FFQ and 24HDR when this data was collected in a clinic setting with adequate time, staff and parental support, and resources. As discussed earlier, collecting accurate food recalls in young children has historically been difficult due to their immature cognitive ability and reporting skills (2). Obtaining food recalls is also challenging in a school setting due to the time and resources required. The results from this study suggest that RRS may be a reliable biomarker of FV intake and could be used in place of or in addition to food records. This may be especially important and useful in school-based studies where FV intake is the outcome of interest or where interventions aim to increase FV intake over a period of time.

This study had strengths and limitations. Most important, this is, to our knowledge, the first study to directly compare RRS measures of skin carotenoids against HPLC analyses of serum carotenoids in children and serves as a validation of the RRS method as an indicator of carotenoid status among children. We tested reliability of this method by testing multiple measures of skin carotenoids at each assessment period and over time. We observed little within person variation in these measures which suggests the method is reliable over repeated measures. We did not find any significant mean

differences in skin or serum carotenoid levels between sex, BMI, sun exposure, or illness. This could be due to the following: We had few children in the high or low BMI groups, the study took place in late fall when sun exposure was minimal, and we did not qualify illness as to the severity or duration. Other limitations may include limited diversity of the sample in terms of race/ethnicity and tobacco exposure.

Based on the present study, we conclude that RRS is valid and reliable as a biomarker of nutritional status for children, supporting its future use as a biomarker for translational research studies.

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Appendix A

Literature Review

Carotenoids

Carotenoids are some of the most abundant antioxidant nutrients present in FV. Carotenoids may be responsible for many of the protective effects of a diet high in FV against cardiovascular disease, certain cancers, and age-related macular degeneration (During, 2004). Carotenoids are a family of some 600 nutrients made up of a common polyisoprenoid structure containing 40 carbon atoms and a system of conjugated double bonds (1,2). The most common dietary carotenoids are beta carotene, alpha carotene, lycopene, lutein, beta cryptoxanthin, and zeaxanthin (3). Beta carotene, alpha carotene and lycopene are provitamin A carotenoids, which means they can be converted in the body to retinol (Vitamin A) (2). Carotenoids are measured in serum using HPLC (4). Fruits and vegetables that have flesh that is deep orange, yellow, red or green have a high carotenoid content include winter squash, carrots, tomatoes, broccoli, kale, spinach, and watermelon.

These essential nutrients provide antioxidant protection and intercellular communication (5). On a biochemical level, increased carotenoid intake has been shown to reduce the pro-inflammatory cytokine, C-reactive protein, in only four weeks (6). Furthermore, higher levels of serum carotenoids appear to be protective against the development of adiposity and metabolic syndrome (7). Taken together, carotenoid status appears to be an indicator of a healthy diet including FV intake, longevity, and antioxidant status.

Serum/plasma Carotenoids as a Biomarker of Fruit and Vegetable Intake

Serum carotenoids have been proposed as a biomarker for usual FV intake (8, 9). In over 3,000 participants in the EPIC study, plasma carotenoids strongly correlated with reported intakes of FV (10). Beta-Cryptoxanthin was most strongly correlated with total fruits (FFQ $r = 0.52$, 24HDR $r = 0.39$, $p<.05$), lycopene with tomato and tomato products (FFQ $r = 0.38$, 24HDR $r = 0.25$, $p<.05$), and alpha-carotene with intake of root vegetables ($r = 0.39$, $p<.05$). In a multi-site study (11) using a 36 item FFQ and 24HDR, FV intake was correlated to total (FFQ $r=.35$, 24HDR $r=.37$, $p<.05$). Additionally, in a study with over 3,000 participants, FV intake as assessed by FFQ correlated to specific serum carotenoids (β-carotene, $r=.26$, alpha carotene, $r=.3$, lycopene, $r=.28$, β-cryptoxanthin, $r=.46$, $p<.05$) (22).

There are known limitations to measuring serum carotenoid levels. Al-Delaimy (12) found that serum carotenoid levels are sensitive to seasonal variation, smoking, stress, illness, and alcohol consumption. Additionally, Body Mass Index (BMI) was inversely correlated to serum carotenoid levels (12). Later analysis found that zeaxanthin and B-Cryptoxanthin levels were not affected by BMI (13).

Research in children found similar correlations between blood carotenoid levels and reported FV intake. In children ages 5-12, Burrows (14) found a significant correlation between reported dietary intake of carotenoids and plasma carotenoid concentrations ($r=.51$ alpha carotene, .32 beta carotene, .56 lutein, $p<.05$). Total carotenoid intake as assessed from FFQ ($r=.46$, $p<.05$) and 24HDR ($r=.45$, $p<.05$) correlated to serum carotenoids (15). Lower scanner scores were found in lower socio-

economic status pre-school children (16), though no statistically significant difference was found based on sex or ethnicity.

RRS as a Non-invasive Assessment of Skin Carotenoids in Adults

RRS is a form of vibrational spectroscopy used to identify and quantify chemical compounds. Carotenoid molecules are especially suitable for Raman measurements since they can be excited with light overlapping their visible absorption bands (17). RRS spectroscopy appears well suitable for quantitative detection of carotenoid antioxidants in living human tissue (18). The RRS unit to be used in this research, the Biophotonic Scanner™, was developed by Pharmanex, now a division of NuSkin. The instrument uses two light-emitting diodes (LEDs) for dual-wavelength excitation and four photomultiplier tubes for multichannel detection. It uses high sensitivity detectors and a high throughput optical system, making it possible to use light sources for RRS in a commercial setting. The carotenoid level is determined by calculating the average height of the peak Raman absorbance signal obtained and quantified from excitation of skin carotenoids using a low-intensity blue (gamma=473 nm) LED light with green light (510 nm) detection (19). Skin carotenoids are reported as Raman intensity counts. The higher the count, the higher the concentration of carotenoid molecules detected at the site of measurement.

The LED RRS instrument is strongly correlated with laser based RRS measurements (20). A typical measurement involves the placement of the palm of the hand against the window of the module and exposing the palm to the LED light for about 90 seconds.

Previous research has found that environmental and physical influences affect RRS scanner scores. A study by Darvin (21) that scanned ten people every work day for a year, found that skin carotenoid levels as assessed by RRS are sensitive to seasonal variation (1.26 higher in summer/fall). Smoking, stress, illness, and alcohol consumption decreased Raman counts and FV intake increased RRS counts. Additionally, Body Mass Index (BMI) was inversely correlated to skin carotenoid levels (21).

Comparison of Skin Carotenoids to Serum Carotenoids

Measures of skin carotenoids by RRS are non-invasive and are well correlated with total serum carotenoid levels in adults (18). In a study of 378 adults, Zidichouski, et al. (22) compared serum carotenoids to RRS three times within an 8-day period. Consistent positive correlations were observed for each of the 3 separate same-day correlation plots ($r=0.80, 0.81, 0.82$; $p<.001$). In addition, RRS skin carotenoid methodology had 0.9% less variance over the 3 tests than serum carotenoids by the HPLC method ($p<0.03$). Mayne et al. (23) compared serum carotenoid levels in 28 participants to skin RRS and skin carotenoids from skin punch biopsy assessed by HPLC. Total skin carotenoids assessed by RRS were significantly correlated with total skin carotenoids assessed by HPLC of dermal biopsies ($r=0.66$; $p<.0001$). Total carotenoid level in skin was also significantly correlated with total carotenoid level in plasma ($r=0.62$, $P<.006$). Currently there are no published studies looking at the comparison of serum or plasma carotenoids to RRS in children.

Skin Carotenoids as a Biomarker for Fruit and Vegetable Intake

Cross-sectional population studies among adults have demonstrated that skin carotenoid levels are higher in individuals with higher fruit and vegetable intake (8, 22-25). Measures of skin carotenoid levels by RRS has the potential to provide valuable data regarding FV intake and its interaction with other environmental factors on health outcomes employing a non-invasive tool suitable for children of all ages. In a study of preschool age children (16), higher skin carotenoid status, measured by RRS, was positively associated with parent-reports of FV consumption ($P=0.02$) and FV preference ($P<0.01$). Skin carotenoids (RRS) correlated in children to reported carotenoid intake ($r=.2$ lutein and zeanthine; $r=.42$ β-cryptoxanthin, $P<.01$) (24, 26).

24-hour Recall Measure in Children

Multiple 24HDR are the preferred method in assessing total habitual dietary intake because they provide the following: (1) estimates of actual intake (as opposed to relative ranking); (2) details on meal and snack consumption; (3) day-of-week consumption; (4) and other correlates of dietary intake (2). A review that looked at 15 studies of children found that the most accurate food recall includes three 24HDR, including two different week days and one weekend day when compared to doubly labeled water (14). The multiple-pass method was developed in 1999. This method is a 5-step dietary interview that includes multiple passes through what the respondent consumed during the 24 hours of the previous day. Initially, a paper-and-pencil version of the method was used in observational validation studies in women (27) and men (28).

A large validation study, Observing Protein and Energy Nutrition (OPEN), used biomarkers to validate this multiple-pass method (29).

Use of 24HDR is limited due to administration time and expenses. The National Cancer Institute recently made available a 24HDR program that is web-based and available for free to researchers. ASA24™ consists of a respondent website used to collect recall data in English or Spanish and a researcher website used to manage study logistics and obtain data analyses. ASA24™ is based upon the USDA Automated Multiple-Pass Method (AMPM) system involving five requests for intake data across the same 24HDR (30). A similar web-based application has been used by researcher since 2002 to collect 24HDR in What We Eat in America (WWEIA), the dietary interview component of the National Health and Nutrition Examination Survey (NHANES) (31). This method has been validated and shown to accurately estimate mean total energy and protein intakes compared to recovery biomarkers (29, 32). The application now has avatars and pictures added to make it user friendly for participants to complete the survey independently. Additionally, a child-friendly version, ASA24™-Kids, has been validated for use in children age 10 and older (31, 33). This version has fewer food selections to simplify the reporting process.

Food Frequency Questionnaires in Children

FFQ provide a list of foods with questions referring to how often the individual ate determined portions of a food over a defined period of time. Several different types of FFQ have been validated for use in children (34, 35.). Paxton et al. (35) used a semi-quantitative dietary recall questionnaire among third, fourth and fifth graders (age 8-11)

that proved to accurately measure school lunch intake. Neuhouser et al. developed and validated a beverage and snack questionnaire (BSQ) for use among seventh-graders (36). The BSQ asks children to report how often they consumed a list of 19 fruits, vegetables, snacks, and beverages over the past week. This BSQ was found reliable by test-retest ($r=.73-.85$) and valid by the correlation of the BSQ to a four day food record ($r=.56-.87$, $p<.05$). Roumelioti (34) validated a similar semi-quantitative FFQ that could accurately measure comprehensive food consumption in fourth-, fifth-, and sixth-graders.

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Appendix B

Recruitment Letter to Parents

Dear Parents,

Researchers in Nutrition (Drs. Heidi Wengreen and Michael Lefevre) and Psychology (Dr. Greg Madden) at Utah State University are conducting a study to determine if carotenoid levels measured on the surface of a child's skin are related to self-reported fruit and vegetable intake and blood carotenoid levels. *Carotenoids* are pigments found in fruits and vegetables and are believed to be responsible for some positive health benefits such as reduced heart disease and some cancers. The results of this study could help us measure children's fruit and vegetable intake in a simple, non-invasive way.

We are recruiting children ages 5-17 to participate in this study. If you are selected to participate, you (one parent) and your child will come to our clinic (1600 N. 650 E., N. Logan) at a scheduled appointment time between 6:00 and 9:00 am on weekdays or 8:00 and 11:00 am on Saturday on three different days within one month. Clinic visits take approximately 45 minutes and include:

- Measurement of height and weight (1st visit only).
- Fasting blood sample (less than 2 teaspoons of blood will be taken each time).
- Skin scan for carotenoid level.
- Breakfast for you and your child.
- Completion of two food records (a record of what your child ate the previous day)

If your child completes the study, they will receive:

- Financial compensation
- Results of carotenoid status
- Diet analysis

If you and your child are interested in participating, please complete the confidential application form at https://anr.usu.edu/htm/available-studies/do-you-qualify. After you complete the application, a clinic representative will contact you if your child qualifies for the study.

Thank you!

The Scan Kidz team
(435) 797- 4226
Nutrition, Dietetics, and Food Sciences
Center for Human Nutrition Studies
Department of Psychology

Appendix C

SCAN Kidz Survey

1. Skin Carotenoids and Nutrition in Kids (SCAN Kidz)

Please complete the following information for one child. If you have multiple children who are interested in participating, complete additional surveys.

***1. Is your child age 5-17?**

☐ Yes ☐ No

***2. Please list your child's birthdate (mm/dd/yyyy)**

2-digit Month (Jan=01) ☐

2-digit Day (01, 15, etc.) ☐

4-digit Year (yyyy) ☐

***3. What is your child's gender?**

○ Female ○ Male

***4. What is your child's race or ethnicity?**

☐ American Indian / Alaska native ☐ Asian ☐ Black / African American ☐ Hispanic ☐ Not Hispanic or Latino ☐ White ☐ Decline / Don't know

***5. Is your child willing to have about two teaspoon of blood drawn on three occasions?**

○ Yes ○ No

***6. Is your child willing to fast overnight (10 hours)? (We will provide breakfast for your child during the clinic visit.)**

☐ Yes ☐ No

***7. Are you and your child able to attend clinic appointments weekdays between 6:00-9:00 am once a week for three weeks?**

☐ Yes ☐ No

***8. Would you prefer to attend clinic appointments on Saturday mornings?**

☐ Yes ☐ No

***9. Does your child have diabetes, asthma, or rheumatoid arthritis?**

○ Yes ○ No ○ Don't know.

***10. Does your child have a chronic disease or condition?**

☐ Yes ☐ No

SCAN Kidz Survey

***11. If yes, please list.**

***12. Please enter the child's name.**

First Name:

Last Name:

***13. Please enter a parent/guardian's name and address.**

First Name:

Last Name:

Address:

Address 2:

City/Town:

State:

ZIP/Postal Code:

Email Address:

***14. Best daytime contact number for parent/guardian xxx-xxx-xxxx:**

Phone Number:

***15. How did you learn about this study?**

- Newspaper
- Flyer
- Other - explain below.
- Radio
- Presentation

Other (please specify)

Appendix D

Health History Form

Center for Human Nutrition Studies
Screening Health Questionnaire

ID label here

Name: _____

Date: _____ / _____ / _____

These questions are used to ensure your safety should you be accepted into the study. Please answer them carefully.

List any ALLERGIES you may have and the resulting symptoms:

Food		**Drug**		**Other**	
Food:	Symptoms:	Drug:	Symptoms:	Substance:	Symptoms:

Have you ever been diagnosed by a health care provider for any of the following conditions? Please check NO if condition does not apply to you. Check YES if you have ever been diagnosed with the condition. If you answer YES, please provide the AGE, <u>or</u> a START DATE and an END DATE for the condition.

Yes	**No**	**Condition**	**Age (or) Start Date**				**Age (or) End Date**			
Examples:										
X		*Thyroid Disease*	*15*				*now*			
X		*Anemia*		*June*		*2010*		*August*		*2010*
		Seasonal Allergies								

		Anemia								
		Anorexia								
		Arthritis, rheumatoid								
		Autism								
		ADHD								
		Bleeding/Clotting Problems								
		Bulimia								
		Cancer (past or present)								
		Constipation								
		Depression								
		Diabetes (specify type)								
		Drug or Alcohol Problems								
		Ear Infections								
		Epilepsy or Seizures								
		Heart Problems- bicuspid valve (genetic)								
		Joint/Bone Problems								
		High Blood Pressure								
		Hypoglycemia								
		Kidney infections								

Yes	No	Condition	Age (or) Start Date Age (or) End Date
		Lung Problems (asthma, bronchitis, etc.)	
		Mental Illness	
		Migraine Headaches	
		Pancreatitis	
		Pneumonia	
		Stomach or Bowel Problems (GERD, IBS, etc.)	
		Thyroid problems	
		Conditions requiring steroids/cortisone (sinus problems, joint problems). Please specify.	
		Frequent urinary tract infections	
		Other (specify)	
		Other (specify)	

Yes	No		
		Are you currently suffering from any cold, flu or allergy symptoms?	Please specify:
		Do you currently smoke?	If yes, what type/amount?
		Does someone in your current household smoke?	
		Are you exposed to environmental cigarette smoke?	Explain:

		Do you currently spend time in a tanning bed?	If yes, how often and for how long?
		Do you currently use self-tanning products?	If yes, when was your last application?
		Do you have sun exposure between 10am and 4 pm?	If yes, how much:
		Have you lost weight unintentionally in the past 3 months?	If yes, please explain:
		Do you eat three meals a day? If no, circle the meals you consume.	Breakfast Lunch Dinner

Have you ever had surgery? If YES, please list below:

Surgery	Date	Surgery	Date
1.		4.	
2.		5.	
3.		6.	

List all **medications** that you have taken in the last 3 months (at least once a week for a month or longer):

Medication	Dosage	How long have you taken?
Example: minocycline	*20 mg/day*	*1 year*

List all **supplements/herbs** that you have taken in the last 3 months (at least once a week for a month or longer):

Supplement (List brand name, if available)	**Dosage**	**How long have you taken?**
Example: Multivitamin-Flintstones	*1 daily*	*3 years*

Appendix E

Food Frequency Questionnaire

ID#_____

Please think about what you ate and drank during the past week. Mark the column that that shows how many times you ate or drank the listed foods and drinks. If you did not eat the food or drink during the past week, please mark "never or less than 1 time per week."

The first section is on beverages (or drinks)

	How often did you drink these beverages in the past week?						
Type of drink	Never or less than 1 time per **week**	1 per **week**	2-4 times per **week**	5-6 times per **week**	1 time per **day**	2-3 times per **day**	4 or more times per **day**
Q1. 100% orange juice, apple juice or other 100% juice	O	O	O	O	O	O	O
Q2. Vegetable juice like V8, carrot, or tomato	O	O	O	O	O	O	O
Q3. Fruit flavored drinks and sports drinks like Capri Sun, Sunny Delight, or PowerAde	O	O	O	O	O	O	O
Q4. Regular soda pop (not diet) or energy drinks like Rockstar, Red Bull or Monster	O	O	O	O	O	O	O

Q5. Plain milk	O	O	O	O	O	O	O
Q6. Chocolate or other flavored milk	O	O	O	O	O	O	O
Q7. Smoothie made with yogurt and fruit	O	O	O	O	O	O	O

The next section is on snack foods.

	How often did you eat these foods in the past week?						
Type of snack food	Never or less than 1 time per **week**	1 per **week**	2-4 times per **week**	5-6 times per **week**	1 time per **day**	2-3 times per **day**	4 or more times per **day**
Q8. Potato or tortilla chips either flavored or plain	O	O	O	O	O	O	O
Q9. French fries or tater tots	O	O	O	O	O	O	O
Q10. Popcorn	O	O	O	O	O	O	O
Q11. Pretzels or salty crackers including gold fish crackers, Ritz crackers	O	O	O	O	O	O	O
Q12. Graham crackers or animal crackers	O	O	O	O	O	O	O
Q13. Candy such as jelly beans,	O	O	O	O	O	O	O

licorice, or gummy bears							
Q14. Chocolate or chocolate candy bars	O	O	O	O	O	O	O
Q15. Cookies, brownies, pies, cake, doughnuts, or pop tarts	O	O	O	O	O	O	O
Q16. Popsicles, Slurpees, or shaved ice	O	O	O	O	O	O	O
Q17. Ice cream or milkshakes	O	O	O	O	O	O	O

The next section is on fruits and vegetables.

	How often did you eat these foods in the past week?						
Type of fruit or vegetable	Never or less than 1 time per **week**	1 per **week**	2-4 times per **week**	5-6 times per **week**	1 time per **day**	2-3 times per **day**	4 or more times per **day**
Q18. Fresh or frozen fruit like apples, banana, orange	O	O	O	O	O	O	O
Q19. Canned fruit like peaches, pears, applesauce, or pineapple	O	O	O	O	O	O	O
Q20. Dried fruit like raisins or	O	O	O	O	O	O	O

Craisins							
Q21. Green salad, spinach, kale or other dark green leafy vegetable	O	O	O	O	O	O	O
Q22. Spaghetti sauce, tomatoes or salsa	O	O	O	O	O	O	O
Q23. Yams, sweet potatoes or winter squash like butternut	O	O	O	O	O	O	O
Q24. Vegetable soup, or stew with vegetables	O	O	O	O	O	O	O
Q25. Carrots	O	O	O	O	O	O	O
Q26. Beans such as baked beans, garbanzo beans, kidney beans, or black beans	O	O	O	O	O	O	O
Q27. Any other vegetables, (string beans, peas, corn, broccoli, celery, cauliflower)	O	O	O	O	O	O	O

Appendix F

Institutional Review Board Approval

Institutional Review Board

USU Assurance: FWA#00003308

Expedite #2

Letter of Approval

FROM:

Melanie Domenech Rodriguez, IRB Chair

True M. Rubal, IRB Administrator

To: Heidi Wengreen, Sheryl Aguilar, Janet Bergeson, Gregory Madden, Michael Lefevre

Date: July 27, 2012

Protocol #: 4556

Title: Skin Carotenoids And Nutrition In Kids (Scan Kidz)

Risk: Minimal risk

Your proposal has been reviewed by the Institutional Review Board and is approved under expedite procedure #2 (based on the Department of Health and Human Services (DHHS) regulations for the protection of human research subjects, 45 CFR Part 46, as amended to include provisions of the Federal Policy for the Protection of Human Subjects, November 9, 1998):

Collection of blood samples by finger stick, heel stick, ear stick, or venipuncture as follows: (a) from healthy, nonpregnant adults who weigh at least 110 pounds. For these subjects, the amounts drawn may not exceed 550 ml in an 8 week period and collection may not occur more frequently than 2 times

per week; or (b) from other adults and children, considering the age, weight, and health of the subjects, the collection procedure, the amount of blood to be collected, and the frequency with which it will be collected. For these subjects, the amount drawn may not exceed the lesser of 50 ml or 3 ml per kg in an 8 week period and collection may not occur more frequently than 2 times per week.

This approval applies only to the proposal currently on file for the period of one year. If your study extends beyond this approval period, you must contact this office to request an annual review of this research. Any change affecting human subjects must be approved by the Board prior to implementation. Injuries or any unanticipated problems involving risk to subjects or to others must be reported immediately to the Chair of the Institutional Review Board.

Prior to involving human subjects, properly executed informed consent must be obtained from each subject or from an authorized representative, and documentation of informed consent must be kept on file for at least three years after the project ends. Each subject must be furnished with a copy of the informed consent document for their personal records.

4460 Old Main Hill | Logan, UT 84322-4460 | PH: (435) 797-1821 | Fax: (435) 797-3769 | WEB: irb.usu.edu | EMAIL: irb@usu.edu

Appendix G

SCAN Kidz Survey

***11. If yes, please list.**

***12. Please enter the child's name.**

First Name:

Last Name:

***13. Please enter a parent/guardian's name and address.**

First Name:

Last Name:

Address:

Address 2:

City/Town:

State:

ZIP/Postal Code:

Email Address:

***14. Best daytime contact number for parent/guardian xxx-xxx-xxxx:**

Phone Number:

***15. How did you learn about this study?**

- Newspaper
- Flyer
- Other - explain below.
- Radio
- Presentation

Other (please specify)

SCAN Kidz Clinic Form Week 1

***8. In the last week have you taken any NEW dietary supplements?**

- ○ Yes (If yes, please explain below)
- ○ No

If yes, please explain:

***9. In the last week, have you used sunless tanning lotion or spent time in a tanning bed?**

- ○ Yes (If yes, please explain below)
- ○ No

If yes, please explain:

***10. Did you eat differently than usual this week?**

- ○ Yes (If yes, please explain below)
- ○ No

If yes, please explain:

***11. Was the amount or the type of your physical activity different than usual this week?**

- ○ Yes (If yes, please explain below)
- ○ No

If yes, please explain: