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Biological models for phytochemical research: from cell to human organism

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Nutrigenomics represents a shift of nutrition research from epidemiology and physiology to molecular biology and genetics. Nutrigenomics seeks to understand nutrition influences on homeostasis, the mechanism of genetic predispositions for diseases, to identify the genes influencing risk of diet related diseases. This review presents some *in vitro* models applicable in nutrigenomic studies, and discuses the use of animal models, their advantages and limitations and relevance for human situation. *In vitro* and *in vivo* models are suitable for performance of DNA microarrays, proteomic and transcriptomic analyses. *In vitro* models (intracellular organelles and suborganellar compartments, cell cultures, or tissue samples/cultures) give insight in metabolic pathways and responses to test stimuli on cellular and molecular levels. Animal models allow evaluation of the biological significance of the effects recorded *in vitro* and testing of the hypothesis on how a specific factor affects specific species under specific circumstances. Therefore, the evaluation of the data in relation to human organism should be done carefully, considering the species differences. The use of *in vitro* and *in vivo* models is likely to continue as the effects of nutrition on health and disease cannot be fully explained without understanding of nutrients action at nuclear level and their role in the intra- and intercellular signal transduction. Through advances in cell and molecular biology (including genomic and proteomic), the use of these models should become more predictively accurate. However, this predictive value relies on an underpinning knowledge of the advantages and limitations of the model in nutrigenomic research as in other fields of biomedical research.

Nutrigenomics: *In vitro* models: Animal models: Human studies

It is widely recognized nowadays that human health is influenced by genetic and environmental factors, and that nutrition is of fundamental importance. Increasing incidence of so called “lifestyle diseases” like obesity, type 2 diabetes, cardiovascular disease (CVD) or cancer is recognized to be related to Western-style diet. Several reports indicate that a diet rich in vegetables and fruits and low in fat may protect against these diseases[1–3]. In the last two decades nutritionists have become increasingly aware of the significance of gene–nutrient interactions, and their possible use as tools to improve the health of the individual. Thus, the application of nutrigenomic analysis in nutrition represents an important paradigm shift in nutrition research, from epidemiology and physiology to molecular biology and genetics. Nutrigenomics (nutritional genomics) represents the junction between the health, diet, and genomics, and is focused upon genetic polymorphism and the interaction of the genome with diet[4]. Nutrients are judged as potential signals, which influence cellular sensor systems to modify gene expression and subsequently metabolite production. Nutrigenomics seeks to understand how nutrition influences homeostasis and to identify the genes influencing risk of diet-related diseases as well as to understand the mechanism of the genetic predispositions for the diseases. For example, a nutrigenomics approach offers promise in modulating the risk of diseases of ageing because of the effects of certain nutrients on gene expression, through both epigenetic mechanisms or modification of transcription factors. Therefore it might be the first step to a personalised nutrition health policy.

It is accepted that the genetic material of each cell is not a rigid but a dynamic structure changing in response to various stimuli including food[5]. The knowledge of the genome and its expression in living organisms, coupled with the tools of molecular biology create the prospect of examining the health benefits of dietary phytochemicals on cellular level in *in vitro* model systems and scrutinizing the most potent compounds in *in vivo* model systems (animal models of cancer, atherosclerosis, diabetes) and in human subjects.

Abbreviations: CVD, cardiovascular disease; ER, endoplasmic reticulum; FAE, follicle associated epithelium; LDLR, low-density lipoprotein receptor; , 3-D cultures, 3-dimensional cultures; WHHL, Watanabe heritable hyperlipidemic; CYP7A1, cholesterol 7-hydroxylase; LDL-C, LDL cholesterol.

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In applying these various approaches there is an over-arching expectation that research is ethically-acceptable, and that researchers apply the three "Rs" (reduction, replacement, refinement)[6] with respect to the use of animals in biological research.

Every human possesses a unique genotype reacting in its own way upon food contituents[7,8]. Clearly, it is impossible for practical reasons to study response to food constituents for each individual separately, and so the interaction of nutrition with mammalian gene expression is routinely studied in model systems, whose cellular and molecular biology can be defined in qualitative and (preferably) quantitative terms. Depending on the question to be elucidated, a model can consist of cell structures, cell cultures, tissue sample/culture (*in vitro* models) or of group of living organisms like animals or humans (*in viv*o models). Both *in vitro* and *in vivo* model systems are suitable for performance of DNA microarrays, proteomics and transcriptomics analyses.

This review presents some of the *in vitro* and *in vivo* models, which are applicable for studies of biological effects of phytochemicals on gene expression, cellular metabolism, activity of enzymes metabolizing xenobiotics, clinical biomarkers and end points of lifestyle diseases (e.g. cancer, atherosclerosis), and discusses the use of animal models, their advantages, limitations and relevance to the human situation.

In vitro models

In vitro models are based on the use of intracellular organelles and suborganellar compartments, cell cultures, or tissue slice/organoid bioassays. Studies in such model systems give insight into responses to the tested factors on a cellular level. This insight and its overall value must, however, be tempered by recognition of the limitations of the model system, not least whether it can be considered of physiological relevance or indicative of the biology of a multi-organellar environment *in vivo*. In this section we review the advantages and limitations of a range of *in vitro* systems in ascending degree of sophistication.

Microsomes, a small inclusion of ribosomes and fragments of the endoplasmic reticulum (ER) serves as example of a sub-cellular *in vitro* model. Microsomes are broadly used to investigate the metabolising actions of the phase I and phase II xenobiotic-metabolising enzymes, which typically include the cytochrome P450 isoforms. Testing of nutritional factors in this cell-free system can indicate likely patterns of metabolism, but is not necessarily indicative of the fate of the test molecule in a more complex environment, not least one in which cellular uptake is a significant factor.

Yeast, as an eukaryotic organism, represents a simple but useful model genome of which is fully sequenced and individual metabolic pathways are well know. At present several precisely defined mutant and transformed lines exist. The advantage of this model system is that growth conditions can be fully controlled and response upon stimuli easily measured. Therefore yeast has been successfully used e.g. as a model system for screening of phytochemicals for estrogenic activity[9].

Cell lines of various origin, some of which may be clonally derived from single cells have some attraction for the study of gene–nutrient interactions. With careful control of cell passaging and culture conditions, the availability of a homogeneous cell population can offer advantages in terms of reproducibility of response and experiment replication. The practical convenience of an easily-handled and renewable test platform is also suited to medium to high-throughput, should screening of large number of test molecules be required. On the other hand, cell lines may have been in culture for extended periods (months, years or decades), and through de-differentiation and/or genetic damage can bear little or no resemblance to their tissue of origin.

One example of a cell line frequently used in nutritional modelling is the Caco-2 cell line. Caco-2 is derived from human colorectal carcinoma but it retains many of the morphological features typical for normal human enterocytes[10]. Caco-2 cells have been extensively studied and there is a considerable literature concerning their characteristics[11–18]. Caco-2 cells may serve as a model system for predicting intestinal absorption of drugs or nutraceuticals[19–20] and are also used for evaluating of dietary constituents as well as food additives, contaminants, toxicants and oxidants and for testing of possible anti-cancer/protective effects of phenolic compounds[21]. The advantage of this test system is that the cells are of human origin, and that experiments can be carried out over a relatively long period permitting the reversibility of any effects to be assessed[22]. The Caco-2 cell line is also the basis of the so-called follicle associated epithelium (FAE), consisting of co-culturing of Caco-2 cells with human B lymphoma Raji cells to induce Caco-2 cells differentiation into M cells[23]. The FAE model may be useful for study of potential toxicological effects on the human gut epithelium of different nanoparticles and their potential capacity to cross the intestinal epithelium.

A second example of a cell line in widespread use for nutritional/metabolic studies is the human hepatoma cell line HepG2[24] and its sub-clones. HepG2 continues to find application in the evaluation of a range of nutritional factors, including spice constituents[25], soybean derivatives[26] and tuber essential oils[27], as well as providing a means of evaluating medicinal materials from natural sources[28,29]. However, whilst a valuable tool, the HepG2 cell line does not necessarily reflect faithfully the performance of test materials *in vivo*. Cytochrome P450 enzyme activities and Phase II metabolic functions are relatively low, and a recent study demonstrated the failure of HepG2 cultures to detect compounds with known toxicity in primary hepatocytes or *in vivo* [30].

Recognition of the limitation of cell lines as models of metabolic performance *in vivo* is increasingly encouraging investment in the production of fresh or cryopreserved primary cell cultures prepared from animal or human tissues. For instance, cultured endothelial cells and macrophages can be applied as *in vitro* model for studying flavonoids in redox-dependent gene expression[31]. The technology for enzymic tissue digestion and cell isolation is well-established for many animal tissues, including liver and intestine, from a range of species[32,34], and with it the potential to cryopreserve cells with retention of xenobiotic-metabolising activity (see, for example, Stevenson *et al.* [35]). Increasing access, with appropriate ethical permission, to human tissue is similarly enabling the isolation and use of human cells which can, under appropriate conditions, retain a differentiated phenotype in culture for days and even weeks[36,37]. The benefit of such a primary cell-culture approach in nutritional research was demonstrated as much as 30 years ago in cultured skin fibroblasts, whose use

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led to discovery of the low-density lipoprotein receptor (LDLR) on the cell surface, and facilitated understanding of endogenous pathways in cholesterol metabolism[38].

It has long been recognised that use of freshly isolated cells is not by itself sufficient to confer physiological relevance on data obtained using these cultures. Primary cell performance is dependent on their microenvironment and their potential for paracrine interaction with neighbouring cells of the same or different cell type. One way to reproduce these microenvironmental influences *in vitro*, not least for nutrigenomic research, is to avoid cell dissociation through the use of organ slices or tissue explants. Organ slices represent a multicellular three-dimensional *in vitro* model, which possesses the biologically-relevant structural and functional features of *in vivo* tissues through the presence of various cell types in an architectural organization that supports both cell–cell and cell–extracellular matrix interactions. Organ slice methodology is readily adaptable to various organs and various species. The availability of human tissue for organ slice studies augments the utility of this model, and provides an important bridge between animal-derived data and the human situation. Precision-cut tissue slices are increasingly being used in toxicology[39,40], to evaluate the toxicity of chemicals[41–43], to evaluate the genotoxic and antigenotoxic potential of chemicals[44,45], to unravel the metabolic pathways of xenobiotics[46], to study the regulation of enzyme systems such as the cytochromes P450[47], to investigate the hepatic uptake of drugs[48], activation of signalling pathways[49] and to determine chemically-induced apoptosis[50]. This broad spectrum of potential applications for precision-cut tissue slices in nutrigenomic research is likely to promote their use in future nutrigenomic research.

In many cases, the demonstrable metabolic performance of tissue slices will outweigh their inherent disadvantage of short functional lifespan and unsuitability for medium to large-scale analysis. In other circumstances, where treatment periods of more than a few hours are required, or volume and throughput of samples are important, technology development has focused on the creation in cell culture of conditions that elicit *in vivo* cellular responses. At its simplest, this involves the use of biological substrata such as collagen or reconstituted basement membrane for cell attachment[51] or through co-culture with helper cells, or both[52]. More recently, biocompatible substrata have offered the possibility of creating architecturally-sophisticated 3-dimensional cell cultures. For study of transport phenomena this may involve the use of a biocompatible membrane, suitably coated with collagen or other extracellular matrix components, onto which the cells of choice are seeded. With this approach primary cultures of epithelial cells can become polarised, displaying distinctive basal and apical surfaces (the former forming the substratum anchorage) and demonstrating the vectorial transport characteristics of an epithelial monolayer. One example of such a system utilises mammary epithelial cells to recreate the lactating epithelium, allowing study of the transport of xenobiotics from the bloodstream into milk (C Wilde; unpublished).

The adaptation of technology aimed principally at tissue regeneration is, in addition, progressively creating 3-dimensional, biocompatible cell scaffolds in which cells can be seeded, grown and encouraged to reproduce the functions of their tissue of origin. This biomaterials technology can take the form of fibrous scaffolds spun in 3 dimensions, and may also incorporate fibre coatings that promote, for example, cell attachment or particular cell functions. Such technology has, for example, been applied to enhance cytochrome P450 activity in primary hepatocyte cultures[53]. Indeed, for hepatocytes alone, numerous other approaches to the creation of 3-D cultures are currently under development, based variously on hydrogels[54], poly-lactic acid[55], alginates[56] or chitosan[57] amongst many other materials.

Combination of biomaterials technology with stem cell science is opening up new opportunities for tissue regeneration, and with it a further *in vitro* option for cell-based analysis through the generation of differentiated cell types from stem cell lineages. With this approach, scaffold composition and architecture can, for example, direct the maturation of hepatocyte progenitor cell lines, with realisation of cytochrome P450 activities[58]. This approach has the considerable attraction of a renewable source of normal human cells with consistent performance, albeit with the caveat that extensive research and development is still required to define the soluble and fixed-scaffold cues that elicit a stable, differentiated phenotype[59,60].

While *in vitro* studies give insight into responses at a cellular level, they are not able to provide definitive information on how the studied factor may affect the organism as a whole. Thus, the biological significance of an effect at the gene level observed *in vitro* should be further assessed in *in vivo* test(s) involving whole organisms, where exposure to a nutritional factor for instance dietary phytochemical is evaluated systemically through application of metabolomics, and pharmacokinetics such that gene–nutrient interactions of the test substance are demonstrably the aggregate of primary action at an organellar level and any secondary response due to inter-organellar cross-talk.

Animals in nutritional research

To bridge the gap between *in vitro* test systems and the whole organism, and to overcome the obvious constraints of human clinical studies, animal studies are widely applied in nutrition research. Animal studies can be performed in a relatively short time, enabling chronic study, potentially from *in utero*-exposure to death, and also research into inter-generational effects. Such pre-clinical studies, which may measure validated biomarkers of nutritional response, can be supported by *post mortem* examination of end-points, and further investigations on molecular level on gene expression patterns in sampled tissues. The influence of environmental factors on the results is minimised by standardization of laboratory conditions and the use of the subjects (organisms) with known genetic and health status, so called "defined animals"[61].

Relevance of animal studies

Nutrigenomics provide powerful approaches to investigate the relationship between dietary factors and genes, providing insight into how test compounds affect gene-expression patterns (transcriptome), organisation of the chromatin (epigenome), protein expression paterns, including post-translational modifications (proteome), as well as metabolite profile (metabolome). In this regard, laboratory animals are used as complex biological instruments to obtain knowledge on

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gene–nutrient interaction, whilst always recognising that extrapolation to human biology must be done with caution and recognition of sometimes-fundamental species differences. While recognising that phenomena studied in one species can sometimes be extrapolated to another[62], and recognising that present knowledge of human biochemistry, physiology, endocrinology and pharmacology relies fundamentally on studies in animal models[63], and that several advances in nutrigenomics were made using murine animal models[64], it is important to stress, that animal studies including nutrigenomic research are not inherently predictive of the human situation. Animal studies can neither confirm nor refute hypotheses about human physiology or pathology; human clinical investigation is the only way to test such hypotheses.

The purpose of animal studies is to test hypothesis on how the specific factor affects the specific species under specific circumstances. Their value is speeding up the process of discovery by generation of ideas or suggesting hypotheses that might be relevant to humans, assistance in predicting effects in humans, and providing support for particular conclusions reached in the population studies. The differences in biological characteristics (e.g. anatomy, physiology, metabolic rate) between animals and man should be kept in mind while studying qualitative questions devoted to identification of the diet component responsible for a specific function in the body, or quantitative questions concerning a dose of a diet component necessary to cause the specific reaction in the body. In this regard a comparative biology continues to be a useful tool for choosing an appropriate animal model.

Animal models

A widely accepted scientific definition of an animal model is as a living organism in which normative biology or behaviour can be studied, or in which a spontaneous or induced pathological process can be investigated, and in which the phenomenon in one or more respects resembles the same phenomenon in humans[65].

Based on this definition, animal models can be categorised in five groups: (1) normative, (2) spontaneous models in which the phenomenon under investigation occurs spontaneously e.g. Apc^{Min} mice a model of human adenomatous poliposis coli[66], or a Watanabe Heritable Hyperlipidemic (WHHL) rabbit a model of human familiar hypercholesterolemia[67], (3) experimental models in which the phenomenon is induced either chemically e.g., 1,2-dimethylhydrazine dihydrochloride or azoxymethane induced colon cancer in rats, or surgically e.g. uraemia induced by nephrotectomy, or by a genetic manipulation e.g. TG.NK mice with MMTV/C-*neu* transgene as a model of breast cancer[68], (4) negative models in which the phenomenon never occurs/is suppressed either as consequence of normative physiology e.g. dog in atherosclerosis research, or due to genetic manipulation e.g. mice lacking the *S100A4(mts1)* gene have suppressed tumour development and lack of metastases[69] and (5) orphan models where a disease is first recognised and described in an animal species, after which a human counterpart may emerge[70].

As an example of application of normative animal model to study biological effects of phytochemicals an investigation of influence of isoflavone intake on mammary gland morphogenesis and gene expression profile in the juvenile mammary gland of mice can be given[71]. A study of soy isoflavones effect on gene expression of endothelial nitric oxide synthase in cerebral arteries in Watanabe heritable hyperlipidemic rabbits[72] is an example of application of a spontaneous model of human disease (familial hypercholesterolemia) to investigate health beneficial effects of phytochemicals. Similarly studies of effect of lignans or soy isoflavone on intestinal neoplasia in *Apc* ^{Min} mice[73,74] are examples of application of a spontaneous model, while investigations of cancer-preventing effect of soy and/or isoflavones in rats induced by dimethylbenz[a]anthracene[75], or DMBA[76,77] or in transgenic TG.NK mice[78] illustrate application of so called experimental models to study cancer-preventive potential of phytochemicals.

Choice of the animal model

The decision to use an animal model in nutritional research will be informed, first, by the non-availability of ethically more acceptable *in vitro* alternatives. Thereafter, the choice of model will depend on their validity with respect to the nature of phenomenon under study and its expression in the biology of the chosen species. An additional consideration will be the practical aspects of the experimental procedure, husbandry and economic considerations. All these factors are routinely considered within legislative frameworks (which vary by country and institution), which ensure sound ethical practice.

When using animal models it is necessary to recognize their predictive validity (performance in the animal study predicts performance in real (non-experimental) conditions), face validity (phenomenological analogy with the modelled condition) and construct validity (the model has a sound theoretical rationale).

Biology of the chosen species

In considering the biology of the chosen species, it is essential that characterization of the biological system is sufficient to allow sound interpretation of the results. Therefore the chosen species should have defined genetic characteristics. The use of outbred and inbred strains should be considered as both have their advantages and limitations. In general outbred strains are recognized to represent populations, which are more likely to mimic the genetic diversity in human populations. The use of inbred strains is believed to minimalize the inter-individual variation in the response to a studied factor allowing smaller experimental groups and with attraction for mechanistic studies.

The anatomy, biochemistry and physiology should be close to those of man with regard to the dietary requirements and function of digestive system (Table 1). The differences in metabolic rates between the laboratory animal species and between the chosen species and humans should be considered. The latter is important in choice of the dose of test compound for animal dietary interventions[79,80], and an appropriate animal *versus* human compensation factor should be applied (Table 2).

The type of experimental diet and feeding regimens (*ad libitum versus* diet restriction or pair feeding) should also be considered[81]. Factors to consider include the choice between the standard fixed open-formula diets based on natural plant ingredients and therefore containing diverse

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Table 1. Selected variables and characteristics relevant when choosing a laboratory species as a model in nutrition research

Species (body weight) Digestive system	Mouse (20–40 g) Omnivore	Rat (150–300 g) Omnivore*	Pig (60 kg) Omnivore	Hamster Herbivore	Man (60–80 kg) Omnivore
Dietary requirement					
Fat intake (g)	0·3	0·5 g	64 g		129 g
Polyunsaturated/S	1·85	1·85	1·85	1·85	0·4
Fat energy %	11	11	10	11	44
Protein energy %	18	18	25	18	14
Carbohydrate energy %	70	70	65	70	42
Volume of blood sampling (ml)[a]	0·13	0·8–1·3	250	0·5	450[b]
Method of blood sampling	Easy	Easy	Difficult	Easy	–
Acquiring and maintaining	Cheap/easy	Cheap/easy	Relatively[c] easy/cheap	Cheap/easy	–
Availability of commercial kits for examination of gene expression	Available	Available	In process of development and validation	In process of development and validation	Available

[a] The presented values are a guidance for repeated blood sampling at weekly intervals. The volume of a sample corresponds to approximately 7.5 % of total blood volume, and to 0·5 % of body weight.
[b] The normal quantum drawn from blood donors.
[c] The cost of a pig exceeds that of laboratory rodent. Pigs need more space and special housing facilities, and large quantities of feed and of test compounds compared to laboratory rodents. Thus availability of facilities for housing and expenses to experimental feed/test compounds should be considered.
* Rats do not have a bile bladder.

phytochemicals like phytoestrogens, isoflavones and lignans, respectively[82,83], or purified semi-synthetic or synthetic diets. These diets are composed of a refined, invariant, and restricted set of ingredients, which offer less variable and more easily controlled experimental conditions. Additionally, the use of purified or synthetic diets instead of natural ingredient chows, gives more precise control over the metabolizable energy, dietary composition of nutrient, and provides better repeatable experimental conditions[84]. On the other hand, casein in purified semi-synthetic diet aggravates hypercholesterolemia in rabbit models of atherosclerosis[85] and can therefore mask potential hypocholesterolemic effects of phytochemicals in a rabbit model of atherosclerosis or naturally occurring phytochemicals in standard laboratory chow may be confounding factors in studies of anti-atherogenic effects of phytochemicals[86] or in studies of other biological effects of test phytochemicals. Switching from standard laboratory chow to purified diet (and *vice versa*) changes the gut flora, which may lead to altered endogenous vitamin synthesis. Furthermore, the occurrence of common contaminants in natural-ingredient non-purified (standard) diets such as heavy metals (e.g. Pb and As), N-nitrosamines, residua of pesticides and antioxidants (BHA and BHT) may serve to confound experiment outcomes[87].

An optimal diet for laboratory animals should have adequate concentrations of all the nutrients for growth and maintenance without substantial excess of high energy and growth-enhancing nutrients such as fat and protein. However, depending on the aim of the study, it is an open question if the experimental diet should be a standard for the chosen species or should mimic the human Western diet with regard to fat, fibre, and calcium content[88]. Feeding a Western style diet instead of a standard chow diet to a rodent model of cancer can exacerbate cancer development[89]. While choosing the feeding regimens it is worthwhile remembering that diet restriction may modify the responses to (phyto)chemicals, and that the practice is labour-intensive.

Another potential source of experimental variation can arise from homeostatic compensatory reactions, which can result in low precision and scattering of results and even misinterpretations[61].

In conclusion, whilst nutrigenomic studies in animal models have value in predicting the human situation, their use must be justified on sound biological criteria, on practical grounds (with respect to housing, feeding and intervention for sample collection) and from an ethical perspective (availability of alternatives, and predictive value). Ultimately,

Table 2. Mouse dosage compensation factor – examples

Factor and value	Basis/comments/examples	Reference
Human to mouse equivalency factor: 9·0	Body surface area and body weight	The US EPA Handbook
Conversion factor from human to mouse from dietary concentrations to dose per kg body weight (bw): 7·5	Food intake, body weight e.g. dose of a compound at dietary concentration 1 ppm (= 1 mg/kg diet) corresponds to 0·02 mg/kg bw for a human and to 0·15 mg/kg bw for a mouse	Modified from OECD, 2000
Energy requirement ratio (per kg bw): 11·4	Human (60 kg) energy intake is 2000 kcal/day ≈ 33 kcal/kg bw. Mouse (40 g) caloric intake (maintenance) is 15 kcal/day ≈ 375 kcal/kg bw	GN Rao (personal communication)
Conversion factor from human to mouse for anticancer agents:12·3	Body surface, metabolism rate and body weight	Freireich *et al.* 1966[79]

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the effects observed in animal models should be demonstrated in humans.

Human studies

Human nutritional studies can be divided into intervention studies (trials), which under defined circumstances (such as clinical trials), apply nutritional intervention and measure the biological outcome (effect), and observational epidemiological studies, divided into descriptive (correlational, case report-series, cross sectional) and analytical experimentation (case–control, cohort), and meta-analyses. Studies, according to study design, may be prospective or retrospective and may use epidemiological instruments to evaluate biological outcome of an identified nutritional factor or nutrition–genomic relationship.

Intervention studies

Intervention studies differentiate between double blind/(non-blinded) and randomized/(non-randomized) controlled trials. Nutritional trials in human volunteers may typically examine the biological effects of nutrient/nutrients [as e.g. glucan enriched fruit juice[90] or polyphenols in olive oils[91]], or whole foodstuffs [e.g. dried cranberry juice[92]] or food commodities [e.g. vegetables and fruit[93,94]] or the effect of different, albeit sometimes imprecisely defined types of diet [e.g.Mediterranean-style[95] or low fat dietary pattern[96]]. The principle of a nutrigenomic approach i.e. an investigation of whether certain nutrients have direct effects on gene expression is exemplified by a randomised controlled trial studying the effect of omega-3, omega-6, and omega-9 unsaturated fatty acids on unstimulated and stimulated monocytes cytokine gene expression[97], and by a trial of conjugated linoleic acid supplementation[98]. Another type of nutrigenomic approach may test a hypothesis that, for example, a particular gene polymorphism is more or less indicative of a biological outcome determined by special type of diet/nutrients. This type of study is represented by work on human volunteers identifying the cholesterol 7-hydroxylase (CYP7A1) gene and its role in determining the LDL-cholesterol (LDL-C) concentration response to a high-fat diet[99].

Observational epidemiological studies

A particularly critical point in nutritional epidemiology is the ability of the epidemiological instrument to measure habitual dietary intake. Dietary-assessment instruments are used in nutritional descriptional or analytical studies. An example of a descriptive cross sectional study is the study dealing with the fruit and vegetable intakes and bone density[100] or a population-based study on diabetes mellitus and serum carotenoids[101].

Analytical, case–control nutritional studies are based on long-term recall of feeding history. They are especially valid for assessment of gene–environment interactions, where there is a critical demand to improve the accuracy in measurements of both genetic and nutritional factors.

Meta-analyses of observational studies try on a comprehensive, systematic bibliographic search of published medical literature to arrive at quantitative conclusions about the contribution of nutritional factors to the occurrence of disease, for example the relationship between fruit and vegetable intake and the occurrence of oral cancer[102].

Summarizing the current knowledge and experience within the nutrition research in human studies indicates that methodological as well as heuristic limits of epidemiological (including interventional) and clinical trials have been reached. The effects of nutrition on health and disease cannot be fully explained without a more comprehensive understanding of how nutrients act at nuclear level and what role they play in the intra- and intercellular signal transduction.

Concluding remarks

Direct, definitive information on the effects of dietary factors, whether they be nutritional or non-nutritional dietary components, on human health can only be obtained through investigation in human subjects. However, the obvious ethical and practical limitations of interventionist human studies limit their applicability. Because of these limitations, intervention trials in healthy subjects and patients often provide information only on early or short-lasting biological effects of the treatment. In consequence, the duration of the intervention is usually much too short to allow study of patho-physiological end-points of interest (e.g. development of tumours or atherosclerotic lesions), and therefore disease indicators are typically measured as clinico-chemical biomarkers. Furthermore, the confounding factors of lifestyle and poor compliance of human subjects with the study protocol may influence the results obtained. Epidemiological studies can be resource-effective but time-consuming, and interpretation of the results is difficult because of the multifactorial nature of the effect. Other more technical obstacles also exist.

The limitations related to human studies are the reason for using alternative model systems in nutritional research and nutrigenetics. Well-characterized *in vitro* model systems give insight in metabolic pathways and responses to test stimuli on cellular and molecular levels, while studies in animal models permit evaluation of the biological significance of the effects recorded in *in vitro* studies. Human nutritional trials may then become justifiable, based on primary knowledge obtained from studies *in vitro*, animal models or observational epidemiological studies.

Nutrigenomics will promote an increased understanding of how nutrition influences metabolic pathways and homeostatic control, how this regulation is disturbed in the early phases of diet-related disease, and the extent to which individual sensitizing genotypes contribute to such diseases. Keeping in mind the limitations of human studies, the use of *in vitro* and *in vivo* models will continue and, through advances in cell and molecular biology (including genomic and proteomic), should become more predictively accurate. However, this predictive value relies on an underpinning knowledge of the advantages and limitations of the model in nutrigenomic research as in other fields of biomedical research.

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