Carotenoids
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Chapter 10

Epidemiology and Intervention Trials

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A. Introduction to Epidemiology

Determining the health effects of carotenoids in humans is a challenging yet high priority area of research. Epidemiology is the study of the distribution and determinants of disease in human populations. Epidemiologists who study carotenoids are thus interested in determining if carotenoid intake or carotenoid status is associated with risk of various disease endpoints. As carotenoids are known to have antioxidant functions in plants, and evidence suggests that oxidative stress could be involved in the aetiology of chronic diseases such as cancer, heart disease, cataract and macular degeneration, much of the epidemiological research on carotenoids has emphasized links with risk of these and other chronic diseases, as summarized in *Chapters 13-15*.

Epidemiological studies all have in common the fact that they examine associations between exposure to some factor, in this case carotenoid intake/status, and the disease outcomes of interest. As will be detailed below, exposure assessment can be undertaken by collecting dietary data (asking subjects to recall their intake of carotenoid-containing foods and supplements) and/or more objective measurements of carotenoid status, including those obtained in the laboratory. Whilst much of the earlier epidemiological research on carotenoids and health used the traditional questionnaire-based approach, current research is relying increasingly on laboratory measurements to determine exposure objectively (biochemical and molecular epidemiology).

B. Types of Epidemiological Studies

Some general types of epidemiological study designs are summarized in Fig. 1. Epidemiological studies include both observational studies and experimental/intervention trials. The distinction between these two types of study design is important. In observational studies, there is no attempt by the researcher to modify the exposure status of the study subjects with regard to carotenoids or any other factor. In contrast, intervention trials are essentially an experimental design where the exposure status of the study subjects to the factor of interest is manipulated. For carotenoids, this includes both carotenoid supplementation trials, and also trials where subjects are asked to increase consumption of carotenoid-rich foods. Observational and intervention research both provide valuable information and are critical to our understanding of the health effects of carotenoids. Both designs also have important limitations that are detailed within each study design discussion.

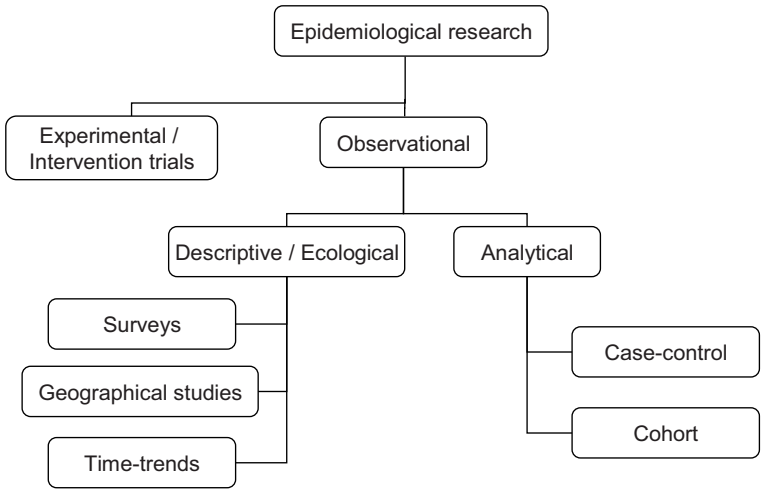


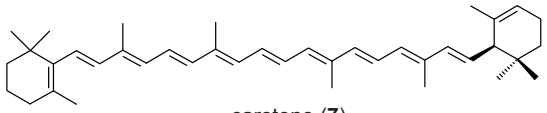
Fig. 1. Summary of epidemiological study designs described in the text.

1. Observational study designs

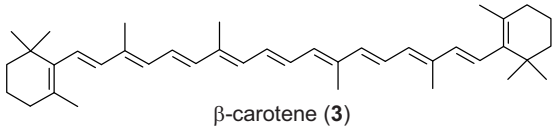
a) Descriptive epidemiology

The aim of descriptive epidemiology is to describe patterns of exposure and/or disease in a population. In carotenoid research, descriptive epidemiology methods are used to describe carotenoid intake patterns in various populations (by age and sex), or to describe typical blood

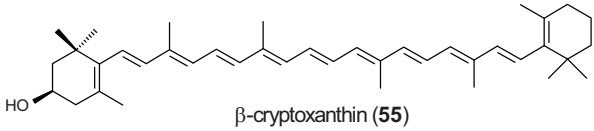
or tissue levels of carotenoids in various populations. Many studies from different parts of the world have set out to ascertain plasma carotenoid concentrations, both for total carotenoids and for individual carotenoids. In the United States, the best source of data for the descriptive epidemiology of carotenoids comes from a national nutrition survey known as NHANES (National Health and Nutrition Examination Survey).



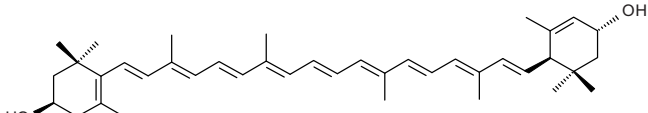
α-carotene (**7**)



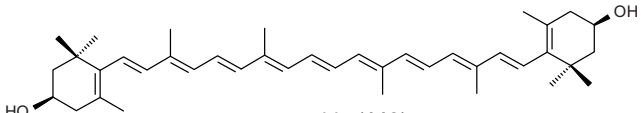
β-carotene (**3**)



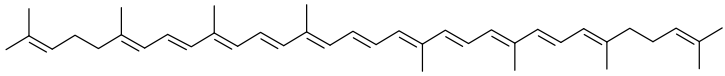
β-cryptoxanthin (**55**)



lutein (**133**)



zeaxanthin (**119**)



lycopene (**31**)

There have been several waves of NHANES surveys; NHANES III included both dietary data and biochemical measurements of various plasma carotenoids for a probability sample, selected to create a population sample from which inferences can be made to the overall U.S.

population. The carotenoid intake data include estimated intakes of α-carotene (**7**), β-carotene (**3**), β-cryptoxanthin (**55**), lutein (**133**) + zeaxanthin (**119**), and lycopene (**31**), and are reported for various age-specific and sex-specific groups [1].

The dietary intake estimates are based on dietary data obtained from nearly 30,000 Americans, and are therefore robust estimates of intake. Median intakes (50th percentile) of carotenoids from NHANES III are summarized in Table 1. The median serum data for these carotenoids are summarized in Table 2.

Table 1. Usual intake of carotenoids (μg/day) from food. The data are taken from the NHANES III survey (1988-1994), showing medians (50th percentile) and selected other percentiles.

Carotenoid	**Percentile**		
	10th	50th	90th
β-Carotene (**3**)	774	1,665	3,580
α-Carotene (**7**)	2	36	1,184
Lutein (**133**) + Zeaxanthin (**119**)	714	1,466	3,021
β-Cryptoxanthin (**55**)	24	88	319
Lycopene (**31**)	3,580	8,031	16,833

Data are based on all individuals excluding pregnant and lactating women (n=28,575) and are taken from reference [1].

Table 2. Serum concentrations of carotenoids (μg/dL) of persons aged 4 years and older. The data are taken from the NHANES III survey (1988-1994), showing medians (50th percentile) and selected other percentiles.

Carotenoid	**Percentile**		
	10th	50th	90th
β-Carotene (**3**)	6.4	14.7	35.1
α-Carotene (**7**)	1.3	3.4	9.2
Lutein (**133**) + Zeaxanthin (**119**)	11.1	18.9	33.0
β-Cryptoxanthin (**55**)	4.0	8.0	16.4
Lycopene (**31**)	11.9	22.4	36.1

Data are taken from reference [2].

These data, compiled by age-specific and sex-specific groupings, are based on a sample size in excess of 20,000 Americans, with all samples analysed in one laboratory [2]. Thus, the NHANES III data are a valuable source of information on typical carotenoid status in a well-nourished population. Carotenoid levels in blood reflect dietary intake, so data for the U.S. may not be an appropriate comparison for countries with different carotenoid intake patterns, but are included here as a reference point.

Descriptive studies have also been done to establish tissue levels of carotenoids [2]; these studies tend to be based upon convenience samples (samples selected for relative ease of access) with a relatively small sample size (usually fewer than 100 subjects).

Descriptive data on a population's typical intake of carotenoids are sometimes used as a basis for ecological studies, in which the intake of carotenoids across populations might be compared with disease patterns across those same populations. Other types of ecological studies include (i) time trends studies, in which trends in carotenoid intake within a population over time might be compared with trends in disease incidence within the same population over time, and (ii) geographical studies, where, for example, carotenoid intake in different parts of a country or region is compared with disease incidence patterns across that country or region. There are many differences other than nutrient intake across populations, so ecological studies are only appropriate for generating new hypotheses, not for suggesting causality.

b) Analytical epidemiology

Analytical epidemiology studies include both case-control and cohort studies. These are the two study designs used most commonly to identify health effects of carotenoids.

i) Case-control studies. In case-control studies, cases with a particular disease are identified and interviewed, as is a comparison group of subjects who do not have the disease of interest. The control group is generally selected to reflect the age and gender distribution of the case subjects. For carotenoid research, the cases are asked to report on their usual consumption of carotenoid-rich foods in some stated period of time **before** the onset of their disease, and the controls for a similar period in the past. Ideally, case-control studies are population-based, meaning that both the cases and the controls are sampled from a defined study population. In contrast to population-based case-control studies, hospital-based case-control studies recruit both cases and controls from one or more hospitals. It is a requirement that controls do not have the disease under study, but they may be afflicted with one or more conditions that led to a hospital admission. One of the limitations with the hospital-based approach for studying carotenoids is that inadequate intake of these nutrients could be related to risk of numerous chronic diseases (not just the one being studied) so that selecting an appropriate control group can be difficult. Thus, population-based case-control studies of carotenoids and disease are considered more informative than hospital-based case-control studies.

Case-control studies are an efficient study design, but the presence of disease in cases might affect the reported carotenoid-containing food intake, as well as affecting circulating carotenoid concentrations, thereby precluding biochemical epidemiological studies of carotenoids. For example, patients with gastrointestinal diseases may have altered their diet in the months preceding diagnosis, because of the disease symptoms; it may be difficult for these cases to recall accurately their normal diets before the onset of disease. This is an important limitation to case-control studies. As case-control studies are less expensive and

more efficient than cohort studies, most of the earlier literature on health effects of carotenoids was derived from case-control studies. More recently, however, data are becoming widely available from numerous large cohort studies of diet and health, conducted around the world.

ii) Cohort studies. The basic cohort design involves recruiting a large population, obtaining dietary and other data on that population, and then following the population forward in time, generally for many years, for the development of future disease. Some cohort studies obtain dietary data only at baseline (when the cohort is constructed), whilst others collect updated dietary intake data at some points during follow-up. There are many well-known cohort studies in the area of nutrition and health; a few of the many that have contributed to the literature on carotenoids and health are the U.S. Nurses Health Study, the U.S. Health Professionals' Follow-Up Study, the Alpha-Tocopherol, Beta-Carotene Cancer Prevention (ATBC) study from Finland, the Women's Health Initiative cohort from the U.S., and the European Prospective Investigation into Cancer and Nutrition (EPIC) study.

In contrast to the case-control approach, in the cohort study design, dietary data are obtained from apparently healthy study participants **before** the development of disease is detected. This provides important temporal information, because the nutrient intake pattern preceded the development of disease rather than being a consequence of the disease. Thus, cohort studies of carotenoids and health are generally considered less biased than case-control studies. In practice, however, it is difficult to study rarer diseases by cohort designs; even the largest cohorts may have too few cases of a particular disease occurring during the follow-up to allow for robust epidemiological research. For these reasons, epidemiologists continue to conduct both case-control and cohort studies to identify health effects of carotenoids, recognizing the strengths and limitations of each approach.

2. Intervention trials

Intervention trials are essentially an experimental design in which the carotenoid exposure status of the study subjects is manipulated, and the resulting effect on some endpoint is evaluated. For carotenoids, this includes both carotenoid supplementation trials with carotenoids alone or in combination with other nutrients, and also trials in which subjects are asked to increase consumption of carotenoid-rich foods, either total fruits and vegetables, or specific sub-groups of fruits and vegetables, such as tomato products.

a) Supplementation trials

β-Carotene was the first carotenoid to be widely available for supplementation purposes; consequently, most of the completed large-scale carotenoid supplementation trials have tested β-carotene. More recently, supplements of lutein and zeaxanthin are being used in intervention trials aimed at reducing progression of eye diseases (see *Chapter 15*), and

lycopene supplements are being used in intervention trials in relation to prostate cancer (biomarker trials to date are summarized in *Chapter 13*). The conduct of these trials is relatively straightforward; subjects are assigned randomly to receive the carotenoid supplement or not. Placebo pills that look identical to the carotenoid supplement provide a comparison group (randomized, placebo-controlled, blinded trial). The doses of carotenoids studied in most human trials are under 50 mg/day and can be formulated into one capsule to be taken daily. Compliance has generally been quite good. In one study that used daily supplementation with 50 mg β-carotene over several years [3], excellent compliance was found, as assessed by returned blister packs, *e.g.* 81% took >90% of the pills and 94% took >75% during the first year of intervention; compliance remained high with 87% taking >90% of their pills during year 4.

Whilst it is straightforward to conduct carotenoid supplementation trials, interpreting the results can be more complex, because the doses studied are often supra-physiological (*e.g.* 50 mg β-carotene per day versus typical median dietary intakes of <2 mg/day, Table 1). Also, resulting plasma concentrations are often vastly in excess of those achieved through normal dietary intake, reflecting both the higher dose and higher bioavailability of carotenoids from supplements. So, results obtained from intervention trials with dietary supplements are necessarily limited to the dose studied. Also, whilst most chronic diseases take decades to develop, most of the carotenoid supplementation trials have a duration of less than one decade, with the exception of the Physicians' Health Study, which studied 12 years of supplementation with β-carotene [4]. So, a lack of effect on a chronic disease endpoint may simply reflect a relatively short intervention duration compared with the period of time involved in development and progression of the chronic disease.

b) Food-based interventions

The other approach for conducting carotenoid intervention trials is *via* food-based interventions. Some trials have randomized subjects to a diet high in fruit and vegetables, or even a specifically high-carotenoid diet, and then followed the study subjects forward in time for either disease development or modulation of some biomarker of interest. Intervention trials are usually only initiated if promising data from observational epidemiology (along with supportive evidence from animal/mechanistic studies) suggests an advantage to high carotenoid intake. Because nearly all of the observational epidemiology research on carotenoids reflects health effects of carotenoids from foods, the food-based design has the advantage of being a more direct test of results obtained in observational studies. However, adherence is a substantial barrier to these interventions. Considering increases in plasma carotenoid as a biomarker of adherence, it is evident that, in some trials, *e.g.* in an ongoing trial involving breast cancer survivors [5], carotenoid intake and status increased substantially with food-based interventions. Other trials, however, have had much less success in producing significant alterations in plasma carotenoids, although some of them have included fruit and vegetable interventions as part of an overall dietary intervention [6]. The

characteristics of the population studied (gender, smoking status, overall health, other behaviour such as alcoholic beverage consumption) as well as the design and intensity of the intervention are all likely to influence adherence to dietary recommendations for increased consumption.

A modification of the food-based approach for carotenoid intervention trials involves a single food source, rather than an overall dietary change, to increase consumption. Examples of this are studies in which interventions based on tomato sauce are used to increase lycopene intake from foods [7], or interventions based on red palm oil to increase intake of carotenes [8,9]. In this design, the researchers typically provide the intervention food to the study subjects, facilitating adherence to the intervention.

3. Exposure assessment in epidemiological studies

a) Dietary assessment

Both observational studies and intervention trials of carotenoids include intake assessment through the diet. The most common method for assessing dietary intake of carotenoids from foods is the food frequency questionnaire, which asks subjects to characterize their usual frequency of consumption of various food items in the diet, including carotenoid-containing foods, primarily fruits and vegetables but sometimes also mixed dishes that contain carotenoids.

i) Food frequency questionnaires. Most food frequency questionnaires assess the overall diet, not just carotenoid-containing foods in the diet. It is important to include mixed dishes in carotenoid intake assessment; for example, in a recent study in a U.S. population, spaghetti/lasagna/other pasta were the top food sources of dietary lycopene [10].

While there are many 'off-the-shelf' food frequency questionnaires in use by nutritional epidemiologists, it must be recognized that investigators working on carotenoids may need to modify existing questionnaires in order better to capture data on the carotenoids of interest. For example, many food frequency questionnaires do not differentiate between different types of lettuces commonly consumed in salads. However, the lutein and zeaxanthin content of darker green 'lettuces' (kale, collard greens, spinach) is substantially higher than that of other green lettuces (butterhead, romaine, iceberg, other green) [11] so modification of the questionnaire may be necessary to distinguish foods that are similar but have different carotenoid content.

Obtaining data on the frequency of consumption is only the first step in intake assessment; the frequency data must then be converted into estimated daily carotenoid intakes by linking the questionnaire data to a food composition database. In the U.S., a carotenoid composition database has been developed [12] that includes data on α-carotene, β-carotene, β-cryptoxanthin, lutein + zeaxanthin, and lycopene in approximately 4,000 food items. This database is publicly available [13] and is updated as new information becomes available.

It is well known that all dietary questionnaires, including food frequency questionnaires, have some measurement error associated with them. Some researchers have even challenged the usefulness of the food frequency questionnaire, given its inherent measurement error [14]. Other dietary assessment methods such as 24-hour recalls and food diaries are also available. Fortunately for carotenoid researchers, blood carotenoid concentrations provide a reference biomarker against which different dietary questionnaires can be assessed for validity. In one recent study, serum carotenoid concentrations were used to examine the validity of fruit and vegetable intake estimated by 14-day weighed records (where subjects are asked to weigh and record all foods consumed over a 14-day period), a 27-item questionnaire and a 180-item questionnaire [15]. The correlation coefficients between serum carotenoids and fruit and vegetable intake were slightly higher for the 14-day weighed records than for the two questionnaires, but no difference was observed between the 180-item and the 27-item questionnaires. Validity coefficients are similar to correlation coefficients but instead use one measurement (in this case plasma carotenoids) as a criterion to evaluate the validity of another measurement (in this case dietary intake). The highest validity coefficients (VC) were observed for vegetable intake (estimated from weighed records, the 180-item questionnaire, and the 27-item questionnaire) when serum α-carotene was used as the criterion biomarker, with VCs of 0.77, 0.58, and 0.51, respectively. These results, along with data from many other studies, suggest that measurement of fruit and vegetable intake, and therefore carotenoid intake, by self-report has acceptable validity within the population studied, especially when combined with biomarkers of carotenoid status.

ii) Dietary supplement questionnaires. Carotenoids can also be consumed as dietary supplements, so dietary intake assessment for epidemiological research often involves a detailed dietary supplement questionnaire, focusing on carotenoid-containing supplements. In the U.S., many multivitamins include β-carotene (as provitamin A), and many now also include lutein. Carotenoids are often also a component of antioxidant-type combination supplements, and some, *e.g*. β-carotene, can be purchased as single nutrient supplements. These sources of carotenoid intake need to be considered in studies of dietary carotenoids and health. There are some particular challenges, however, to doing this properly. In some supplements, the actual amount of vitamin A as β-carotene is not always indicated, so some assumptions may have to be made about carotenoid content. Also, compared to foods, supplements are often consumed erratically, with periods of use and non-use, and frequent switching of supplement brands. This presents some challenges to the accurate estimation of 'usual' carotenoid intake values.

iii) Combined intake assessment. Once intake estimates from foods and supplements have been obtained, it is not clear how that information should be used for exposure assessment. For many nutrients, it is entirely appropriate to combine nutrients from foods with those from supplements in order to arrive at a total intake level of that nutrient. For carotenoids, this is not appropriate, because the bioavailability of carotenoids from supplements is dramatically

better than that from most food sources (*Chapter 7*). For example, the absorption of β-carotene in supplements in a form solubilized with emulsifiers and protected by antioxidants can be 70% or more [2], whereas less than 5% bioavailability has been reported for carotenes from raw foods such as carrots [2]. So, it may be logical to keep intake of carotenoids from supplements separate from that from foods when making intake assessments. It must be noted, however, that some foods contain both naturally occurring carotenoids and carotenoids added to supplement the food source either as a source of vitamin A or for food colouration. In this case, the food label does not differentiate between the two, making it difficult, in practice, to derive an estimate of carotenoids from the foods themselves, while excluding those added during food fortification.

iv) Pooling and correlation of data. As mentioned earlier, cohort studies are being used increasingly to identify diet/disease relationships. A challenge in conducting large cohort studies of dietary nutrients like carotenoids is that different dietary questionnaires may need to be used. For example, in the European Prospective Investigation into Cancer and Nutrition Study (EPIC), dietary data are being collected in many countries across Europe, some of which have quite distinct dietary patterns [16]. It is difficult to be sure that intakes in one region (*e.g.* Southern Europe) are assessed similarly to intakes in another region (*e.g.* Northern Europe), due to different dietary patterns in these regions. This type of measurement error is called non-differential measurement error, and means that it may be more difficult to discover a true association between carotenoid intake and disease risk.

A related concern involves the pooling of data from several different case-control and/or cohort studies of carotenoids and health, into a larger study (pooled analyses or meta-analyses). Most of the dietary questionnaires are considered to have some validity for assessing relative levels of intake within a population (*i.e.* classifying who is a high-consumer and who is a low-consumer), but these same questionnaires have limitations in terms of assessing intakes quantitatively; portion sizes are difficult to estimate, more extensive food lists tend to produce over-reporting, *etc*. However, the pooled analyses of carotenoids or carotenoid-containing foods in relation to health need some common measurement of intake (*e.g.* grams of vegetables consumed per day) to compare and combine studies. It is not appropriate simply to categorize into 'high' or 'low' from a particular population, because a low intake in one population (such as lowest quartile of lycopene in the U.S.) may actually be a similar intake to the highest quartile in another population (*e.g.* lycopene in China). Thus, pooled analyses require a level of quantitative measurement that does not exist in most dietary questionnaires today. For these reasons, pooled analyses based on dietary intake data must be interpreted with great caution.

Despite these challenges in measuring dietary intake for studies of carotenoids and health, dietary measurements do correlate, albeit not highly, with measurements of blood carotenoids by HPLC. Correlation coefficients between dietary carotenoid intake and carotenoid concentrations in blood tend to be modest (approximately 0.2-0.4 in most studies). These coefficients,

however, are better than those obtained for other nutritional factors, such as energy, where intake estimates correlate poorly, if at all, with objective biomarkers of intake [17].

b) Biomarker assessment

i) Analysis of blood samples. Given the inherent difficulties in assessing quantitatively carotenoid intakes for human studies, biomarkers are an attractive alternative for determining carotenoid status. To date, blood carotenoid concentration has been the most commonly used biomarker. Carotenoids in plasma or serum can readily be analysed by HPLC, but at significant cost. For large epidemiological studies and clinical intervention trials, involving tens of thousands of subjects, this cost may be prohibitive. Also, the use of blood samples requires study subjects to agree to submit to venipuncture, which may reduce rates of participation and possibly introduce participation bias. The blood sample has to be protected from light and processed relatively quickly to separate the plasma/serum, which then has to be stored frozen to await analysis, adding to the cost and complexity. Furthermore, carotenoid concentrations in blood fluctuate in response to recent dietary intake. Thus, plasma carotenoid concentrations have the advantage of being an objective biomarker of intake, but there are some practical and economical limitations to their use for epidemiological studies.

For investigators who choose to measure plasma carotenoids for large epidemiological studies, laboratory quality control becomes very important, because it may take months, if not years, to complete all the biochemical analyses for large studies, and avoiding drift over time in the laboratory assay is essential. Most biochemical epidemiological studies that measure plasma carotenoid concentration are cohort studies or intervention trials, although some case-control studies will use this approach, more often for diseases that are not likely to affect systemic nutrient levels. If samples from case-control studies are measured, it is imperative to include in each batch samples from both cases and controls, in the same ratio of cases to controls as in the overall study, in order to avoid potential artifacts. Sometimes, in cohort studies, blood samples are collected from all participants at baseline, then subjects are monitored over time to determine who develops the disease of interest. Only the samples from those cases who developed the disease and a sub-sample of the remaining cohort who remained free of disease are then retrieved and analysed. This modified cohort design is called a nested case-control study, as the case-control study is nested within a larger cohort study. As with traditional case-control studies, samples from both cases and controls should be included in each batch of laboratory analyses.

There are some formal quality control programmes in place for laboratories that determine carotenoids for epidemiological studies and other purposes. In the U.S. a government agency, the National Institute of Standards and Technology (NIST), has coordinated a micronutrient quality assurance programme for participating laboratories. Blinded samples are sent to the laboratories, and results are fed back to the NIST programme to assess both the accuracy (in comparison to other laboratories, are the values correct?) and the reproducibility (if a sample is sent at one time point and then again several months later, how closely do the laboratory

results agree?). Such quality control programmes have greatly improved the quality of laboratory data obtained on the most commonly occurring carotenoids in human blood.

ii) Analysis of tissue samples. Whilst blood is most commonly used to assess carotenoid status of humans, other tissues can be used. Adipose tissue is thought to be a more stable depot of carotenoids than blood, reflecting the strongly lipophilic nature of carotenoids, and a few epidemiological studies have utilized adipose tissue to assess carotenoid exposure status [18,19]. This approach, however, requires biopsies, more extensive sample preparation, *e.g*. saponification to remove excess lipids, and HPLC analysis. Thus, for large population studies, carotenoid concentrations in adipose tissue are more difficult and more expensive to use than blood carotenoid concentrations as a marker of systemic carotenoid concentrations.

Other tissues that have been used to monitor carotenoid exposure status in humans, by HPLC, include exfoliated oral mucosa, and tissue-specific biopsies (*e.g*. lung biopsies), where researchers measure carotenoids in a target tissue of interest [2]. These approaches are not typically used in epidemiological research.

iii) Non-invasive methods. A newer research approach to assessing carotenoid status of humans involves non-invasive assessment by spectroscopic methods. Resonance Raman (RR) spectroscopy has recently been developed for the non-invasive measurement of carotenoids in the macula (see *Chapter 15*) and also in the skin [20] (see *Chapter 16*). The obvious advantage of this approach is that it is non-invasive, as no biopsies or venipuncture are required. Also, the measurement is very quick, with results obtained almost instantaneously. A limitation to this approach, however, is that, with the exception of lycopene, it is not possible to separate out the contributions of individual carotenoids to the Raman signal.

Before RR spectroscopy measurements of dermal carotenoids can be used as a suitable biomarker in human studies, data on intra-subject and inter-subject variability, and validity are critically needed. A recent study [21] assessed the reproducibility and validity of RR spectroscopy measurements of dermal carotenoids in 75 healthy humans. Exciting light of 488 nm was used to estimate total carotenoids, and light of 514 nm to estimate lycopene separately. Measurements were taken from three sites, the palm, inner arm and outer arm, at baseline and after 1 week, 2 weeks, 1 month, 3 months and 6 months, to maximize seasonal variation. Reproducibilty was assessed by intra-class correlation coefficients (ICCs). For total carotenoids, ICCs across the three body sites for each time point ranged from 0.85 to 0.89, and the ICCs across time were 0.97 (for palm), 0.95 (inner arm) and 0.93 (outer arm).

In a second part of this study, 30 healthy subjects were examined. Dietary carotenoid intake, HPLC analyses of blood carotenoids and RR spectroscopy measurements of dermal carotenoid status (back of hip) were determined. Dermal biopsies (3 mm) were performed and the dermal carotenoids were analysed by HPLC. Total back-of-hip dermal carotenoids assessed by RR spectroscopy were highly and significantly correlated with total dermal carotenoids determined by HPLC of dermal biopsy samples. Correlation with blood

carotenoid content determined by HPLC was also good. Similarly lycopene assessed by RR spectroscopy with exciting light of 514 nm was highly and significantly correlated with lycopene assessed by HPLC of dermal biopsies. These studies show that the RR spectroscopy method is reproducible and valid for use as a suitable biomarker for human studies.

Other non-invasive approaches are possible; recently an optical method based on light reflection spectroscopy has been proposed as a method to assess carotenoid levels in skin [22].

The development and validation of biomarkers of carotenoid status that can be used for epidemiological research is a very important priority, because dietary data are known to have significant errors, and can be biased. Due to social desirability biases, subjects may report that they are consuming more carotenoid-containing foods than they truly are. This makes it difficult to interpret studies based solely on dietary measurements of carotenoid intake. Having non-invasive measurements of carotenoid status as objective indicators, to support or refute self-reported dietary data, is important to furthering our understanding of carotenoid and health/disease associations.

c) Assessment of multiple antioxidant nutrients: Antioxidant indices

Interactions between antioxidants are important in biological systems [23]. Examination of multiple antioxidants simultaneously may, therefore, capture antioxidant and disease associations more effectively than other approaches that focus on single nutrients. A dietary antioxidant index has been constructed that summarizes the combined intake of individual carotenoids, flavonoids, tocopherols (vitamin E), vitamin C, and selenium [24]. The index was created by use of principal components analysis, a sophisticated statistical approach that reduces a large number of highly correlated variables (nutrients in this case; correlated because several nutrients such as carotenoids and flavonoids and vitamin C share similar food sources) to a smaller set of components that capture as much of the variability in the data as possible. The index was evaluated in terms of its ability to predict lung cancer risk in a cohort of Finnish male smokers. Risks of lung cancer were lower among men with higher antioxidant index scores. Of note was the finding that the composite index predicted risk similarly to total fruit and vegetable intake, but better than alternative nutrient measurements, including direct summation of intakes of groups of related nutrients, such as carotenoids.

C. Interpretation of Diet-Disease Associations Relevant to Carotenoids

1. Interpreting results of observational studies with carotenoid-containing foods

As most of the carotenoids consumed by typical human populations come from foods, an issue of great importance is to what extent observed effects are due to the carotenoids in the foods, or to the food sources themselves. For example, carrots are the leading food source for α-carotene in the U.S. diet so, in studies that examine α-carotene as a possible protective

factor for chronic disease risk, it is difficult to isolate effects of α-carotene from effects of carrots. This is true even for studies that use plasma analysis; plasma α-carotene is a biomarker of carrot consumption. As another example, lycopene is consumed in the diet primarily from tomatoes and tomato products. While lycopene is found in some other foods, *e.g.* watermelon, pink grapefruit, the frequency of consumption of these foods is such that, in many populations, they contribute only modestly to lycopene intake at a population level. Thus, dietary lycopene and plasma lycopene are generally markers of tomato product intake, so it is difficult to know whether associations are driven by lycopene or by tomato products.

Much of the older literature on carotenoids and health failed to recognize this distinction carefully, so that effects were often attributed to specific carotenoids, without appreciation that results could be attributable to other components found in those same carotenoid-rich foods, or from the combination of nutrients found naturally in carotenoid-rich foods, *e.g.* one carotenoid interacting with other carotenoids or other phytochemicals. Today's research should recognize this and be more careful in the interpretation of associations with carotenoids when these are derived from carotenoid-rich foods.

A method that aims to separate the effects of carotenoids from those due to their primary plant food sources has been suggested [25]. In this study, it was found initially that higher intakes of β-carotene, β-cryptoxanthin, lutein + zeaxanthin, and total carotenoids were each associated with lower risks of lung cancer in women residing in rural America. After including total vegetable intake, which is the strongest predictor of lung cancer risk among all fruit and vegetable groupings, in the statistical models, however, the attributed protective effects of carotenoids disappeared. Importantly, vegetable intake remained significantly inversely associated with lung cancer risk in these same models. The authors concluded that vegetable consumption was more strongly associated with a lower risk of lung cancer than intake of any individual carotenoid or total carotenoids, which was concordant with two other studies that also used statistical testing to separate formally the effects of carotenoids from those of plant foods [26,27]. Future epidemiological studies of carotenoids could attempt this approach to understand better if protective effects are more likely to be due to carotenoids *per se*, or reflect the food sources rich in those same carotenoids, as subsequent intervention strategies, *e.g.* provision of nutrients *versus* foods, may differ.

2. Interpreting results of intervention trials with carotenoid-containing foods

Observational studies often find that people who consume more of the carotenoid-rich foods (fruits and vegetables) are at lower risk of various chronic diseases than are people who eat less of these same foods. It is impossible, however, to know whether or not associations are causal in these observational studies. People who eat more fruits and vegetables are less likely to smoke [28] and to be obese, and are more likely to engage in health-promoting behaviour such as physical activity. These 'confounding' factors make it difficult to assert causality from observational research. Researchers attempt to control statistically for confounding, but

there remains the possibility that associations are not due to dietary intake specifically, but rather to correlated behaviour, *e.g*. smoking or not.

In order to overcome this limitation, intervention trials can be used, wherein study participants are randomly assigned to a dietary intervention or not. In this randomized trial design, the researchers strive to achieve balance in the intervention and control arm with regard to important confounders such as smoking. Ideally, the only variable being manipulated in these designs is the dietary pattern or specific dietary factor of interest. This is true in principle but, because diets are complex, it turns out that dietary manipulations tend to affect multiple nutrients simultaneously. For example, interventions aimed at increasing the consumption of carotenoid-containing foods in a population are likely to alter not only carotenoid status, but also intake of many other plant-based nutrients (folate, fibre, vitamin C, *etc*.), several of which are under investigation for their own health-promoting properties. Whilst plasma carotenoids are typically used as the biomarker of adherence to trials aimed at increasing intake of fruits and vegetables, it is obvious from the above that concentrations of many other nutrients and phytochemicals are also being modified. If such a dietary intervention is shown to affect rates of chronic disease in comparison to a usual diet group, then it remains inappropriate to conclude that it is carotenoids *per se* that are having disease-fighting properties. For these reasons, supplementation trials are a stronger design for truly evaluating relationships between carotenoids and chronic disease.

3. Interpreting results of carotenoid supplementation trials

Randomized trials of carotenoid supplements have been done with the goal of clearly identifying causal relationships between carotenoids and disease. As noted earlier, β-carotene is by far the most widely studied carotenoid in supplementation trials. Despite the rigour of this experimental design, results must also be interpreted cautiously. This is because, typically, only one dose level of carotenoid can be evaluated within a trial, and results obtained with this may not predict what may happen at a different dose level. As an example, two lung cancer prevention trials that used high-dose supplements of β-carotene (at least 20 mg β-carotene/day) unexpectedly indicated adverse effects on lung cancer risk [29,30]. In the setting of lung cancer prevention, other trials with either lower doses [31,32] or preparations of high-dose β-carotene with lower bioavailability [4] have not revealed this adverse effect. In addition to dose, lifestyle characteristics of the population under study may affect disease prevention efficacy. Thus a combination antioxidant supplement including β-carotene may well have a beneficial effect against cancer in a poorly nourished population from rural China [31], but not in a better nourished French population [32]. Tobacco use [33,34] and alcohol consumption [33] are other factors that may substantially modify the efficacy of carotenoids in disease prevention [35]. These considerations suggest that single trials are inadequate to test associations between carotenoids and disease, and that multiple trials that use different

doses in differing populations are needed to allow better understanding and prediction of health effects of carotenoids in diverse populations.

The practicality of conducting multiple trials of carotenoid supplements in diverse populations is questionable, however, due to limited resources and concerns about additional adverse effects. For example, whilst β-carotene has been shown to interact with concurrent tobacco exposure to produce adverse effects, it is possible that adverse interactions with tobacco could extend to other carotenoids such as lycopene, lutein and zeaxanthin. Just as β-carotene was found to exacerbate harmful effects in the lungs of heavy smokers (who are under significant oxidative stress), the same could hold true in the retinas of patients with early macular degeneration (wherein the macula is under significant oxidative stress) with higher-dose supplementation of the carotenoids lutein and zeaxanthin. For these reasons, carotenoid supplementation trials must proceed with great caution and with appropriate Data and Safety Monitoring Committees to monitor the progress of the trial overall.

As any intervention has possible harms and benefits, study designs that maximize the information obtained but expose relatively fewer study subjects to potential harm are an attractive option. For example, considering cancer prevention trials, an attractive study design to gain evidence of efficacy is to limit the trial to persons who have previously had a cancer of interest, and then aim to prevent second cancers, for which they are often at higher risk [36]. This design has been used in carotenoid supplementation trials, for example to evaluate efficacy in the prevention of second cancers of the head and neck [3], or skin [37]. Likewise, trials of carotenoid supplementation for efficacy in prevention of age-related macular degeneration have enrolled patients who already have early stages of the disease, with the goal of slowing disease progression [38]. The benefit of this type of design is that efficacy (at least in disease progression) can be evaluated while exposing fewer study subjects to unknown potential harms associated with higher-dose supplementation.

While carotenoid supplementation trials continue, the limited but disappointing results to date suggest a cautious approach to conducting such trials in the first place, and a careful interpretation, as results (both beneficial and harmful) obtained in one population may not predict the experience in diverse populations.

4. Interpreting results of trials with intermediate endpoints

Trials that are designed to study modulation of a chronic disease endpoint such as cancer incidence or development of macular degeneration are necessarily very large trials, with typically thousands of participants enrolled. As noted above, sample size requirements can be reduced to some extent by choosing populations at very high risk, such as patients with prior cancers. An alternative design is to conduct trials that use intermediate endpoints as a ‘signal’ of possible preventive or therapeutic efficacy. For example, lycopene has not yet been evaluated in a trial aimed at prostate cancer prevention, but some preliminary evidence of efficacy comes from trials that use various biomarkers of risk for prostate cancer prevention

(*Chapter 13*). These types of biomarker trials are a logical step to take before launching larger disease-prevention trials. However, it is critical that biomarker trials are not used as the final arbiter of efficacy, as it is still not clear that modulation of a biomarker of interest, *e.g.* decreased prostate-specific antigen levels following carotenoid supplementation, is predictive of a decreased risk of prostate cancer.

In some cases, intermediate endpoints may be shown to predict preventive efficacy for carotenoids, but may be based on too few subjects to reveal the full risk-benefit ratio associated with carotenoid supplementation. As an example, supplementation with β-carotene was shown to regress oral precancerous lesions in several trials of patients with such lesions [39], and a subsequent efficacy trial also observed a 31% (non-significant) decreased risk of oral/pharynx/larynx cancers in β-carotene-supplemented subjects [3]. However, the trials aimed at oral precancerous lesions failed to identify the increase in lung cancer risk that was identified in the efficacy trial that had a larger sample size. So, biomarker trials are a useful, but incomplete, approach to evaluating preventive efficacy for carotenoids and other agents.

D. Future Directions

The study of health effects of carotenoids in humans has proven to be a difficult area of research. It is challenging to separate effects of carotenoids from those of the plant food sources in which the carotenoids are concentrated. There are some possible approaches for epidemiologists to take to make progress in this area, to help to understand, from observational studies, whether observed risk reductions are likely to be specific to carotenoids, or to carotenoid-rich foods. Intervention trials, including biomarker trials, trials in high-risk populations, and experimental animal studies, provide additional information about whether observed risk-reducing effects of carotenoids are real, are specific to carotenoids, and are biologically plausible. Thus, it is all of the study designs together that contribute to the totality of evidence concerning health effects of carotenoids in humans. Despite all our best efforts, we must realize that clinical intervention trials are only undertaken when there is a state of equipoise; that is, sufficient evidence to warrant further evaluation of health effects of carotenoids, balanced against sufficient scepticism or possible concern about the use of carotenoids as an interventional approach. So, sometimes we will be right and the intervention will be found to be beneficial, and sometimes we will be proven wrong and the intervention will have no effect or even be proven harmful.

In order to increase the likelihood that carotenoid health effects are successfully identified, the following approach to research development is suggested.

(i) Perform careful observational epidemiological research with measurements of both dietary intake and objective biomarkers of carotenoid status in diverse populations to distinguish carotenoid effects from those of fruit and vegetables.

(ii) Complement these with animal studies, using appropriate animal models of disease, of food extracts *versus* single carotenoids, as has been done, for example, with lycopene, tomato powder, and prostate cancer [40], with the goal of clarifying efficacy and mechanisms of action.

(iii) If evidence continues to support carotenoid-specific effects, embark on human intervention trials cautiously, with intermediate endpoint trials in relevant populations of interest (considering smoking, alcohol drinking, and baseline nutritional status of the population), using more than one dose if possible to examine dose-dependency of effects.

(iv) Embark on secondary prevention trials/therapeutic trials in populations at risk, to identify efficacy and possible adverse effects.

(v) Conduct primary prevention trials in more general populations as the final step in the research process.

Some of these steps could be concurrent (especially steps i-iii) in order to keep research moving forward on several fronts simultaneously, but the key to this more cautious and necessarily more time-consuming approach to elucidate health effects of carotenoids depends on a clearer understanding of carotenoid actions before large, primary prevention trials in human populations are launched.

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