PART 1

STUDY DESIGN

CHAPTER 1

THE SCIENTIFIC RATIONALE OF HUMAN FEEDING STUDIES

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Half of all the chronic illness in America is estimated to be a direct consequence of the national diet (1, 2). This is a remarkable figure, given that the most common chronic illnesses and debilitating conditions—cancer, coronary heart disease, arthritis, gallstones, obesity, osteoporosis, diabetes, hypertension, and liver disease—have widely differing causes and treatments. Although our understanding of the relevant biological processes is incomplete at present, diet probably acts through multiple mechanisms to enhance or diminish the propensity for disease in susceptible individuals. This chapter reviews the various types of research designs involved in postulating and testing relationships between diet and disease, or between diet and metabolic effects. The chapter also describes the specialized but essential role of human feeding studies in providing evidence for these relationships.

RESEARCH METHODOLOGY FOR TESTING DIET-DISEASE RELATIONSHIPS

Concluding that some aspect of diet causes a disease requires a diverse body of information collected by epidemiologists, laboratory scientists, and clinical researchers. These individuals use the specialized methods of their scientific disciplines to assess a variety of outcomes, including measures of early pathology, occurrence of subclinical and clinical disease, and, for some diseases, death. Nondisease (intermediate) outcomes are also assessed; these are sometimes referred to as *risk factors* if they have been associated with likelihood of disease or are good predictors of disease (3–6). The following are some lines of scientific evidence used to generate and test a diet-disease hypothesis:

- Between-country data demonstrating relationships between food balance sheets or food disappearance data and national rates of disease.
- Studies of migrants demonstrating that the disease rates of migrants gradually shift toward the rates typical of the adopted country.
- Studies of identical twins demonstrating greater concordance of disease in twins reared together than in twins reared apart.
- Case-control studies suggesting that diet-related risk factors and characteristics of diet differ between cases with disease and controls without disease.
- Prospective cohort studies demonstrating that individuals with differing dietary intakes have different levels of a risk factor or different rates of disease.
- Animal studies describing dietary effects on the development of disease and the mechanisms involved.
- Human feeding studies examining the effects of intake of specific dietary constituents on risk factors or other intermediate outcomes.
- Large, randomized controlled trials demonstrating that altering dietary intake alters risk factors, disease incidence, or mortality.

Each research method has its strengths and limitations for assessing the relationship between diet and disease. In particular, the various lines of research differ in their ability to demonstrate strong associations between diet and disease

risk. Doing so requires the investigators to establish the following: proper temporal sequence (ie, the postulated cause precedes the effect); independence of effect through control of confounding and other sources of experimental error; consistency of results; and biological plausibility. In addition, the various types of studies differ in the generalizability of their findings (7–10). All of the lines of research described in this section can contribute information to the totality of evidence that allows for conclusions about causality.

Epidemiologic Studies

Observational Investigations

Observational epidemiologic studies can be classified as ecologic (observations are made on groups of people); cross-sectional (information on individuals is obtained in a defined population at 1 point in time); case-control (individuals are selected on the basis of their disease status); or prospective (individuals from a defined population are selected, exposure such as diet is determined, and the individuals are observed over time).

Observational studies comparing disease rates between genetically similar migrant and nonmigrant populations, or comparing twins reared together or apart, can evaluate the relative importance of genetic and environmental factors (11–13). Epidemiologic studies can also be experimental in character, in which case they are called *randomized controlled clinical trials* or *intervention trials.*

Types of Observational Studies

Ecologic studies compare disease rates in populations and are useful in generating hypotheses. For example, ecologic studies have found that countries characterized by different dietary intakes also experience different rates of disease; those nations whose populations have diets high in saturated fat and cholesterol and low in polyunsaturated fat have correspondingly high rates of coronary heart disease (14–16). *Cross-sectional epidemiologic observations,* in which information is obtained on individuals at one point in time, also can identify associations between diet and disease or risk factors. For example, cross-sectional studies have shown that blood pressure is related directly to dietary salt intake and inversely to dietary potassium intake (17, 18). In *case-control studies,* the nutrient intakes of individuals who have disease are compared with those who do not have disease. For example, blood levels of homocysteine, which are strongly related to folate intake, are higher in patients with coronary heart disease (cases) than in healthy individuals (controls), supporting the hypothesis that hyperhomocysteinemia is a risk factor for coronary heart disease (19, 20).

Prospective cohort studies gather information on a population sample (cohort) at baseline (the beginning of the study period) and then make sequential observations for an extended period of time, usually years (the follow-up period). For example, research on a cohort of men living in Framingham, Massachusetts, has found that a diet high in fruits and vegetables is associated with a reduced risk of stroke after 20 years of follow-up (21).

Strengths and Limitations of Observational Studies

Because epidemiologic investigations typically study large numbers of individuals and because the study sample often is chosen to be representative of the underlying population, the results usually are widely applicable and generalizable. Prospective cohort studies also can provide strong evidence of causality in the relationship between diet and disease risk because dietary intake is measured at the start of the study, prior to any disease onset, and disease rates are measured prospectively (4, 6, 7, 14). This allows the temporal sequence of cause and effect to be established.

Observational studies have several limitations, however. One problem is that, in cross-sectional and some case-control studies, the temporal sequence is unknown; that is, it cannot be determined whether the postulated causal factor (such as dietary exposure) preceded the disease, or whether diet was altered in response to the diagnosis or initial symptoms. Associations identified solely from these study designs cannot be used to draw conclusions about causality, but they are useful for generating hypotheses. They also provide some evidence that can contribute to causal inference (ie, drawing conclusions concerning cause and effect). Another limitation of observational studies is the inability to characterize or otherwise control the many relevant genetic, behavioral, and environmental factors that could influence the interpretation of the results. Differences among populations or individuals in these factors, which may be unmeasured or unknown, could account for observed differences in disease rates.

It therefore is desirable, in both cross-sectional and prospective observational studies, to measure as many diet- and disease-related characteristics and factors as possible in order to adjust for them in the data analysis and avoid drawing wrong conclusions.

Experimental Studies

Randomized Controlled Trials

Randomized controlled trials, which also are called *clinical trials,* are studies in humans that provide strong evidence of causality. In these studies, individuals are assigned in random order to one or more experimental treatments or to a control condition or treatment, and disease or risk factor outcomes are measured prospectively. (Also see Chapter 2, ''Statistical Aspects of Controlled Diet Studies.'')

Dietary Counseling Trials

The most common method of delivering a dietary intervention in a randomized controlled setting is by counseling participants to follow the diet. Adherence to the diet is assessed in order to confirm that dietary exposure or treatment differs between the intervention and control groups. Intervention trials using dietary counseling have the potential for long treatment periods and large sample sizes. An example is the Trials of Hypertension Prevention (TOHP) study, which tested the effects of sodium reduction and weight loss, singly and in combination, on blood pressure and risk of hypertension over a 3- to 4-year period in 2,382 men and women (22). Another example is the Dietary Intervention Study in Children (DISC) study, a nutrition counseling trial that followed 663 children for as long as 7 years and examined the effects of a reduced-fat diet on blood cholesterol concentration (23).

Human Feeding Studies

Another method of delivering a dietary intervention using a randomized controlled design is the feeding study, wherein participants consume prepared foods of specified composition. Two main approaches are used for assignment of participants to treatment: (1) random assignment to a control diet or a test diet (note: there may be several such diets) (parallel-arm design); and (2) assignment to a random sequence of test and control diets (crossover design). In addition to diet, many other aspects of the participants' lives, such as physical activity and medications, are also tightly regulated during the study. Specific dietary effects on pathologic processes or on risk factors, rather than on development of disease, are the primary outcomes. For example, in a study of the effects of salt and potassium intake on blood pressure, 20 men received, in random order for 2 weeks at a time, each of 4 diets: a control (typical) diet low in potassium and high in salt, and 3 test diets—high-potassium/high-salt, low-potassium/low-salt, and high-potassium/low-salt (24). Primarily for feasibility, a feeding study often has small numbers of participants and a short duration, typically ranging from days to months, but occasionally as long as one year. (Also see A Study Design to Test the Hypothesis later in this chapter.)

Strengths and Limitations of Randomized Controlled Trials

The randomized controlled trial design has the cardinal feature of ensuring that the exposure (such as diet) precedes the disease-related outcome, thus providing strong evidence of causation. In addition, all other factors, either known or unknown, that may influence the outcome are equally likely to be found in the intervention or treatment group(s) as well as in the control group(s). Therefore, any differences in disease rates or risk factors observed between the study groups can be attributed to the diet and not to other factors (25), provided other sources of bias are also minimized during the study. Results that are generalizable to a target population generally require trials that have large sample size and long duration, such as dietary counseling trials; achieving large sample size in the context of feeding studies usually requires multicenter designs or the enrollment of successive cohorts (see the discussion of study design later in this chapter and in Chapter 25, ''The Multicenter Approach to Human Feeding Studies'').

Other Types of Human Feeding Studies

Many feeding studies are conducted as clinical investigations that do not require a randomized controlled design. Either the control group is lacking or the test diets are not assigned to the participants in random order. The sample sizes of clinical studies are generally small, their duration is limited, and the outcomes usually relate to some biological parameter or risk factor rather than disease risk. The population sample for clinical studies tends not to be broadly representative of the general population, and the lack of randomization limits the ability to draw definitive cause-and-effect conclusions (26). Instead, the value of these studies lies in their ability to provide detailed information about specific dietary components and about physiologic processes and mechanisms. For example, one clinical study gave 10 women sequentially increasing doses of vitamin B-6 during 4 test diet periods of 12 days each; although the design was not randomized, the study provided useful information regarding the relationship between vitamin B-6 status and the dietary vitamin B-6:protein ratio (27).

Animal Studies

Animal research conducted by laboratory scientists contributes other types of information, especially for elucidating the mechanisms whereby diet may exert biological effects, such as: high sodium intake → hypertension → arterial wall stress → arterial wall injury → atherosclerotic plaque. The advantage of such studies is that, in a relatively short period of time (several weeks to several years, depending on the animal model), a diet → mechanism → disease relationship can be tested, wherein outcomes consist of pathologic changes confirmed by necropsy examination (28). Just as with the human studies described earlier, the ability to draw definitive conclusions is limited if the animals are not randomly assigned to the experimental conditions.

Dietary Assessment Methodology

Apart from experimental design, an important feature of the various types of research methods is the procedure for ascertaining dietary intake (29, 30). Ecological studies typi-

cally use food balance data for this purpose (31, 32). The intake estimates are calculated from food supply statistics, such as foods grown or processed in the country, foods imported and exported, and changes in food stocks. The amounts of food used for other purposes, such as livestock feed, are then subtracted. To calculate per capita consumption, the mean available quantities are divided by the population size (31, 32). It can be difficult to estimate average per capita intake of populations because data on food supply and population size may be incomplete.

For observational and intervention studies, the unit of observation is not a population but rather an individual. Thus, the available information on diet is based primarily on the individual's recall of foods consumed. Several methodological approaches for the collection of dietary intake data are available; the intake estimates that they yield have varying degrees of precision, reliability, and accuracy (32–37). The intake of some nutrients (such as energy, fat, or protein) may be relatively easy to estimate. Estimating the intake of other nutrients (such as certain vitamins or minerals) may be more difficult, especially if the nutrient is found in a large number of foods or in greatly varying concentration. The methods also depend on the availability of high-quality food composition databases (38). Nutrient intake in feeding studies can be measured with relatively high accuracy because the food provided to the participants has been purchased by the study staff and has been prepared, weighed, and measured in a research kitchen. Furthermore, the nutrient composition of the menus is often verified by chemical analysis.

Lines of Scientific Evidence: The Example of Diet and Coronary Heart Disease

Studies of the relationship between diet and coronary heart disease provide a good example of the process of examining the totality of the evidence based on various types of research to provide evidence of causality. Virtually the full scope of research methods has been used. Early ecologic investigations of dietary intake in different countries yielded observations that average dietary fat intake is correlated with coronary heart disease rates (14). Studies of Japanese migrants who moved to Hawaii or San Francisco and adopted the dietary habits of their surroundings showed that those individuals experience coronary heart disease rates typical of their adopted, rather than of their native, environments (39). Case-control studies provided suggestive evidence, and longitudinal cohort studies provided strong evidence, that risk factors such as high blood cholesterol, high blood pressure, and smoking are associated with, precede, and increase the probability of developing the disease (40, 41). Cross-sectional and longitudinal epidemiologic studies further showed that intakes of saturated fat, cholesterol, and sodium are associated with various risk factors, notably serum cholesterol levels and blood pressure, as well as disease (42–46). Human feeding studies indicated that specific dietary saturated fatty acids, as well as the cholesterol content of the diet, play a major role in determining serum cholesterol levels (47, 48). Animal and human pathology studies provided information about mechanisms of disease development by demonstrating clear influences of diet on blood cholesterol levels and blood pressure, and subsequent anatomical changes in disease progression when these factors are modified (49, 50). Lastly, intervention trials of individuals with hypercholesterolemia demonstrated that lowering serum cholesterol levels with either diet or drug therapy can lower disease rates (51–54).

Key Aspects of Conducting a Human Feeding Study

Four broad conceptual and practical issues must be considered before researchers embark on a human feeding study: a testable, well-founded hypothesis; a study design that can test the hypothesis; appropriately selected outcome measures; and a feasible study protocol.

A Testable, Well-founded Hypothesis

Hypotheses amenable to testing with feeding studies are those for which one or more dietary constituents, given in a known amount, are expected to alter one or more outcome variables. The variables usually are risk factors or surrogate measures for disease. It must be both necessary and possible to test the efficacy of the dietary variable under conditions of high adherence to the diet. In addition, the effects of diet must be expected to occur in a relatively short time frame—days, weeks, or months—and must be unlikely to cause harm to long-term health.

The tight dietary control and adherence conditions of a feeding study yield a high probability that the participants will receive the intended experimental treatment (ie, diet). These conditions lead to high precision in determining the effects of specific dietary constituents.

A Study Design to Test the Hypothesis

Feeding studies are hypothesis-testing studies. (Note: As mentioned earlier, the strongest evidence for a cause-and-effect relationship is provided by a randomized design.) Because feeding studies provide conditions of high adherence, they can quantify precisely the independent effects of a small number of dietary constituents on one or more outcome variables. The specific study design and the characteristics of the experimental diets are purposefully chosen to allow a specific hypothesis to be tested. Following are examples of design elements that must be defined by the study's hypothesis:

Participants
Disease status
- Healthy volunteers
- Individuals with established risk factors
- Individuals with disease

Special populations
- Demographic subgroups
- Older adults
- Children
- Pregnant women

Diet
Type of diet: liquid formula or conventional food
Dose of test nutrient needed to achieve desired effect
Sources of test nutrient and how they are integrated into diet
Macronutrient content of diet
Distribution of energy sources
Micronutrient content of diet
Other nutrient requirements
Dietary and nondietary factors balanced across feeding periods or diet groups to avoid confounding

Time Factors and Statistical Issues
Length of study needed to achieve steady state in endpoint measurements
Need for washout periods
Anticipated effect size
Variability and reliability of endpoint measurements
Sample size calculations to estimate number of participants and measurements
Number of participants feasible to study at one time
Concurrent or successive cohort enrollment

Defined test and control diets are fed to individuals over a specified period of time and all known factors that might alter measured outcomes are balanced across the treatment groups. For example, the foods comprising the diet must be selected in a way that controls for constituents that may alter nutrient absorption (such as the dietary content of vitamin D and oxalate in calcium studies). Extraneous sources of nutrients also must be controlled because they may accidentally influence the results of the study (such as water and toothpaste in calcium studies, or sunlight exposure in a calcium study with controlled vitamin D intake).

The hypothesis of a diet study can be generally phrased as follows: In subjects Q, compared to subjects R, or compared to a control condition, what are the effects of a change in nutrient X on outcome Y, while the confounding variables P, S, and T, known to influence X and Y, are controlled? The hypothesis should have biologic plausibility and should generate data that would fit the time sequence of a causal relationship. The experimental design should be simple, able to produce definitive information, and amenable to standard statistical approaches. Study designs enrolling a relatively small number of participants need to provide sufficient statistical power to detect effects of defined magnitude. (See Chapter 2, ''Statistical Aspects of Controlled Diet Studies.'')

The type of diet administered should be the one that best fits the hypothesis. For example, liquid formula diets can be effectively used to test a hypothesis requiring a direct comparison of proteins or fats but may be inappropriate when a study compares natural dietary fibers. The dose of the nutrient may deliberately be set to be higher than is commonly consumed in order to detect small biological effects. Conversely, a smaller difference in dose could be used to evaluate effects of typical intakes.

The statistical power for testing the hypothesis can be enhanced by appropriate criteria for participant selection. For example, if the anticipated effect of the test diet is relatively small, it may be desirable to use participants who exhibit a more marked response to dietary modification. Study participants also can be specifically selected to help demonstrate particular biological effects or applications of research. Participants fitting specific entry criteria (such as high-normal blood pressure) may be preferred if the experimental diets (with varying sodium levels, for example) offer information on how to alter disease risk. Participants with established disease (such as osteoporosis) may be chosen when the goal of the intervention is to obtain specific information about how to modify existing disease (such as prevention of subsequent fractures).

The duration of the diet periods should be chosen so that there is sufficient time to achieve first a nutritional steady state for each diet and then a change in outcome measurements. Studies of lipoprotein metabolism might require a feeding period of 2 to 5 weeks to achieve steady state. Other studies may be shorter (such as 5-day periods for sodium balance) or longer (such as a year to evaluate dietary effects on bone mineral density).

Feeding studies tend to enroll small numbers of participants because usually only 5 to 25 individuals can be brought into the facility at one time (although there are a small number of facilities with higher capacity). If larger sample sizes are needed to achieve adequate statistical power, successive cohorts can be studied using the same protocol. Another approach is to use a concurrent multicenter protocol of the type developed for large clinical trials. (Also see Chapter 25, ''The Multicenter Approach to Human Feeding Studies.'')

Appropriately Selected Outcome Variables

In feeding studies the diet itself is the primary independent design variable: the diet is ''well-controlled.'' The effects of the diet are measured as dependent outcome variables, which may include biochemical assays of blood or tissue samples, physical characteristics of the study participants, objective or subjective assessments of behavior, or clinical symptoms of disease confirmed by physical examination or tissue pathology. Although certain rapidly progressing diseases or metabolically labile conditions may allow researchers to make short-term assessments of diet effects on observable

clinical outcomes, chronic diseases generally are symptomatic only after decades-long pathologic processes have taken place (50). This time lag obviously makes it difficult to study dietary effects on chronic diseases in a prospective manner during the lifespan of the investigator. Sometimes this problem can be circumvented with methods that detect occult or early disease (such as exercise tolerance testing or coronary angiography for coronary heart disease; or colonoscopy or barium enema to evaluate the presence of colonic polyps, precursors of colon cancer). Alternatively, study participants can be selected who are at high risk for developing disease within a few years. The typical time course of a feeding study, however, is usually too brief to produce measurable changes in preclinical disease status. Yet another approach (discussed earlier in Research Methodology for Testing Diet-Disease Relationships) is to identify intermediate outcomes or risk factors for the biological process of interest. Risk factors also can suggest plausible mechanisms by which disease processes become manifest.

Outcome parameters are useful in the conduct of a human feeding study only if the available measurement techniques are reliable and precise. In addition, the study design should address how often the outcome variable must be measured after achieving the steady state to reduce imprecision from both analytic and biologic variation. For example, bone densitometry measurements are relatively constant within individuals or with repeated observations. Plasma cholesterol levels, however, have 3% to 5% analytic variation and 9% to 19% within-individual biologic variation and require multiple measurements to estimate true effects (55). The need for multiple measurements can extend the length of the study and greatly increase the cost of the project.

A Feasible Study Protocol

The distinguishing features of a feeding study are the high degree of precision in executing the diet and the ability to monitor adherence. In a dietary counseling study, the participant is instructed about how to select a diet to achieve study goals, but it can at best only be estimated how well those goals are achieved. In a feeding study, however, all of the food given to the participant is of known composition, the participant is observed while consuming the food, and energy intake is adjusted if needed to maintain the patient's weight. Thus, dietary counseling studies only approximate the desired diet due to varying levels of adherence, whereas feeding studies literally define the diet. These elements of control establish feeding studies as providing the best estimate in quantifying the relationship between a specific dietary constituent and a specific outcome.

It must be practical to execute the experimental diet design. An adequate nutrient database must be available to determine sources of the test nutrients, and methods must be available to confirm dietary composition. Essential information about the nutrients includes their natural variability in specific foods (such as the vitamin C content of tomatoes) and whether their biologic effects can be altered through processes such as storage (such as the antioxidant content of vegetable oils) or food preparation (such as the fatty acid profile in oils used for frying). It is also necessary to calculate the nutrient dose needed to produce an outcome effect (such as the amount of fiber from psyllium vs other natural sources) and whether this designated dose can be reasonably consumed by participants with typical energy requirements for their age, sex, and state of health. Nutrient-nutrient interactions (such as vitamin C enhancement of nonheme iron absorption) must be considered, so that they can be controlled for when study designers construct the test diets. The diets must be visually attractive, pleasing in taste, and reasonably varied, yet within the production capabilities of the kitchen. The composition of the diet must meet study goals, yet also be nutritionally adequate in other respects.

Conclusion

A strong association between diet and disease or measured intermediate outcome is determined through multiple lines of investigation, including epidemiologic studies, animal studies, dietary counseling intervention studies, and human feeding studies. Each line of investigation has its strengths and weaknesses. Epidemiologic studies poorly quantify diet but carefully quantify manifest disease, whereas feeding studies precisely quantify diet but can only approximate disease through risk factors or surrogate endpoints for disease. Because there are many problems inherent in estimating the nutrient intake of individuals consuming self-selected diets, animal and clinical studies are used to identify more precisely which dietary factors, in which quantities, might be implicated in altering disease processes or disease risk. Large randomized controlled trials, such as dietary counseling trials, usually provide the best generalizable test of whether altering diet can alter risk factors and subsequently risk of disease.

Because their great precision demands great effort, human feeding studies are undertaken only when the weight of the scientific evidence is sufficiently strong to justify hypothesis-testing research concerning the biological effects or mechanisms of dietary constituents, individually and in combination, on given outcomes. Feeding studies thus will always be essential in clarifying how diet influences risk factors and disease processes, even though many other types of research will also be needed to define cause-and-effect relationships between diet and disease, and to determine the populations to which the results may be generalized. Feeding studies with well-controlled diets provide a scientific viewpoint that is like looking at the world through a keyhole: the perspective may be narrow but the picture is clear.

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