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The design of probiotic studies to substantiate health claims

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The EC Regulation No. 1924/2006 on Nutrition and Health claims made on foods has generated considerable debate and concern among scientists and industry. At the time of writing, the European Food Safety Authority (EFSA) has not approved any probiotic claims despite numerous human trials and meta-analyses showing evidence of beneficial effects.

On the 29th and 30th of September 2010, 10 independent, academic scientists with a documented record in probiotic research, met to discuss designs for future probiotic studies to demonstrate health benefits for gut and immune function.

The expert panel recommended the following: (1) always formulate a precise and concrete hypothesis, and appropriate goals and parameters before starting a trial; (2) ensure trials have sufficient sample size, such that they are adequately powered to reach statistically significant conclusions, either supporting or rejecting the a priori hypothesis, taking into account adjustment for multiple testing (this might necessitate more than one recruitment site); (3) ensure trials are of appropriate duration; (4) focus on a single, primary objective and only evaluate multiple parameters when they are hypothesis-driven.

The panel agreed that there was an urgent need to better define which biomarkers are considered valuable for substantiation of a health claim. As a first step, the panel welcomed the publication on the day of the meeting of EFSA's draft guidance document on immune and gut health, although it came too late for study designs and dossiers to be adjusted accordingly. New validated biomarkers need to be identified in order to properly determine the range of physiological functions influenced by probiotics. In addition, validated biomarkers reflecting risk factors for disease, are required for article 14 claims (EC Regulation No. 1924/2006). Finally, the panel concluded that consensus among scientists is needed to decide appropriate clinical endpoints for trials.

There is an abundance of scientific literature (human trials, meta-analyses) providing evidence for the beneficial effects of the intake of probiotics on the intestinal microbiota. For example, certain strains have shown positive results in terms of prevention and treatment of diarrhoea, allergy and inflammatory disorders in children and adults.[1-3] The precise mechanisms that underpin the health-promoting effects of probiotics are not fully understood but it is believed that a wide range of metabolic processes and mechanisms are involved, including the modulation of the intestinal microbiota, reduction/normalisation of intestinal permeability, maintenance of mucosal barrier function, protection against pathogen invasion, production of beneficial metabolites and antimicrobial substances, stimulation of immunity and a shift from Th2 to Th1 response.[4,5] There is also evidence demonstrating that probiotic strains can communicate with the host by modulating key signalling pathways, enhancing or suppressing their activation and hence influencing downstream pathways.[6-8]

There is a vast body of peer-reviewed scientific publications that describe beneficial effects associated with probiotic consumption in well-designed studies, which is why the recent negative opinions on probiotics health claims by the European Food Safety Authority (EFSA) have provoked considerable debate and confusion. The opinions sparked critical responses, not just from industry[9] but also from scientists representing a range of appropriate and relevant expertise.[10-13]

The EC Regulation No 1924/2006 on Nutrition and Health claims made on foods[14] enables two different kinds of proprietary health claims to be made: those for beneficial physiological effects on body functions (according to article 13.5) and claims for reduction of disease risk (according to article 14). In the latter, the regulation actually refers to a reduction of a risk factor and not to actual endpoints of disease. In this way, the regulation is able to differentiate between health claims appropriate for food and medical claims that can only be used for drugs. It is evident, however, that at present, EFSA experts do not accept that sufficient data are available to substantiate health claims on the beneficial effects of any probiotic strains.

In the opinions published before this meeting (held at the end of September 2010), EFSA's reasons for rejection of probiotics health claims (for both beneficial physiological effects or reduction of disease risk factor) included the following: lack of available data on characterisation of the microorganism(s) in question, the experimental daily intake and the duration of the trial were not in accordance with the proposed use of the food

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item, insufficient number of subjects in human trials, lack of adjustment for multiple testing, significant effects found only in a subpopulation, risk factor (pathogen) assessment only in those who were ill, and no biologically plausible mechanism of activity to explain the observed effect. EFSA also questioned the scientific status and validity of certain biomarkers, and the definition of clinical symptoms assessed in some submitted studies.

There are several valid reasons to explain these outcomes. Firstly, the submission requirements and evaluation criteria for such health claims were unknown, or at least very unclear, at the start of the procedure. Secondly, food and food ingredients are, by their nature, part of complex lifestyle and eating patterns and therefore their effects may be more difficult to show. On the other hand, smaller effects may be more relevant for foods compared to pharmaceuticals, since foods may enable a population-wide long-term approach whereas drugs are administered to selected patients based on their diagnosis or medical indication. This may make a less efficacious principle more efficient. Thirdly, food is a complex matrix and not just one single active component (as is often the case in pharmaceutical applications). Finally, many of the probiotic studies were conducted prior to this legislation and thus were not designed for substantiating very specifically worded health claims. In fact, most scientific publications to date have derived from academic sources and have usually been aimed at further understanding the physiological mechanism of a disease, with no intention of product testing. Often these studies have demonstrated a reduction of relative risk (RR) of disease endpoints (e.g., *Clostridium difficile*-associated diarrhoea). These are valuable data with regard to evidence of reduction of risk of disease, but EFSA might not necessarily consider this as appropriate evidence for the weaker risk reduction *factor* claim allowed under the EU regulation.

Taking these observations together, the question needs to be asked: "How can one design probiotic studies to substantiate health and disease risk reduction claims, which will fulfil the requirements stipulated by EFSA and other regulatory bodies?" To address this topic and to discuss the current level of evidence for probiotic health benefits, ten independent academic European scientists with a documented record in probiotic research, were invited to a meeting. On the day of the meeting, EFSA issued a draft guidance on the scientific requirements for health claims related to gut and immune function; this document was considered during the panel's discussions. Subsequently, in April 2011 EFSA issued a final version of this guidance document.[15]

To facilitate the flow of discussion, prior to the meeting each panel member was asked to review different aspects of probiotic research. These were: the best design for human studies; how best to demonstrate beneficial effects of host-microbe interactions; probiotics and immune modulation; probiotic research in different target groups; the efficacy of probiotics in reducing the risk of infection; the potential role for probiotics in health maintenance; the impact of probiotics on colonic metabolism; and measurement of probiotic effects in healthy people—appropriate biomarkers and clinical endpoints.

The discussion was wide-ranging therefore only the main points are included here. This paper is not intended as an exhaustive review of current evidence but to inform and advise on the best way to conduct future probiotic studies, particularly those intended for claim dossiers.

Probiotics and Health

How to measure health. The human large intestine is recognised as one of the most metabolically active organs of the human body due to its extremely complex and dynamic resident microbiota. The metabolic activities of this microbial community play an important role in maintaining host health and well-being, which have been shown to respond to metabolic challenges and dietary factors. In the context of probiotic influence on health, either in general or to gut health in particular, it is actually quite difficult to define 'health'. The World Health Organization defines health as "… not only the absence of infirmity and disease but also a state of physical, mental and social well-being." Probiotic foods are functional foods intended for normal, healthy people and it may be difficult to show short term beneficial health effects in such a target group, whether these relate to the function of the gut or to systemic body functions. Here, challenge models of persons seen to be at risk of gut problems (e.g., elderly, athletes, subjects under stress, frequent traveller's, persons in care centres) could be of value in showing beneficial effects of probiotics on the homeostatic function of the body.

Studies on health benefits of probiotics in the past frequently focussed on changes in markers and/or parameters of disease rather than health. The panel embraced a concept that beneficial effects of probiotics could be better identified and described in terms of 'functionality.' Such functionality could be related to the gut in strictu sensu, but also could be extended to the functionality of the entire human body. For describing functionality, a dynamic "resilience model" may be helpful, in which the response to challenges is indicative of the adaptive capacity of the gut and/or body and the ability to maintain homeostasis.

Probiotic effects in the gut. A number of factors influence the composition and the metabolic activity of the colonic microbiota. Nutrient availability is one important regulator of bacterial metabolism, especially the ratio of available carbohydrate to nitrogen, which determines the degree of saccharolytic versus proteolytic fermentation. In the proximal colon, substrates are abundantly available and the microbiota will preferentially ferment carbohydrates because it is energetically more favourable to produce ATP from carbohydrates than from proteins. The metabolic endpoint of carbohydrate fermentation is the generation of short-chain fatty acids (SCFA), such as acetate, propionate and butyrate. These provide energy for the colonic mucosa and are associated with host health.[16] Along the length of the large intestine, the ratio of available carbohydrate to nitrogen progressively decreases and the bacterial composition in the more distal parts of the colon changes towards a more proteolytic, methanogenic and sulphate-reducing type of microbiota. Anaerobic metabolism of peptides and proteins by the microbiota produces SCFA and branched-chain fatty acids but, at the same time, it generates a series of potentially toxic substances including ammonia, amines, phenols, thiols and indoles. As a consequence, dietary

interventions with probiotics (and prebiotics) should focus on enhancing the saccharolytic activity in the colon, while decreasing the degree of proteolysis[17] (currently it is not known whether this could be an acceptable endpoint for substantiation of benefit, either by scientific consensus or secondarily by EFSA). In fact, probiotics should be acid-producers and not producers of ammonia or H_2S. There is also convincing evidence that specific probiotic strains can modulate other metabolic pathways, such as the activity of bacterial enzymes, the conversion of primary to secondary bile acids, the metabolism of phytochemicals and/or oxalate.

With advancing age the barrier function and permeability of the gut changes.[18] Adhesion of bifidobacteria to the gut mucosa also decreases with age, which may be explained by the reduction in mucus quality that occurs with advancing age.[19] The panel concluded that probiotics have an effect on the intestinal microbiota both in terms of its composition and its metabolic activity, and this may prevent some negative effects of the ageing process on gut function. However, the extent and nature of health effects associated with this still need to be confirmed.

Probiotics and immune effects. Interactions between the intestinal microbiota and the immune system via the gut-associated lymphoid tissue can have a profound influence on overall health.[20] Several areas of benefit relating to infection, inflammatory disease and allergic disease have been linked to probiotic immunomodulation.[21] The panel discussed one area of evidence: allergic disease.

Specific probiotics downregulate the immune response associated with allergic disease by providing maturational signals for the gut-associated lymphoid tissue and affecting the ratio of pro- and anti-inflammatory cytokines and the balance between Th1 and Th2 response.[22] Specific strains of the healthy intestinal microbiota have been shown to promote normalisation of increased intestinal permeability. Promotion of normal gut barrier functions by probiotics also includes the normalization of the gut micro-ecology and the alleviation of inflammatory responses, both locally and systemically.[23] In addition, probiotic bacteria reduce the dietary antigen load by degrading and modifying macromolecules. This all contributes to a healthy host-microbe cross-talk. Thus, immune regulation in the gut depends on establishment of its commensal microbiota. Lifestyle factors, such as eating habits, influence the composition of the gut flora. Disruption of this microbial population and impairment of the gut barrier function may increase health risks. The panel came to the conclusion that reduction of risk of allergic disease is a potential probiotic benefit in terms of immune function; a few trials with certain strains, in children and adults, have indicated this.[24]

Optimal Study Design

Study design. In functional food research, nutritional and clinical sciences use similar methodology. Experimental scientists have already published on several aspects of probiotic trials.[25] The panel agreed that the best study design is a randomised, placebo-controlled and double-blinded human trial, either of parallel or crossover design. The latter studies, however, must ensure sufficient washout before crossover is conducted. In certain circumstances a crossover design may not be appropriate, as the functional effect of a probiotic may not disappear during the intervening washout period (e.g., in children if maturation of the gut barrier has improved). The length of study and duration of intervention should be appropriate for the endpoint as well as reflecting consumption patterns recommended for the probiotic. An acute effect associated with intervention may be seen in short-term interventions, but if people are recommended to take a product for a longer period of time, it might be better to test the efficacy of intervention over a more extended period.

Goals, target groups, conditions. A likely consequence of current regulatory approaches is a change in the future design of human trials on probiotics. In the past, trials were designed to maximise the chances of detecting an effect by, for instance, testing several doses per day, testing high dosages, controlling background diet and other life style factors, using a homogeneous test population (often patients) with strict inclusion and exclusion criteria (sex, age, body mass index) and sensitive to the intended effect (to reduce sample size and costs). The scientific opinions published by EFSA on probiotics claims indicate that there is a difference between the currently available scientific data and the level of evidence mandated by EFSA or the data needed to substantiate probiotic benefits for regulatory purposes. With respect to the study design of human trials, EFSA has based some of its negative opinions on the inappropriate sample size but also by their rejection of extrapolation of data from subjects with pathological conditions to the healthy population. For instance, the application of probiotics in antibiotic-associated diarrhoea (AAD) has positive outcomes in meta-analyses;[26,27] a Cochrane review on the prevention of pediatric AAD with probiotics gave promising results[28] and the use of *Saccharomyces boulardii* as a therapeutic probiotic has been considered as evidence-based for both efficacy and safety for several types of diarrhoea,[29] yet this did not convince EFSA. The decisions by EFSA were based on the facts that *S. boulardii* was not appropriately identified and that a significant effect on AAD was not considered for legislative reasons (disease-endpoint) and that the risk factor *Clostridium difficile* toxin was only measured in those who fell ill.

The expert panel agreed that the health relationship for a probiotic must be carefully identified: the starting point for any trial should be a definition of the desired health claim. EFSA regards double-blinded, placebo-controlled human studies and meta-analyses of such trials as the highest level of scientific evidence. The trials must follow the rules of good clinical practice (as given in the ICH-GCP guidelines), including proper randomisation, blinding of interventions to participants and investigators, use of an appropriate control group and crossover or parallel design as appropriate. A suitable 'dosage' should be chosen, i.e., similar to the recommended daily intake and with a realistic intervention period. Whilst it is important to demonstrate a biologically plausible mechanism of activity, unnecessary physiological and clinical parameters should be avoided or allocated to exploratory parameter level in a hierarchy of primary, secondary and exploratory parameters.

Subjects. If the claim is intended to be meant for the general population, the study population should be representative of this target group thus subjects should be of different age, sex and body mass index. Normal eating habits (i.e., a normal diet and not controlled) are recommended to reflect the real life situation. The panel came to the conclusion that inclusion criteria should be kept as broad as possible. The best way to do this would be to choose healthy subjects or people at risk, or particularly susceptible, to certain illnesses, e.g., for gut health it would be appropriate to study subjects with irritable bowel disease-type symptoms and/or those with slow transit time or low or high frequency of bowel movements.

The issue of responders and non-responders in nutritional research needs to be investigated further. Identifying non-responders may give valuable insight into the mechanisms of activity of an intervention and increase the likelihood of a positive outcome, but it may restrict the eventual target group for a claim.

Reduction of statistical variance leads to an increased sensitivity and subsequently could limit the sample size and costs of a trial. Another approach may be to focus on a part of the population in a first step, even if the claim will have to be limited to this target group.

Product. Published EFSA opinions indicate that probiotic strains used in the studies for substantiation of health claims, should be well characterised. The genome of strains should be sequenced and deposited in an authentic and publicly accessible culture collection. The expert panel concluded that the conditions of use in human studies (probiotic strains, dose and matrix) should be the same as in daily practice and as recommended commercially. An ideal study design could include a dose response curve and be placebo-controlled, although it was appreciated this may sometimes be difficult due to subject numbers and cost. Simple dose response curves may be of less value for probiotic studies, where there could be multiple underlying mechanisms of activity. It was also suggested that it is easier to perform placebo-controlled studies with powder formulations (as compared to whole food products), but in practice fermented milk is often used as the vehicle of delivery for probiotic strains.

Dietary assessment. The need for nutritional assessment was discussed. The panel agreed that a record of dietary intake can be useful. However, one has to realize that the accuracy of such a record can be compromised. For instance, underreporting and change in eating pattern can occur during keeping records, as has been reported for multiple food records or 24-hour dietary recall methodology.[30] These behavioural aspects result in bias and thus limit the validity of the data obtained. It would be better to use a Food Frequency Questionnaires (FFQs) to record dietary intake retrospectively. The advantages of FFQs are that the questionnaires are filled out at home, that the respondent burden is usually low and that food consumption is not affected.[31] There still may be limits to the use of FFQs in a way that there is concern that FFQs are too much affected by measurement error to detect weak associations between diet and health outcome.[32] Whilst comparatively easy for paediatric studies, a dietary questionnaire is frequently omitted in human trials.

The conclusion of the panel was that dietary assessment should not be mandatory, because it could influence the behaviour of the test persons. However on the other hand, dietary assessment could help to document equal distribution among the *verum* and control group in terms of dietary behaviour when entering the trial, and also help emphasize the importance of compliance of the test product and dietary habits during the study.

Questionnaires. The panel was of the opinion that self-administered questionnaires for Quality of Life (QoL) are acceptable as a tool and that general symptoms scores (as in pharmaceutical trials) should be allowed. In practice this would mean, for instance, that measurements of abdominal pain (which might be relevant for certain gut function claims) could be done using validated, subjective, global symptom severity questionnaires. Moreover, questionnaires which have been demonstrated in previous trials to detect differences between intervention groups seem to be accepted by EFSA. Although not discussed by the panel members during the meeting, validated questionnaires can be found in the literature for upper respiratory symptoms,[33] constipation,[34] intestinal transit time,[35] gastrointestinal discomfort[36] and quality of life for digestive disorders.[37]

Biomarkers for beneficial physiological effects. In the absence of hard clinical endpoints, there is clearly a need for validated biomarkers and, vice versa, in the absence of unambiguous surrogate markers a clinical outcome or endpoint is needed for interpreting an alteration as beneficial. Several studies supporting probiotic claims relating to gut function have used changes in gut metabolism as surrogate biomarkers. At present, these biomarkers are insufficiently validated.

The panel agreed that maintenance of gut barrier function was one mechanism involved in many observed positive effects associated with probiotic consumption. One example of a surrogate marker for gut barrier function was discussed: a study where a *Lactobacillus* strain was shown to induce changes in the small bowel mucosa that mirror an improvement in the epithelial tight junction function.[38] Although there is ample evidence that an increase in barrier function is beneficial,[39] the EFSA presently does not accept reduction of permeability for a health claim.

There was also discussion of newly developed "*omics*" techniques such as metabonomics which enable the evaluation of colonic metabolism to be approached from a top-down perspective, bypassing the need to define an a priori hypothesis and offering opportunities to detect new pathways affected by probiotic intervention. Such an approach enables changes in a wide range of metabolites to be monitored simultaneously. Depending on the chosen matrix, either the microbial *or* human metabonome can be evaluated. Despite the demonstrable impact of probiotics on bacterial metabolism in the colon, concrete health benefits associated with modulation still need to be established. Possible health benefits for the general "healthy" population may consist of risk reduction of long term diseases such as cancer. In patient groups suspected of an abnormal colonic metabolism, e.g., with inflammatory bowel disease, irritable bowel syndrome or chronic kidney failure, an improvement of their clinical condition may indicate that there is a reduced risk to health for the general population.

Relating changes in metabolite concentrations to relevant health benefits will be an important challenge for future research. New and validated tests, for example, might include the effects of probiotics on subjects taking aspirin or beta-blockers[40] but the restricted nature of such test groups would severely limit the target group and usefulness of the claim. The panel remarked that a search of the medical literature would be very helpful in identifying new markers for food claims and development of specific markers validated by clinical associations would be a major step forward for functional foods. Until now, the emphasis has been on disease-related applications but future research needs to focus on the apparently healthy general population.

Disease risks factors and disease endpoints. The efficacy of probiotics for reduction of disease risk has been shown in several studies. Meta-analyses have concluded there are positive effects associated with probiotics for infectious diarrhoea,[41] traveller's diarrhoea[42] and common cold infections.[43] It is questionable, however, whether EFSA would consider a claim worded as "enhances defence/resistance against infection" which is only based on evidence relating to infection endpoints e.g., relative risk or severity or duration of episodes. The panel came to the conclusion that EFSA would probably require evidence of both a change in an immune parameter known to mediate immunity *and* an effect on infection endpoints (as supporting evidence).

It is not always possible to make a clear distinction between disease symptoms and clinical outcomes. For specific claims, EFSA is likely to accept a number of disease-related symptoms as supporting evidence and, in fact, has stated certain examples of this, e.g., reducing gastrointestinal discomfort may improve quality of life and be beneficial to human health. EFSA has further stated that improvement of intestinal transit might be beneficial to human health. Intermediate endpoints such as body functions or risk factors with supportive evidence from clinical outcomes, however, are still preferred. It is of the utmost importance for future disease risk (factor) reduction claims that consensus is reached among the scientific and medical community with respect to useful clinical intermediate parameters and symptoms that can be used to evaluate health and disease risk.

Immune studies. In the area of immune function, the panel agreed that this is an area where the effects of probiotics are most likely to be strain specific, therefore it is important to identify the appropriate strain for a specific target. Salivary IgA or specific IgG responses to vaccination were cited as relevant immune parameters to show probiotic benefit, together with evidence of reduction of disease risk.

Statistics. It is essential that statistically significant data are the outcome from a human trial therefore it is vital that a statistical plan is defined *before* development of the study protocol. The involvement of a statistician is certainly recommended. A priori power calculations are essential, for instance, to determine sample size. Larger study populations may be needed that are of sufficient size for subsequent statistical calculations; analyses of subgroups may also be necessary, even post hoc, but in that case a confirmatory trial will be required for substantiation. Primary endpoints should be decided at the beginning of the study; multiple endpoints dilute study results and therefore need adjustment. Right from the first stages of planning a study, a clear view and definition of the claim are needed, as well as identification of the number and type of study endpoints to be investigated. The panel recommended limiting the number of parameters studied, as multiple endpoints weaken the significance of the statistical analysis at the end of the trial. As part of their evaluation process, EFSA judges whether there is appropriate use of statistics in the submitted evidence and have previously rejected statistically significant results due to a lack of adjustment for multiple variables, even though there was a low level of error probability.[44]

Biobanking. As far as is possible, the panel advised that biological samples from trials should be stored, in the event that they could be used in future mechanistic investigations. It is a waste of resource to discard samples that could prove useful for a posteriori analyses. For reasons of privacy protection test persons need to be informed ahead of time of the potential use of their stored samples for additional measurements. This potential future use of samples should also be clearly reflected in the informed consent forms.

Legislation. Equivalent products can make use of approved claims that are generic, provided conditions of use are met. Probiotics are an entirely different case, however, as many health effects are considered to be strain-specific and bioequivalence needs to be demonstrated. Moreover, a search for equivalent effects among probiotic strains (as suggested by EFSA) will be limited if evaluated dossiers remain confidential.

Introduction of the concept of responder and non-responder (which is common in nutritional trials) will affect application of the health claim regulation. This concept implies that it is not the general population in the pure sense that is investigated in a study, but a more susceptible part of the population or a population at higher risk. It is uncertain to what extent such a population may be representative of the risk of the general population, when substantiating a health claim. The concept of responders and non-responders in nutritional research needs to be investigated further. Identifying non-responders may give valuable insight into the mechanisms of activity of an intervention and may increase the likelihood of a positive outcome, but it may restrict the eventual target group for a claim. It should be noted, however, that there are precedents in nutrition for general recommendations to be made that are based on evidence of benefit for a subset of responders (e.g., dietary sodium and benefit shown for hypertensive people; folic acid supplementation and benefit during pregnancy).

Conclusions

Scientific evidence supports the role of probiotic bacteria in the maintenance of health. The EFSA panel, however, has not yet accepted current knowledge as sufficient for the substantiation of specific probiotic claims (either for health maintenance or disease risk reduction). The main reason for this seems to be the fact that many nutritional studies, even if well designed and published in peer-reviewed journals, were not designed specifically for the

substantiation of health claims within the particular frame of the European health claim regulation. The expert panel strongly advised that future studies should be designed, executed and evaluated by a multi-disciplinary team, consisting of microbiologists, nutritionists, statisticians, etc., although cost is an obvious issue. Future focus should be on three aspects of research: study design, biomarkers and clinical endpoints.

Study design. According to the expert panel the preferred study design would include (a) large (multicentre), randomized controlled trials with sufficient statistical power, (b) an a priori hypothesis with a well-characterized probiotic strain to support the hypothesis and (c) a limited number of endpoints to show either a beneficial effect on body function or a reduction in a risk factor and to indicate a working mechanism of activity.

Biomarkers of physiological beneficial effect. There is an urgent need to better define which biomarkers are considered valuable for substantiation of a health claim and there is a need for researchers to develop and validate new biomarkers that would be generally accepted as appropriate and valid indicators of health status. In this respect, the panel was of the opinion that the increased availability of genome sequences together with systems biology approaches will facilitate elucidation of probiotic mechanisms of activity.

Clinical endpoints. The expert panel concluded that consensus is needed among the scientific and medical communities with respect to new and useful clinical intermediate endpoints to measure the extent, duration or severity of disease. In the absence of unambiguous biomarkers, these generally accepted clinical endpoints will be of importance for the approval of future disease risk reduction claims, and in some cases for health maintenance claims.

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