AQUABIOPRO-*FIT* at a glance

PART II

Nutritional supplements bioactivity, functional properties and safety: *in vitro* & *in vivo* studies

PART II: Nutritional supplements bioactivity, functional properties and safety: in vitro & in vivo studies

Summary:	Part II includes 7 courses devoted to in vitro and in vivo assays for testing the bioactivity, functional properties and safety of nutritional supplements. Dietary supplements deliver essential nutrients, including vitamins, minerals and amino acids. Microminerals, macrominerals and bioactive food components are equally important. They can only be legally sold with a warning statement not to exceed the recommended dose. The legal and regulatory framework regarding the safety, efficacy and labeling of food supplements is outlined. This Part devotes special attention to in vitro and in vivo assays as fundamental steps before any safe conclusions can be drawn for the effectiveness and safety of a dietary supplement. Cell-culture based models and animal models for testing the bioactivity of nutritional supplements (anti-inflammatory, wound-healing, anti-cancer, cytostatic or pro-apoptotic properties, anti-obesity properties etc.) are described. In line with the growing interest in gut microbiome manipulation using nutritional supplements as pre-, pro- or post-biotics, animal models for assessing changes in the gut microbiome and methods for assessment of such changes are presented, with special emphasis on modern molecular techniques. This Part also educates readers on how to design and conduct clinical studies aiming at establishing the effectiveness of nutritional supplements on human health, well-being, and performance.

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Course 2.1: Nutritional supplements in general

Author: Zoi Georgiou

2.1.1 Introduction

A dietary supplement is a manufactured product intended to supplement the diet when taken by mouth as a pill, capsule, tablet, or liquid. A supplement can provide nutrients either extracted from food sources or synthetic, individually or in combination, in order to increase the quantity of their consumption. The class of nutrient compounds includes vitamins, minerals, fiber, fatty acids and amino acids (https://en.wikipedia.org/wiki/Amino_acid). Dietary supplements can also contain substances that have not been confirmed as being essential to life, but are marketed as having a beneficial biological effect, such as plant pigments or polyphenols. Animals can also be a source of supplement ingredients, as for example collagen (https://en.wikipedia.org/wiki/Collagen) from chickens or fish. These are also sold individually and in combination, and may be combined with nutrient ingredients.

The scope of their use is to deliver nutrients that humans may not consume in sufficient quantities. People following the modern way of living, supplement their diets for reasons as uncertainty about the nutrient sufficiency of their diets, a desire for an upgraded health status, or following medical consultation, and desire to treat themselves for an illness. However, in order human body functions to be in balance only a certain amount of each nutrient is needed and some substances may have adverse effects, and become harmful at high doses. The use of dietary supplements is probably fostered by their wide availability, aggressive marketing, and media reports on studies suggesting that supplements may help to prevent or treat common health problems. The use of dietary or nutritional supplements now days, is extensive and noticeably increasing, as they are available in different doses and combinations. For safeguarding consumers' health, supplements can therefore only be legally sold with an appropriate daily dose recommendation, and a warning statement not to exceed that dose. In the United States and Canada, dietary supplements are considered a subset of foods, and are regulated accordingly. The European Commission (https://en.wikipedia.org/wiki/European_Commission) has also established harmonized rules to help insure that food supplements are safe and properly labeled and the EU's food safety authority, EFSA, is currently reviewing health claims for the various nutrients.

2.1.2 Bioactivity and functional properties of Nutritional Supplements

Vitamins are natural components of foods that are essential in very small amounts for supporting normal physiologic function. It is recognized that vitamin deficiencies can create or intensify chronic health conditions. They have an essential role for body physiologic functions such as growth, reproduction, etc. Vitamins and their derivatives often serve a variety of roles in the body and one of the most important is their function as cofactors for enzymes, e.g. coenzymes NAD and FAD require vitamins B3 and B2, and red blood cell synthesis demands

vitamin B9, B6, B12, but also for energy releasing processes Vitamin B1, B2, B3, B5, B6, biotin are necessary.

Water soluble vitamins (Table 2.1.1) are absorbed by both passive and active mechanisms, based on specific molecular carriers, are not stored in big quantities in the body and are excreted in the urine with their metabolites. There is a constant need for their regular intake via food.

Table 2.1.1 Nine water-soluble vitamins

Vitamin	Deficiency	Toxicity	Sources
Vitamin B1 (Thiamine)	Burning feet, weakness in extremities, rapid heart rate, swelling, anorexia, nausea, fatigue, and gastrointestinal problems.	None known.	Sunflower seeds, asparagus, lettuce, mushrooms, black beans, navy beans, lentils, spinach, peas, pinto beans, lima beans, eggplant, Brussels sprouts, tomatoes, tuna, whole wheat, soybeans
Vitamin B2 (Riboflavin)	Cracks, fissures and sores at corner of mouth and lips, dermatitis, conjunctivitis, photophobia, glossitis of tongue, anxiety, loss of appetite, and fatigue.	Excess riboflavin may increase the risk of DNA strand breaks in the presence of chromium. High-dose riboflavin therapy will intensify urine color to a bright yellow (flavinuria)	Almonds, soybeans, mushrooms, spinach, whole wheat, yogurt, mackerel, eggs, liver
Vitamin B3 (Niacin)	dermatitis, diarrhea, dementia, and stomatitis	Niacin from foods is not known to cause adverse effects. Supplemental nicotinic acid may cause flushing of skin, itching, impaired glucose tolerance and gastrointestinal upset. Intake of	Mushrooms, asparagus, peanuts, brown rice, corn, green leafy vegetables, sweet potato, potato, lentil, barley, carrots, almonds, celery, turnips, peaches, chicken meat, tuna, salmon

		750 mg per day for less than 3 months can cause liver cell damage. High dose nicotinamide can cause nausea and liver toxicity	
Vitamin B5 (Pantothenic acid)	Very unlikely. Only in severe malnutrition may one notice tingling of feet.	Nausea, heartburn and diarrhea may be noticed with high dose supplements.	Broccoli, lentils, split peas, avocado, whole wheat, mushrooms, sweet potato, sunflower seeds, cauliflower, green leafy vegetables, eggs, squash, strawberries, liver
Vitamin B6 (Pyridoxine)	chelosis, glossitis, stomatitis, dermatitis (all similar to vitamin B2 deficiency), nervous system disorders, sleeplessness, confusion, nervousness, depression, irritability, interference with nerves that supply muscles and difficulties in movement of these muscles, and anemia. Prenatal deprivation results in mental retardation and blood disorders for the newborn.	High doses of supplemental vitamin B6 may result in painful neurological symptoms.	Whole wheat, brown rice, green leafy vegetables, sunflower seeds, potato, garbanzo beans, banana, trout, spinach, tomatoes, avocado, walnuts, peanut butter, tuna, salmon, lima beans, bell peppers, chicken meat
Vitamin B9 (Folic acid)	Anemia (macrocytic/megaloblastic), sprue, Leukopenia, thrombocytopenia, weakness, weight loss, cracking and redness of tongue and mouth, and diarrhea. In pregnancy there is a risk of low birth weight and preterm delivery.	None from supplements or food	: Green leafy vegetables, asparagus, broccoli, Brussels sprouts, citrus fruits, black eyed peas, spinach, great northern beans, whole grains, baked beans, green peas, avocado,

			peanuts, lettuce, tomato juice, banana, papaya, organ meats
Vitamin B12 (Cobalamin)	Symptoms include pernicious anemia, neurological problems and sprue	None known from supplements or food. Only a small amount is absorbed via the oral route, thus the potential for toxicity is low.	Fortified cereals, liver, trout, salmon, tuna, haddock, egg
Vitamin H (Biotin)	Very rare in humans. Keep in mind that consuming raw egg whites over a long period of time can cause biotin deficiency. Egg whites contain the protein avidin, which binds to biotin and prevents its absorption.	Not known to be toxic.	Green leafy vegetables, most nuts, whole grain breads, avocado, raspberries, cauliflower, carrots, papaya, banana, salmon, eggs
Vitamin C (Ascorbic acid)	Bruising, gum infections, lethargy, dental cavities, tissue swelling, dry hair and skin, bleeding gums, dry eyes, hair loss, joint paint, pitting edema, anemia, delayed wound healing, and bone fragility. Long-term deficiency results in scurvy.	Possible problems with very large vitamin C doses including kidney stones, rebound scurvy, increased oxidative stress, excess iron absorption, vitamin B12 deficiency, and erosion of dental enamel. Up to 10 grams/day is safe based on most data. 2 grams or more per day can cause diarrhea.	Guava, bell pepper, kiwi, orange, grapefruit, strawberries, Brussels sprouts, cantaloupe, papaya, broccoli, sweet potato, pineapple, cauliflower, kale, lemon juice, parsley

Fat soluble vitamins (Table 2.1.2) are mostly absorbed passively and must be transported with dietary fat. These vitamins are usually found in the portion of the cell which contains fat, including membranes, lipid droplets, etc.

Table 2.1.2 Four fat soluble vitamins

Vitamin	Deficiency	Toxicity	Sources
Vitamin A (Retinoids) Carotenoids that can be converted by the body into retinol are referred to as provitamin A carotenoids.	Difficulty seeing in dim light and rough/dry skin.	Nausea, headache, fatigue, loss of appetite, dizziness, and dry skin can result. Excess intake while pregnant can cause birth defects	Carrots, sweet potato, pumpkin, green leafy vegetables, squash, cantaloupe, bell pepper, Chinese cabbage, beef, eggs, peaches
Vitamin D (Calciferol, 1,25-dihydroxy vitamin D) Cholecalciferol = vitamin D3 = animal version; ergocalciferol = vitamin D2 = plant version	In children: rickets, deformed bones, retarded growth, and soft teeth. In adults: osteomalacia, softened bones, spontaneous fractures, and tooth decay. At risk for deficiency: infants, elderly, dark skinned individuals, those with minimal sun exposure, fat malabsorption syndromes, inflammatory bowel diseases, kidney failure, and seizure disorders	Excessive supplement use will elevate blood calcium levels and cause loss of appetite, nausea, vomiting, excessive thirst, excessive urination, itching, muscle weakness, joint pain and disorientation. Calcification of soft tissues can also occur	Sunlight, fortified foods, mushrooms, salmon, mackerel, sardines, tuna, eggs
Vitamin E (tocopherol)	Only noticed in those with severe malnutrition. However, suboptimal intake of vitamin E is relatively common.	Minimal side effects have been noted in adults taking supplements in doses less than 2000 mg/day.	Green leafy vegetables, almonds, sunflower seeds, olives, blueberries, most nuts, most seeds,

		There is a potential for impaired blood clotting. Infants are more vulnerable.	tomatoes, avocado
Vitamin K	Tendency to bleed or hemorrhage and anemia.	No known toxicity with high doses.	Broccoli, green leafy vegetables, parsley, watercress, asparagus, Brussels sprouts, green beans, green peas, carrots

Most minerals are considered essential and comprise a vast set of micronutrients. There are both macrominerals (Table 2.1.3) (required in amounts of 100 mg/day or more) and microminerals (Table 2.1.4) (required in amounts less than 15 mg/day).

Table 2.1.3 Five macrominerals

Macrominerals	Deficiency	Toxicity	Sources
Calcium	Low bone mineral density, rickets, osteomalacia and osteoporosis.	Nausea, vomiting, constipation, dry mouth, thirst, increased urination, kidney stones and soft tissue calcification	Dairy, green leafy vegetables, legumes, tofu, molasses, sardines, okra, perch, trout, Chinese cabbage, rhubarb, sesame seeds
Phosphorus	Very rare. Those at risk include premature infants, those who use antacids, alcoholics, uncontrolled diabetes mellitus and refeeding syndrome	Very rare. May result in soft tissue calcification	Legumes, nuts, seeds, whole grains, eggs, fish, buckwheat, seafood, corn, wild rice
Potassium	Not a result of	Found with kidney	Sweet potato,

	insufficient dietary intake. Caused by protein wasting conditions. Diuretics can also cause excessive loss of potassium in the urine. Low blood potassium can result in cardiac arrest.	failure and potassium sparing diuretics. Oral doses greater than 18 grams can lead to toxicity. Symptoms include tingling of extremities and muscle weakness. High dose potassium supplements may cause nausea, vomiting and diarrhea.	tomato, green leafy vegetables, carrots, prunes, beans, molasses, squash, fish, bananas, peaches, apricots, melon, potatoes, dates, raisins, mushrooms
Magnesium	Very rare due to abundance of magnesium in foods. Those with gastrointestinal disorders, kidney disorders, and alcoholism are at risk.	None identified from foods. Excessive consumption of magnesium containing supplements may result in diarrhea (magnesium is a known laxative), impaired kidney function, low blood pressure, muscle weakness, and cardiac arrest	Legumes, nuts, seeds, whole grains, fruits, avocado
Salt (sodium chloride)	Does not result from low dietary intake. Low blood sodium typically results from increased fluid retention. One may notice nausea, vomiting, headache, cramps, fatigue, and disorientation.	Excessive intake can lead to increased fluid volume, nausea, vomiting, diarrhea and abdominal cramps. High blood sodium usually results from excessive water loss.	Any processed foods, whole grains, legumes, nuts, seeds, vegetables

Table 2.1.4 Nive microminerals

Microminerals	Deficiency	Toxicity	Sources
Iron	Anemia with small and pale red blood cells. In children it is associated with behavioral abnormalities.	Common cause of poisoning in children. Excessive intake of supplemental iron is an emergency room situation. Cardiovascular disease, cancer, and neurodegenerative diseases are associated with iron excess	Almonds, apricots, baked beans, dates, lima beans, kidney beans, raisins, brown rice, green leafy vegetables, broccoli, pumpkin seeds, tuna, flounder, chicken meat, pork
Zinc	Decreased immunity cellular damage from increased oxidative stress, diarrheal disease Growth retardation, lowered immune statue, skeletal abnormalities, delay in sexual maturation, poor wound healing, taste changes, night blindness and hair loss. Those at risk for deficiency include the elderly, alcoholics, those with malabsorption, vegans, and those with severe diarrhea.		abdominal pain, diarrhea, nausea, and vomiting. Long-term consumption of excessive zinc can result in copper deficiency.
Copper	is hypochromic anemia unresponsive to iron therapy. Neutropenia and leucopenia. Hypopigmentation	Symptoms include abdominal pain, nausea, vomiting, and diarrhea. Long-term exposure to lower doses of copper	Mushrooms, green leafy vegetables, barley, soybeans, tempeh, sunflower seeds,

	of skin and hair. Those at risk for deficiency include premature infants, infants fed only cow's milk formula, those with malabsorption syndromes, excessive zinc consumption and antacid use.	can result in liver damage.	navy beans, garbanzo beans, cashews, molasses, liver
Chromium	Symptoms include impaired glucose tolerance and elevated circulating insulin	Limited to industrial exposure. Long-term supplement use may increase DNA damage. Rare cases of kidney failure have also been documented.	Lettuce, onions, tomatoes, whole grains, potatoes, mushrooms, oats, prunes, nuts, brewer's yeast
Fluoride	Increased risk of dental caries.	Mottled tooth enamel nausea, abdominal pain, and vomiting.	Water, tea, fish
Iodine	Impairs growth and neurological development, decreased production of thyroid hormones and hypertrophy of the thyroid.	Burning mouth, throat and stomach. Fever and diarrhea can also result.	Sea vegetables, iodized salt, eggs, strawberries, asparagus, green leafy vegetables
Selenium	Limited glutathione activity, juvenile cardiomyopathy and chondrodystrophy.	Multiple symptoms including dermatologic lesions, hair and nail brittleness, gastrointestinal disturbances, skin rash, fatigue, and nervous system abnormalities.	Brazil nuts, mushrooms, barley, salmon, whole grains, walnuts, eggs

Manganese	Not typically observed in humans.	Generally, from industrial exposure.	Green leafy vegetables, berries, pineapple, lettuce, tempeh, oats, soybeans, spelt, brown rice, garbanzo beans
Molybdenum	Never been observed in healthy people.	Never been observed in healthy people.	Legumes, whole grains

Vitamins and minerals keep a 'k' role in normalizing physical functions and an adequate intake from food and/or supplements is necessary to prevent deficiency, promote optimal health, improve nutrient partitioning and promote fat loss and muscle gain.

Some other important bioactive food components are polyphenols, anthocyanidins, carotenoids, flavonoids, glucosinolates, isoflavonoids, limonoids, lycopenes, omega-3 and 6 fatty acids, phytoestrogens, phytosterols, polyphenols, probiotics, resveratrol and terpenoids. They play specific pharmacological effects in human health as anti-inflammatory, anti-allergic, antioxidants, antibacterial, antifungal,antispasmodic, chemopreventive, hepato-protective, hypolipidemic, neuroprotective, hypotensive, anti-ageing, antidiabetic, osteoporosis, protection of DNA damage, cancer and heart diseases, induce apoptosis, diuretic, CNS stimulant, analgesic, protects from UVB-induced carcinogenesis, immuno-modulator and carminative.

The European Responsible Nutrition Alliance (ERNA) has published an information book on the scientific facts relating to the safety and health benefits of vitamins, minerals, and other food components in Oct 10, 2011. The fact book, which can also be downloaded from the ERNA website, was developed to provide an overview of information available in scientific literature, and it is presented in the next Table 2.1.5.

Table 2.1.5. Facts about vitamins, minerals and other food components with health effects ERNA

Facts about vitamins, minerals and other food components with health effects	
	It is an illustrative communication tool underlining the importance for health of vitamins (A, B, C, D, E and folate); minerals (calcium, iron, magnesium, selenium, and zinc); carotenoids (beta carotene, lycopene, and lutein), and a number of bioactive substances, including polyunsaturated fatty acids (PUFA), conjugated linoleic acid, L-carnitine, Coenzyme Q10 and Epigallocatechin gallate (EGCG).

In addition, manufacturers of nutritional supplements need a broad knowledge of the bio-availability of the active ingredients and their effects to be able to make high-quality products. There are major variations in the body's uptake and the effect of the active ingredients depending on the form in which they are supplied. The regulations on nutritional supplements have appendices that are intended to ensure that the chemical compounds used as a source of vitamins and minerals are safe and present in a form that the body can absorb.

2.1.3 Dietary Reference Values (DRVs) for nutrients

Each nutrient has a particular function in the human body, and a balanced diet provides adequate amounts of all necessary nutrients to maintain health and well-being. The amount of each individual nutrient needed to maintain an individual's health is called the nutrient requirement. Nutrient requirements vary depending on age and gender, level of physical activity, physiological status (such as pregnancy), dietary habits and genetic background. Dietary reference values (DRVs) for Vitamins (Table 2.1.6) and (DRVs) for Minerals (Table 2.1.7) are quantitative reference values for nutritional intakes derived for different population groups, based on health criteria.

Table 2.1.6 DRVs for Vitamins. Summary of Tolerable Upper Intake Levels (UL) of Vitamins and Fatty acids (EFSA Summary of Tolerable Upper Intake Levels – version 4 (September 2018))

	Unit	Age/Life-stage group 0-1 y	1-3 y	4-6 y	7-10 y	11-14 y	15-17 y	Adults	Pregnancy	Lactation
Boron	mg/d		3	4	5	7	9	10	10	10
Calcium	mg/d	No adequate data to derive a UL						2500	2500	2500
Chloride		No adequate data to derive a UL								
Chromium (trivalent)		No adequate data to derive a UL								
Copper	mg/d		1	2	3	4	4	5	Insufficient data	
Iodine	µg/d		200	250	300	450	500	600	600	600
Iron		No adequate data to derive a UL								
Magnesium[(a)]	mg/d		Insufficient data	250	250	250	250	250	250	250
Manganese		No adequate data to derive a UL								
Molybdenum	mg/d		0.1	0.2	0.25	0.4	0.5	0.6	0.6	0.6
Nickel		No adequate data to derive a UL								
Phosphorus		No adequate data to derive a UL								
Potassium		No adequate data to derive a UL								
Selenium	µg/d		60	90	130	200	250	300	300	300
Silicon		No adequate data to derive a UL								
Sodium		No adequate data to derive a UL								
Tin		No adequate data to derive a UL								
Vanadium		No adequate data to derive a UL								
Zinc	mg/d		7	10	13	18	22	25	25	25
	Unit	**Age/Life-stage group 0-1 y**	**1-3 y**	**4-8 y**	**9-14 y**		**15-17 y**	**Adults**	**Pregnancy**	**Lactation**
Fluoride	mg/d		1.5	2.5	5		7	7	7	7

d, day; y, year

Table 2.1.6 DRVs for Minerals. *Summary of Tolerable Upper Intake Levels (UL) of minerals (EFSA Summary of Tolerable Upper Intake Levels – version 4 (September 2018))*

	Unit	Age/Life-stage group 0-6 mo	6-12 mo	1-3 y	4-6 y	7-10 y	11-14 y	15-17 y	Adults	Pregnancy	Lactation
VITAMINS											
Biotin		No adequate data to derive a UL									
β-Carotene		No adequate data to derive a UL									
Folic acid (synthetic)	µg/d			200	300	400	600	800	1000	1000	1000
Niacin											
Nicotinamide	mg/d			150	220	350	500	700	900	Inadequate data	
Nicotinic acid	mg/d			2	3	4	6	8	10	Inadequate data	
Pantothenic acid		No adequate data to derive a UL									
Vitamin A[(a)]	µg RE/d			800	1100	1500	2000	2600	3000[(b)]	3000	3000
Vitamin B1		No adequate data to derive a UL									
Vitamin B12		No clearly defined adverse effects									
Vitamin B2		No adequate data to derive a UL									
Vitamin B6	mg/d			5	7	10	15	20	25	25	25
Vitamin C		No adequate data to derive a UL									
Vitamin D	µg/d	25	35	50	50	50	100	100	100	100	100
Vitamin E	mg/d			100	120	160	220	260	300	300	300
Vitamin K		No adequate data to derive a UL									
FATTY ACIDS											
DHA, EPA, DPA		No adequate data to derive a UL									

d, day; DHA, docosahexaenoic acid, DPA, docosapentaenoic acid; EPA, eicosapentaenoic acid; mo, month; RE, retinol equivalents; y, year

DRVs are used for the estimation of quantities of energy and nutrients needed to support adequate growth, development and health, while reducing the risk of deficiencies and non-communicable diseases. DRVs are developed for different life-stages and gender groups, and for different age ranges, depending on the available data. Within any population group, nutrient requirements vary between individuals. DRVs are intended for healthy people. Those who suffer from diseases may have different needs. Health professionals provide guidance to individuals or groups with specific needs. DRVs were set in the EU Food Information for Consumers Regulation 1169/2011, and are also used as the basis for information on food labels and for establishing dietary guidelines, to help consumers make healthy dietary choices. In Europe, terminology has been harmonised and EFSA used the following set of defined DRVs.

- ✓ Population Reference Intake: level of intake adequate for virtually all people, i.e. an optimal intake for the population as a whole.
- ✓ Average Requirement: level of intake adequate for half of the people, assuming a normal distribution of requirements
- ✓ Lower Threshold Intake: level of intake below which, on the basis of current knowledge, almost all individuals would have an inadequate intake.
- ✓ Adequate Intake (when evidence is insufficient to set a Population Reference Intake): the average level of a nutrient consumed by healthy populations (i.e. assumed to be adequate).

Since DRVs not only aim to ensure sufficient intakes of essential nutrients, but also guard against over-consumption, the following reference values also exist:

- ✓ Reference intake ranges for macronutrients: expressed as a proportion of daily energy intakes, to reflect intakes that are adequate for maintaining health and are associated with a low risk of chronic disease. For example, the reference intake range set for dietary fat is 20–35% of total daily energy intake.
- ✓ Tolerable Upper Intake Level (or Upper Level): the maximum level of chronic daily intake of nutrients unlikely to have adverse health effects

Dietary intakes can be estimated and assessed against DRVs to determine whether diets are at risk of inadequate, or indeed excessive, intakes of nutrients. These values guide professionals on the amount of a nutrient needed to maintain health in an otherwise healthy individual or group of people. In the next Table 2.1.7 is presented an EFSA's extended report on different Dietary Reference Values (DRVs) for nutrients.

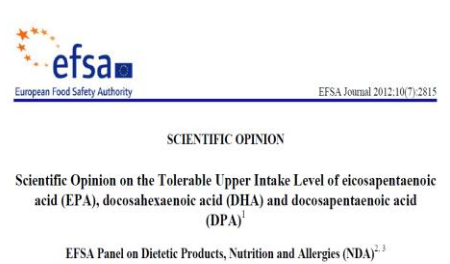
Table 2.1.7 EFSA's extended report on different Dietary Reference Values (DRVs) for nutrients.

EFSAs Technical Report on Dietary Reference Values for Nutrients	
TECHNICAL REPORT efsa European Food Safety Authority Approved: 4 December 2017 doi: 10.2903/sp.efsa.2017.e15121 **Dietary Reference Values for nutrients** **Summary report** European Food Safety Authority (EFSA)	Are presented: reference values for nutrients, dietary nutrient intake, tolerable upper intake levels for nutrients, food health claims, bioactive nutrients.

Regulatory authorities around the world need to ensure that levels of micronutrients in the total diet are safe, and that the cumulative intake from all sources does not lead to excessive intakes and any adverse effects in the population, including sensitive groups such as children, the elderly and women during pregnancy and lactation. Major physiological changes in the velocity of growth and in endocrine status occur during childhood and adolescence. The onset of puberty is an extremely anabolic period that is influenced by a marked rise in hormonal activity, which results in a number of physical changes that characterize adolescence. Although most nutraceuticals currently used are known vital nutrients for the human body, many details such as dose, drug-drug

interaction, nutraceutical-drug interaction, and their effects on individuals under certain health conditions remain elusive. In the next Table 2.1.8. is presented scientific opinion of EFSA on the tolerable Upper intake level of EPA, DHA, DPA.

Table 2.1.8. Scientific Opinion on the Tolerable Upper Intake Level of EPA, DHA, DPA EFSA Journal 2012

Scientific Opinion on the Tolerable Upper Intake Level of EPA, DHA, DPA	
 efsa European Food Safety Authority EFSA Journal 2012:10(7):2815 SCIENTIFIC OPINION Scientific Opinion on the Tolerable Upper Intake Level of eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA) and docosapentaenoic acid (DPA)[1] EFSA Panel on Dietetic Products, Nutrition and Allergies (NDA)[2, 3]	Following a request from the European Commission, the Panel on Dietetic Products, Nutrition and Allergies was asked to deliver a scientific opinion on the Tolerable Upper Intake Level of eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA) and docosapentaenoic acid (DPA).

Increasing knowledge from food chemistry, nutritional and clinical studies is providing more insight into our understanding of biological functions, usage and potential adverse effects of nutritional elements.

There are three major and complementary ways of delivering the essential micronutrients for human health and well-being:

- ✓ by promoting the consumption of nutrient-dense foods
- ✓ by increasing the availability and intake of foods with added nutrients (fortified foods)
- ✓ by the appropriate use of food (dietary) supplements

However, it is necessary to ensure that levels of micronutrients in the total diet are safe and that the cumulative intake from all sources does not lead to excessive intakes and any adverse effects.

The Population Reference Intakes (PRIs), also called Recommended Daily Allowances (RDAs), are based on the principle that most, if not all, individuals of a population or a specific population group should obtain an adequate nutrient intake to satisfy their requirements. Such recommended values are generally based on the principle of the average requirement plus two Standard Deviations (SD) for the nutrient in the population group. In 1992 an SCF opinion on nutrient and energy intakes for the European Community3 defined the PRIs for several nutrients. These values were mainly determined on the basis of the concept of optimal nutrition which implied that these values were determined among other, to prevent deficiencies, to optimize body stores and to reduce the risk of diseases.

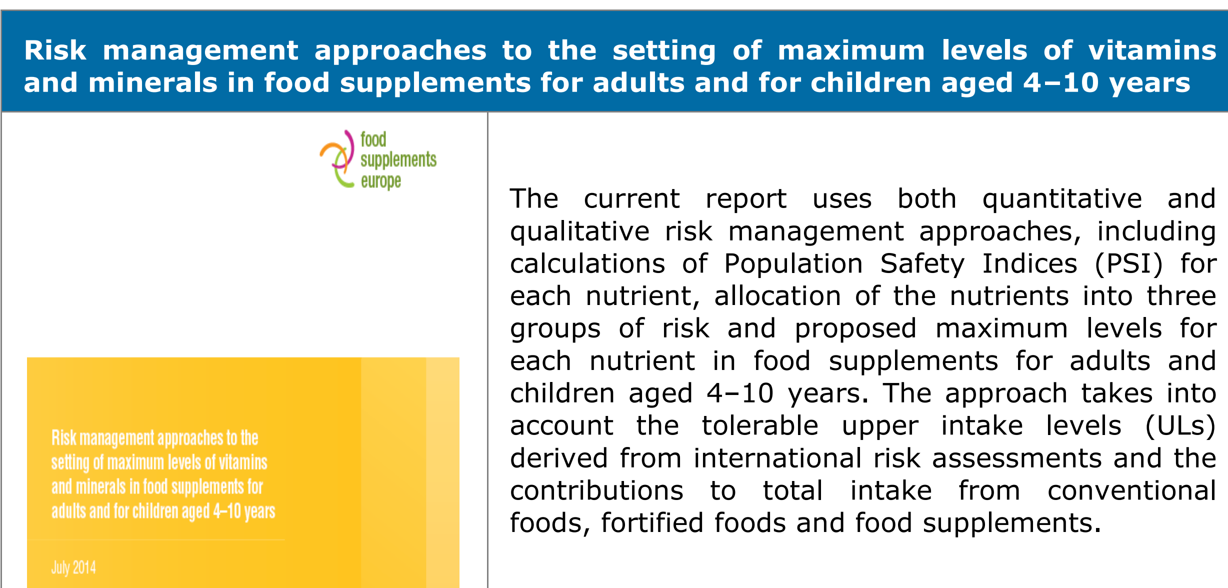
Risk analysis for nutrients differs from other substances in foods because vitamins and minerals are essential for life, and consequently adverse effects can result from suboptimal intakes and deficiencies as well as from excessive intakes. Risk analysis comprises three distinct but closely linked components: risk assessment, risk management and risk communication.

The quantitative and qualitative risk management approaches described in this report attempt to address the many difficulties, limitations and inconsistencies

surrounding the establishment of ULs, particularly for children e.g. the limited nutrient intake data from conventional foods, fortified foods and food supplements, the uncertainties and biases in the estimation of habitual nutrient intake distributions and the care needed not only to address the risk of excessive intakes but also the risk of suboptimal intakes and micronutrient deficiencies in vulnerable groups.

The Food Supplements Europe society published in July 2014 an extensive report on Risk management approaches to the setting of maximum levels of vitamins and minerals in food supplements for adults and for children aged 4–10 years presented in the next Table 2.1.9.

Table 2.1.9. Food Supplements Europe report on Risk management approaches to the setting of maximum levels of vitamins and minerals in food supplements for adults and for children aged 4–10 years



Risk management approaches to the setting of maximum levels of vitamins and minerals in food supplements for adults and for children aged 4–10 years	
food supplements europe Risk management approaches to the setting of maximum levels of vitamins and minerals in food supplements for adults and for children aged 4–10 years July 2014	The current report uses both quantitative and qualitative risk management approaches, including calculations of Population Safety Indices (PSI) for each nutrient, allocation of the nutrients into three groups of risk and proposed maximum levels for each nutrient in food supplements for adults and children aged 4–10 years. The approach takes into account the tolerable upper intake levels (ULs) derived from international risk assessments and the contributions to total intake from conventional foods, fortified foods and food supplements.

Obtaining adequate nutrients from various foods plays a vital role in maintaining normal function of the human body. New concepts have appeared, such as nutraceuticals, nutritional therapy, and these functional or medicinal foods play positive roles in maintaining well being, enhancing health, and modulating immune function to prevent specific diseases.

2.1.4 Do we need nutritional supplements?

Changing culinary and social habits have led to low intakes for some vitamins and minerals compared to those being recommended for certain groups of the population, although the vitamins and minerals and the groups of the population concerned may vary from Member State to Member State. In addition, some argue that optimal health may depend on higher levels of vitamins and minerals than those recommended today on the basis of avoiding deficiencies. Partly due to contemporary lifestyle, people are not following a healthy diet. In Europe, dietary surveys have suggested that there are sub optimal intakes for several micro nutrients. The EU-funded EURRECA 2 project found inadequate intakes for

vitamin C, vitamin D, folic acid, calcium, selenium and iodine. A recent comparison of national surveys showed widespread concern about vitamin D intakes, whereas certain age groups are more likely to have low intakes of minerals (https://www.eufic.org/en/healthy-living/article/food-supplements-who-needs-them-and-when - ref2). For example, there is concern about adequate intakes of iron among teenage girls in Denmark, France, Poland, Germany and the UK. Poor iron status in young women also increases the risk of infants being born with low-birth weight, iron deficiency and delayed brain development. Folate status is also critical for women who may become pregnant. They are advised to take folic acid before conception, and continue for the first 12 weeks of pregnancy. An adequate folate status can decrease the risk of having a baby with neural tube defects such as spina bifida. Recent research suggests that 50–70% of Europeans including habitants of Mediterranean countries have poor vitamin D status, despite their bigger exposure to UV light. In some countries (including UK, Ireland, the Netherlands and Sweden) there are already recommendations for certain groups in the population to take a vitamin D supplement. Other common concerns are shown in Table 2.1.10, although groups considered at risk are not the same in different countries.

Table 2.1.10 Examples of population groups requiring specific advice about supplements

Population group	Nutrients
People over age 50	Vitamin D, Vitamin B12, folate
Women of childbearing age	Folic acid and vitamin D, possibly iron
Children under age 5	Vitamin A, vitamin C, vitamin D, although children with a good appetite who eat a wide variety of food may not need them.
Breastfeeding individuals	Vitamin D
People with insufficient sun exposure or darker skin	Vitamin D
Vegans	Vitamin B12, vitamin D2

Supplement use varies in Europe. For example, it is common in Germany and Denmark (43% and 59% of the adult population respectively) but is less so in Ireland and Spain (23% and 9% respectively). Women use supplements more than men. Despite having a role in the health of some individuals, not all supplements are useful for everybody. In fact, for some people, it is not advisable to take certain supplements, in particular in high doses. Some studies show multivitamins can contribute to an increased risk of excessive nutrient intakes, and it has been suggested that multivitamins should be formulated with greater consideration for the intakes of micronutrients from foods. The Norwegian dietary recommendations advise people with a very low energy intake (< 6.5 MJ/d or < 1 550 kcal/d) to use a multivitamin/mineral supplement. This applies in particular to elderly persons who have a small food intake. In addition, people of dark complexion and people who are little exposed to sunlight should take a daily supplement of 10 µg of vitamin D in addition to what is ingested through food.

According to a German cohort study the use of vitamin and/or mineral supplements was significantly associated with higher age, being non- or ex-

smoker, lower BMI, higher physical leisure time activity, and higher educational level. After adjustment for these factors, there was observed positive associations between supplement use and the consumption of milk, milk products, and fish as well as the intake of vitamin C and beta-carotene. In contrast, the supplement use was related to lower meat and meat product consumption, saturated fat intake, and n6/n3-fatty acid ratio in the diet, both in women and men.

The benefits of nutritional supplements are a source of constant debate. Debates have included whether large doses of vitamins and minerals in the form of nutritional supplements may have undesired health effects. Products in which the content of vitamins and minerals exceeds the maximum values defined by the regulations are classified as pharmaceuticals.

Nutritional supplements contain nutrients that can also be ingested through a healthy diet. According to the regulations, it is illegal to claim or to render the impression that a balanced and varied diet in general will not provide a sufficient supply of vitamins and minerals. The Directorate of Health has defined the recommended intake of various vitamins, minerals and other nutrients. If the diet does not provide these, nutritional supplements can increase the body's level of them, and thereby prevent symptoms of deficiencies. For example, the Norwegian regulations define minimum and maximum limits for the various vitamins and minerals. The maximum limit for vitamin C and vitamin E are 200 mg and 30 mg respectively since Sweden has different regulations, which do not define maximum limits, and therefore their nutritional supplements contain far higher doses of nutrients.

Therefore, food supplements are intended to correct nutritional deficiencies, maintain an adequate intake of certain nutrients, or to support specific physiological functions. Medication use, forces the body to react in certain ways in order to achieve a desirable effect, since nutritional supplement gives the body the actual tools it needs to build health at the cellular level. Nutritional supplements are actually vitamins and minerals that the body needs, to function properly. They are not medicinal products and as such cannot exert a pharmacological, immunological or metabolic action. Therefore, their use is not intended to treat or prevent diseases in humans or to modify physiological functions.

Consequently, it is illegal to claim that nutritional supplements can prevent, heal or alleviate diseases, symptoms of diseases or pain. Such marketing of a nutritional supplement entails its classification as a drug. Dishonest suppliers who market their products with such claims contribute to spreading misconceptions about nutritional supplements. There are also examples of producers who mix undeclared drugs and/or unapproved active substances and herbs into their products.

While these products must comply with a series of European laws, the composition of these products is still largely subject to national legislation, resulting in numerous trade barriers even between European Union (EU) member states. So, while the calls for further regulatory harmonization of food supplements rings loudly, travel along the road to harmonization is slow and difficult.

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Course 2.2: Legislation issues about nutritional supplements use

Author: Zoi Georgiou

2.2.1 The European Legislative Framework

In the EU, "food supplements" are concentrated sources of nutrients or other substances with a nutritional or physiological effect, aiming to supplement the normal diet. They are marketed in many shapes and sizes and they come in dose form, such as tablets, capsules, liquids etc. Safety and Efficacy of Food Supplements are evaluated by the European Food Safety Authority (EFSA), (http://www.efsa.europa.eu/en/topics/topic/food-supplements) an entity set up in January 2002 as an independent source of scientific advice that produces opinions which then are used by the European Commission to adopt legislation.

Since 2002, the EU has created a legal and regulatory framework for these products with the Food Supplements Directive 2002/46/EC. EC legislation includes the following:

- ✓ general requirements for food safety, responsibilities for producers and obligations for traceability, information provision and recall of harmful products (Regulation (EC) No 178/2002)2
- ✓ preparation and hygiene of foodstuffs based on the principles of Hazard Analysis and Critical Control Points (HACCP) (Regulation (EC) 852/2004)3
- ✓ food labeling with the aim of adequately informing the consumer about the composition, properties and use of foodstuffs (Regulation (EU) 1169/2011)4
- ✓ use of nutrition and health claims, which must be authorized before they can be used (Regulation (EC) 1924/2006)5
- ✓ conditions for the use of additives (Regulation (EC) 1333/2008)6
- ✓ maximum levels for residues and contaminants (Regulation (EC) 369/2005 - Regulation (EC) 1881/2006)7,8
- ✓ approval of novel foods and food ingredients not on the European market before 15 May 1997 (Regulation (EU) 2015/2283)9

With this Regulation the EU outlined the general principles and requirements of the Food Law. The food supplements safety for human health was adopted as the leading principle in the field of food supplements. The Directive 2002/46/EC partially harmonized the rules for placing on the market of food supplements including the label information. This Directive is applied to all food supplements of any composition that are marketed as foods and presented as foods, reaching to the end user only in prepackaged and prelabeled dose form.

The Directive calls for the establishment of harmonized minimum and maximum dosage amounts; however, this has yet to be done and remains a competence of EU member states. Also, substances other than vitamins and minerals are not directly covered by the directive, and rules regulating these substances are still governed by individual EU member states. In the next Table 2.2.1. is presented a List of competent authorities of the Member States within the meaning of Article 4(6) of Directive 2002/46 on food supplements.

Table 2.2.1 List of competent authorities of the Member States Prepared by: Health and Food Safety Directorate-General Unit E1 - Last revision: February 2019

List of competent authorities of the Member States	
  List of competent authorities of the Member States within the meaning of Article 4(6) of Directive 2002/46 on food supplements	List of competent authorities of the Member States responsible for: Nutrition, food composition and information

In Art. 5 of the Directive2002/46/EC are set requirements to the maximal and minimal amounts of vitamins and minerals in food supplements based on scientific evidence concerning health safety.

Vitamins and minerals may be ingested from a variety of foods that are eaten as part of the daily diet. They can be naturally present in foods or added to them both for technological or nutritional purposes. Innovation in the food sector tends to take increasingly into account the relationship between diet and health and may lead to an increase of the percentage of foodstuffs fortified with vitamins and minerals on the market. The amounts contributed by these various sources have to be taken into account when setting maximum levels.

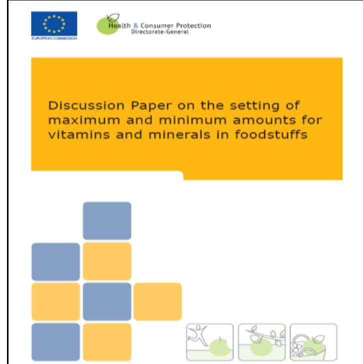
On the other hand, is obvious that vitamins and minerals used in food supplements or added to foods should result in a minimum amount being present. Otherwise, the presence of too small amount would not offer any benefit to consumers, namely to supplement the normal diet in the case of food supplements, and would be misleading.

Council Directive 90/496/EEC on nutrition labeling requires that in order that vitamins or minerals may be declared in nutrition labeling they have to be present in a significant amount, which, as a rule, is the amount in 100 g or 100 ml of the food representing 15% of the recommended allowance specified in the Annex to Directive 90/496/EEC. The same amount has to be present if a nutrition claim is made that the food is a "source" of a vitamin or a mineral, according to the proposed Regulation on nutrition and health claims made on foods, expected to be adopted in September/October 2006. The proposed Regulation on the addition of vitamins and minerals to foodstuffs foresees that, in order to guarantee consumers of the presence of vitamins and minerals in fortified foods in at least a meaningful amount, their addition shall result in the presence of at least a significant amount where this is defined according to the Annex to Directive 90/496/EEC on nutrition labeling. However, this provision allows discretion to set minimum amounts that are different from that significant amount for specific foods or categories of foods.

While the use of significant amounts as minimum amounts may be considered generally acceptable for fortified foods, some argue that it would not be adequate for food supplements. Food supplements are by definition concentrated sources of nutrients. Therefore, some argue that these products should be providing minimum amounts of those nutrients, per recommended daily consumption dose, higher than the abovementioned significant amounts. In the next Table 2.2.2. is offered a Discussion Paper on the setting of maximum and

minimum amounts for vitamins and minerals in foodstuffs where are presented Examples of existing models for the setting of Maximum amounts of vitamins and minerals in foods.

Table 2.2.2. Discussion Paper on the setting of maximum and minimum amounts for vitamins and minerals in foodstuffs. Health and Consumer Protection Directorate-General June 2006.

	This paper identifies the issues to be considered in the setting of maximum and minimum amounts of vitamins and minerals in foods. It also describes the use of different theoretical models to assess the safety of the (voluntary) addition of vitamins and minerals to foods.

Additionally, regulations regarding vitamins and minerals were issued: Regulation (EC) No 1925/2006 of the European Parliament and of the Council of 20 December 2006 on the addition of vitamins and minerals and of certain other substances to foods and Commission Regulation (EC) No 1170/2009 and Regulation (EC) No 1925/2006 of the European Parliament as regards the lists of vitamin and minerals and their forms that can be added to foods, including food supplements.

The Food Supplements Directive (FSD) Directive 2002/46/EC, establishes a definition for food supplements, establishes a list of allowable vitamins and minerals, and sets labeling requirements.

Currently the European Community is concerned about food products, including nutritional supplements present in the market aiming to offer to its citizen's reliable protection of their health.

- ✓ Quality and post-marketing control of nutritional supplements cannot be neglected, and it is necessary to set more precise quality and safety criteria in future strategies associated with those products.
- ✓ Guidance on safety evaluation of sources of nutrients
- ✓ Quality assurance and full traceability of ingredients are essential to ensure consumer safety.

2.2.2 Vitamins and Minerals

Harmonised legislation regulates the vitamins and minerals, and the substances used as their sources, which can be used in the manufacturing of food supplements as presented in the next Table No 2.2.3.

Table 2.2.3. Vitamin and mineral substances which may be used in the manufacture of food supplements

A. Vitamins and the substances used as their sources	
1. VITAMIN A	**7. NIACIN**
(**a**) retinol	(a) nicotinic acid
(b) retinyl acetate	(b) nicotinamide
(c) retinyl palmitate	**8. PANTOTHENIC ACID**
(d) beta-carotene	(a) D-pantothenate, calcium
2. VITAMIN D	(b) D-pantothenate, sodium
(a) cholecalciferol	(c) dexpanthenol
(b) ergocalciferol	**9. VITAMIN B6**
3. VITAMIN E	(a) pyridoxine hydrochloride
(a) D-alpha-tocopherol	(b) pyridoxine 5′-phosphate
(b) DL-alpha-tocopherol	**10. FOLIC ACID**
(c) D-alpha-tocopheryl acetate	(a) pteroylmonoglutamic acid
(d) DL-alpha-tocopheryl acetate	**11. VITAMIN B12**
(e) D-alpha-tocopheryl acid succinate	(a) cyanocobalamin
4. VITAMIN K	(b) hydroxocobalamin
(a) phylloquinone (phytomenadione)	**12. BIOTIN**
5. VITAMIN B1	(a) D-biotin
(a) thiamin hydrochloride	**13. VITAMIN C**
(b) thiamin mononitrate	(a) L-ascorbic acid
6. VITAMIN B2	(b) sodium-L-ascorbate
(a) riboflavin	(c) calcium-L-ascorbate
(b) riboflavin 5′-phosphate, sodium	(d) potassium-L-ascorbate
	(e) L-ascorbyl 6-palmitate
B. Minerals and the substances used as their sources	
1. Calcium	**6. Zinc**
calcium carbonate	zinc acetate
calcium chloride	zinc chloride
calcium salts of citric acid	zinc citrate
calcium gluconate	zinc gluconate
calcium glycerophosphate	zinc lactate
calcium lactate	zinc oxide
calcium salts of orthophosphoric acid	zinc carbonate
calcium hydroxide	zinc sulphate
calcium oxide	**7. Manganese**
2. Magnesium	manganese carbonate
magnesium acetate	manganese chloride
magnesium carbonate	manganese citrate
magnesium chloride	manganese gluconate
magnesium salts of citric acid	manganese glycerophosphate
magnesium gluconate	manganese sulphate
magnesium glycerophosphate	**8. Sodium**

magnesium salts of orthophosphoric acid	sodium bicarbonate
magnesium lactate	sodium carbonate
magnesium hydroxide	sodium chloride
magnesium oxide	sodium citrate
magnesium sulphate	sodium gluconate
3. Ferrous	sodium lactate
ferrous carbonate	sodium hydroxide
ferrous citrate	sodium salts of orthophosphoric acid
ferric ammonium citrate	**9. Potassium**
ferrous gluconate	potassium bicarbonate
ferrous fumarate	potassium carbonate
ferric sodium diphosphate	potassium chloride
ferrous lactate	potassium citrate
ferrous sulphate	potassium gluconate
ferric diphosphate (ferric pyrophosphate)	potassium glycerophosphate
ferric saccharate	potassium lactate
elemental iron (carbonyl+electrolytic+hydrogen	potassium hydroxide
reduced)	potassium salts of orthophosphoric acid
4. Cuprum	**10 Selenium**
cupric carbonate	sodium selenate
cupric citrate	sodium hydrogen selenite
cupric gluconate	sodium selenite
cupric sulphate	**11. Chromium**
copper lysine complex	chromium (III) chloride
5. Iodide	chromium (III) sulphate
sodium iodide	**12. Molybdenum**
sodium iodate	ammonium molybdate (molybdenum (VI))
potassium iodide	sodium molybdate (molybdenum (VI))
potassium iodate	**13. Fluor**
	potassium fluoride
	sodium fluoride

The Community Register on the addition of vitamins and minerals and of certain other substances to foods has been established as laid down by Article 9 of Regulation (EC) No 1925/2006. The European Commission is conferred with the task of establishing, publishing and maintaining this Register, which is updated regularly. In it are presented rules about:

- ✓ The permitted addition of vitamins and minerals and of certain other substances to foods
- ✓ Maximum and minimum amounts of vitamins and minerals which may be added to foods and any associated conditions set in accordance with Article 6 of Regulation (EC) No 1925/2006
- ✓ Restrictions on the addition of vitamins and minerals as set out in Article 4 of Regulation (EC) No 1925/2006
- ✓ Substances whose use in foods is prohibited, restricted or under community scrutiny

For ingredients other than vitamins and minerals, the European Commission has established harmonised rules to protect consumers against potential health risks and maintains a list of substances which are known or suspected to have adverse effects on health and the use of which is therefore controlled. Efforts are made on the categorisation of substances currently existing on the EU market, market data and the presentation of regulatory and non-regulatory models in 27 EU Member States.

2.2.3 Other substances used in food supplements in the EU.

It is estimated that there may be in excess of 400 other substances with nutritional or physiological effect on the EU market in food supplements. The categorisation and characterisation of substances:

- ✓ Other substances: substances other than vitamins and minerals, ie
- ✓ Botanicals and other bioactive substances;
- ✓ Botanicals: includes herbs, plants, fungi, algae and extracts
- ✓ Other bioactive substances: any substance with a nutritional or physiological effect, other than vitamins, minerals and botanicals.

Six categories were identified and 31 substances were chosen for evaluation. The categories and substances are presented in the next Table 2.2.4.

Table 2.2.4. Categorization and characterization of substances present in Nutritional supplements

Categories	**Substances**
Amino acids	- L-arginine - Other essential amino acids - Non-essential amino acids
Enzymes	- Lactase - Papaine
Pre- and Probiotics	- Inulin - Lactobacillus acidophilus - Bifidobacterium species - Yeast species
Essential fatty acids	- Gamma-linoleic acid - EPA/DHA - Evening Primrose oil (Oenothera biennis (L.) - Borage oil (Borago officinalis) - Flax seed oil (Linum usitatissimum (L.)
Botanicals & botanical extracts	- Aloe (Aloe vera (L.) - Ginkgo (Ginkgo biloba) - Ginseng (Panax ginseng)

	- Garlic (Allium sativum (L.) - Green tea extract (Camellia sinensis) - Garcinia extract (Garcinia cambogia) - Guarana extract (Paullinia cupana)
Miscellaneous bioactive substances	Lycopene - Lutein - Coenzyme Q10 - Taurine - Carnitine - Inositol - Glucosamine - Chitosan - Spirulina - Soy isoflavone

Since there is no legal categorization of other substances currently used in food supplement products in the EU, the above categorization was made based on defined biochemical classes of molecules (*e.g.* amino acids, enzymes and fatty acids), or on their nature and origin (*e.g.* botanicals, extracts, oils etc). In addition, commonly used terms such as pro- and prebiotics characterizing specific ingredient categories were taken into consideration. There is always potential overlap between the categories. For example, although borage is a botanical, borage oil falls within the fatty acids' category. It is important to highlight that herbal oils classified under the "essential fatty acids" category do not refer to essential oils but only to commonly extracted oils. The substances in each category were chosen based on their significance in the EU food supplement market and/or the extent to which they could illustrate effectively the different regulatory approaches taken by the Member States.

2.2.4 Evaluation of sources of nutrients

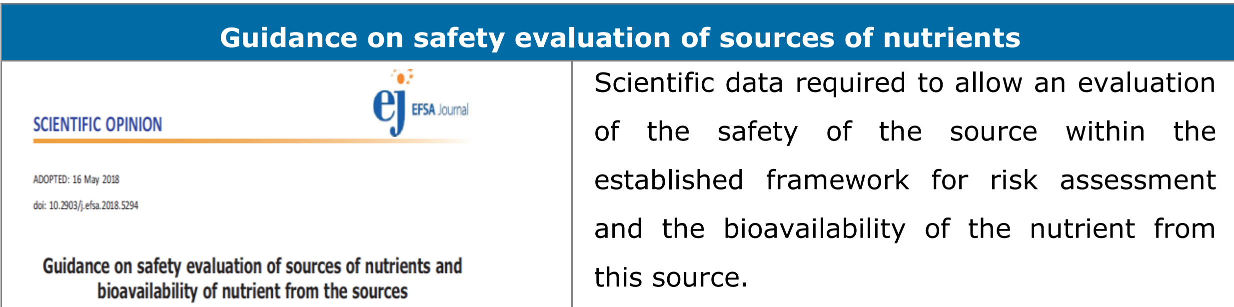
Whenever new substances are proposed for use as sources of nutrients in food supplements, foods for the general population or foods for specific groups, EFSA is requested by the European Commission to perform an assessment of their safety and of the bioavailability of the nutrient from the proposed source. The chemical substances used as sources which may be added to food, including food supplements and foods for specific groups, should be safe and also bioavailable, a property which is described, in the relevant legislation, as 'available to be used by the body'.

The use of chemical substances as 'sources' of vitamins and minerals in food is regulated in the

European Union (EU) by the establishment of positive lists of substances, annexed to the relevant

sectorial legislation. In the next Table 2.2.5 is presented the new guidance document proposed by EFSA which refers to the data needed for the assessment of sources of nutrients proposed for use in the manufacture of foods.

Table 2.2.5. Guidance on safety evaluation of sources of nutrients and bioavailability of nutrient from the sources



Guidance on safety evaluation of sources of nutrients	
SCIENTIFIC OPINION EFSA Journal ADOPTED: 16 May 2018 doi: 10.2903/j.efsa.2018.5294 Guidance on safety evaluation of sources of nutrients and bioavailability of nutrient from the sources	Scientific data required to allow an evaluation of the safety of the source within the established framework for risk assessment and the bioavailability of the nutrient from this source.

For the safety assessment of the source, data requirements which should be covered in all applications relate to the description of the source, manufacturing process (including possible residuals or contaminants), technical specifications, proposed uses and use levels, and anticipated intake of the source and the corresponding intake of the nutrient. There are described the:

- ✓ Technical data aimed at characterizing the proposed source and at identifying potential hazards resulting from its manufacture and stability in food;
- ✓ Existing authorizations and evaluation, providing an overview of previous assessments on the proposed source and their conclusions;
- ✓ Proposed uses and exposure assessment section, allowing an estimate of the dietary exposure to the source and the nutrient based on the proposed uses and use levels;
- ✓ Toxicological data, describing approaches which can be used to identify (in conjunction with data on manufacture and composition) and to characterize hazards of the source and any relevant breakdown products.
- ✓ Bioavailability focuses on determining the extent to which the nutrient from the proposed source is available for use by the body in comparison with one or more forms of the same nutrient that are already permitted for use on the positive lists.

2.2.5 A global scope

The importance of the diet for a healthy life has been amply demonstrated. Individual nutrients have received variable attention and vitamins and minerals are among them. The levels of intake of the latter have been cause of concerns both for being potentially on the low side but also because of the adverse effects that excessive intakes of certain vitamins and minerals may cause. It should, however, be noted that it is increasingly difficult to develop accurate assessments of the pattern of overall diets, since these vary across the regions, between population groups and over time.

The fundamental challenge in any discussion about the regulation of food supplements is that there is no global consensus on how the category of products known as dietary supplements, natural health products (NHPs), complementary medicines or food supplements in different countries is defined. For example, a product considered to be a dietary supplement and regulated as a food in the USA, or EU in another country may be considered a therapeutic good (complementary medicine) or a therapeutic good (prescription medicine). The situation is even more complicated when countries like China or India that have an existing regulatory framework for traditional medicine or phytomedicine that includes crude botanicals are considered. Even in countries with similar cultures, legal systems, and levels of economic development, regulations applying to dietary supplements vary considerably. The vast variety of global organizations regulating (Table 2.2.2) nutritional supplements should be harmonized, as consumers rely more and more on food supplements it is crucial to guarantee that they access safe products and they are able to make informed choices.

Table 2.2.2 Global Organizations for Nutritional Supplement Regulation

Name	**URL**	**Comment**
WHO World Health Organization World Self Medication Industry International Alliance of Dietary/Food Supplement Associations (IADSA)	who.int/medicines/areas/traditional/en/ www.wsmi.org www.iadsa.org	Provides links to on-going work by the WHO including the Traditional Medicine Strategy 2014-2023, the International Regulation on the Cooperation of Herbal Medicines and various technical guidelines. Industry association website providing details on international approaches to over-the-counter medicines including dietary supplements. Industry association website providing details on international approaches to dietary supplements.
EU European Union EU Parliament and Council European Food Safety Authority (EFSA)	ec.europa.eu/health/human-use/herbalmedicines_en www.efsa.europa.eu	Details on the traditional herbal medicine directive: member states. Provides details and links to regulation of foods and food supplements.
USA FDA Food and Drug Administration Dietary Supplements	www.fda.gov/food/dietarysupplements/	Details on regulations, policies and guidelines dealing with dietary supplements
Canada	www.canada.ca/en/health-canada/services/drugshealth- products/natural-non-prescription.html	Details on the existing NHP regulations, policies and guidelines as well as work underway with regards to a comprehensive approach to self care

Health Canada	www.canada.ca/en/health-canada/services/foodnutrition/legislationguidelines/guidancedocuments/category-specific-guidance-temporarymarketing-authorization-supplemented-food.html	products Information on supplemented food category.
Australia Therapeutic Goods Administration (TGA)	www.tga.gov.au/complementary-medicines www.foodstandards.gov.au/Pages/default.aspx	Details of existing complementary medicine regulations, policies and guidelines Food Standards Australia and New Zealand Details on food standards, policies and guidelines.
New Zealand Medsafe	www.medsafe.govt.nz/regulatory/ DietarySupplements/Regulation.asp	Provides information related to regulation, policies and guidelines dealing with dietary supplements.
China China Food and Drugs Administration (CFDA) Special Administrative Region of Hong Kong Health Ministry—Chinese Medicine Division	eng.sfda.gov.cn/WS03/CL0755/ www.cmd.gov.hk/html/eng/important_info/regulation.html	Information on health food regulations including 'blue hat' process. Information on policies and regulation related to Chinese proprietary medicines.
Singapore Health Sciences Authority	www.hsa.gov.sg/content/hsa/en.html	Information on policies, regulation and guidelines related to health products and Chinese proprietary medicines. As a member state, resource to access work on regulatory harmonization of products within Association of South East Asian Nations (ASEAN).
India Food Safety and Standards Authority of India (FSSAI) Ministry of Ayurveda, Yoga, Unani, Siddha andHomeopathy (AYUSH)	fssai.gov.in/home ayush.gov.in	Government direction, standards and regulation of health supplements and nutraceuticals. Health supplements are intended to supplement the diet of healthy individuals over 5 year, and levels of nutrients should not exceed RDA amounts. Policies, guidelines and regulations dealing with Indian traditional medicines.

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5. **Error! Hyperlink reference not valid.**

Course 2.3: Cell Culture models for bioactivity testing of extracts

Authors: Christina Efraimoglou, Christos Tsatsanis

2.3.1 Cell culture models of effects on cell proliferation and toxicity

Cell culture is a good in vitro model for bioactivity analysis since there is wide availability and easy handling for high-throughput testing. Experiments can be repetitive and targeted since not many environmental factors can affect the results. A cell-based bioassay is important to determine toxicity or potential anti-cancer or anti-inflammatory applications. Selection of cell type, culture medium, culture system (2-D or 3-D), nutrient concentrations and type of bioassay are crucial for reliable testing.

Murine macrophage cell line RAW 264.7 is easy to propagate and constitutes a good model for cytotoxicity or anti-inflammatory tests such as Nitric Oxide (NO) and inflammatory cytokines measurements. RAW 264.7 can be cultured in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% heat inactivated Fetal Bovine Serum and 1% penicillin streptomycin at 37°C incubator with 5% CO_2. Nutrient supplements in powder form are diluted in the appropriate amount of sterile water or 1X PBS while liquid supplements are initially dried using speedvac vaccum concentrator before diluted. Supplement concentration varies usually between 5 μg/mL and 500 μg/mL.

Cytotoxicity can be verified with MTT, a colorimetric assay which includes Thiazol Blue Tetrazolium Bromide and can measure cell viability and proliferation. It is based on the reduction of a yellow tetrazolium salt (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide or MTT) to purple formazan crystals by metabolically active cells in a NADH – NADPH dependent manner. To perform this assay, 5×10^5 RAW 264.7 mouse macrophages should be seeded in 96-well plate and cultured overnight. The next day, cells should be treated with the respective nutrient supplement concentration and incubate for 24, 48 or 72 hours at 37°C. Thiazol Blue Tetrazolium Bromide (MTT) must be added to cell culture in a final concentration of 500 μg/mL and incubated at 37°C plus 5% CO_2 for 4 hours. Supernatant should be discarded and cells lysed with 0.4% v/v HCl in isopropanol. Absorbance can be measured in a microplate reader between 500 and 600nm. To evaluate results, the average OD of each treated sample should be normalized to the absorbance of the control sample and statistical analysis could be performed via GraphPad Prism or any related tool.

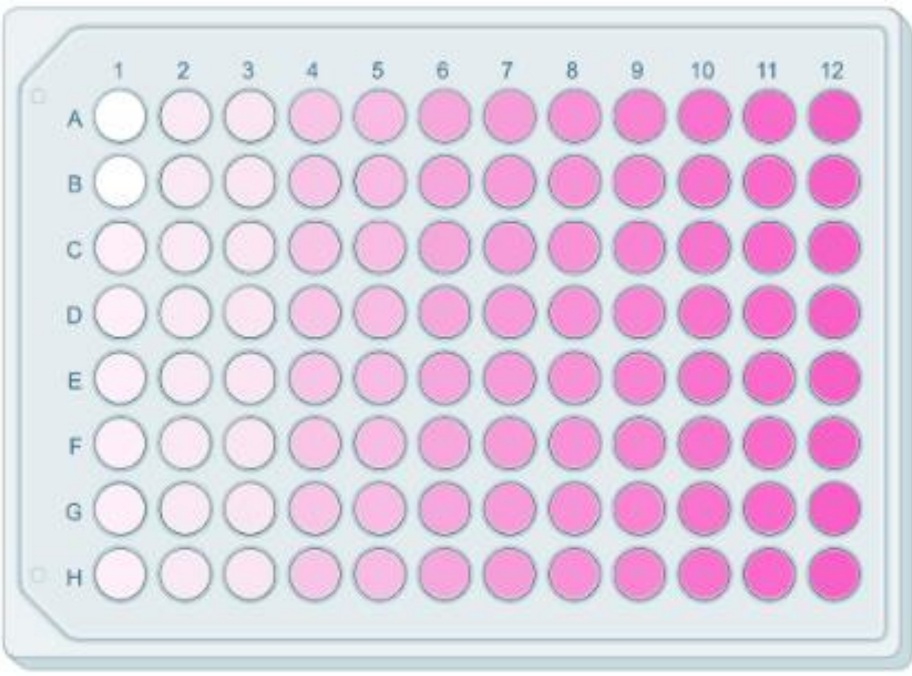

Figure 2.3.1: 96-well plate after MTT assay

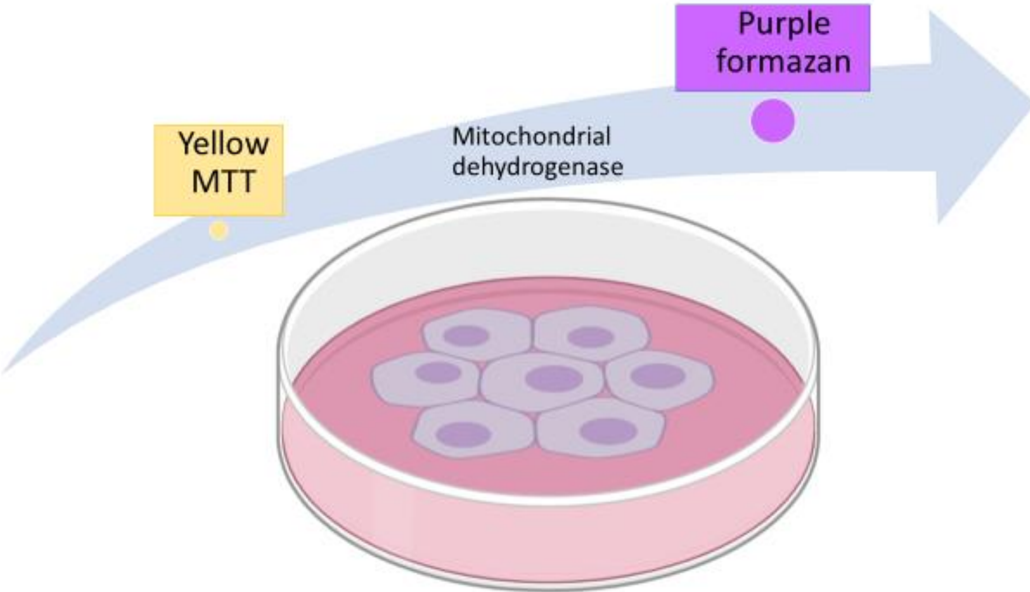

Figure 2.3.2: MTT schematic representation of color development

Comet assay, alternatively known as single gel electrophoresis assay, is a simple and inexpensive technique for DNA damage evaluation. It was first introduced by Ostling and Johanson in 1984 and was furtherly modified by Singh and co-workers in 1988. A well described protocol has been published by Olive and Banath. The concept is that damaged DNA, containing breaks, would migrate further towards anode during gel electrophoresis compared to large and cumbersome undamaged DNA. This assay is important for the evaluation of toxicity of some supplement, in the DNA level. Cells are embedded in low-melting point agarose 1% w/v in distilled water, cast onto mini-slides and lysed before electrophoresis. Slides can be stained either with Ethidium Bromide or

other fluorescent dye (e.g. SYBR-green, DAPI) and observed under UV-light. Tail intensity and length can be analyzed to determine the DNA damage.

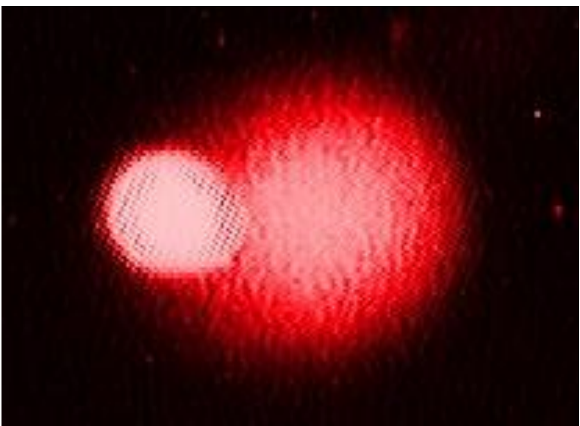

Figure 2.3.2: Imaging comet assay. Source of image: https://npc.univie.ac.at/en/research/lab-equipment/techniques/comet/

The cell culture model is also useful for evaluation of apoptotic events after nutritional supplements treatment. Pro- (caspase-3, Bax) and anti-apoptotic (BCL-2) proteins can be detected and quantified in both mRNA level with real time PCR and protein level with western blot. Additionally, annexin-V is a well-established assay for apoptotic cell death and mitochondria damage measurement. Human vascular anticoagulant, annexin-V, is a Ca2+-dependent phospholipid-binding protein with high affinity with phosphatidylserine residues on the cell membrane. Physiologically, these residues are on the inner part of the cell membrane, thus inaccessible to annexin-V. However, early during apoptosis, phosphatidylserine residues are flipped on the outer part of the cell and annexin-V is able to react with these cells. When annexin-V is hybridized with fluorescein isothiocynate conjugate (FITC) or other fluorescent dye, signal will depict apoptotic cell ratio and can be measured with flow cytometry. After incubation of cells with the desired supplement, the method includes discarding supernatant of cell culture, washing with PBS and detaching cells from the bottom, using scratcher, trypsin/EDTA or other way, depending on the cell type. For RAW 264.7, scratching the bottom is sufficient. If trypsin is used, addition of 10% fetal calf serum (FCS) in media, is necessary for the inactivation of trypsin. Cells are then centrifuged at 1000 rounds per minute for 5 minutes and resuspended in fresh DMEM. Three controls are recommended for each assay: unstained cells to for background florescence, cells stained only with Propidium Iodine (PI), cells stained with only annexin-V/FITC. Treated cells are resuspended in 500 μL 1X binding buffer and 5 μL of annexin-V/FITC and 5 μL of Propidium Iodine are added. Samples are incubated at room temperature for 5 minutes in dark. Flow cytometer is customized and unstained cells are used to adjust red and green fluorescence until all cells are inside lower left quadrant. Next, the green annexin-FITCH is adjusted to the lower half display and red background is equilibrated. Promidium Iodine sample is adjusted to the left side of the display and green fluorescence background is equilibrated. Then, treated samples can be tested and analysed based of the four populations that will be on display. An alternative is to detect apoptosis by fluorescence microscopy by putting cell suspension on a glass slide fixed or not. Incubation with annexin-V/FITC should be performed prior to fixation. The slide can be observed with a dual filter for

FITC and rhodamine. Green plasma membranes have bound annexin-V/FITC while cells with disrupted membrane integrity will appear red (PI) in the nucleus and green on the cell membrane (https://www.abcam.com/protocols/annexin-v-detection-protocol-for-apoptosis).

Poly(ADP-ribose) polymerase (PARP) cleavage is another way to estimate apoptosis since it occurs as a result of the activity of caspase-3 and is associated with chromatin condensation, a hallmark of apoptosis. Specifically, the 116 kD PARP protein is cleaved into 89 kD and 24 kD polypeptides by caspase-3. This event can be detected and quantified with western blot since specific antibodies can detect both 116 kD and 89 kD fragments.

2.3.2 Cell culture models for testing anti-inflammatory action

Chronic inflammation is a harmful condition that might have a role in many diseases such as: asthma, rheumatoid arthritis, type 2 diabetes. Thus, anti-inflammatory properties of food supplements could be beneficial for human health, motivated by the fact that many anti-inflammatory drugs can cause testinal toxicity or other side effects. In order to examine potent anti-inflammatory potential, RAW 264.7 cells treated with nutrient supplement extract in the presence or absence of bacterial liposaccharide (LPS), which triggers the production and secretion of Nitric Oxide (NO) and inflammatory cytokines. RAW 264.7 cell line is a good system for such treatment since macrophages play an important role in immune regulation, initiation and resolution of inflammation. Major pro-inflammatory markers such as NO and tumor necrosis factor alpha (TNFa) or other secreted cytokines can be detected and evaluated in cell culture supernatants, either with specified assays (e.g Griess reaction) or with enzyme-linked immunosorbent assay (ELISA). More pro-inflammatory and anti-inflammatory macrophage phenotype markers can be validated in mRNA level, through Real Time PCR.

For nitric oxide measurement, RAW 264.7 cells are cultured overnight in 24-well plates and then are pre-treaded for 1 hour with different concentrations of extracts. Subsequently, macrophages are stimulated with 100 ng/mL LPS for 48 hours before nitric oxide measurement using Griess reaction. 50 μL of cell culture supernatant from each sample are transferred to a 96-well plate and mixed with equal volume of suldanilamide solution (1% sulfanilamide in 5% H_3PO_4) and mix is incubated at room temperature for 5 minutes, in dark conditions. Subsequently, 50 μL of NED solution (0.1% N-1-napthylendiamine dihydrochlorite in H2O) is added and absorbance is measured at 540 nm in an automated microplate reader. A sodium nitrite standard curve can be used for the evaluation of nitride concentration.

ELISA is a biochemistry immunoassay first described by Engvall and Perlmann in 1971 and is used to quantify antigen concentration within a sample. The basic principle is that flat-bottom 96-well plates coated with a capture antibody for the antigen of interest, bind the protein which is then detected with another antibody specified for a different epitope. Signal is produced after substrate solution addition. TNF-α cytokine is a good candidate for checking inflammatory actions of a food supplement using ELISA since it is secreted by macrophages in high levels after stimulation. The assay should be performed according to the manufacturer's instructions. Here we describe an example of a BioLegent ELISA MAX™ mouse TNF-α kit. Cell culture supernatants can be diluted or used as they

are. It is generally proposed that when highly differential levels of target are detected, samples should be diluted accordingly. On the first day, plate bottoms are coated with Capture Antibody and incubated overnight between 2°C and 8°C. The following day, plate is washed 4 times and Assay Diluent is added for blocking at room temperature for 1 hour with shaking. Plate is washed 4 times and Detection Antibody solution is added and incubated for 1 hour with shaking. Plate is washed 4 times and Avidin-HRP solution is added and incubated at room temperature for 30 minutes with shaking. Plate is washed well 5 times and TMB Substrate Solution is added and incubated in dark for 15 to 30 minutes or until desired color develops. Reaction is stopped with Stop Solution (2N H_2SO_4) and absorbance is red at 570nm. Control sample and standard curve should always be included in such assays in order to quantify applicably (https://www.biolegend.com/Files/Images/media_assets/pro_detail/datasheets/430901_R03_MuTNFa_Standard.pdf).

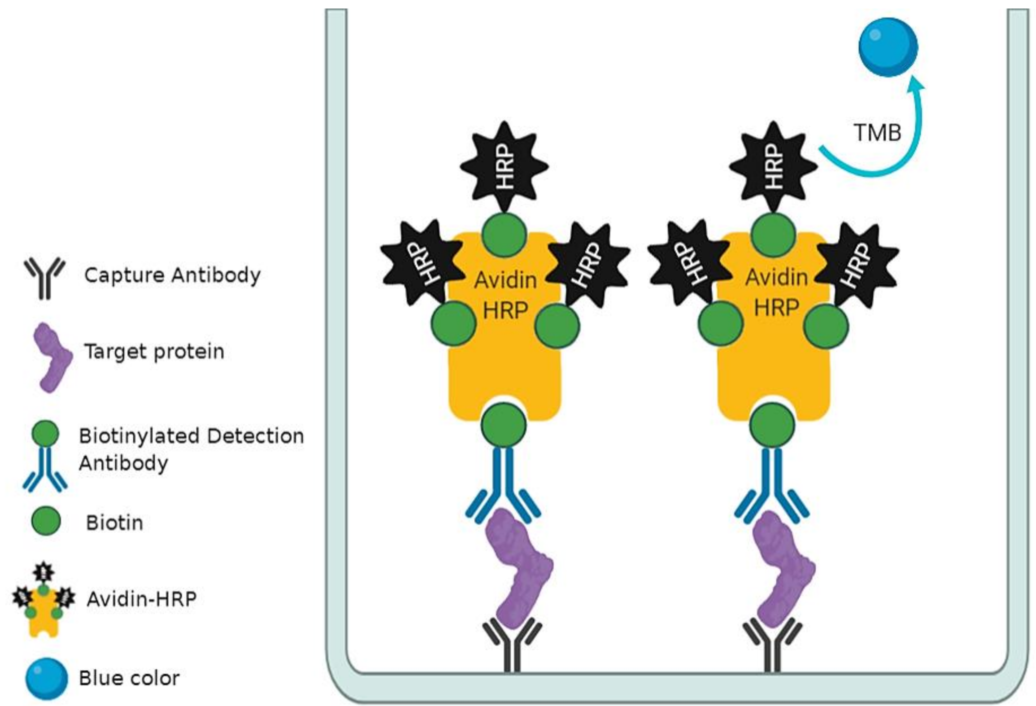

Figure 2.3.4: Schematic representation of ELISA MAX assay

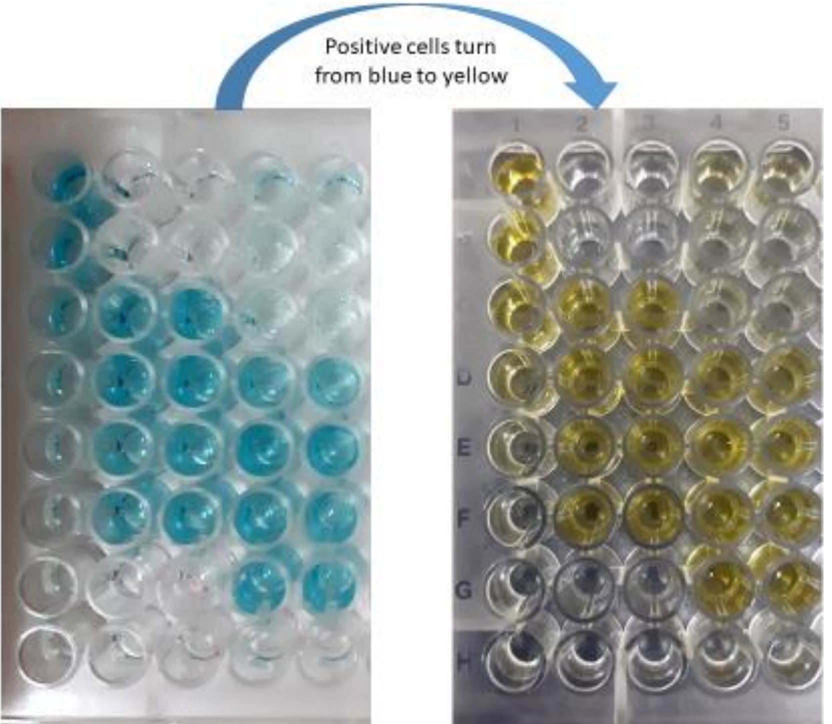

Figure 2.3.3: Positive samples turn from blue to red after stop solution is added to ELISA assay. Column 1 represents the standard curve with reducing concentration from A to H.

Another useful approach for evaluation of anti-inflammatory action of nutritional supplements, is the quantitative polymerase chain reaction for pro- and anti-inflammatory markers after LPS stimulation. RAW 264.7 cells are treated with LPS for 24 of 48 hours and then harvested and have their RNA extracted. Reverse transcription and Real Time PCR follow, using primers specific for M1 – M2 markers, depicting the pro- and anti-inflammatory phenotypic states of macrophages accordingly. The main pro-inflammatory markers are iNOS, TNF-a, IL-12, IL-1, IFNγ, mir155 while anti-inflammatory are Arginase 1, Ym1, Fizz1, IRAKm, mir146a.

The aforementioned cell culture assays can also be performed in peritoneal primary macrophages derived from mice fed with the nutritional supplements for a period of time. In particular, thioglycolate broth is injected to mice intraperitoneally, and macrophages are harvested 3 to four days later in Dulbecco's Modified Eagle's Medium (DMEM), centrifuged for 5 minutes in 1000 rounds per minute and resuspended in fresh DMEM under sterile conditions. Then, peritoneal macrophages can be plated, cultured and treated just as cell lines.

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Course 2.4: Animal and cell culture models of Obesity, Metabolic Diseases and Intestinal Inflammation

Authors: Konstantinos Axarlis, Christos Tsatsanis

2.4.1 Cell culture models of obesity and metabolic diseases

Obesity and metabolic disease have arisen to be of paramount concern in public health all over the world. There is a strong link of the former with the adipose tissue, whose role as an endocrine organ, besides that of excess energy and nutrient storage, has gained much attention during the last years. Adipokines and proinflammatory cytokines are secreted by the adipose tissue and exert systemic metabolic and immunological effects. Low levels of chronic inflammation are considered hallmarks of plenty metabolic pathologies. A fundamental trait of obesity is the extravagant concentration of fat mass in white adipose tissue (WAT), which is achieved through the expansion of adipocyte volume (hypertrophy), increase in adipocyte number (hyperplasia) or both.

Adipocytes originate from mesenchymal stem cells (MSCs), which initially differentiate into lipoblasts, consequently into preadipocytes, and finally into mature adipocytes. Primary cell lines, such as mature adipocytes, MSCs, and preadipocytes can be easily isolated from adipose tissue homogenates. Additionally, numerous relative cell culture models have been established, including human, but mostly rodent, for the *in vitro* research of the adipogenic differentiation processes linked to obesity, as well as the potential antiobesity effects of dietary and drug compounds. A necessary step of such models is the use of differentiation cocktails to induce adipogenesis. Therefore, the bioactivity of nutritional or drug compounds can be tested by assessing their impact on the adipogenesis process itself, as well as adipocyte metabolism (i.e. lipogenesis, lipolysis, oxidation of fatty acids, browning), gene expression profile, and cell survival (Table 2.4.1).

Table 2.4.1 Selected murine cell lines used in adipogenesis process research. Adapted from Ruiz-Ojeda et al. (2016).

Cell Model	Characteristics
3T3-L1 (preadipose cells)	Most frequently used model / Homogenous response to treatments and experiments
3T3-F442A (preadipose cells)	Similar to 3T3-L1 cells, but more committed
C3H10T1/2 (multipotent cells)	Preferred for studies of multipotent cells commitment towards the adipocyte lineage
OP9(preadipose cells)	Rapid adipogenic differentiation / Suitable for high-throughput screening

MEFs (mouse embryonic fibroblasts)	Unlimited, undifferentiated proliferation in vitro

For example, the 3T3-L1, a well-characterized and reliable cell model for studying the conversion of preadipocytes into adipocytes developed from murine Swiss 3T3 cells, when treated with curcumin (5–20 μM) for 24 h, resulted in suppressed preadipocyte differentiation, increased adipocyte apoptosis, as well as elevated fatty acid oxidation and reduced fat accumulation.

Adipocytes may also be examined in co- or 3D- cultures with other cell types, such as macrophages, endothelial, or muscle cells, in order to research the relationship of fat with other tissues. Most often, macrophages, which are physiologically found in adipose tissue, are co-cultured with adipocytes, due to the high interest and need of examining interactions among obesity or insulin resistance and inflammation. The aforementioned example of 3T3-L1 cell line has been excessively exploited in such experimental setups. For instance, it has been exhibited that, when differentiated medium from 3T3-L1 cells was used to culture macrophages, the macrophage inflammatory response was inhibited if 3T3-L1 cells had been treated with phloretin and phlorizin (natural substances for diabetes combating).

2.4.2 *In vivo* models of metabolic disease

Importance of in vivo testing of nutritional supplement bioactivity

In vivo testing of the bioactivity of nutritional compounds is fundamental before any safe conclusion can be drawn for the effectiveness (and safety) of a dietary supplement. Surely, *in vitro* or *in silico* trials often display important advantages, such as reduced time and financial demands, compared to the corresponding *in vivo* experiments and can provide useful insights into the molecular mechanisms of action of the examined compound or, at least, give a rough estimation about its activity (i.e. anti-inflammatory). Extremely rarely are they sufficient, however. That is due to a plethora of reasons, a selection of which will be subsequently considered.

- ✓ **Physiological process of digestion**

To begin with, a determinant factor that is usually completely omitted during *in vitro* or *in silico* experiments is the physiological process of digestion. The bioactivity of an orally administrated nutritional supplement is immensely dependent on the bioavailability, distribution, metabolism, and excretion properties of its compounds. Frequently, it is noted that promising bioactivity of a substance *in vitro* is hindered *in vivo* because of its low bioavailability, as is the case with tea catechins or resveratrol. Many compounds are prone to extensive digestion by the enzymes of the gastrointestinal tract and do not even reach the absorption stage in the duodenum and jejunum or are subjected to chemical modifications affecting their activity. For instance, polyphenolic compounds which can act as inhibitors potent pancreatic lipase inhibitors exert their inhibitory effect in regards to their structural conformation as well as the degree of

polymerization and elimination of glycosylation during digestion. Some compounds may also exert synergistic effects when combined with other dietary substances that could be lost at the level of a single substance, such as curcumin. Some others may exhibit desired effects by interacting with the digestion process itself, like polyphenols, which influence glucose metabolism by inhibiting carbohydrate digestion and absorption.

- ✓ **Complex systemic responses to multifactorial pathologies**

Especially when examining bioactivity in regards to such multifactorial pathologies like obesity and metabolic disease, intestinal inflammation, and cancer, where complex systemic responses are implicated and influence each other, including endocrine, immune and metabolic signaling, *in vivo* experiments are deemed mandatory. Taking as an example the immune system, it alone is thought to have a central role in all of the aforementioned diseases. For instance, obesity activates Kupffer cells, which then produce chemokines that trigger the accumulation of pro-inflammatory liver macrophages, which contribute to insulin resistance and hepatic steatosis. There is a variety of immune cell types with many different roles, assembling innate and adaptive immunity, whose interactions are vastly complicated. Even with co-cultures, *in vitro* conditions are yet nowhere near simulating real-life conditions of the perplexing immune system, therefore the effect of a nutritional substance on the above context should be also examined *in vivo*.

- ✓ **Gender dependence**

Gender itself seems to be pervasive in disease susceptibility, progression, and response to treatment. Gender differences can emerge from reversible or irreversible hormonal effects and from differential gene expression of the sex chromosomes, which, again, have a notable impact on major systems, such as the immune and metabolic system. For example, obesity-induced low-grade inflammation in the visceral adipose tissue (VAT), which contributes to the development of metabolic disease, is limited by estrogen in females, whereas in males is heightened. Therefore, a nutritional substance with an observed anti-inflammatory effect *in vitro* could potentially exhibit significant improvement in VAT inflammation in males but not in females *in vivo*.

- ✓ **Intestinal microbiome**

Intestinal flora, which is also neglected in vitro, is, nonetheless, a key factor by itself when testing dietary supplements. It is noteworthy that gut microbiota tend to differ between the two genders. The bioavailability and bioactivity of nutritional substances can be greatly impacted by the gut microbiome's metabolic activity during digestion. Dietary compounds shape gut flora's composition and influence the metabolites the microbiome excretes, through which the metabolism, the immune system, and inflammation, as well as the behavior itself of the host organism and even its circadian clock, among others, are modulated. For instance, gut bacteria metabolize choline and L-carnitine, which are provided mainly from animal sources, producing trimethylamine N-oxide, which, when elevated, the risk of developing pathologies like obesity or type 2 diabetes is shown to increase, too.

- ✓ **Other factors**

Finally, in vivo experiments collectively take into account so many other factors potentially affecting the effectiveness of a nutritional supplement in health or a disease context, such as strain, age, stress, physical exercise, feeding behavior, diet composition, the timing of supplementation, dietary-induced physiologic effects (i.e. satiety), including many more. Whatever that case, in vivo testing of the bioactivity of dietary supplementation is obligatory for the safe conclusion of the latter's actual effectiveness.

Animal models of obesity and metabolic disease

A vast number of animal models of metabolic disease have been developed, granted the high pressure of contemporary's pandemic of metabolic disease. An obvious categorization of such models would be the different animal groups used in obesity and diabetes research, that is, non-mammalian, rodent, large animal, and non-human primate models. There is a vast variety of genetic animal models, as well as non-genetic animal models, which result from interventions such as dietary, chemical or surgical. Needless to say, all of the aforementioned models present both advantages and drawbacks, and their choice strictly depends on the research question under investigation. Herein, rodent animal models and dietary treatments developed for the study of metabolic disease will be compactly presented, as their preclinical use on the field of metabolic disorders is the most extensive (Table 2.4.3).

Table 2.4.3 Selected rodent models potentially useful in obesity, insulin resistance, and type 2 diabetes mellitus research. Adapted from Kleinert et al. (2018).

Strain or method	Species	Diet	Obesity	Hyperglycaemia	IR	T2DM	Dyslipidaemia	Pathologic islet changes
Monogenic								
C57BL/6J–ob/ob	Ms	SD,HFD	++	-	++	-	+	-
C57BLKS/J–db/db	Ms	SD,HFD	++	+	++	+	++	+
fa/fa	Rat	SD,HFD	++	+	++	-	+	-
Zucker Diabetic Fatty	Rat	SD,HFD	++	++	++	+	++	++
Koletsky	Rat	SD,HFD	++	-	+	-	+	-
Polygenic								
C57BL/6J	Ms	HFD	+	–	+	–	+	–
NZO	Ms	CHO	++	+	++	+	+	+
TALLYHO/Jng	Ms	SD, HFD	+	+	++	+	++	+
DIO-sensitive Sprague Dawley	Rat	HFD	+	–	+	–	+	–
DR Sprague Dawley	Rat	HFD	–	–	–	–	–	–

Sand rat	Gerbil	SD, HFD	+	–	+	+	+	+
Goto–Kakizaki	Rat	SD, HFD	–	+	+	+	+	+

–, absent; +, mild; ++, severe; CHO, carbohydrate enriched diet; DIO, diet-induced obesity; DR, diet-resistant; HFD, high-fat diet; IR, insulin resistance; Ms, Mouse; SD, standard diet; T2DM, type 2 diabetes mellitus.

A classic approach to diet-induced obesity (DIO) and insulin resistance in rodent animals is the unconstrained feeding of the latter with high-calorie high-fat foods (referred to as high-fat diets, HFDs), possibly enriched in other dietary compounds, such as sugar or salt. This procedure simulates adequately the human obesity pathogenesis, involving progressive body weight increase and the consequential insulin resistance development.

Monogenic rodent models have arisen from mutations in the hypothalamic leptin-melanocortin feeding pathway, especially in genes encoding leptin and its receptors. The latter is the case with the historic mildly diabetic but critically obese ob/ob mice (C57BL/6J), which have a spontaneous mutation in *Lep* gene that prevents the secretion of bioactive leptin, and the relatively obese but severely diabetic db/db mice (C57BLKS/J), whose leptin receptor is mutated and defective. Both were initially developed in the 1960s and are used as preclinical models to this day. Various genes of the aforementioned pathway have been associated with human monogenic obesity syndromes.

Equivalent rat models also exist. Notably, the obese Zucker fa/fa rat has its leptin receptor trapped intracellularly due to a missense mutation in *Leptr*, resulting in weakened signaling. The Koletsky rat suffers from an inoperative leptin receptor stemming from a nonsense point mutation in *Leptr.* The ZDF rat presents malfunctioning β-cell transcription apparatus because of an autosomal recessive flaw.

Taking into account that GWAS studies have linked around 100 genes to obesity, polygenic models for researching such conditions are deemed necessary. An excessively used polygenic model of metabolic disease is the C57BL6/J mouse strain due to its susceptibility to hyperphagia-induced obesity when treated with HFD, although important heterogeneity in the increased body weight phenotype is noted, attributed mainly to epigenetic differences.

The New Zealand Obesity (NZO) mouse exhibits moderate hyperphagia, decreased physical activity, and energy expenditure, thus imitating human obesity. Plenty of other polygenic models are available, such as the TALLYHO/Jng mouse – a non-insulin-dependent diabetes mellitus model, the DIO-sensitive or diet-resistant Sprague Dawley rat and the sand rat - a nutrition-dependent early-onset obesity model. Genetic models for primary (instead of obesity-induced) pancreatic β-cell dysfunction, a key feature in the progression of diabetes, are also at one's disposal, like the Goto–Kakizaki (GK) rat or the FVFPBF^{DHom} mice, which constitute a sexually dimorphic diabetic model[13,14].

Assessment of nutritional supplement effects in metabolic disease at a glance

A classic experimental setup for the *in vivo* testing of nutritional supplements in a metabolic pathology context usually includes HFD feeding of a selected animal model for a minimum duration of 3 weeks. Key points that should be considered in order to minimize intra-group differences include the use of genetically identical animals of similar age, the same gender ratio among the different animal groups, a minimum of five animals per group and perfectly matched control groups, which should include both a HFD and a SD group. A typical dietary supplement assessment in regards to obesity, metabolic disease, and inflammation includes the following:

Weight measurement: animal body weight is monitored tactically throughout the duration of the in vivo experiment, including pre- and after- fasting time points.

Measurement of Food Intake: food weight is measured before and after refill, in order to plot food consumption normalized to total weight.

Glucose tolerance test (GTT): for the GTT test, mice are fasted overnight (6-12h) and then injected i.p. with D-glucose (dextrose). Glucose measurements are received by mildly cutting the tail and measuring glucose in the blood with a glucometer at the following time points: 0, 15, 30, 60, and 120 min.

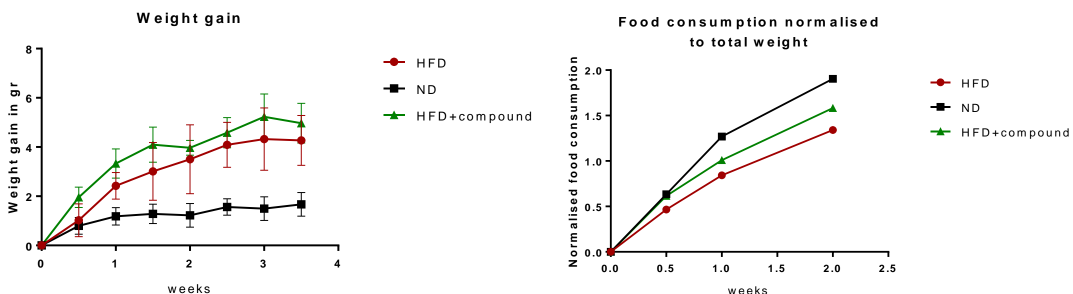

Figure 2.4.1 Example of graphs illustrating animal weight gain and food consumption. HFD: high fat diet, ND: normal diet.

Insulin tolerance test (ITT): for the ITT test, mice are fasted overnight (6-12h) and then injected i.p. with insulin. Glucose measurements are received by mildly cutting the tail and measuring glucose in the blood with a glucometer at the following time points: 0, 15, 30, 60, and 120 min.

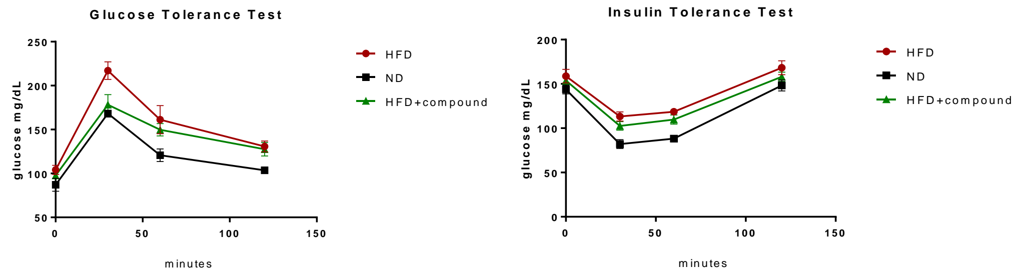

Figure 2.4.2 Example of graphs illustrating animal glucose and insulin tolerance tests. HFD: high fat diet, ND: normal diet.

Abdominal fat mass measurement: when mice are sacrificed, their abdominal fat tissue is isolated and its weight measured. The latter is normalized to total body weight. The same can be done for other tissues or organs of interest. Alternatively, the entire body composition can be measured using magnetic resonance imaging (MRI) and dual-energy X-ray absorptiometry scanning (DEXA).

Inflammation measurement: levels of inflammation are defined by quantifying markers of inflammation, mainly inflammatory cytokines, such as TNF-α or IL-6. Homogenized fat tissue is used for quantitative PCR or ELISA assay, and blood serum for ELISA assay.

A more detailed approach can be found here.

2.4.3. Models of Intestinal Inflammation

Intestinal inflammation constitutes the fundamental characteristic of celiac disease and inflammatory bowel diseases (IBD), such as Crohn's disease or ulcerative colitis, which usually involve severe diarrhea, pain, fatigue, and weight loss. In that context, it is considered to be the consequence of chronic dysregulated immune response in the gastrointestinal tract, stemming from the host's genetic predisposition, as well as inciting environmental factors, including gut microbes and possibly dietary components. Several intestinal inflammation animal models are available, deepening our understanding of enteric pathogenesis and the ways to combat it. These can be categorized as spontaneous colitis, chemically inducible colitis (Table 2.4.1), genetically modified (Table 2.4.2), and adoptive transfer models[17,18]. Chemically induced colitis models are widely exploited because they effectively resemble human intestinal pathologies morphologically, symptomatically, and histologically, therefore they will be briefly presented.

The most extensively utilized animal model is that of DSS colitis, which relies on epithelial damage induction by dextran sodium sulfate (DSS), a chemical colitogen with anticoagulant properties that is added to drinking water (table). The latter’s concentration and frequency of administration can be adjusted in order to simulate acute or chronic intestinal inflammation, as well as relapsing versions of it. DSS proposedly behaves as a chemical toxin that disrupts the epithelial monolayer lining of the intestine, resulting in the ingress of proinflammatory luminal content, including microbes and their metabolites, in the underlying tissue. Symptoms include gross bleeding in the stool, diarrhea, and weight loss. Elevated TNF-α levels is the hallmark of DSS-induced colitis, accompanied by changes in Th1/Th2 cytokine profile. Generally, the particular model of intestinal inflammation exhibits plenty of benefits, as the followed protocols are fast, simple, and reproducible.

DSS can also be exploited to cause colitis-associated cancer (CAC). Colorectal cancer (CRC) is the third most common cancer in the world and it has been demonstrated that individuals with colitis are predisposed to colorectal tumor formation. Therefore, it is worth considering a very well established model of inflammation-induced intestinal carcinogenesis which combines DSS with azoxymethane (methyl-methylimino-oxidoazanium, AOM): the AOM/DSS model, whose core principle is to chemically induce DNA damage and repeated cycles of colitis. AOM is converted by cytochrome p450 into the carcinogenic form of methylazocymethanol (MAM), a highly reactive alkylating species, which is received by enteric epithelial cells when excreted into the bile, causing DNA

mutations. The AOM/DSS model is reproducible, relatively inexpensive with a moderate timeline (~10 weeks), and simulates accurately CAC. The protocol is initiated with an intraperitoneal injection of mice with AOM and then follow cycles of DSS uptake though water intervened by recovery phases. A detailed approach is presented in "AOM/DSS Model of Colitis-Associated Cancer" by Parang et al. (2017).

Another vastly used model of both acute and chronic intestinal inflammation, especially Crohn's disease, constitutes TNBS colitis, which utilizes hapten reagent 2,4,6-trinitrobenzene sulfonic acid (TNBS), a typical skin contactant (Table 2.4.4). This chemical is mixed with ethanol, which disrupts the intestinal barrier, and administered intra-rectally. Thus, TNBS interacts with colon tissue proteins, resulting in increased leukocyte infiltration and excessive Th1 inflammation, which involves IL-12 and TNF-α as effector cytokines. Due to the hypersensitivity immune response, the above proteins are rendered immunogenic to the host immune system. After treatment, animals present various symptoms of acute colitis, such as edema, ulceration, inconsistent stool formation, and bloody diarrhea.

Table 2.4.4 Selected models of chemically-induced intestinal inflammation. Adapted from Westbrook et al. (2010) and Randhawa et al. (2014).

Model	Description	Method for induction of colitis
DSS	Epithelial damage resulting in acute and chronic colonic inflammation.	Mice: 3 to 5 % DSS (w/v) (36,000~ 50,000 Da) is diluted in the drinking water for 5~ 8 days.
TNBS	Delayed type hypersensitivity immune response leading to acute and chronic inflammation. Simulates Crohn's disease.	Mice: 200 mg/kg TNBS dissolved in 30% ethanol is instilled via a catheter approximately 3~ 4 cm proximal to anus.
Oxazolone	Th2 immune response. Simulates ulcerative colitis.	Mice: Presensitization of abdominal skin with 50~ 150 μl 3% oxazolone in 100% ethanol followed by rechallenge after 5~ 8 days with intrarectal administration of 70~ 150 μ l of 0.75~ 1% oxazolone in 45~ 50% ethanol solution. Presensitization by transdermal application of 200 μ l of a 3% (w/v) oxazolone solution in acetone/olive oil (4:1) followed by rechallenge with 100 μ l of 1% oxazolone in 50% ethanol on 8th day.
Acetic acid	Non-transmural inflammation.	Mice: 1 ml of acetic acid (4~ 5%, v/v) in 0.9% saline is instilled into the lumen of the colon approximately 4 cm proximal to the anus.
Carrageenan	Mucosal inflammation of the cecum	2% lambda-carrageenan is added into the drinking water of rats for 6 weeks. Presensitization of rats by parenteral administration of 1.5 percent solution of degraded lambda carrageenan followed by oral administration of the same solution for 30 days.

Due to the polygenic and heterogeneous nature of intestinal inflammation, numerous genetic models have been established (Table 2.4.5). One of the primarily focused areas of research is the epithelial barrier function and involve mice deficient in essential structural elements of the protective mucosal layer (e.g. Muc2 or O-glycans), in transporters and exchangers of intestinal epithelial cells (e.g. Octn2, a carnitine transporter), and in pattern recognition receptors (e.g. Nod2, TLR4). Immune regulation is also extensively examined, highly involving cytokine responses and their regulators. For example, there are widespread models of deficiencies in T cells (like TGF-β1 and its signal transducer Smad3, IL-2, and IL-10), as well as models with modifications in important signal-transduction factors, like STAT3, NF-κB, and SOCS1, which exhibit types of spontaneous enterocolitis.

Moreover, models of intestinal stress responses have also been developed, since the latter are fundamental for maintaining enteric homeostasis. A representative example would be the double Gpdx1-Gpx2 (antioxidant enzyme glutathione peroxidase) knockouts, which lead to ileocolitis and adenocarcinoma, or XBP1 transcription factor deficiency in gut epithelial cells, resulting in intestinal inflammation[18]. Regarding intestinal carcinogenesis, a widely used for over 25 years genetically engineered model is the *Apc$^{Min/+}$* mouse, which carriers an autosomal dominant loss of function mutation in the *Apc* - a tumor suppressor gene and the most common driver mutation for colorectal carcinoma in humans.

Table 2.4.5 Experimental Models of Inflammatory Bowel Disease Due to Specific Genetic Defects. Adapted from Kiesler et al. (2015).

Model	Description
Muc2 (and related models)	Increased intestinal permeability due to deficiency of essential structural elements of the epithelial barrier leading to inflammation and enhanced susceptibility to colitis and corectal cancer.
MDR1a colitis	Increased intestinal permeability and translocation of bacteria into the lamina propria, and the development of colitis, driven bt Th1 cytokines.
TRUC model	Spontaneous colitis induced by "colitogenic" intestinal microflora, associated with abnormal levels of TNF-a and IL-23.
NEMO colitis	Spontaneous and severe chronic intestinal inflammation, due to disrupted NF-κB signaling.
SAMP1/Yit mouse	Severe inflammation of the terminal ileum and caecum. The former is the primary location of Crohn’s disease lesions.
XBP1 model	Induction of ER stress through disruption of XPB1 causes small intestinal inflammation.
STAT3 colitis	Spontaneous chronic enterocolitis, linked to polarized Th1 response and defective IL-10 signaling in macrophages.
IL-7 transgenic mice	Spontaneous chronic colitis, linked to Th1 response.

Assessment of nutritional supplement effects at a glance

A number of factors are examined while testing the effectiveness of nutritional supplements in the context of intestinal inflammatory diseases. These are briefly described below:

- ✓ **Disease activity index (DAI) score:** body weight and disease symptoms, such as diarrhea and hematochezia are tactically monitored.
- ✓ **Colon length:** right after animal sacrifice, the intestine is isolated and measured
- ✓ **Inflammatory cytokines:** suitable parts of stool-free colon (e.g. 10mg from the ascending colon and 10mg from the descending colon) are homogenized in appropriate ice-cold lysis buffer. Suspensions are centrifuged (e.g. 13,000 rpm, 15min) and the resulting supernatant is used for ELISA assay of the desired inflammatory cytokines. Alternatively, the expression of relative genes can be measured with real-time PCR.
- ✓ **Histopathological analysis:** proximal and distal colonic sections are fixed and embedded in paraffin. Haematoxylin and eosin are used for staining. Microscopic colonic epithelial damage is assessed, alongside with inflammatory cell infiltration. For a detailed approach, refer to "Intestinal Preparation Techniques for Histological Analysis in the Mouse" by Williams et al. (2016).
- ✓ **Cell population analysis:** cell populations expressing distinct levels of suitable markers are isolated by flow cytometry or fluorescence-activated cell sorting (FACS). These can then be used for gene expression profiling with real-time PCR.

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Course 2.5: Animal and cell culture models of skin homeostasis and repair, and cancer

Authors: Ioanna Lapi, Christos Tsatsanis

2.5.1 Models of acute and chronic wound healing

A wound is defined as a disruption in the epithelial lining of a living tissue due to physical, chemical, thermal damage, microbial or immunological insults. This break in the epithelial integrity can be divided into two categories, acute and chronic wound, based on the duration and nature of the healing process. Specifically, an acute wound is the result of an accident or surgical injury, while the time of its healing process is usually 8-12 weeks, dependent on the size, depth, and the extent of damage. On the other hand, conditions such as decubitis ulcer and leg ulcer, create chronic wounds that are not able to heal through the normal stages of healing in a predictable timeframe as acute wounds.

Wound Healing Stages

The dynamic and complex process of wound healing consists of four different phases; the coagulation and hemostasis phase (immediately after injury), the inflammatory phase, (shortly after injury, when swelling takes place), the proliferation period, where new tissues and blood vessels are formed, and the maturation phase, in which remodeling of new tissues. After a tissue injury, blood vessels are disrupted and exposed platelets play a crucial role in the first step of wound healing, known as hemostasis. Hemostasis consists of two major processes, the development of a fibrin clot and coagulation. Platelets release chemoattractants and growth factors that trigger the initiation of the second phase of the healing process. Recruited leukocytes, first neutrophils and later macrophages, lymphocytes, and mast cells, infiltrate the wound and clean the area by removing damaged tissue debris and foreign particles. Upon activation, macrophages also release several important growth factors and cytokines. Thereafter, resident dermal fibroblasts nearby proliferate and migrate into the wound clot to produce collagen-rich matrix, resulting in the formation of contractile granulation tissue that assist the wound margins to close together. Meanwhile, the epidermal cells (keratinocytes) on the wound edges migrate to fill the wound space, reconstituting the epidermis (re-epithelization).

Factors Affecting Wound Healing Process

Consequently, wound healing is a multi-step process that relies on interactions among cytokines, growth factors, blood and the extracellular matrix (ECM). The ECM forms a three-dimensional structure and is composed of extracellular macromolecules, such as collagen, elastin, fibronectin, vitronectin, integrins and laminins, providing structural and biochemical support to surrounding cells. In the wound, ECM molecules act either as substrate scaffolds to facilitate processes such as differentiation, migration and proliferation, or interact with cells through transmembrane cell surface receptors, mediating signal transduction. During the

hemostasis phase, platelets release platelet-derived growth factor (PDGF), transforming growth factors (TGF)-a and -b, fibroblast growth factor (FGF)-2, epidermal and endothelial growth factors as well as chemotactic factors. Additionally, fibroblasts produce vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF), while lymphocytes modulate healing through the release of several growth factors including interleukin-1 (IL-1), tumor necrosis factor (TNF), fibroblast-activating factor, and macrophage-activating and inhibitory factors. Therefore, ECM molecules combined with cytokines and growth factors regulate distinct processes in the overlapping phases of wound healing.

2.5.2 Skin Inflammatory diseases: The model of Psoriasis

Psoriasis is an autoimmune chronic skin disease with features, such as abnormal growth and differentiation of keratinocytes, increased vasodilation with local release of nitric oxide (NO), and skin infiltration by leukocytes. The most common form, psoriasis vulgaris, is characterized by erythematous plaques covered by thick white scales. Although psoriatic skin lesions are mostly found on the extensor surfaces of the body, any part of the body including the scalp and nails can develop a lesion. It is well established that psoriatic lesions are triggered by trauma, also known as Koebner phenomenon. Psoriasis and wound healing share common features. In both conditions, the expression of keratins I and X is reduced, while keratins VI and XVI are upregulated in comparison with the normal skin. Moreover, the psoriatic skin lesion expresses antimicrobial peptides as well as the antimicrobial protein REG3A that regulates keratinocyte proliferation and differentiation after skin injury. However, in psoriasis keratinocytes are not able to differentiate due to elevated intracellular Ca^{++}concentration; this leads to keratinocyte hyperproliferation and subsequently increased rate of wound healing.

2.5.3 Animal models of Wound Healing and cutaneous repair

To understand better the complexity of the wound healing process and relative diseases, such as psoriasis, experimental models have been developed, both *in vivo* and *in vitro*.

In vivo models of wound healing

In general, *in vivo* models for studying wound repair use mostly laboratory animals, specifically rats, mice, rabbits and pigs, each offering advantages and shortcomings. The animal that will be chosen depends on several factors, such as cost, availability, ease of handling, investigator familiarity, and similarity to humans. Traditionally, a wound experiment involves wounding the animal and monitoring gradually the wound closure. However, the protocols for the animal testing should follow the principles of the 3Rs (replacement, reduction and refinement), that verify the animal welfare. The animal model provides valuable information regarding skin biology and human pathophysiology, but has its own limitations. For instance, there are differences in skin structure and wound healing time between rodents and humans. Furthermore, these animal models

are also utilized to evaluate the effect of several factors, such as nutrition supplements and medications, on wound healing.

Animal models: Chronic & Acute wound models

To study chronic wounds, emerged from impaired wound healing, animal models have been developed from an acute wound by inducing diabetes, mechanical pressure or ischemia. By contrast, acute wounds have been more extensively analyzed via well-established models, such as the excisional, incisional, and burn model. All these models are summarized in *Table 2.5.1M* (Table 2.5.1).

Table 2.5.1 Features & Protocols of Chronic and Acute wound models used in wound healing studies.

Animal models	Species	Comments	Related protocols
Chronic wounds	Mouse, rat, rabbit, pig, dog	Impaired wound models generated from an acute wound by inducing diabetes, mechanical pressure, ischemia, or reperfusion injury.	Ischemia model (mouse) https://pubmed.ncbi.nlm.nih.gov/15235597/. Pressure ulcer model induced by ischemia-reperfusion injury (mouse) https://pubmed.ncbi.nlm.nih.gov/18219277/ or by implanting a metal plate (mouse) https://pubmed.ncbi.nlm.nih.gov/19614912/. Diabetic wound models (pig) https://pubmed.ncbi.nlm.nih.gov/18318812/ & (rat) https://pubmed.ncbi.nlm.nih.gov/18585672/. Biofilm-infected wound model (rabbit) https://pubmed.ncbi.nlm.nih.gov/21518094/.
Acute wounds			
Incisional wound	Mouse, rat, rabbit, pig	For wound tensile strength studies. Subdivided into primary and secondary closure model based on the use of suture or not.	Back incisional model (mouse) https://pubmed.ncbi.nlm.nih.gov/15454143/. Incisional wound models for: investigation of cellular mechanisms (knockout iNOS mouse) https://pubmed.ncbi.nlm.nih.gov/12464872/, genome-wide microarray analysis (red Duroc pig) https://pubmed.ncbi.nlm.nih.gov/24739276/, testing a diacetyl chitin surgical suture (rat)

			https://pubmed.ncbi.nlm.nih.gov/25677094/.
Excisional wound	Mouse, rat, rabbit, pig	*Full-thickness model*: Primary closure model. Well suited for testing the effect of a compound on wound repair. *Other excisional wounds*: partial-thickness, splinting, ear punch, head punch models.	Excisional wound splinting model https://pubmed.ncbi.nlm.nih.gov/15260814/. Ear punch model (rabbit) https://pubmed.ncbi.nlm.nih.gov/17617336/ & (mouse) https://pubmed.ncbi.nlm.nih.gov/9683548/. Head punch model (mouse) https://pubmed.ncbi.nlm.nih.gov/15454143/. Partial-thickness excisional wounds (pig) https://pubmed.ncbi.nlm.nih.gov/31715245/ .
Burn wound	Mouse, rat, rabbit, pig, dog	Burn injuries created by hot water, hot metal tools, electricity, and heated paraffin.	Burn injury induced by hot metal plate (rat) https://pubmed.ncbi.nlm.nih.gov/25955320/, by burn apparatus containing aluminum cylindrical heads (rat) https://pubmed.ncbi.nlm.nih.gov/25440857/, and by water scalding (mouse) https://pubmed.ncbi.nlm.nih.gov/29755839/.

✓ **Acute models**

i) Incisional wound model

The incisional skin healing model, the second most frequently employed model, can be beneficial for studying the wound tensile strength (Fig. 2.5.1). Incisional wounds are generally full thickness and scalpel induced by using a blade, that causes bleeding but minimal tissue damage, or an electrocautery or laser surgical device. This type of wound can be subdivided into primary and secondary closure model. The basic difference between these two models of closure is the use of suture in the primary model to close the wound margins. The primary closure model (or first intention) of incisional wound is ideal for biomechanical analysis of wound strength, whereas the second model (second intention) is better suited for investigating scarring at late time points. However, the size, periodicity, and type of suture used has been shown to importantly affect the tensile forces across a sheet of wounded skin.

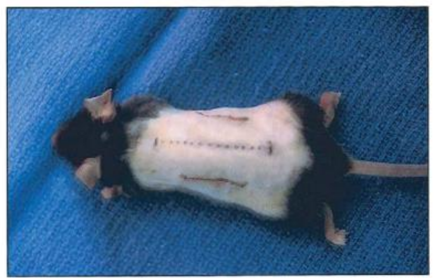


Figure 2.5.1 Incisional wound model. Two incisions are made on the mouse back along the paramedian (pre-operative marking), allowing tensile strength measurements by tensiometry. Adapted from: https://pubmed.ncbi.nlm.nih.gov/15454143/.

ii) Excisional wound model

Excisional wound models are the most commonly used owing to their similarity with the acute clinical wounds. Since the wound edges are not sutured, they are considered as secondary closure models. Excisional wounds are generated through the surgical removal of all skin layers (epidermis, dermis, and subcutaneous fat) from the animal. Excisional wound models provide valuable information. The wound healing rate measures how the wound area closes relative to the original dimensions through photographs. The simplest method of determining wound area is by using a ruler, or other accurate scale, photographed near the wound, enabling the user to calibrate the software for the analysis. The average diameter of the wound, expressed as percentage, over the time period (Day 0 diameter/Day X diameter) is depicted in the wound closure curve (Fig. 2.5.2). The ability to generate more than one wounds in each animal increases the statistical value of this model. In addition, the wound bed is easily accessed, allowing agents, such as biomaterials and pharmaceuticals, to be tested for their effect on the repair process.

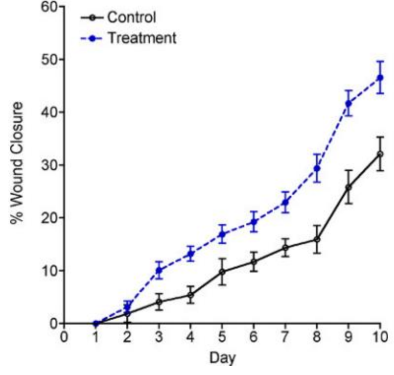


Figure 2.5.2 Wound closure graph. A therapeutic compound or vehicle control was applied to the wound bed on a daily basis. https://pubmed.ncbi.nlm.nih.gov/23748713/.

Excisional wound protocols

The protocols for creating an excisional full-thickness wound have variations; the size of excision (2 -20 mm diameter) and the number of wounds per animal differ as well as the tools employed, such as biopsy punches, surgical scissors and lasers. In the mouse model, after anesthesia, hair is shaved on the mouse back, and the loose skin is lifted and cut, generating a wound. Apart from the full-

thickness excisional model, which is widely used, there are also other excisional wound models, such as the partial-thickness, the head punch, the ear punch, and the excisional wound splinting models. In the mouse, around 70-80% of the wound healing occurs via contraction due to increased skin laxity. In contrast, human wound healing relies primarily on re-epithelization. To control for contraction and isolate epithelialization, "doughnut"-shaped rings (splints) can be sutured around the perimeter of the wound to splint the skin of the mouse (Fig. 2.5.3). Another approach to overcome wound contraction is choosing anatomical sites with securely attached dermis and subcutaneous tissue (e.g. rabbit ear). The excisional wound splinting model is explained below, while other excisional wound protocols are summarized latter in this Unit.

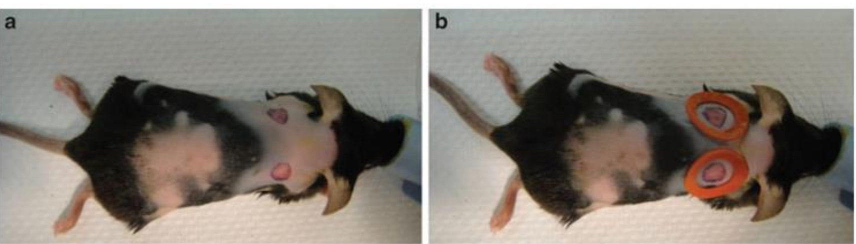


Figure 2.5.3 Excisional wound splinting model in mouse. (a) Full thickness skin wounds are generated at the same level on the dorsum of mice. (b) "Doughnut"-shaped rings are sutured around the perimeter of the wounds to splint the skin and isolate re-epithelialization. https://pubmed.ncbi.nlm.nih.gov/24029931/ .

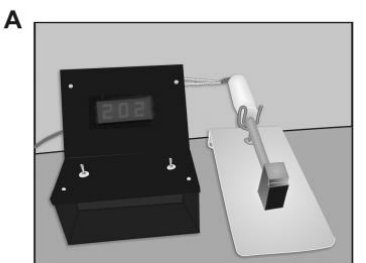
iii) Excisional wound splinting model in mouse

To create the "doughnut"-shaped rings, thick silicone rubber sheet is cut to form two silicone disks and an aperture in the dimensions of the excisional wound is opened in the middle of each silicone disk using a biopsy punch to form the "doughnut"-shaped ring that will be used as a splint. After mice are anesthetized using a gas anesthetic (e.g. isoflurane) or an intraperitoneal injection of sodium pentobarbital (50 mg kg^{-1}), the fur is cut on the dorsal and side skin with an electric clipper and a depilatory cream is applied on the skin for a couple of minutes. The cream and skin are then completely removed by wiping with sterile cotton balls soaked in warm water or an alcohol swab, followed by two applications of 10% povidone-iodine. The mice are kept individually to avoid scratching and chewing. On the day of surgery, mice are anesthetized and the position of the wound is marked either by drawing or using the biopsy punch to outline the pattern of the wound on either side of the mouse midline. The dorsal skin is lifted and one full-thickness wound is created using the biopsy punch or iris scissors on either side of the midline. The wound size is dependent on the mouse strain as well as research aims. Each splint is covered with cyanoacrylate adhesive (e.g., Krazy glue) and is placed around the wound. The splint is secured to the skin with 6 or more interrupted sutures of 6.0 nylon. At this step, bioactive factors can be applied to the wound bed or injected into the tissue to determine their effects on wound healing; the bioactive compound to one wound and the vehicle control to the other (https://pubmed.ncbi.nlm.nih.gov/23748713/ & https://pubmed.ncbi.nlm.nih.gov/23329003/).

iv) Burn skin models

In burn skin healing models, full- or partial-thickness wounds are generated by four different methods, hot water, hot metal tools, electricity, and heated

paraffin. Different species, such as mouse, rat, pig, rabbit and dog, can be utilized as experimental burn injury models, each with its own advantages and limitations. Before the burn injury, animals are anesthetized usually with a mixture of ketamine/xylazine, the back of the mouse is shaved using a hair clipper and then hair removal cream is applied to remove the hair completely. The first model, known as water scald model, uses a template with an aperture, through which part of the body is immersed into a water bath with controlled temperature. The second method involves a hot metal plate, which directly apply heat to the skin, producing partial thickness wounds (Fig.2.5.4). In both models, the blister can be de-roofed, exposing the dermis and leaving the wound opened. To produce third-degree burn injuries, the aforementioned hot metal plate or an electric coil are utilized as these devices reach high temperatures, 170°C and 400°C respectively.



B

Figure 2.5.4 Burn wound model. (A) The experimental wounds were generated using this burn device with the metal plate. (B) Initial burn injury on dorsal surface of the rat. Adapted from https://pubmed.ncbi.nlm.nih.gov/25955320/.

Animal models in psoriasis

In the context of psoriasis, apart from these "normal" laboratory animals, animals with spontaneous mutations and genetically modified animals with artificially introduced mutations are also used. In addition, xenograft models, generated by transplantation of human skin fragments to immunodeficient mice, are another useful experimental model that resemble the clinical manifestations of psoriasis. Currently, imiquimod (IMQ)-induced skin inflammation is the most widely accepted psoriasis animal model since imiquimod treatment exert similar cytokine expression patterns, histopathological alterations and cellular infiltrates as those observed in psoriatic patients. All these models have been developed to study the pathophysiology of the disease, such as the aetiologia of the increased wound healing rate, as well as the effect of potential therapeutic targets.

2.5.4 *In vitro* cell culture models of wound healing

Undoubtedly, an *in vivo* wound model is the best possible model system, offering multiple benefits. It allows the study of interactions between multiple cell populations and the whole-body system during repair. In addition, the animal model also allows selective depletion of specific genes to determine their effect on wound healing, while enables the generation of multiple wounds within one animal. However, ethical issues, cost, and the fact that these models often show high degree of variability, must be taken into consideration. Therefore, *in vitro*

models also exist; rapid, low cost and reproducible systems that produce important preliminary results regarding one or few components of the skin at a time. Most *in vitro* models are performed to answer basic questions regarding cell signaling in response to cell stress or injury.

Migration assays. Scratch assays use dermal fibroblasts, keratinocytes, or endothelial cells, creating a monolayer cell culture. A 'wound' is generated in the cell layer by scraping the dish surface with either rubber devices or the tip of a pipette. The rate of wound closure, namely how fast the cells divide and migrate into the 'wound' area, is determined by imaging. An alternative migration assay is the Boyden chamber/transwell assay. In this model, cells such as endothelial or keratinocytes are seeded on top of an insert, optionally coated with ECM components, with a porous membrane (upper compartment). Cell migration is quantified via measuring the number of cells that have passed through the pores during a defined time interval into the lower compartment.

Three-dimensional (3D) *in vitro* models. In contrast to these 2D models, 3D models represent better the 3D macroscopic fibrotic tissue structure of a scar. Thus, 3D models have been developed for studying the replacement and remodeling of ECM during wound healing. One such model is a 3D collagen construct with embedded fibroblasts, providing information about cell migration as well as cell-induced matrix reorganization. In this model, the compressed collagen matrix containing cells is placed within an acellular, lower density outer matrix. In another model, the fibroblast-populated collagen lattice (FPCL), fibroblasts are seeded in a collagen solution (usually type I acquired from rat tail), which is solidified to gel, forming a cell-encapsulated three-dimensional lattice. Being unsuccessful as a skin graft, FPCL became valuable for investigating cell–connective tissue interactions within a three-dimensional matrix. This model is based on the principle that cell-generated forces reduce the water mass (reduction in FPCL volume) between collagen fibers, resulting in compaction (collagen matrix compaction). In addition, organotypic skin-like three-dimensional cultures can also be used for studying interactions between different cell types. In this model, typically keratinocytes are grown on top of a collagen matrix that contain fibroblasts, creating epidermal equivalents. Moreover, human epidermal equivalents can be also developed using either human adult primary keratinocytes, isolated from surplus healthy skin or patient skin, or immortalized keratinocyte cell lines, such as N/TERT-1 and N/TERT-2G.

Wound histopathology. For histopathological analysis, skin biopsy is performed, followed by embedding, sectioning and staining in order to observe the healing progress in the course of treatment. Haematoxylin and eosin (H&E), the most widely used stain, as well as other non-H&E stains, trichromes (stain collagen fibers and muscle) and immunohistochemical markers allow quantification of white blood cells, blood vessel, fibroblasts, and collagen.

Histopathological analysis

For histological analysis, animals can either be euthanized (eg mice, rats) or locally anesthetized (eg rabbit ear model) and biopsies are collected. The fresh isolated wound tissue is fixed in 4% (wt/vol) paraformaldehyde in PBS at 4°C overnight. The next day, the tissue is placed in a tissue culture plate and washed 5 times with PBS (30 min each) on a shaker. The fixed tissue is then incubated in 20% and 40% (wt/vol) sucrose in PBS at room temperature for 2 h each. After cryoprotection, the tissue is embedded in OCT using a cryomold and freezed immediately at -70°C. A cryosectioning machine is used to cut cryosections of the desired thickness on superfrost plus slides. For analysis of wound granulation

(recruited cell populations, vascularity and matrix alterations) and re-epithelialization, the sections can be stained with H&E and examined under a light microscope (Fig. 2.5.5) https://pubmed.ncbi.nlm.nih.gov/23329003/ .

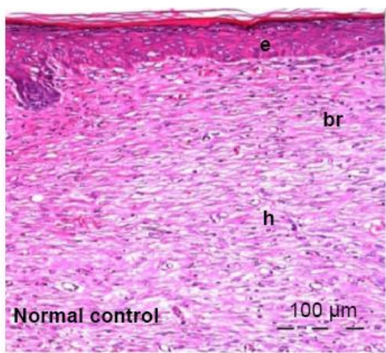


Figure 2.5.5 Hematoxylin–eosin histological sections of excisional wound site (Wistar rat on day 21 after wounding). (h): healed area with multiple layers of fibrous connective tissue; (br): boundary between unhealed and healed tissue; (e): epithelium. https://pubmed.ncbi.nlm.nih.gov/27307703/ .

Biochemical & Immunological methods. The progression of the wound healing can be also monitored through biochemical assays such as hydroxyproline, myeloperoxidase assay (MPO), N-acetylglucosaminidase (NAG) and oxidative stress profile. As previously mentioned, during wound healing cell-to-cell communication is mediated via soluble or membrane-bound factors (cytokines and growth factors), which provide the necessary signals to stimulate endogenous repair mechanisms. These signaling molecules can be quantified by different techniques such as immunohistochemistry and enzyme-linked immunosorbent assay (ELISA).

2.5.5 Conclusion on animal and cell culture models of skin homeostasis and repair

In summary, currently available *in vivo* and *in vitro* models have improved our understanding of wound healing process. A complex process that integrates multiple cell types and repair phases, including inflammation, proliferation, re-epithelialization and remodeling. A variety of *in vivo* skin healing models, including incisional, excisional, burn models combined with *in vitro* migrations assays and histopathological, immunological and biochemical methods have provided valuable information regarding the basic mechanisms of wound healing. It is of high importance to develop optimal strategies in the future to help patients with adverse wounds or related diseases, such as psoriasis.

2.5.6 Animal and cell culture models of Cancer

Cancer, a devastating large group of diseases, can occur in almost any organ or tissue of the body when cell cycle is disrupted. In the late stages of cancer, the

abnormal cells break through normal tissue boundaries and invade other organs (metastasis). According to the World Health Organization (WHO), cancer is considered the second leading cause of death globally, accounting for an estimated 9.6 million deaths, or one in six deaths, in 2018. Lung, prostate, colorectal, stomach and liver cancer are the most common types of cancer in men, whereas breast, colorectal, lung, cervical and thyroid cancer are the most common among women. The Cancer Research UK organization estimates that there will be 27.5 million new cases of cancer each year worldwide by 2040. The cancer therapeutic treatments, currently available, are highly expensive and none of them is completely safe. In particular, these treatments often involve surgical removal and radiation of the biomass in combination with systemic chemotherapy. Although chemotherapy is the most widely used treatment in all types of cancer, may result in recurrence of cancer accompanied with drug resistance as well as severe side effects, making patient's life difficult. Therefore, new non-invasive therapeutic approaches are required to impede the development of cancer.

Bioactive compounds and Cancer

Cancer chemoprevention generally refers to the pharmacologic intervention with synthetic or naturally occurring chemicals to prevent, inhibit or reverse carcinogenesis or prevent the development of invasive cancer. Up to now, multiple studies have examined the potential anticancer properties of distinct nutritional supplements. These nutritional supplements contain isolated compounds or extracts with bioactivity. As stated by the Office of Dietary Supplements at the NIH, bioactive compounds are defined as constituents in foods or dietary supplements, other than those needed to meet basic human nutritional needs, which are responsible for changes in health status. But what kind of approaches are being utilized to assess the health and safety of bioactive food components? In the context of cancer, the first step is a deep comprehension of health promotion and disease condition. Secondly, the bioactivity of the compound is assessed through appropriate research models, both *in vitro* and *in vivo*. Carcinogenicity testing, crucial to assess the safety of a substance, includes short-term *in vitro* assays, short-term *in vivo* assays, and the 2-year rodent bioassay.

Models for assessing Cytotoxicity of bioactive compounds

In vitro assays for assessing cytotoxicity

The cytotoxicity assays utilize cell lines of different origins, in both 2D and 3D systems, and predict the acute toxicity by estimation of LC_{50} values (lethal concentration for 50% of the cells). In general, these assays are fundamentally colorimetric, involving optical activity of organic dyes (Table 2.5.1).

Table 2.5.1 In vitro cytotoxic tests used to measure dead cells.

Cytotoxic assays	Short Characteristics	Protocols
Trypan blue dye exclusion assay	Trypan blue dye stains cell membrane-damaged cells. Cell counting: manually (hemocytometer) or automatically	Trypan blue assay: https://pubmed.ncbi.nlm.nih.gov/21468962/ , https://pubmed.ncbi.nlm.nih.gov/18432654/ , https://pubmed.ncbi.nlm.nih.gov/26529666/ , https://pubmed.ncbi.nlm.nih.gov/27371594/.
Lactate dehydrogenase (LDH) assay	Measurement of LDH release due to cellular membrane permeabilization (rupture).	LDH assay: https://mitofit.org/images/4/47/MiPNet08.18_LDH.pdf , https://pubmed.ncbi.nlm.nih.gov/21722700/ , https://pubmed.ncbi.nlm.nih.gov/23070760/ , https://pubmed.ncbi.nlm.nih.gov/29858337/.
AlamarBlue assay	Resazurin dye (dark blue, non-fluorescent) is reduced to resorufin (pink, highly fluorescent) via metabolic enzymes (absorbance reading).	AlamarBlue assay: https://pubmed.ncbi.nlm.nih.gov/10951200/ , https://pubmed.ncbi.nlm.nih.gov/17307808/ , https://pubmed.ncbi.nlm.nih.gov/28807796/.
Microculture tetrazolium assays	Tetrazolium salts (MTT, MTS, XTT, WST-1, WST-3, and WST-8) cleaved by mitochondrial enzymes to form colored products (absorbance reading).	MTT assay: https://pubmed.ncbi.nlm.nih.gov/14634227/ , https://pubmed.ncbi.nlm.nih.gov/21516412/ , https://www.ncbi.nlm.nih.gov/books/NBK144065/ , https://pubmed.ncbi.nlm.nih.gov/24300943/ , https://pubmed.ncbi.nlm.nih.gov/29858338/ .

Trypan blue dye exclusion assay

In the trypan blue dye exclusion assay, which is the most commonly used test for cell viability, only cell-membrane damaged cells take up the dye. Thus, the proportion of colored damaged cells can be counted using a hemocytometer. However, cell membrane damage does not necessarily mean cell death. Another disadvantage of this technique is that the user estimates what is a dead cell or stained debris, measuring only one sample at a time. In the Lactate dehydrogenase (LDH) assay, LDH activity is spectrophotometrically measured in the cell culture supernatant as this cytoplasmic enzyme, found in all cell types, is rapidly released when the plasma membrane is damaged. LDH catalyses the reduction of NAD+ to NADH in the presence of L-lactate and the produced NADH can be measured in a coupled reaction in which the tetrazolium salt, INT, is reduced to a colored product with properties that are quantitated. This assay also measures cell membrane damage and not necessarily total cell death. The AlamarBlue assay is also performed to establish relative cytotoxicity of compounds. This method is based on a resazurin dye (dark blue, non-fluorescent), which is reduced to resorufin (pink, highly fluorescent) through a redox reaction. In addition to mitochondrial reductases, other enzymes, such as diaphorases, NAD(P)H:quinone oxidoreductase and flavin reductase, present in

the cytoplasm may be able to reduce AlamarBlue. The quantified fluorescence intensity is consistent with the cellular metabolic activity of living cells. Since damaged and dead cells have lower metabolic activity, they generate a proportionally lower signal than viable cells.

Microculture tetrazolium assays

Microculture tetrazolium assays (MTAs), based on colorimetric determination, use a broad number of tetrazolium salts, including MTT, MTS, XTT, WST-1, WST-3, and WST-8, to detect viable cells. The tetrazolium salts are cleaved by a mitochondrial enzyme, succinate dehydrogenase, resulting in the formation of a colored product, formazan, which can be easily quantified by spectrophotometry 8. This assay is based on the principle that the mitochondrial activity of viable cells is constant and, thus, a change in the number of living cells is related to metabolic activity in a linear relationship. Measuring formazan by the optical density (OD) indicates any increase or decrease in viable cell number.

In the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) tetrazolium reduction assay, the yellow water-soluble tetrazolium MTT dye forms a purple colored insoluble formazan product that precipitates. Hence, the use of a solubilization solvent is necessary before recording absorbance readings. A variety of different MTT reagents, MTT solutions as well as solubilization reagents are commercially available.

MTT assay protocol

Firstly, the MTT is dissolved in Dulbecco's Phosphate Buffered Saline, pH=7.4 (DPBS) at a final concentration of 5 mg/ml and subsequently the MTT solution is filtered through a 0.2 μm filter. It can be stored at 4°C for frequent use or at -20°C for long term storage (light-sensitive). Next, the solubilization solution (pH 4.7) is prepared by adding 16% (wt/vol) sodium dodecyl sulfate (SDS) into a solution containing 40% (vol/vol) dimethylformamide (DMF) in 2% (vol/vol) glacial acetic acid under a ventilated fume hood. To avoid precipitation, the solution is stored at room temperature. On the day of experiment, the cells are seeded into a 96-well plate and incubated with the test compounds (100 ul/well) for the desired exposure time. Next, 10 ul MTT solution is added per well (0.45 mg/ml); the final concentration ranges from 0.2-0.5 mg/ml. After a 1-to 4- hour incubation (37 oC), 100 μl solubilization solution is added to each well to dissolve formazan crystals. The absorbance is then measured at 570 nm (and sometimes at 630 nm - reference wavelength) using a plate reading spectrophotometer, which estimates the formazan concentration that is proportional to the number of viable cells. *https://www.ncbi.nlm.nih.gov/books/NBK144065/*.

2.5.7 Models for assessing Genotoxicity of bioactive compounds

The term genotoxicity refers to the ability of agents to alter the genetic information within a cell. This DNA alteration can be in the form of single- and double- strand (ssDNA and dsDNA) breaks, point mutations, deletions, chromosomal aberrations, micronuclei formation, DNA repair and cell-cycle interactions. Genotoxicity testing for hazard identification and risk assessment is crucial for evaluating the ability of a chemical agent to induce genetic alterations.

Currently, there is no single validated test able to provide information on the three genotoxicity critical end-points, which are mutation induction (permanent, transmissible changes in the DNA), clastogenicity (structural chromosomal damage), and aneugenicity (numeric chromosomal abnormalities).

In silico & *in vitro* genotoxicity testing

Genotoxicity assessment can be determined through computational *in silico* methods that predict biological activities of a molecule from its physicochemical properties. These methods are based on computational tools, mathematical calculation, and analysis of predicted or experimental data through computer-based models. Thus, the *in silico* approach is widely used to assess toxicological effects relevant to human health as well as to prioritize chemicals for *in vitro* or *in vivo* testing.

A battery of *in vitro* genotoxicity assays provides simple, robust and time- and cost-effective testing of toxicity and the subsequent underlying mechanisms. To assess the risk of cancer, the most commonly utilized methods to determine genetic damage include the bacterial Ames test, DNA strand break measurements in cells (e.g. comet assay, alkaline unwinding and hydroxyapatite chromatography, alkaline elution), and cytogenetic assays (micronucleus and chromosomal aberration assays). Three *in vitro* genotoxic tests, the Ames test, the Comet assay and the Chromosomal aberration test, are explained in Table 2.3.2 and in the following sections where all tests are summarized as well.

Table 2.5.2. Different in vitro assays used for detecting genetic damage in genotoxicity studies.

***In vitro* genotoxic assays**	**Short Characteristics & Protocols**
Ames test	Use of *Salmonella typhimurium* and *E. coli* bacterial strains. Fast, sensitive, and economic method for detecting mutagenicity *https://doi.org/10.1787/9789264071247-en* , *https://pubmed.ncbi.nlm.nih.gov/23653807/*.
Alkaline unwinding and hydroxyapatite chromatography	Rapid test for detecting DNA-strand breaks *https://pubmed.ncbi.nlm.nih.gov/2836728/* , *https://pubmed.ncbi.nlm.nih.gov/1436982/*.
Alkaline elution	Flexible and sensitive assay for detecting ssDNA and dsDNA breaks as well as other forms of DNA damage (e.g. DNA – protein cross-links). Standard procedure High-throughput procedure
Comet assays	Simple, sensitive, and versatile technique. Subdivided into the neutral method (detecting ssDNA breaks) and the alkaline method (ssDNA and dsDNA breaks). Detailed protocols: *https://pubmed.ncbi.nlm.nih.gov/10737956/* , *https://pubmed.ncbi.nlm.nih.gov/12473734/* , *https://pubmed.ncbi.nlm.nih.gov/16673888/* ,

	https://pubmed.ncbi.nlm.nih.gov/17406208/ , https://pubmed.ncbi.nlm.nih.gov/21772771/.
Mammalian gene mutation tests	Thymidine kinase (TK) Mouse lymphoma assay (MLA) Hypoxanthine phosphorybosyl transferase (HPRT) gene mutation test
Chromosomal aberration test	Detects structural chromosomal abnormalities (breaks or exchanges) https://pubmed.ncbi.nlm.nih.gov/32084673/ , https://pubmed.ncbi.nlm.nih.gov/32025500/ , https://pubmed.ncbi.nlm.nih.gov/23896876/.
Micronucleus aberration assays	Assays for determination of chromosome damage (chromosomal loss or chromosome breakage) Subdivided into (https://pubmed.ncbi.nlm.nih.gov/32102335/): Cytokinesis-block micronucleus assay https://pubmed.ncbi.nlm.nih.gov/17546000/ , https://pubmed.ncbi.nlm.nih.gov/21057931/ ; Mammalian Erythrocyte micronucleus assay Buccal micronucleus assay Micronucleus assay in other cell types, e.g. urine-derived cells

Ames test (bacterial reverse mutation assay)

The Ames test (bacterial reverse mutation assay), the most frequently used genotoxicity test, assess the mutagenic potential of a substance by using *Salmonella typhimurium* and *E. coli* bacterial strains. Specifically, each strain possesses a different mutation in an endogenous gene required for synthesis of an amino acid, histidine in *S. typhimurium* or tryptophan in *E. coli*. The test substance is considered mutagenic if enables bacteria to synthesize either histidine or tryptophan, needed to form visible colonies on an agar plate in the absence of the previously required amino acid.

Comet assay

The comet assay, or single cell gel electrophoresis (SCGE) assay, is a rapid, sensitive, and relatively inexpensive method to detect irradiation-induced DNA damage in different cell types. DNA damage is detected at the level of an individual eukaryotic cell as DNA breaks cause relaxation of supercoiled DNA which then extends to form comet-like tails under electrophoresis. Briefly, radiated cells are embedded and lysed in a thin low melting point agarose gel on a microscope slide. After incubation of the slide with alkaline buffer, the DNA is electrophoresed and stained with a fluorescent DNA binding dye. The quantified relative fluorescence intensity of DNA in the tail indicates the DNA break frequency.

Chromosomal aberration test

Numerus studies shows that chromosomal damage causing alterations in oncogenes and tumor suppressor genes of somatic cells are implicated in

carcinogenesis in both humans and experimental animals. Hence, the chromosomal aberration test is often performed to identify agents that cause structural chromosomal or chromatid breaks in cultured mammalian cell lines or primary cultures. The test substance, both with and without metabolic activation, is added to the cells, followed by a metaphase-arresting substance (e.g. colcemid or colchicine) treatment before harvest. Metaphase cells are then analyzed microscopically for the presence of chromosomal aberrations.

In vivo genotoxicity testing

Consequently, *in silico* analysis as well as *in vitro* genotoxicity testing, provide valuable preliminary information, evaluating the initial safety of a substance; however, *in vivo* testing is also required. *In vivo* testing is conducted using animal tissues to verify the potential safety or the mutagenic effect of a substance in the animal's whole physiological system. In 1970, the two-year chronic exposure bioassay in laboratory rodents became the gold standard method for the identification of chemicals or physical agents with carcinogenic activity. To date, it is still the primary method requested by regulatory organizations across the world. In these long-term bioassays, rodents, usually rat and mouse, are exposed to multiple doses of the agent over their life span and are observed for the development of neoplastic lesions.

Nevertheless, the carcinogenicity testing conducted in rodents is not necessarily translated into humans. For instance, these studies usually use higher doses of substances in comparison with those to which humans would normally be exposed. Additionally, the standard carcinogenicity studies utilize a large number of animals, approximately 400-500 of each species, with increasing concern surrounding the principles of 3Rs (Reduction, Replacement and Refinement) of animals in research. Therefore, alternative animal models are under investigation for evaluating their utility in safety assessment of nutritional supplements and/or hazard identification. For example, distinct genetically modified mouse strains have been generated that develop tumors in a rapid way compared to wild type ones through genetic modifications in genes critical to the carcinogenic process. Commonly used strains that are under evaluation include the p53+/- hemizygous knockout mouse, the rasH2 model, the Tg:AC skin model as well as the Xpa-/- and Xpa-/- p53 +/- transgenic mouse models. However, yet none of them has been defined as a genuine alternative to 2-year rodent bioassay.

2.5.8 Animal Models of Cancer for assessing bioactivity of therapeutic compounds

Genetically engineered mouse models

The main challenge in preclinical oncology is the translation of human cancer into the animal models. Recent technological revolution has made possible the generation of genetically engineered mouse models. The genetic profile of these mice is altered in order to develop *de novo* tumors in a natural immune-proficient microenvironment. The developed tumors closely mimic the genetic composition as well as the drug response, and resistance observed in human cancer.

However, the targeted gene modifications cannot fully simulate the complexity of the human tumor, while it is also a costly model, requiring years of work.

Orthotopic models of cancer

In contrast to GEM models, the human tumor xenograft is widely used, allowing rapid analysis of human tumor response to a specific therapeutic regime *in vivo*. In the ectopic tumor xenograft model (or subcutaneous xenograft model), an established human cancer cell line is subcutaneously injected into the hind leg or back of immunocompromised mice e.g., athymic nude mice (nu/nu) or severe combined immune-deficient mice (scid/scid) (Fig.2.5.5). Anticancer activity can be evaluated by easily monitoring tumor growth. Hence, this model is very reproducible and amenable to use, but it cannot be applied to tumors that show necrosis or are not solid.

Figure 2.5.4 Injection of cultured tumor cells into the subcutaneous space in an ectopic xenograft mouse model.

Another type of animal model to test anti-tumor compounds is the orthotopic tumor xenograft model, in which the human cancer cells are engrafted into the relevant organ of tumor origin (Fig.2.5.5). As cancer cell lines used in this model express fluorescence or luciferase, the tumor growth can be monitored by optical imaging, computerized tomography (CT) or magnetic resonance imaging (MRI). In contrast to the subcutaneous xenograft model, the orthotopic tumor model is more clinically relevant owing to the establishment of an organ-specific tumor microenvironment, which can differ in each cancer type. However, surgical skills as well as specialized expensive equipment are required to monitor tumor progression and metastatic activity.

Patient-derived xenograft (PDX) models

Since the xenograft models using cancer cell lines are not sufficient to represent the complex tumor heterogeneity, patient-derived xenograft (PDX) models have emerged. The establishment of a PDX is accomplished by transplanting freshly isolated tumors from patients into immunocompromised mice. This personalized approach, which preserves the heterogeneity of the original tumor, is a powerful tool because of its predictive therapeutic value. On the other hand, these models have technical constraints, and are expensive and time-consuming. Moreover, PDX models cannot completely recapitulate the human tumor immune microenvironment; thus, humanized mice have been developed by engrafting cultured $CD34^{+}$ hematopoietic stem cells (HPCs) or cultured precursor cells

(HSPCs) that resemble better the human immune system. Improved humanized mice have been generated by combining the humanized mouse with either a human tumor xenograft or a patient-derived xenograft (Fig. 2.5.6) .

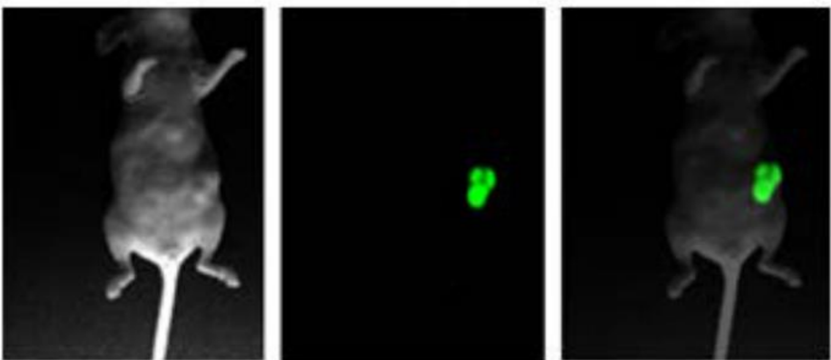


Figure 2.5.5 [An orthotopic xenograft model](). Surgical orthotopic implantation via laparotomy of human pancreatic cancer cells genetically engineered to express the green fluorescent protein (GFP) and the use of fluorescent in vivo small animal imaging to detect the GFP signal.

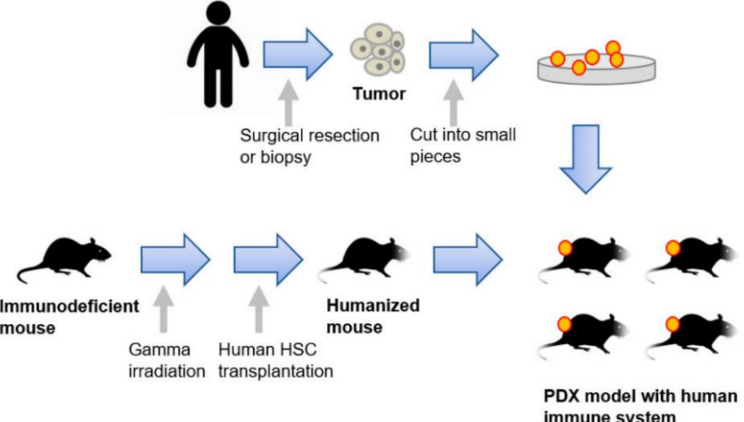


Figure 2.5.6 The development of a PDX model engrafted with a human immune system. Humanized mice have been derived by engrafting human hematopoietic stem cells (HSC) transplantation into irradiated immunodeficient mice. Patient-derived tumors obtained by surgical resection or biopsy are then transplanted into the humanized mice. Adapted from https://pubmed.ncbi.nlm.nih.gov/31226846/.

2.5.9 Conclusions on animal and cell culture models of cancer

In summary, carcinogenicity testing combined with animal models are invaluable in understanding the complexity of cancer and in chemoprevention research. To quote George Box, ''All models are wrong, but some are more useful than others'' (Box, 1975). Each model has intrinsic advantages and limitations, therefore, a combination of *in silico*, *in vitro* as well as *in vivo* approaches should be utilized to evaluate the bioactivity of nutritional supplements in terms of cancer.

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Course 2.6: Models for assessing the impact of nutritional supplements in the gut microbiome

Authors: Maria Daskalaki, Christos Tsatsanis

2.6.1 Animal models for assessing changes in the gut microbiome

For millions of years, immediately after birth humans and other mammals are turned into a scaffold for microorganisms' colonization. Environmentally exposed tissue like skin, mouth, gut and vagina are the most prominent colonized surfaces for microorganisms. It is estimated that over 100 trilion bacteria colonize the distal gut, fact that makes the human gut one of the most complex ecosystems of the world. It has been known for long that, gut is mainly colonized by symbiotic bacteria known for their beneficial properties to the host such as combating potential pathogenic microorganisms, providing nutrients, metabolizing undigested compounds and even promoting the development and function of the immune system. However, microbial imbalance, dysbiosis, can be caused by external factors such as antibiotic consumption, dietary changes, physical and psychological stress, resulting in gut enrichment with opportunistic microorganisms impairing host's wellness and homeostasis. The gastrointestinal microbial composition and host immune tolerance are subjected to a continuous interplay. Dysbiosis promotes the induction of host inflammatory responses and the onset of pathogenesis of a broad spectrum of diseases such as Inflammatory Bowel Disease (IBD), celiac disease, obesity, colorectal cancer, autism spectrum disorder and it is even associated with the modulation of host's brain function and epigenome. To this point, microbiome based new treatments have newly emerged, among them microbiome transplantation, prebiotic and probiotic consumption and metabolite-based (postbiotics) administration such as short chain fatty acids (SCFAs) have gained interest in combating above-mentioned diseases. Although, humans and especially socially close individuals share quite common gut microbiota composition, individuals genetic background and dietary habits play distinct role on personal gut microbiota. New diet acquisition has been a fundamental driver for the evolution of new species and the co-evolution of gut colonizing microorganisms. Hence, personalized medicine based on individual's microbiome (enterotype) and the development of precise dietary supplements is of imperative interest.

For the past years, new technologies have emerged allowing for the phylogenetical identification of gut microbiota by analyzing extracted nucleic acids (DNA and RNA). The majority of identification techniques are based on the amplification of 16S ribosomal RNA gene (rRNA) from genetic material isolated directly from stool samples. Although it is estimated that over 10^{14} microorganisms consist human gut microbiome including bacteria, viruses, fungi and protozoa, the most well studied group of organisms is bacteria. Metagenomics is defined the study analyzing all microbiota members found in environmental or biological specimens and entire ecosystems. In general, it includes the isolation of DNA from specimens, the construction of a metagenomic librady and then screening for target genes. This new field of metagenomics by-passes classical microbiology techniques as it enables sequencing and research

of individual microorganisms without their previous isolation and cultivation. Application of metagenomic analysis is increasingly prominent in biotechnology, ecology, pharmaceutical and medical science providing a powerful tool to study all genomes of a biocommunity and its interactions improving the final products and accurate characterization of an ecosystem.

2.6.2 Methods for assessing changes in the gut microbiome

In order to assess potential gut microorganism variations between populations of mice treated with different nutritional supplements, experimental design is of crucial importance as there are many different factors that are implicated in gut microbial consistency like sex, age and genetic background. Then, depending on the experimental design nutritional supplements can be tested as protective additives in healthy individuals or whether they possess beneficial properties against pathological conditions. In the first case, a shorter experimental protocol is preferred where mice are fed 2-3 weeks with the nutritional supplements and then sacrificed in order to isolate fecal samples, blood serum and organ tissues in order to test for macrobiota changes and potential cytotoxicity of the supplement. In addition, mice weight gain as well as food consumption is advised to be monitored during the experiment. In the second case, where pathological phenotypes have to be induced and then nutritional supplements are tested for their potential beneficial outcome, the experimental protocol is modified accordingly. For example, in case of obesity, metabolic condition largely growing in the recent years, microbiome consistency can be changed upon High Fat Diet (HFD) consumption even in 3 weeks upon the start of the experiment but it can be extended to 8 weeks for the obesity phenotype to be fully established. In order to monitor the progression and establishment of the obese phenotype it is preferred to closely monitor mice weight gain, food consumption as well perform regular Glucose Tolerance Test (GTT) to mice. GTT test provides a clear image of insulin tolerance in obese mice, an expected outcome of type-2 diabetic mice, induced by the obese phenotype. All tested nutritional supplements should be compared to HFD obese mice as well as Lean Diet (LD) counterparts. In the end of the experiment, fecal samples are collected, blood serum, organ tissue and especially abdominal fat where adipose chemokines and inflammatory mediators are expected to be elevated.

- ✓ **Fecal Isolation**

In order to diminish cross-DNA contamination during the isolation of fecal samples, proper tool sterilization should be conducted and wear of protective lab coat, goggles and gloves throughout the whole procedure. On the day of experimental termination, mice are weighted and euthanatized using standard procedure, then 70% ethanol is sprayed on the body of the mouse and it is carefully opened by a ventral midline incision. The peritoneum membrane is carefully lifted and cut so the intestine is exposed. Colon, cecum and small intestine are isolated by separating them from the stomach at the duodenum and from the anus at the distal end of the rectum. The majority of the bacterial load of the gastrointestinal track is found in the terminal ileum, cecum and large intestine mainly due to lower pH values in the small intestine closer to stomach eliminating bacterial growth (Fig. 2.6.1). After Intestine is isolated, it is put in sterile PBS and then placed in sterile petri dish where terminal ileum, cecum and

large intestine are opened with successive incisions and the fecal samples are collected in 2mL eppendorfs. Samples are weighted (approximately 200mg of sample is needed) and either stored in 80°C or proceeded immediately for DNA isolation. Excellent DNA quality is essential for further DNA sequencing analysis. To this point, DNA isolation step is optimized according to DNA extraction kit. For this project, NucleoSpin® DNA stool kit (Macherey Nagel) has been used and DNA extraction of stool samples was conducted according to manufacturer's instructions. In the end, the quantity and quality of the isolated DNA is measured using nanodrop spectrophotometer.

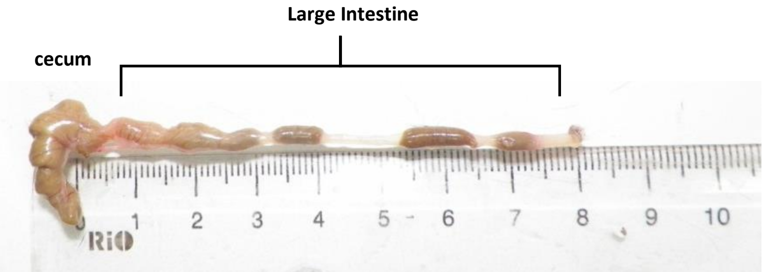

Figure 2.6.1 Mouse cecum and large intestine, the part of the gastrointestinal track with the majority of colonized microorganisms.

- ✓ **16S sequencing**

Metagenomic analysis of sample DNA was conducted by isolating and enhancing bacterial reads using for sequencing the V3 – V4 hypervariable region of the 16S rRNA gene (≈ 460bp) and applying the Illumina protocol for 16S post-genomic analyzes described in 'Illumina's 16S Metagenomic Sequencing Library Preparation' (15044223 B) manual. To boost V3 – V4 sequencing region polymerization primers designed by Klindworth et al., 2013 were used adding an Illumina adapter sequence at the 5' end of each primer. The size and quality of the generated library was evaluated using the Fragment Analyzer (Agilent Technologies Inc. Santa Clara, United States). Sequencing was performed in INEB genome analysis infrastructure using MiSeq platform (Illumina Inc. San Diego, California) and the MiSeq® reagent kit v3. Bioinformatic analysis of interpreted reads was performed using Qiime2 software (Quantitative Insights into Microbial Ecology, https://qiime2.org/) and prokaryotic organisms were identified. Phylogenetic analysis of these organisms occurred using OTUs (Operational Taxonomic Units) based on 99% nucleotide homology to SILVA 132 database. Examples of generated graphs are represented in figure 2.6.2. The generated Stacked bar charts (Fig. 2.6.2A) and heatmaps (Fig. 2.6.2B) are then used to analyze results and conclude to box plots (Fig. 2.6.2C) for individual genus and other taxonomic units of bacteria of interest.

- ✓ **Data Interpretation – Microbial composition**

Metagenomic analysis of the 16S rRNA of gut microbiome provides a plethora of data which are usually difficult to interpretate. Bacteria are taxonomically classified according to phyla, classes, orders, families, genera and species. Hypervariable regions of the 16S rRNA gene provide the necessary nucleotide variability to enable microorganism classification and generation bar graphs and heatmaps of microorganism's abundance according to taxonomic rank. Although, 6 phyla are predominantly found in gut microbiome Firmicutes, Bacteroidetes, Actinobacteria, Proteobacteria, Fusobacteria and Verrucomicrobia, Firmicutes and Bacteroidetes represent over 90% of gut microbiota (Fig. 2.6.3) and a gut

microbial core of 37 genera has been identified as prominent in healthy mice. As mentioned above, changes in gut microbiome composition can trigger diseases; nevertheless, changes in the context of healthy microbiome are expected due to age, lifestyle, genetic background and dietary habits. As the continuous cross-talk between gut microorganisms and host immune system forms a "super-organism", certain phylogenetic taxa of microorganisms begin to be profound is health and disease. In a healthy gut microenvironment, prebiotic species from the lactic acid bacteria group are found, with the most prominent to be Lactobacillus. In addition, short chain fatty acid (SCFAs) such as acetate, propionate and butyrate producing microbes have also great beneficial functions in immune, neuronal and metabolic regulation of the host. There is a long list of butyrate producing bacteria such as members of Actinobacteria, Bacteroidetes, Fusobacteria, Proteobacteria, Spirochaetes, and Thermotogae, although the main butyrate bacteria are members of the Firmicutes, Faecalibacterium prausnitzii and Clostridium leptum of the family Ruminococcaceae, and Eubacterium rectale and Roseburia spp. of the family Lachnospiraceae. In contrast, in pathological conditions a loss in microbial richness is observed with certain microbial groups to be more prominent. For example, in Inflammatory Bowel Disease (IBD) patients an increase in Proteobacteria families has been observed whereas Lachnorpiraceae and Bacteroidetes populations are found to be decreased. Even, between ulcerative colitis (UC) and Crohn's disease (CRD) patients' distinct microbial variations have been observed. Indeed, in UC patients decreased abundance of butyrate producing bacteria Roseburia hominis and Faecalibacterium prausnitzii has been observed, whereas the opposite has been observed in CRD patients, indicating the constant dynamics between microbiome and host.

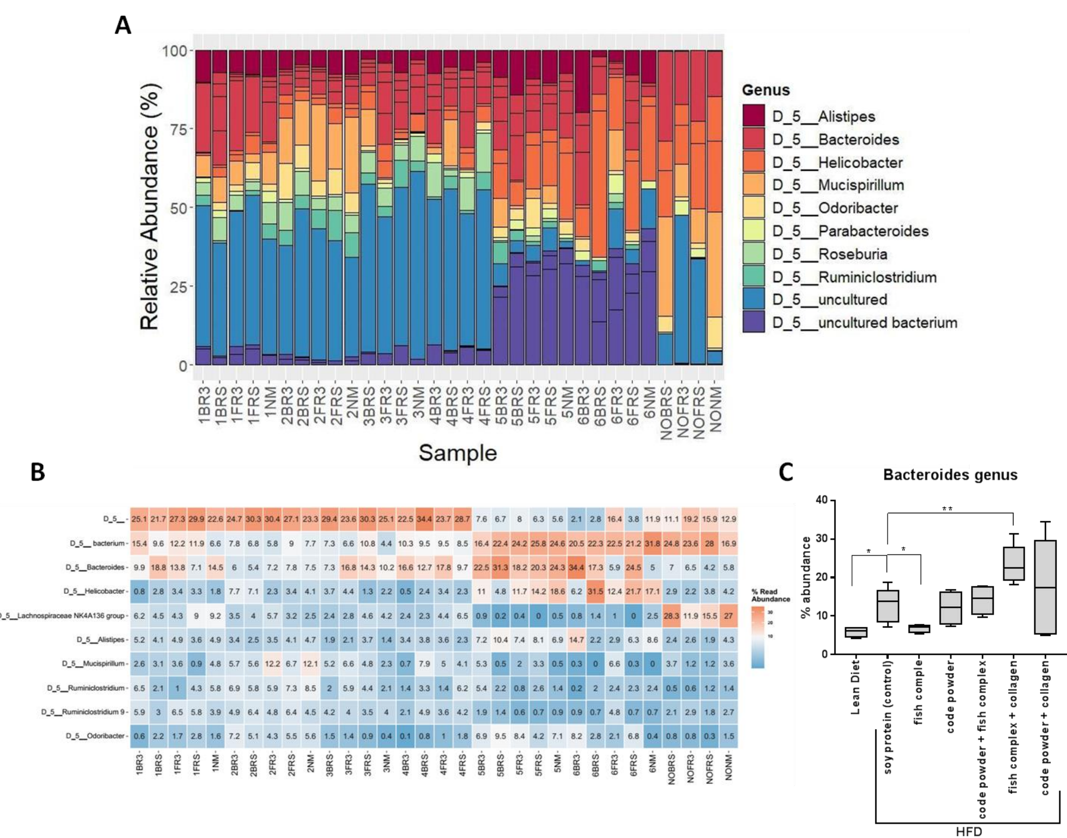

*Figure 2.6.2 Example of sequencing data analysis pipeline. **A**. Stacked bar chart of 10 top abundant genera. **B**. Heatmap of 10 top genera. **C.** Box plot of genus of interest with differences among nutritional supplements.*

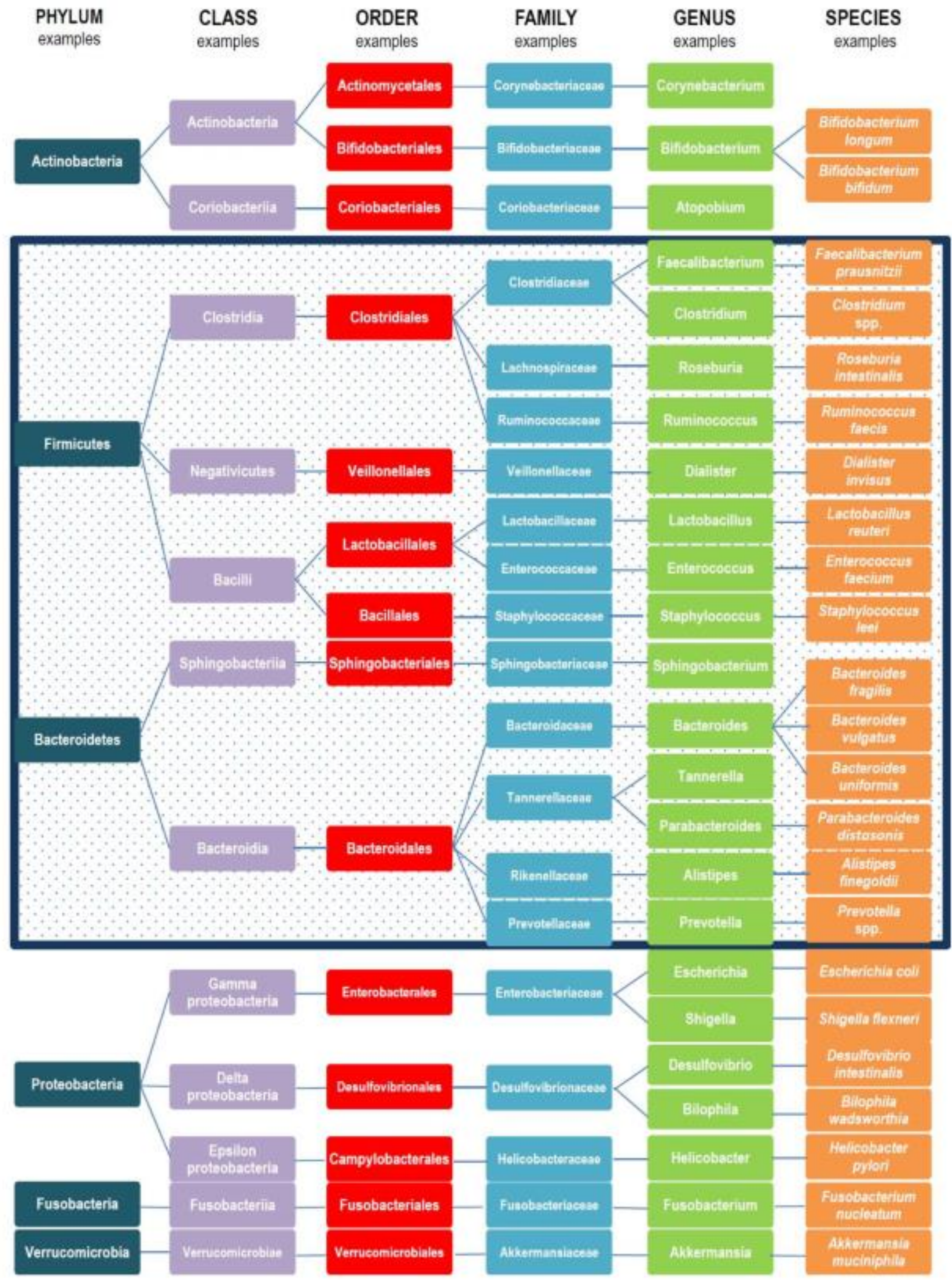

Figure 2.6.3 Examples of taxonomic gut microbiota composition. In the box are cited examples of bacteria belonging to Phyla Fermicutes and Bacteroidetes, representing [90% of gut microbiome consistency](#).

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Course 2.7: Design of human clinical studies

Authors: Ioannis Kosmidis, Vasilis Mougios

2.7.1 Introduction

This Unit will educate readers on how to design and conduct clinical studies aiming at establishing the effectiveness of nutritional supplements on human health, well-being, and performance. Readers will receive information on the necessary actions before beginning a clinical study, guidelines on how to run the study in a way that ensures valid results, and instructions on how to analyze the results.

2.7.2 Preparatory steps for a human clinical study on a nutritional supplement

Searching the literature

Search authoritative bibliographic databases, such as PubMed and Scopus to find scientific papers about the experimental nutritional supplement that you want to test and/or about the ingredients that it contains. Look in particular for similar clinical studies. Bibliographic information that relates to your research will help you design a study that is valid and original.

Defining inclusion and exclusion criteria

Decide what the inclusion and exclusion criteria for the volunteers in your study will be. Criteria to consider include gender, age, lifestyle (e.g., sedentary or active), training status (referring to athletes), body mass index, smoking, diseases (acute and chronic), allergies, dieting (e.g., mixed isoenergetic diet, intermittent fasting), regular use of medication or supplements, etc.

Note: Since an exclusion criterion may become an inclusion criterion if stated in the opposite way, you may wish to have only inclusion criteria. For example, rather than establishing “Illness” as an exclusion criterion, you may establish “Absence of illness” as an inclusion criterion. On the other hand, it doesn’t look nice if you have only exclusion criteria, so you should have some inclusion criteria.

Designing the study

Your findings regarding the effectiveness of your experimental supplement will be no good unless you compare it to a placebo (that is, an inert supplement) or to an established (commercial) supplement as control. This is called a *controlled study,* and you need to decide what your control supplement will be. Next, you need to decide whether your study will be an *acute* or *prolonged* one. Some supplements (such as caffeine) are effective when taken once, whereas others (such as creatine) require repeated doses over days or weeks to show an effect. Your literature search will help you decide whether to perform an acute or prolonged study and, in the latter case, how long the supplementation will be. In

additional, you need to decide whether your study will be a *parallel* or *crossover* one. In a parallel study, you will need two groups, one taking the experimental supplement and another taking the control supplement. In a crossover study, all volunteers will take both formulations, except that half will take the experimental supplement first and the control afterward, while the other half vice versa. The dilemma whether to perform a parallel or crossover study usually holds in prolonged studies, since a parallel study has the advantage of a shorter duration (the two groups take their supplements during the same period). The disadvantages are that you need more volunteers and that differences between groups may threaten the validity of your findings. Conversely, a crossover study has the disadvantage of a longer duration and the advantages of fewer volunteers and the fact that each volunteer will be compared to himself/herself. Thus, you need to weigh the pros and cons of each case before you decide. On the other hand, in the case of an acute study, it is advisable to use a crossover design, since the issue of time becomes of minor importance.

Note: In a crossover study, you will need to consider some time for washout, that is, for the effects of the first supplement to go before you administer the second one. In the case of acute administration, you usually need a couple of days. In the case of prolonged administration, you usually need one to four weeks. Again, consult the literature on this.

Choosing the dosage

Search authoritative bibliographic databases, as mentioned above, about the dosage of your experimental supplement and the control supplement. Then, decide the formulation that you will administer to the volunteers. Generally, formulations are classified as solid (such as capsules) or liquid (such as drinks). In prolonged studies, decide the duration (that is, how many days, weeks, or months they will consume the supplement) and frequency (that is, how many times during the day they will consume the supplement).

To blind or not to blind?

Decide whether your study will be *open-label* or *blinded*. In case you implement a blinded study, you must decide whether it will be *single blind* or *double blind*. In double-blind studies, volunteers and researchers do not know what the volunteers are taking, whereas in single-blind studies the volunteers or the researchers (not both) are blinded. Double-blinded studies are preferred because they are more objective. However, they are more difficult to implement. For example, the volunteers may identify supplements by taste or smell. It may also be difficult to conceal the packaging of different supplements. On the other hand, single-blinded studies are more applicable because they involve fewer difficulties. Nevertheless, single-blinded studies are less objective, since researchers or volunteers will know what is being administered, which introduces the possibility of bias. This is even more so with open-label studies.

It is worth mentioning that the necessity of blinded trials has been questioned recently. However, until there is a consensus on the matter, applying some form of blinding remains an important safeguard of the validity of such studies.

Looking for volunteers

Decide where you will look for volunteers. Ideally, you should perform a random selection from among the population (just like pollsters do when they perform opinion polls), but this is seldom done in biological research. Usually, we resort to a "convenience" sample, that is, volunteers that are easy to find through public announcements, acquaintances, clienteles etc. Either way, you should describe how you picked your sample when you prepare a report or publication of your study.

Choosing a randomization method

Decide how you will randomize the volunteers between groups (in the case of a parallel study) or order of treatments (in the case of a crossover study). Examples of randomization include assignment in order of recruitment in an alternating fashion, by drawing lots, or through a Random number generator.

Choosing the outcome measures, materials, methods, and statistical tests

Carefully choose the measurements you will perform, the timing of measurements, the facilities you will use, the staff you will need, and the equipment. Also decide on the statistical tests you will use to analyze your data.

Creating a questionnaire and consent form

Prepare a detailed questionnaire to be filled in and a consent form to be signed by the volunteers. The questionnaire must include the inclusion and exclusion criteria. The consent form must contain:

(1) the study title and aims;

(2) a summary of the study design with focus on what the volunteers will be asked to do;

(3) the benefits of participating in the study;

(4) potential risks of the procedures that will be used;

(5) how the privacy of the volunteers' personal data will be protected;

(6) the right to withdraw from the study at any time and with no obligation to give explanations;

(7) contact information of the researchers for further clarification and reporting of problems;

(8) a place at the end for the date and for the volunteer to sign;

(9) a place for the principal investigator to sign as guarantor.

Submitting the study for approval

Apply to an appropriate Institutional Review Board for approval of the study design by carefully fulfilling all requirements. Do this well in advance of the anticipated start of the study, considering that your study design might not be approved on first submission (much like a scientific paper!).

2.7.3 Conducting a human clinical study on a nutritional supplement

Registering the study in a clinical trial database (optional)

You may register your study in a clinical trial database. There are several available databases (Table 2.7.1).

Table 1 List of available databases

Word/Phrase	Definition
PubMed	https://pubmed.ncbi.nlm.nih.gov/
Scopus	https://www.scopus.com/
Random number generator	https://www.random.org/
A list and a detailed description of databases	https://en.wikipedia.org/wiki/Clinical_trial_registration
The United States Department of Agriculture	https://fdc.nal.usda.gov/

Registration is not mandatory for clinical studies such as those on nutritional supplements. However, it is recommended as a good research practice in order to avoid publication bias and selective reporting. It also increases transparency in research and is considered a sign of a study of good quality.

Carrying out a pilot study

It is highly advisable to carry out a pilot study that will include the same steps as the main study (the next 10 steps described below), except that it will have fewer subjects and may last less (in the case of a prolonged study). The purpose of the pilot study is to check that everything works as planned, to locate and solve unanticipated problems and improve the design of the main study. Remember to include the pilot study in your application for approval. If the pilot study forces you to make substantial changes to the study design, you need to apply to the Institutional Review Board for an amendment.

Selecting your volunteers

Recruit your volunteers according to the guidelines given above and according to your predefined inclusion and exclusion criteria. Arrange your first meeting with each one of them in your research facilities. Also, give them the opportunity to meet the researchers who will participate in the study. Help the volunteers complete the questionnaire. As they do, make sure they meet all inclusion criteria and do not meet any exclusion criterion; otherwise, you will need to exclude them. Apart from asking questions (such as age, history of disease, and medication), you may need to make measurements (such as body weight, height, and blood pressure). See also item Creating data forms. Thoroughly

inform them about the study and answer any questions they may have. At the end, have them sign a copy of the consent form and keep it in a safe place. Sign another copy of the consent form and give it to the volunteer as guarantee.

Providing detailed instructions

Provide the volunteers with detailed written and oral instructions about what to do during the study. Pay special attention to that in order to ensure maximal compliance and correct execution of each step. You do not need to give instructions about everything at once, especially if your study design is complex. Instead, you may find it more effective to provide instructions in doses, just before they are needed.

Randomizing the supplementation

Choose which volunteers will receive what or when as described. In case you implement a single-blind study, the individuals (researchers or volunteers) who are to be blinded must not know the results of randomization. In case you implement a double-blind study, both (researchers and volunteers) must not know the results of randomization. If the researchers are to be blinded (single-blind or double-blind study), ask a third person (one who will not be involved in the study) to do the randomization and keep a record of it in two copies in different safe places.

Creating data forms

Create electronic forms to record the data from each volunteer's measurements. The forms will be about the medical examination, the screening measurements, the baseline measurements, the measurements you will perform during the experimental protocol and after the experimental protocol. Also, you may need to design a dietary record form for the volunteers to record what they consume every day or on the days before the experimental measurements. This decision depends on how strict a dietary control you wish to apply.

Note: If any of the measurements are made in another laboratory, the other laboratory may have their own forms.

You may input the data either directly into a computer or on printouts of the forms.

Making the medical examination and screening measurements

Measurements may include anthropometric data (e.g., height, weight, body mass index and waist circumference), vital signs (e.g., blood pressure and heart rate), and a blood sample for biochemical screening (under fasted conditions). These measurements may or may not be made during the first meeting with the volunteers, depending on what is more convenient. More than one visit may be necessary to complete all measurements. Also, the volunteers may need to visit another laboratory for some measurements because your research facilities may not have the necessary equipment.

Carrying out baseline measurements

Before you administer the supplements, you will need to obtain baseline values of all the outcome measures of the study. These may be part of the screening measurements (such as body mass index) or may be measurements not needed

for screening (such as body composition and resting energy expenditure). Baseline measurements may have preparatory steps that the volunteers must observe. For example, body composition assessment by bioelectrical impedance has specific hydration requirements, and blood sampling may require previous fasting.

Packaging and distributing the supplements

If the volunteers are to be blinded (single-blind or double-blind study), package the supplements in identical containers. If the researchers are to be blinded (single-blind or double-blind study), have the person who did the randomization do the packaging as well. Then distribute the supplements to the volunteers after the baseline measurements. If the volunteers are to take the supplements on their own (such as during a prolonged study), the detailed instructions must contain information about the dosage and timing of supplementation. If needed, also provide instructions about taking the supplement before or after a meal. A form in which they record their daily doses will help.

Applying dietary control

To ensure that the volunteers' habitual diet will not affect the outcome measures, you need to apply some sort of dietary control. The kind of dietary control depends on the kind and aims of the study. Generally, it can be in the form of a dietary plan, instructions to consume or avoid certain foods, or instructions to keep dietary records. In an acute study, it is relatively easy to give the volunteers dietary plans for 1 or 2 days before the experimental measurements and also ask them record what they actually consumed. Then, in a crossover design, you can ask them to replicate the diet before the next measurements. This way, you will reduce interindividual and intraindividual variance. However, in a prolonged study, it may not be enough to apply dietary control for just 1 or 2 days before the measurements. Rather, you will need dietary control over the entire duration of the study. If following a prescribed dietary plan during a prolonged period is not feasible, you may limit yourselves to providing recommendations on what to consume and what to avoid, in combination with keeping dietary records.

Conducting the experimental protocol, making measurements, and collecting samples

Ask the volunteers to faithfully follow the instructions they received, including consumption of the supplements. In prolonged studies, you need to regularly check with them to ensure that everything proceeds according to schedule. The volunteers must, on specific days based on the protocol, visit the research facilities for the experimental measurements, such as anthropometric parameters, physical examination, performance tests, and sampling of biological fluids. Biological samples may need to be processed before storage at low temperature to preserve the substances that will be measured later. For example, you may need to centrifuge blood samples to obtain plasma or serum.

This includes possible intermediate measurements and, of course, the final measurements. Data need to be carefully collected and stored. It is important to point out that volunteers must follow the same preparatory steps at the experimental measurements as they did for the baseline measurements.

Reporting adverse events

Throughout the study, encourage the volunteers to contact you if they experience adverse events. When they do so, discuss their experiences with them thoroughly and openly, trying to find solutions.

2.7.4 Analyzing the results of a human clinical study on a nutritional supplement

Analyzing the diet

As mentioned, the volunteers need to keep dietary records during the study, either acute or prolonged. Analysis of their diet will help you to validate that they followed the dietary plans or instructions given and that there are no unacceptable differences between measurements. Dietary records can be analyzed for energy, macronutrient, and micronutrient intake through the use of commercially available or custom-made software, based on existing food databases. An excellent and freely available food database is the one from the United States Department of Agriculture.

Excluding non-conforming volunteers

If some volunteers failed to follow important parts of the instructions, they must be excluded from the study. Examples of failure to follow important parts of the instructions include failure to follow a dietary plan or failure to consume the dietary supplement as instructed. Also, absence from a measurement because of illness or other commitments is an important reason for exclusion from the study.Data from such volunteers may not be valid and may distort the analysis.

Analyzing biological samples

If the study design included collection and storage of biological samples, it is preferable to analyze all of them together in the end to eliminate or reduce day-to-day variability and save time and money. Schedule the analysis by deciding how many samples you will analyze each day and what you are going to measure. Analysis must be done according to established laboratory methods and good laboratory practices to ensure validity. If it is not possible to analyze all samples for a certain analyte in one day or one instrument run, make sure to analyze all samples from each volunteer in the same batch to increase their comparability. If you need to give samples to another laboratory for the analysis of some or all the analytes, make sure they are transported in a way that preserves their integrity (for example, frozen in dry ice).

Unblinding the study and assigning the data to treatments

When all measurements are completed, you must unblind the study (if it is a blinded one). Therefore, ask the person who did the randomization to give you the copies he/she has made. Then, assign each volunteer's data to the experimental or control treatment.

Carrying out the statistical analysis

When all data have been assigned to treatments, you need to perform the statistical analysis. Your basic question is this: “Do the effects of the experimental supplement differ from those of the control supplement?” However, there may also be interesting questions regarding, for example, gender differences or correlations between variables. Numerous statistical tests exist to answer these questions (depending on the design of the study and structure of the data), which cannot be covered here. If you are not familiar with statistics, we highly recommend seeking the assistance of an expert.

Reviewing the literature

Search authoritative bibliographic databases, such as those mentioned above, to find new relevant scientific papers that may have been published since your original search. Then, you will need to compare your findings with those of similar clinical studies and try to find what is novel, what is similar, and what is different (and why). At the end, draw the conclusions deriving from your findings.

Disseminating your findings

Congratulations, your study is now finished! However, you will need to disseminate your findings to let other people know. This can be done in several ways:

(1) You may be required to write an official report to your funding authority or to write a thesis (if the study is part of a master’s or doctoral degree)

(2) It is highly advisable to present the study at a pertinent scientific conference

(3) It is highly advisable to publish the study as an article in a pertinent scientific journal.

Of the three dissemination activities mentioned, the latter is usually the most painful and time consuming. First, writing a concise manuscript according to the journal’s requirements is quite demanding. Then, most probably, your manuscript will not be approved immediately. You may be asked to make changes according to the editors’ and/or reviewers’ comments or they may altogether reject the manuscript, in which case you will need to look for another journal. You should not be discouraged by all these difficulties and have the necessary patience and perseverance to see the publication of your study through.

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AQUACULTURE AND AGRICULTURE BIOMASS SIDE STREAM PROTEINS AND BIOACTIVES FOR FEED, FITNESS AND HEALTH PROMOTING NUTRITIONAL SUPPLEMENTS

Grant agreement:	790956
Duration:	April, 2018 – December, 2022
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