Monitoring immune modulation by nutrition in the general population: identifying and substantiating effects on human health

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Abbreviations: CRP, C-reactive protein; DTH, delayed-type hypersensitivity; ILSI, International Life Sciences Institute; NK, natural killer; sIgE, specific IgE.

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Optimal functioning of the immune system is crucial to human health, and nutrition is one of the major exogenous factors modulating different aspects of immune function. Currently, no single marker is available to predict the effect of a dietary intervention on different aspects of immune function. To provide further guidance on the assessment and interpretation of the modulation of immune functions due to nutrition in the general population, International Life Sciences Institute Europe commissioned a group of experts from academia, government and the food industry to prepare a guidance document. A draft of this paper was refined at a workshop involving additional experts. First, the expert group defined criteria to evaluate the usefulness of immune function markers. Over seventy-five markers were scored within the context of three distinct immune system functions: defence against pathogens; avoidance or mitigation of allergy; control of low-grade (metabolic) inflammation. The most useful markers were subsequently classified depending on whether they by themselves signify clinical relevance and/or involvement of immune function. Next, five theoretical scenarios were drafted describing potential changes in the values of markers compared with a relevant reference range. Finally, all elements were combined, providing a framework to aid the design and interpretation of studies assessing the effects of nutrition on immune function. This stepwise approach offers a clear rationale for selecting markers for future trials and provides a framework for the interpretation of outcomes. A similar stepwise approach may also be useful to rationalise the selection and interpretation of markers for other physiological processes critical to the maintenance of health and well-being.

The overall aim of this article is to provide further guidance for the assessment and interpretation of immune modulation by nutrition in the general population. To this end, the European Branch of the International Life Sciences Institute (ILSI) established a group of experts from academia, government and the food industry to agree upon criteria to evaluate the usefulness of immune function markers in a structured manner. Over seventy-five markers were scored and evaluated within the context of three distinct domains of immune function: defence against pathogens; avoidance or mitigation of allergy; control of low-grade inflammation mainly focusing on metabolic inflammation. Other aspects of immune function such as autoimmunity and surveillance against tumours were not included at this stage. The most useful markers were classified depending on whether they by themselves demonstrate clinical relevance and/or involvement of immune function. In addition, five theoretical scenarios were drafted describing potential changes in the values of markers compared with a relevant reference range. These include (significant) modulation within the reference range (a very common scenario for modulation due to nutrition), modulation from outside the reference range back into the range, modulation from within the reference range out of the range, prevention of modulation induced by other factors, and modulation from a less favourable range to the reference range of a comparator group with a more desired immune function (e.g. from bottle-fed to breast-fed infants). Finally, the expert group combined all of the above-mentioned information, providing a framework to aid the design and interpretation of studies assessing the effects of nutrition on immune function. An early draft of this report was discussed with a wider group of experts at a workshop held in Nice, France, 16–17 April 2012. Additional information about the workshop discussions and participants is available on the ILSI Europe website (http://www.ilsi.org/Europe/Pages/HomePage.aspx).

The main function of the immune system is to help maintain homeostasis by providing protection against infections. On the other hand, inappropriate or improperly controlled immune functions contribute to pathophysiological processes such as allergic manifestations and chronic inflammatory responses. Development and maintenance of a normal immune system are thus essential for a healthy and active life. Functioning of the immune system is influenced by a variety of inherited, environmental, behavioural, social and individual factors[1]. Therefore, it is no surprise that solutions to help develop, restore or optimise immune functions are much sought after by scientists, consumers and industry alike.

One of the major modifiable factors affecting immune function is nutrition (the primary factor being vaccination); undernutrition is often related to decreased immune function, whereas overnutrition and obesity can contribute to chronic low-grade inflammatory changes. Whole diets, individual nutrients and food components such as phytochemicals, prebiotics and probiotics have all been shown to influence distinct aspects of the immune system. These effects have been reviewed extensively in a number of recent papers[2–21]. Several other papers have provided some guidance on how best to assess specific immune functions and which confounding factors and methodological aspects to consider[1,18,22–28]. Moreover, the European Food Safety Authority panel on dietetic products, nutrition and allergies has recently issued a guidance document on the scientific requirements for the substantiation of health claims related to gut and immune function[29]. This document offers an excellent starting point for the assessment and interpretation of immune modulation by nutrition. However, some critical elements are still missing, and especially when focusing on the assessment and optimisation of immune function in the general population, some questions remain largely unanswered. These include the following: (1) How can 'optimal immune function(s)' be identified and characterised? (2) Which markers are most informative to describe (optimal) immune function(s)? (3) How can changes in the values of these markers be interpreted? (a) Can immune function(s) in a general population be optimised? (b) How to assess improved immune function(s)? (c) How to identify risk associated with the modulation of immune function(s)?

This article aims to provide some further guidance on these aspects by providing criteria for the selection of immune function markers, ranked lists of markers that best describe the (modulation of) specific immune functions in the general population, reference to normal values and ranges established in laboratory medicine and daily clinical practice for selected

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markers, and rational approaches for the design of studies, selection of markers and interpretation of changes observed in these markers due to exogenous factors such as nutrition.

Paradigm relating immune function to health

The basic paradigm of the relationship between immune function and health is illustrated in Fig. 1. Regardless of the marker or assay used, immune functions vary between subjects and they fluctuate over time within a subject. In a healthy population, these fluctuations define the boundaries of a 'normal range', and immune function(s) within this 'homeodynamic bandwidth' are postulated to support the maintenance of 'optimal health'. Genetic make-up and past experiences contribute to differences between subjects, and environmental or lifestyle-associated factors can temporarily or permanently move specific immune function(s) outside these ranges, thus failing to support optimal health. If this is sustained or becomes more extreme, it may contribute to pathogenic processes and modify disease risk. Within this paradigm, optimising immune function encompasses the return of such function(s) back into the normal range and/or strengthening of the resilience of the function(s), thus reducing the amplitude of fluctuations and reinforcing the homeodynamic regulation within the optimal range[2,30]. Consequently, at times, it may be beneficial to down-regulate hyperactive immune functions or up-regulate hypoactive immune functions or to strengthen the resilience of immune functions to respond to external 'stressors'.

Brief overview of major immune functions

The immune system helps to maintain homeostasis by mounting non-specific innate and specific adaptive responses against potentially pathogenic micro-organisms. At the same time, the immune system should tolerate self-antigens and innocuous non-self-antigens and allergens as uncontrolled or inappropriate responses to such antigens contribute to the pathogenic processes underlying various non-communicable diseases. Inappropriate or exaggerated immune responses to allergens lead to allergic manifestations such as allergic rhinitis (hay fever), allergic asthma, atopic dermatitis (eczema) and food allergy. Sustained responses to persistent antigens, such as autoantigens or those that are derived from commensal micro-organisms, lead to tissue remodelling and loss of function of the affected tissue and contribute to the symptoms of chronic inflammatory diseases such as rheumatoid arthritis, inflammatory bowel disease and psoriasis[2,31]. More recently, it has also become apparent that metabolic stresses (e.g. in visceral adipocytes of obese subjects) trigger low-grade asymptomatic inflammatory responses, which contribute to the comorbidity of metabolic disorders[32,33].

These different aspects of immune function are highly relevant in the general population, affecting the maintenance of health and vitality. Immune function markers are, therefore, considered within the context of the following distinct physiological function domains of the immune system: defence against pathogens; avoidance or mitigation of allergy; reduction of asymptomatic low-grade metabolic inflammation.

Defence against pathogens relates to the physiological function of the immune system to deal with (common) pathogens such that the infection does not establish itself, or if it does, then with no or minimal symptoms. Avoidance or mitigation of allergy relates to the ability of the immune system to tolerate potentially allergenic substances without symptoms of allergy. The beneficial effects of nutrition on this activity could thus, in theory, lead to reduced sensitisation to an allergen or mitigation of the severity of allergic responses to non-food-related allergens such as respiratory or contact allergens and so are not necessarily restricted to food allergy. Reduction of asymptomatic low-grade inflammation relates to the ability of the immune system to control low-grade inflammatory responses that are triggered by metabolic stresses and that, if improperly controlled, become chronic and contribute to the pathophysiology of various diseases.

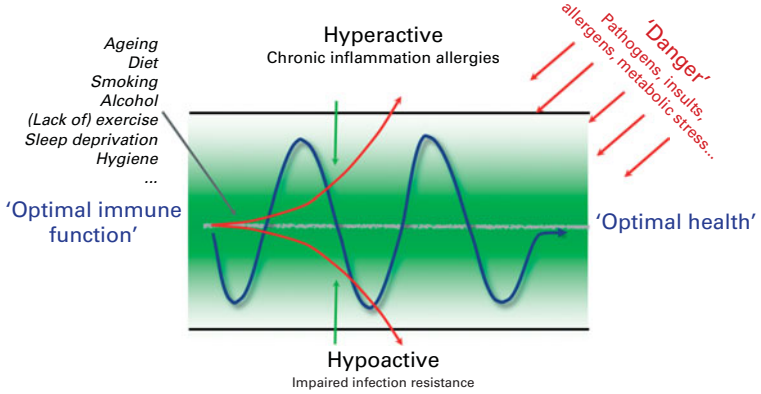

Fig. 1. Illustration of the general paradigm postulating that fluctuations within the boundaries of a normal range support the maintenance of optimal health. Regardless of the marker or assay used, immune functions vary between subjects and they fluctuate within subjects over time (⟶), although apparently within some normal limits (green zone) that may be individually defined. Certain (combinations of) factors can drive immune function(s) to a state of hypo- or hyperactivity (⟶). The objective of a (nutritional) intervention is to restore functions to the normal range (⟶) and/or to strengthen the resilience of these function(s), reducing the amplitude of fluctuations and thus reinforcing the homeodynamic regulation within the normal range. Adapted from Hamer *et al.*[30].

To exert its functions, the immune system deploys a range of structural, cellular and molecular components. A wide variety of methods and markers are being used to measure the magnitude of variation of these immune functions. Such markers range from the assessment of clinical symptoms and integrated responses to particular *in vivo* challenges and *ex vivo* assessment of isolated functions of the immune system to more basic enumeration of a particular (sub)type of cell or measurement of the concentrations of specific factors without any defined challenge to the system. Building on a previous publication[1], we have classified the markers from the most integrated/physiologically relevant to the most isolated/mechanistically insightful as illustrated in Fig. 2.

It is acknowledged that new technologies, such as genomics, proteomics and so forth, are continuously being developed. Such techniques may, in the future, provide new biomarkers and also more insight into relevant intracellular responses. Future evaluation of such new biomarkers, however, can follow the same criteria as for the current biomarkers.

The following definitions are used in this work:

Symptom: sensation or change in bodily function or appearance experienced by a person that suggests a disorder or pathology (e.g. runny nose as a symptom of a respiratory infection or rhinitis and watery stool as a symptom of diarrhoea).

In vivo response: integrated response to a (standardised) *in vivo* challenge (e.g. response to vaccination, prick and patch tests or oral provocation test).

Ex vivo response: (nutritional) intervention or comparison occurring *in vivo*; cells or blood isolated from participants in a study is assayed for functionality using a defined *in vitro* challenge (e.g. phagocytosis, natural killer (NK) cell activity or production of cytokines by *ex vivo* stimulated peripheral blood mononuclear cells or whole blood).

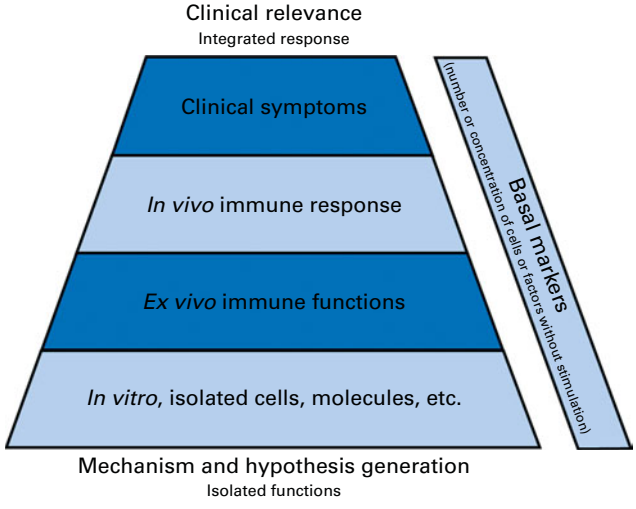

Fig. 2. Graphical representation indicating the classification of immune function markers from the most integrated/physiologically relevant to the most isolated/mechanistically insightful, with the basal markers being positioned on the side as they do not indicate a function by themselves, but aid in the interpretation of the functional markers.

In vitro response: (nutritional) intervention or comparison occurring *in vitro*; cells or blood isolated from subjects not participating in a study is exposed *in vitro* to the compounds, nutrients and so forth to be compared and is subsequently assayed for functionality using a defined *in vitro* challenge. This use of functional assays is intended not as a biomarker but rather as a tool for screening or mechanistic studies. This approach is outside the scope of the current activity.

Basal markers: cellular or molecular components of the immune system that are measured without a defined preceding challenge (e.g. cell types or cytokines). Not to be confused with the basal level (which is the actual value).

Methodological and technical considerations

Study design, randomisation and selection of appropriate control groups are critically important aspects when designing human studies for any outcome. Generic and more specific considerations for studies focusing on the effects of nutrition on immune function have been described in detail in earlier publications[1,23–25]. The effects of nutrition are potentially important in the longer term, but are typically modest and often difficult to observe in the short term. To assess these modest effects, it is important to carefully consider other factors known to influence immune function as these may otherwise obscure the effects of nutrition. Such confounders include stress[34], age[35,36], sex[37], ethnicity, physical fitness[18], circadian[38,39] and seasonal[40] influences, and sleep deprivation[41]. Using carefully selected procedures for randomisation and clear criteria for (non)inclusion[42], these confounders should be controlled for as much as possible. In some cases, they may also be used to select representative at-risk subpopulations to increase the sensitivity of the study. Examples of this include the selection of children from atopic parents[43], children in day-care centres exposed to a high infection load[44], people complying with specific exercise regimens[18], shift workers[45], people suffering from irritable bowel syndrome[46] or food intolerances[47,48], people with a BMI above a particular threshold[49], elderly individuals above a particular age[50] and so forth. Although this can greatly increase the sensitivity of the study to detect modulation by nutrition, care should be taken that the selected subpopulation is still sufficiently representative of the general population to allow meaningful extrapolation. For the purpose of the current activity, we focus on the selection of markers of immune functions to assess the effects of nutrition in the general population assuming the use of properly controlled, well-designed observation or intervention studies that take all of these critical elements into account.

When selecting markers to assess effects on immune function, it is important to realise that the identification, development and (clinical) validation of markers are mostly done in the context of diseases. Whereas the relevance of particular markers for the diagnosis or prognosis of specific diseases may be well established, their relevance in a general population is typically less clear as very few prognostic studies have carefully assessed the predictive value of these markers

for maintenance of health. Moreover, in the context of disease, markers are commonly used to determine the diagnosis or prognosis for individual patients. Based on a comparison of a patient's marker with a defined normal or reference range, conclusions are drawn for the individual patient. In contrast, in the context of nutritional effects in the general population, immune function markers are used to establish meaningful changes that occur at the group level. Typically, average (changes in) values of markers in one group are compared with (changes in) the values of markers in a control or reference group. This can be done observationally to assess associations between particular nutritional traits and immune functions or experimentally by applying a specific nutritional intervention in one of two groups to assess the impact of that intervention. In both cases, conclusions are drawn regarding the effect of the nutritional difference on immune function at the group level. Despite these differences between the use and interpretation of markers in the context of disease or in the context of the general population, essentially similar criteria apply when determining the usefulness of individual markers, although the relative weight of the individual criteria may be somewhat different.

In all cases, it should be clear as to what marker is measured and the assay used should measure it reliably; procedures and assays should be properly standardised and analytically validated. This includes the use of standardised operating procedures, including details of sampling, transport, storage and measurement of the analytes. Many analytes are sensitive to sampling conditions such as time during the day (circadian rhythm), fasting *v.* non-fasting, subject anxiety or stress level and even whether a blood sample is taken using a venepuncture or an indwelling catheter. Conditions for sample processing, transport and storage should, therefore, be clearly established and standardised, and the laboratory measurement should be analytically validated. This includes specificity, sensitivity, limit of detection, precision, robustness and linearity involving proper quality control and international gold standards if available. Several excellent papers have provided more specific information on the critical aspects of analytical validation and general laboratory quality management systems (e.g. EN ISO 15 189 and EN ISO 17 025) or on those described in the good clinical laboratory practice guidelines[51–55].

Some immune function markers such as C-reactive protein (CRP), differential cell counts or lymphocyte subset distributions are commonly used in clinical settings; for these properly validated procedures, assays and defined normal ranges and threshold values have been established (see, for instance, http://labtestsonline.org/). An example of reference ranges for lymphocyte subsets in different age groups has been given in several publications[56–59]. However, most of these markers are basal markers involving the enumeration of cells or factors in a sample without controlled experimental stimulation to assess a specific functional response. Such markers can aid the interpretation of more functional assays, but by themselves have limited value in nutrition studies, as they are rather insensitive to detect a modest modulation due to nutrition and are difficult to interpret in the general population. Unfortunately, more useful markers involving a functional response to a challenge such as *in vivo* challenge tests and *ex vivo* cellular function assays are much less standardised, have not been validated to the same extent and results can generally not be compared very well between laboratories. Moreover, assays and procedures involved are frequently tailored, for instance, by using suboptimal stimulation conditions (suboptimal concentration or incubation time) in *ex vivo* functional cell assays or by assessing responses after a suboptimal vaccination protocol (low level of adjuvant, low vaccine dose, only fraction of a multiple-dose antigen given, and assessment of the response during its early exponential phase). Although these modifications make good sense to increase the sensitivity and dynamic range to detect modulatory effects by avoiding saturation of the responses, they have led to a broad variety in protocols being used, many of which are not yet sufficiently standardised or validated to allow comparisons between different laboratories. There is a clear need for further standardisation and validation of these markers using protocols optimised to assess (alterations in) immune function in the general population. This should include further standardisation, analytical validation and ring testing between sites. Once this has been established, it will become more feasible to include such markers in prospective cohorts aiming to establish normal ranges and threshold values as well as their predictive values in the general population.

Normal ranges and thresholds are commonly used in clinical settings to interpret (changes in) markers of individual patients. Despite the different contexts and the limitations indicated above, essentially similar principles may be applied to devise a rational framework to aid the interpretation of nutritional effects on immune function at the group level in the general population. In the absence of more generically applicable normal ranges, (changes in) immune function markers are commonly compared with the following: the value of the markers in the same subjects before the intervention, the values of the markers in an appropriate control group matched for the most relevant criteria (age, sex, body weight, etc.) within the same study or combinations of the above (e.g. comparing the change within subjects between groups).

The strengths and limitations of these approaches have been discussed previously, and selection of the most appropriate reference group needs to be done on a case-by-case basis[1,18,22–25,27]. Importantly, for markers without established normal ranges and threshold values, comparison with the reference range obtained in an appropriate control group can be used as the basis for the interpretation of effects. This can be done by using statistical significance after appropriate analysis to determine the threshold for relevance as discussed in more detail below.

Criteria to select immune function markers

As has been indicated above, a wide variety of markers can be used to assess different aspects of the immune system, and multiple assays based on diverse technical principles are often available. We aimed to define generic criteria to select

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the most useful markers. It is acknowledged that markers may respond in clusters or that ratios of different markers may be calculated. Such marker clusters or ratios have not been included (except for an example of an immune risk profile defined in elderly individuals) in order not to further increase the size of the tables. The reader can, however, compile such composite markers and assess their usefulness following the criteria set out below.

The criteria and scoring system used are based on the work by Albers *et al.*[1] involving criteria that cover clinical relevance, biological sensitivity, feasibility and practical aspects for use in nutrition studies. Scores range from 0 to +++, where +++ is the highest score for the most solid evidence. More details on the standardised scaling used for each of these criteria are provided in Table 1.

Because the analysis focuses on the evaluation and ranking of markers and not on assays used to measure these markers, criteria for analytical validation were not included. Instead, it was assumed that markers are assessed using the optimal assay, according to the relevant standards discussed in the following sections on methodological and technical considerations and the selection and prioritisation of markers. In addition, whether a marker has previously been shown to be sensitive to nutrition was not included as a criterion because this is severely confounded by what has been tried and does not help to assess the intrinsic usefulness of a marker to define the (modulation) of immune function. However, when designing a specific study, it would be wise to consider the possibility that a particular marker is modulated by that particular nutritional modification (e.g. based on existing data or on mechanistic insights). The criteria that were selected to evaluate the generic usefulness of markers can be grouped into three clusters: clinical relevance; biological sensitivity; feasibility. In addition, practical aspects including costs and logistic implications were scored because these are important aspects to know; however, as these are non-scientific arguments that may weigh very differently in different settings, these practicalities were not included in the integrative assessment of the overall usefulness of the markers.

Clinical relevance

Clinical relevance is the weight that one would give to a specific biomarker to reflect an immune system-mediated clinically detectable health status in the general population

Differentially expressed in normal and high-risk individuals. This criterion should be linked to the different functional domains (defence against pathogens, allergens and inflammation). Scores given in Tables 2 and 3 are for the functional domain for which the marker has most relevance. While the level of a given marker may be important, the way it is measured is also relevant because the evaluation can be made based on single measurements as well as the kinetics of a marker response. Markers without any known or anticipated relevance for the general population are outside the scope of this article (e.g. specific leucocyte tumour markers).

Correlates with relevant clinical endpoint. Note that correlation does not necessarily imply causality. Markers without a known correlation with a given immunological function/status for the general population are outside the scope of this work for further evaluation.

Experimentally linked to causal pathway. The markers rating the highest would be those for which proof of causality has been clearly established using human data.

Biological sensitivity. Biological sensitivity is the level to which the marker is influenced by and linked to the biological process. This factor is highly influenced by the 'normal' inter- and intra-subject variation as well as by the amplitude of the studied effect. A distinction is made between explainable variation and unexplainable variation; it is assumed that it would be possible to (partially) correct for the former.

Reasonable within-subject variation. The higher the within-subject variation, the larger the number of subjects required to observe an effect within a subject and the lower the score. As many immune function markers have (substantially) lower intra- than inter-subject variation, comparison of changes within subjects between groups (instead of comparing individual measurements per subject) often helps to increase sensitivity to detect effects.

Reasonable/explainable between-subject variation. The higher the between-subject variation, the larger the number of subjects required to observe an effect between groups and the lower the score.

Feasibility. Feasibility pertains to how feasible/practical it is to measure the biomarker.

Technical feasibility. The sensitivity of the assay available should be sufficient to detect the effect. The assay can be used repeatedly if needed (e.g. no saturation of response as with vaccination). The criteria include aspects such as stability of the marker, storage of the sample (storage possible *v.* need to work with fresh material), limitations of sample transportation, level of preanalytical processing and so forth.

Robustness. This criterion makes a general assessment of the precision and accuracy of the best assay available to measure a given marker. This reflects the level of variation between assays performed by different people, with different instruments (if relevant) or in different laboratories as well as the degree of uncertainty about the result obtained. The best score is given to a standardised assay with an acceptable CV that enables the detection of the expected effect size. This is reflected by approval or non-approval by regulatory authorities and/or wide distribution. Note that an assay only used in a single research laboratory can be very robust, but strong evidence would not be accessible to prove so.

Practicality. Practicality pertains to how practically feasible it is to measure the biomarker in nutrition studies. Although cost and logistic aspects involved in the assessment of particular markers affect the feasibility to include markers in particular studies, these aspects were not taken into consideration when determining the overall usefulness of a marker, which was based on scientific criteria. As these practical aspects are nonetheless very important, a score is provided for information based on aspects such as the number of visits the subject is required to make, the availability and cost of the assay, the degree of expertise needed to perform the assay and so forth.

Table 1. Criteria for the evaluation of markers

	Clinical relevance			Biological sensitivity		Feasibility		
Levels	Differentially expressed	Correlates with clinical endpoint	Linked to causal pathway	Within-subject variation	Between-subject variation	Technical	Robustness	Practicality
Proven (+++)	Reproducibly proven association of differential expression with differential risk	Generally accepted as a risk factor (correlation with onset/resolution of the clinical endpoint)	Proven explanation backed by human data	Minimal variation and relevant effects highly superior to variation: effects likely to be observed between groups of tens of people	Minimal variation and relevant effects highly superior to variation: effects likely to be observed between groups of tens of people	Marker is stable and validated and highly available assay and can easily be done repeatedly with high throughput (e.g. CRP)	Approved diagnostic test available (IVD; e.g. CE marked or FDA approved)	Minimally invasive, done at bedside (a general practitioner can do it) (e.g. symptoms, faeces, urine and saliva) – single interaction with the subject
Strong (++)	Direct evidence linking differential response to differential risk (e.g. vaccination)	Described as a cause and effect relationship, but not (yet) generally accepted as a risk factor, needs more studies or not specific	Plausible mechanistic hypothesis with some human data	High variation explainable (e.g. circadian cycle) and possible to correct it and relevant effects reproducibly superior to variation: effects likely to be observed between groups of fifties to hundreds of people	High variation explainable (e.g. age, sex, BMI, ethnicity and genotype) and possible to correct it with stratification and relevant effects reproducibly superior to variation: effects likely to be observed between groups of fifties to hundreds of people	Sample can easily be made stable and limited processing (e.g. preparation of PBMC), or sample can be refrigerated for a limited time (e.g. ELISA of cytokines)	Service commercially available in accredited laboratories (e.g. LDT through CLIA laboratories in the USA)	Somewhat invasive (blood sample) – may require several interactions with the subject (vaccination, skin prick test, etc.) and sample sent to a laboratory
Medium (+)	Indirect evidence linking a change in function to a change in risk	Body of evidence suggesting correlation, but cause and effect not established	Plausible mechanistic hypothesis backed by animal data	High variation explainable (e.g. circadian cycle) and possible to correct it and relevant effects reproducibly close to variation: effects may be observed between groups of fifties to hundreds of people	High variation explainable (e.g. age, sex, BMI, ethnicity and genotype) and possible to correct it with stratification and relevant effects reproducibly close to variation: effects may be observed between groups of fifties to hundreds of people	Sample needs to be frozen, or assay can only be done once (e.g. response to vaccination)	Commercially available RUO kits	Expert and/or expensive material needed (MRI, X-ray and routine flow cytometry)
Low (0)	Plausible hypothesis with supporting animal data	Plausible hypothesis, in use as an exploratory marker, but no substantial body of evidence (yet)	Plausible mechanistic hypothesis backed only by *in vitro* data	High and unexplained variation in a short time span and relevant effects likely to be observed between groups of thousands of people	High and unexplained variation in a very short time span and relevant effects likely to be observed between groups of thousands of people	Sample needs to be extensively processed or stored at (80°C or analysed fast (e.g. in-line functional assays)	No commercially available LDT locally (in-house) and validated/published protocols available	Requires an expert outside the laboratory, medical surveillance and/or specific equipment (e.g. colonoscopy, biopsies, investigative flow cytometry and chemical sensitisation)

CRP, C-reactive protein; IVD, *in vitro* diagnostics; CE Mark, a mandatory conformity mark for products placed on the market in the European Economic Area; FDA, US Food and Drug Administration; PBMC, peripheral blood mononuclear cells; LDT, laboratory-developed tests; CLIA, Clinical Laboratory Improvement Amendments; RUO, research use only.

Selection and prioritisation of markers

Over seventy-five commonly used or recommended markers were evaluated according to the criteria specified in Table 1. For these markers, Tables 2 and 3 indicate the following: (1) whether a marker has relevance for the general population or is mainly relevant for specific subpopulations; (2) scores given for each of the defined criteria detailed in Table 1, all scored from 0 to +++; (3) an overall marker score indicating the subjective expert judgement on the usefulness of each marker based on the weighed evaluation of scores for individual criteria (□, not very useful; ■, low suitability; ■■, medium suitability; and ■■■, high suitability); (4) practicalities associated with the assessment of a marker (need for expensive, specialised equipment, need for repeated assessments, etc.); (5) plausible link to the three domains of immune function (scored not relevant (0) to most relevant (+++) for each domain), including defence against pathogens, avoidance or mitigation of allergy, and control of low-grade metabolic inflammation; (6) references illustrating the use of a particular marker in nutrition studies.

Table 2 summarises the clinical symptoms and *in vivo* markers for the three domains of physiological immune function. Symptoms of conditions that are relatively common in the general population are sometimes used as indirect markers of immune function. Clearly, they provide the most clinically relevant indication of intervention effects, but it is important to realise that symptoms by themselves may not necessarily indicate altered immune function because other non-immune system-mediated mechanisms may be responsible for the changes in symptom scores. As self-assessment has been criticised for being subjective, unspecific and therefore unreliable, it is important that symptoms be scored by qualified persons blinded to the intervention. Alternatively, properly validated questionnaires can be used, which may be complemented by confirmation of symptoms by qualified persons. Examples of validated questionnaires include the Jackson[60–62] and Wisconsin[63–65] scores for respiratory infections, the Vesikari[66] or WHO[67] scores for diarrhoea, SCORing Atopic Dermatitis (SCORAD)[68–70] for eczema, Allergic Rhinitis and its Impact on Asthma[71] for rhinitis, Asthma Control Test[72] or Test for Respiratory and Asthma Control in Kids[73] for asthma, and Mini Nutrition Surveys for the health and well-being of elderly individuals[74,75]. Because symptoms of naturally occurring infections or allergies develop at unpredictable moments and relatively infrequently in the general population, they are only useful as markers in studies of sufficient size and duration. To some extent, this can be addressed by performing the study in periods with increased incidence (e.g. winter for respiratory infections and spring/summer for hay fever) or by selecting (sub)populations more prone to develop symptoms such as groups with a higher prevalence of getting infected (e.g. children in developing regions, elderly individuals in nursing homes, children attending day-care centres, shift workers, etc.) or populations predisposed to develop allergies (e.g. children of atopic parents). However, in such cases, it is important to evaluate to what extent the outcome of the study can still be extrapolated to the general population. Contrary to (common) infections and allergies, inflammatory responses do not lead to symptoms that can be usefully assessed in the general population. Low-grade metabolic inflammation associated with (visceral) adiposity or inflammation linked to ageing is quite common, but by itself does not lead to overt symptoms.

Instead of waiting for symptoms to occur due to natural causes, they can also be elicited as part of the study design using an experimental infection with (attenuated) pathogens or a provocation with allergens. For instance, studies have successfully assessed symptoms elicited by an experimental infection with rhinoviruses[76–78], *Shigella*[79], respiratory syncytial virus[80] or enterotoxigenic *Escherichia coli*[81]. Likewise, acute symptoms elicited by ingestion[82,83] or nasal application[84,85] of allergens such as those used for the diagnosis and monitoring of allergy can also be used as markers in nutrition studies, be it that they will only result in symptoms in subjects who are allergic to a particular allergen. Importantly, unlike naturally occurring symptoms in which the time of occurrence and the exact eliciting trigger are unknown, experimental challenges make it feasible to combine the assessment of symptoms with that of the markers of contributing immune function(s) and even the kinetics of responses can be monitored to help establish cause–effect relations.

Although it is scientifically very attractive to use experimental challenges leading to symptoms that can be associated with changes in specific immune functions, such an approach clearly has ethical constraints. In particular studies, it may be more feasible to use somewhat weaker or more localised challenges that do not lead to symptoms but still modulate the relevant immune functions. Such *in vivo* markers of immune function include responses to vaccination (as a model for the response to an infection), to dermal recall antigen application (as a model for immune surveillance of the skin), to (local) allergen challenge (as a model for allergic responsiveness) and to transient inflammatory responses triggered by a pro-inflammatory challenge (as a model for the resilience of inflammatory control).

Vaccines trigger *in vivo* immune responses almost without eliciting symptoms of disease that would result from inoculation with live virulent pathogens. Specific immune responses to vaccines that are part of a national vaccination schedule can be used as *in vivo* indicators of the integrated immune response to these vaccines. Alternatively, one or more selected vaccinations can be integrated into the design of a study. Selection or stratification of subjects based on pre-existing responsiveness and careful consideration of the vaccine used (e.g. oral *v.* injected, type and dose of adjuvant, primary *v.* booster, single *v.* multiple dose, and T-cell independent *v.* T-cell dependent) and time point(s) selected to assess the response (early exponential phase *v.* later plateau phase, *v.* detailed analysis of the kinetics of the response) in relation to the postulated mechanism of action and the population in which the study will be performed can help to increase the sensitivity to detect the modulation of responsiveness due to nutrition. Seroprotection is defined as an antibody titre superior to an established threshold for clinical protection specific to each vaccine[86,87]. Seroconversion is defined as a

Table 2. Clinical symptoms and *in vivo* immune function markers

		Clinical relevance*				Biological sensitivity*		Feasibility*			Plausibly linked to†				
Functions	Markers	Mainly relevant for specific subpopulations	Differentially expressed	Correlates with clinical endpoint	Linked to causal pathway	Within-subject variation	Between-subject variation	Technical	Robustness	Arbitrary marker score‡	Practicality*	Pathogens	Allergy	Inflammation	Example references
Defence against natural infections§	Incidence of symptoms	No	+++	+++	+++	+	+	+++	+++	■■■	++	+++	0	++/0‖	120–132
	Duration of symptoms	No	+++	+++	+++	+	+	++	++	■■■	++	+++	0	++/0‖	
	Severity of symptoms	No	+++	+++	+++	+	+	+++	+++	■■■	++	+++	0	++/0‖	
	Pathogen load¶	No	++	++	+++	+	+	+	+	■■	+	+++	0	0	
	Pathogen-specific immune response¶	No	++	+	++	NA	+	0	0	■	0	+++	0	++/0‖	
Defence against experimental infection**	Incidence of symptoms	No	+++	++	+++	NA	+	+	+++	■■■	0	+++	0	0	80,81,133–135
	Duration of symptoms	No	+++	++	+++	NA	+	+	++	■■■	0	+++	0	0	
	Severity of symptoms	No	++	++	+++	NA	+	+	+++	■■■	0	+++	0	0	
	Pathogen load	No	+++	+++	+++	NA	+	+	++	■■■	+	+++	0	0	
	Pathogen-specific immune response	No	+++	++	+++	NA	+	+	+	■■■	++	+++	0	0	
Response to vaccination††	Seroprotection	No	+++	+++	+++	NA	+	+	+++	■■■	++	+++	0	0	136–141
	Seroconversion	No	+++	+++	+++	NA	+	+	+++	■■■	++	+++	0	0	
	Vaccine-specific antibodies (concentration and titre)	No	++	++	++	NA	+	+	+++	■■■	++	+++	0	0	
	Vaccine-specific T-cell responsiveness	No	++	+	++	NA	+	+	+++	■■■	++	+++	0	0	
Immunosurveillance of the skin	DTH response to local recall antigen application	Yes (sensitised)	++	++	+++	+	+	++	++	■■	++	+++	++	++	137,142–146
	Experimental CHS	No	++	++	++	+	+	++	0	■■	0	++	+++	++	90,91
	Migration of Langerhans cells	No	+	0	+	+	+	+	0	■	0	++	+++	0	92,147
GI barrier function‡‡	Sugar permeability	No	+	++	+	+	+	++	0	■	++	+++	+	+++	122,148–152
	Bacterial translocation	No	+	++	+	+	+	+	0	■	0	+++	++	+++	153–155
	Serum endotoxins	No	+	++	+	+	+	++	+	■	+	+++	+	+++	154,156
Tolerance to allergens	Incidence of symptoms	Yes (allergic subjects)	++	+++	+++	+	+	++	0	■■■	++	0	+++	+	68,69, 71–73,98, 157–163
	Duration of symptoms	Yes (allergic subjects)	+	0	+	+	+	+	+	■	0	0	+++	+	
	Severity of symptoms (e.g. peak flow, SCORAD, ARIA, Asthma Control Test (ACT) and TRACK)	Yes (allergic subjects)	+++	+++	+++	+	+	+++	+	■■■	+++	0	+++	+	
Response to an allergen challenge	Prick test	Yes (allergic subjects)	+++	+++	+++	+	+	++	++	■■■	++	0	+++	0	164–167
	Contact hypersensitivity/patch test	Yes (allergic subjects)	+++	+++	+++	+	+	++	++	■■■	+	+	+++	+	157,166, 168–172
	Respiratory (nasal) provocation test	Yes (allergic subjects)	+++	+++	+++	+++	++	++	+	■■■	++	0	+++	0	173,174
	Labial/nasal/oral provocation test	Yes (allergic subjects)	+++	+++	+++	+	+	++	+	■■■	++	0	+++	0	82,83,175

Table 2. *Continued*

		Clinical relevance*				Biological sensitivity*		Feasibility*				Plausibly linked to†				
Functions	Markers	Mainly relevant for specific subpopulations	Differentially expressed	Correlates with clinical endpoint	Linked to causal pathway	Within-subject variation	Between-subject variation	Technical	Robustness	Arbitrary marker score‡	Practicality*	Pathogens	Allergy	Inflammation	Example references
Symptomatic inflammation	Incidence of symptoms	Yes (patients)	0 (+)§§	0 (++)§§	0 (+ +)§§	+	+	++	++	□ (■■)§§	++	+	+	+	2,32,156
	Duration of symptoms	Yes (patients)	0 (+)§§	0 (++)§§	0 (++)§§	+	+	++	++	□ (■■■)§§	++	+	+	+	
	Severity of symptoms	Yes (patients)	0 (++)§§	0 (++)§§	0 (++)§§	+	+	++	++	□ (■■■)§§	++	+	+	+	
Response to inflammatory challenges‖‖	Kinetics and amplitude of induced inflammatory response (assessed as acute-phase protein, cytokine or gene expression)	No	+	+	++	+	+	++	+	□ (■)¶¶	++	+	+	+++	31,176–179

NA, not applicable (cannot be assessed repeatedly in the same subject due to the development of immunological memory); DTH, delayed-type hypersensitivity; CHS, contact hypersensitivity; GI, gastrointestinal; ARIA, Allergic Rhinitis and its Impact on Asthma; TRACK, Test for Respiratory and Asthma Control in Kids.

* See Table 1 for score interpretation.

† +++, Most relevant; ++, next most relevant; +, somewhat relevant; 0, not relevant.

‡ Arbitrary marker score is based on subjective expert judgement on the usefulness of a marker based on weighed evaluation of individual criteria. □, Not very useful; ■, low suitability; ■■, medium suitability; ■■■, high suitability.

§ Response to natural acute infections of respiratory tract (e.g. influenza virus and rhinovirus) or gastrointestinal tract (e.g. *Clostridium difficile*, enterotoxigenic *Escherichia coli* (ETEC) and rotavirus) or to natural chronic infection (e.g. cytomegalovirus (CMV), Epstein–Barr virus or *Helicobacter pylori*).

‖ ++ Indicates natural chronic infections (e.g. CMV and Epstein–Barr virus); 0 indicates acute infections.

¶ Pathogen-specific immune response such as pathogen-specific antibody titre or seroconversion or pathogen-specific T-cell response. Note that as with most natural infections, it is difficult to identify the responsible pathogen.

** Response to experimental infection (e.g. experimental rhinovirus infection, experimental infection with attenuated ETEC or experimental infection with respiratory syncytial virus).

†† Response to injected (systemic) or oral (mucosal) vaccination.

‡‡ Mainly relevant in GI patient populations.

§§ First score for low-grade metabolic inflammation, given in parentheses for patients with inflammatory conditions.

‖‖ Response to injected endotoxin, oral fat load, oral glucose load and exercise challenge and initial innate (inflammatory) response to vaccination. Still mainly experimental.

¶¶ Responses to inflammatory challenges seem promising, but relevance remains to be largely established.

Table 3. *Ex vivo* and basal immune function markers

		Clinical relevance*				Biological sensitivity*		Feasibility*				Plausibly linked to†			
Functions	Markers	Mainly relevant for specific subpopulations	Differentially expressed	Correlates with clinical endpoint	Linked to causal pathway	Within-subject variation	Between-subject variation	Technical	Robustness	Arbitrary marker score‡	Practicality*	Infection	Allergy	Inflammation	Example references
Systemic immune function markers															
Ex vivo (integrated)															
Immune risk profile (specific to elderly individuals)	Predefined profile (e.g. CD4:CD8 ratio, B-cell count, proliferative response, naive cell counts, NK-cell activity and phagocyte function)	Yes§	++	++	+	+	+	+	+	■■	+	+++	0	++	180–183
Ex vivo innate															
Phagocyte function	Phagocytosis	No	++	++	++	+	+	+	+	■■	+	+++	0	0	184–193
	Oxidative burst	No	+	++	++	+	+	+	+	■■	+	+++	0	0	
	Migration of cells	No	0	++	++	+	+	+	+	■■	+	+++	0	+	
NK-cell function	NK-cell activity‖	No	++	++	++	+	+	+	+	■■	+	+++	0	0	15,194–202
	LAK cell activity‖	No	++	++	++	+	+	+	+	■■	+	+++	0	0	
APC function	Expression of activation and differentiation markers (e.g. CD83, CD80, CD86, CD40 and HLA-DR)	No	++	+	++	+	+	+	+	■■	+	+++	++	++	15, 203–210
	Expression of TLR	No	++	+	++	+	++	0	+	■■	+	+++	++	++	
Bioactive mediator production (by PBMC or whole blood)	Production of cytokines (pro-/anti-inflammatory profiles)	No	++	+	++	+	++	++	++	■■	++	+++	++	+++	127,211–218
	Production of eicosanoids	No	0	+	+	+	+	+	0	■	+	+++	+++	+++	
Ex vivo adaptive															
T-cell function‖	Proliferation¶	No	++	++	++	+	+	++	+	■■	++	+++	++	++	219–221
	Expression of activation markers (e.g. CD25, CD69, CD95 and HLA-DR)	No	++	++	++	+	+	++	+	■■	++	+++	++	++	127,143,144,203,222–224
	Production of cytokines (e.g. Th1/Th2/Th17)	No	++	++	++	+	+	++	+	■■	++	+++	+	+++	225–230
	Cytotoxicity	No	++	++	++	+	+	++	+	■■	+	+++	+	++	231,232
	T_{reg} function	No	++	++	++	+	+	+	+	■■	++	+++	+	+++	233–238
B-cell function‖	Production of Ig (polyclonal or specific)	No¶	+	+	+	+	+	++	+	■	++	++	++	+	120,239
	Ig class switch	No	0	+	0	+	+	+	+	□	++	++	+	+	123,127,240,241
Specific IgE sensitisation	Basophil activation test	Yes (allergic)	+++	+++	++	+	+	++	+	■■■	++	0	+++	0	111,112,115,222,242–244
Basal markers (numbers or concentrations in blood or plasma)															
Cells	Differential cell counts	No	++	++	+	++	++	++	+++	■■	++	++	+	++	181,243,245
	Basic lymphocyte subsets (e.g. T, B, NK and CD4:CD8 ratio)	No	+	+	+	++	++	++	++	■	++	+++	+	++	28,59,222
	Sophisticated subsets (e.g. CD45RA/RO, T_{reg}, Natural Killer T-cells (NKT), pDC and mDC)	No	+	+	+	++	++	++	+	■	+	++	+	++	28,127,222,246
	Expression of activation markers (e.g. CD25, CD69, CD95 and HLA-DR)	No	+	+	+	+	++	++	++	■	++	+++	+	++	243,247
	T- and B-cell repertoires (clonality)	No**	+	+	+	+	+	+	+	□	++	+++	++	++	123,126,248,249
Mediators	Acute-phase proteins (e.g. CRP and fibrinogen)	No	++	++	+	+	+	+++	+++	■■	++	++	0	+++	116,250–253
	Antigen-specific antibodies	No	++	+	++	++	++	++	+	■■	++	++	++	+	254
	Allergen-specific IgE	Yes (allergic)	++	++	++	++	++	++	++	■■■††	++	+	+++	0	114,255–261
	Ig isotypes (including total IgE)	No	+	+	+	++	++	+++	+++	■	++	++	++	+	25,262
	Complement components	No	+	+	+	+	+	+++	+	■	++	++	+	+	263,264

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Table 3. *Continued*

Functions	Markers	Clinical relevance*				Biological sensitivity*		Feasibility*			Plausibly linked to†				
		Mainly relevant for specific subpopulations	Differentially expressed	Correlates with clinical endpoint	Linked to causal pathway	Within-subject variation	Between-subject variation	Technical	Robustness	Arbitrary marker score‡	Practicality*	Infection	Allergy	Inflammation	Example references
	Cytokines, chemokines and matrix metalloproteinases	No	+	+	+	+	+	++	+	■	++	+++	++	+++	183,212,265,266
	Profiles of cytokines (e.g. pro-/anti-inflammatory and Th1/Th2/Th17)	No	++	++	+	+	+	++	+	■■	++	+++	++	+++	267–269
	Soluble receptors (e.g. sCD14, sVCAM1 and sICAM1)	No	+	+	+	+	+	++	+	■	++	+	0	+++	204,270,271
	Adipokines (e.g. adiponectin, leptin and IGF)	No	++	+	+	+	+	++	+	■	+	+	0	+++	265,272–277
	Serum calprotectin	No	+	+	+	+	++	++	+	□	++	+	0	+	278,279
	Tryptase	Yes (allergic)	+++	+++	+	++	+	++	++	■■■	++	0	+++	+	113,280–283
Local immune function markers															
Ex vivo markers															
Local immune function	Functional assays on biopsy material (e.g. from the intestine, adipose tissue and skin)	Yes	++	++	++	+	+	0	+	■■	0	++	0	+++	284–286
	Functional assays on nasal or bronchoalveolar lavage	Yes	++	++	++	+	+	0	+	■■	0	++	+++	0	287–289
Basal markers (numbers and/or concentrations in blood, plasma, saliva and faeces)															
Cells	Cellularity of biopsies or lavage	Yes	++	++	++	+	+	0	+	■■	0	++	++	+++	287–289
	Homing markers on circulating cells	No	+	+	++	+	+	++	0	■	++	++	+	0	285,290
Soluble mediators	Stool calprotectin	No‡‡	+	++	+	+	+	+	+	■■	+	+++	0	+++	291–293
	Secretory and stool Ig (mucosal IgA)	No	+++	+++	++	++	+	+	++	■■■	++	+++	+	+++	40,103,104,107,109, 294–296
	Cytokine concentration, e.g. in faecal water/BAL/sputum	No	+	+	+	+	+	+	+	□	+	+	+	++	297–304
Mucus	Amount of degradation of mucus	Yes	+	+	+	+	+	++	+	□	+	+++	++	+++	13,305–307

CD, cluster of differentiation; NK, natural killer; LAK, lymphokine-activated killer cells; APC, antigen-presenting cells; HLA, human leucocyte antigen; TLR, Toll-like receptor; PBMC, peripheral blood mononuclear cells; Th, T helper; T_{reg}, regulatory T cell; pDC, plasmacytoid dendritic cells; mDC, myeloid dendritic cell; CRP, C-reactive protein; sVCAM, soluble vascular cell adhesion molecule; sICAM, soluble intracellular adhesion molecule; IGF, insulin-like growth factor; BAL, bronchoalveolar lavage.

* See Table 1 for score interpretation.

† +++, Most relevant; ++, next most relevant; +, somewhat relevant; 0, not relevant.

‡ Arbitrary marker score is based on subjective expert judgement on the usefulness of a marker based on weighed evaluation of individual criteria. □, Not very useful; ■, low suitability; ■■, medium suitability; ■■■, high suitability.

§ Is associated with all-cause mortality in elderly individuals; relevance in other (sub)populations to be established.

‖ Practical feasibility: 0 for Cr, ++ for flow cytometry.

¶ Assessed after polyclonal, oligoclonal or antigen-specific stimulation.

** Less clarity on relevance and much more variability in infants.

†† Specific IgE (sIgE) clearly indicates the involvement of immune function, but its relevance is controversial in the absence of concurrent clinical assessment. Some see it as a clinically relevant marker also used to guide therapy, whereas others (including the European Food Safety Authority) emphasise that not all allergic subjects have sIgE, not all subjects with sIgE have allergic symptoms and changes in sIgE are not always associated with changes in symptoms.

‡‡ Mainly relevant in gastrointestinal patients.

certain fold increase in specific antibody titres before and after vaccination[86], e.g. at least a fourfold rise in the case of influenza vaccines[88].

Delayed-type hypersensitivity (DTH) and contact hypersensitivity responses are local cell-mediated inflammatory responses triggered in sensitised individuals by the cutaneous administration of an antigen. Such responses can be measured 24–48 h after antigen application as epidermal induration[89]. For diagnostic purposes, DTH responses such as the prototypic Mantoux test have been largely replaced by more specific methods; however, as indicators of integrated *in vivo* cellular immune responsiveness, they remain valuable markers of immune function. However, standardised application has become complicated since the Cell Mediated Immunity (CMI) Multitest, in which seven different common antigenic preparations are administered simultaneously, is no longer available. Rigorously standardised application of antigenic material by syringe or prick is occasionally used; however, since application only yields DTH responses in sensitised subjects, it is important to apply a range of antigens. These problems have been overcome in several studies by experimentally sensitising subjects to uncommon chemical antigens not normally encountered. In this way, all subjects will respond, and the effect of nutrition on both the sensitisation and the elicitation phase of contact hypersensitivity responses can be assessed[90,91]. Although this is scientifically very attractive, there are clear ethical constraints. The same holds true for the assessment of Langerhans cells in skin biopsies, which can be used to assess temporal depletion of Langerhans cells due to migration induced by UV radiation. This is associated with increased susceptibility to (skin) infections, and mitigation of this depletion can, therefore, serve as a relevant marker of skin immune function[92].

The physical barrier of the gastrointestinal tract is central to the protection of the body against infections, allergens and inflammatory stimuli alike[93–96]. Tests designed to examine the integrity of the intestinal barrier typically involve the appearance of marker substances in plasma or urine after oral application of such substances (most often a non-metabolised sugar) and/or of resident bacteria or components thereof such as endotoxins after translocation from the lumen of the intestine into the circulation. These markers are considered useful to assess alterations in intestinal barrier function, which is considered important in gastrointestinal infections and (food) allergies as well as low-grade inflammation. However, in the strict sense, such markers do not necessarily demonstrate alterations in (mucosal) immune function as the permeability may be altered by non-immunological mechanisms.

In vivo provocation tests with allergens aim to reproduce allergic reactions in conditions as close as the natural exposure and using a lower dose and standardised conditions of exposure. Allergen provocation tests include intradermal injection or trans-cutaneous patches on the skin[97,98], oral provocation with food allergens[99,100] and mucosal (nasal, conjunctival, bronchial or labial) provocation with allergens[101,102]. While allergen provocation tests are quite safe to be used for most people, the possibility of anaphylaxis does exist and such tests should, therefore, only be done under clinical supervision.

Responsiveness to experimental inflammatory challenges has been suggested as a useful marker of inflammatory resilience. This involves the assessment of transient inflammatory responses induced by metabolic stressors (e.g. oral glucose or lipid load), infection stressors (e.g. injection with lipopolysaccharide, TNF, or IL-6 or the early response to the adjuvant components of vaccines) or tissue damage (e.g. acute exercise and exposure to UV radiation). Such dynamic responses to inflammatory challenges are promising markers of inflammatory resilience and are likely to be more relevant indicators of the ability to maintain inflammatory homeostasis than the mere static assessment of acute-phase proteins (e.g. CRP) or cytokines. However, there is a clear need for further standardisation of experimental protocols, and relevance in the general population remains to be established in prospective studies[31].

The evaluation of a range of *ex vivo* and basal markers of immune function is summarised in Table 3. It is beyond the scope of this article to provide detailed technical information on the assessment of all of these immune function markers as they are quite commonly used and specific considerations have been discussed in a detailed manner elsewhere[1,18,22–25,27]. The references listed in the table provide pointers to specific details on the use of individual markers in nutrition studies.

Clustering of markers according to clinical relevance and involvement of immune functions

A selection of the most relevant markers given in Tables 2 and 3 are further categorised in Table 4 according to the most relevant functional domain (horizontally) while indicating (vertically) whether the markers by themselves are classified into the following groups. Group A is indicative of clinical relevance and involvement of immune function(s) (e.g. response to vaccination). Group B is indicative of clinical relevance but not necessarily of the involvement of immune function(s) (e.g. symptoms of diarrhoea). Group C is indicative of the involvement of immune function(s) and is associated with clinical relevance in specific (sub) populations (selected markers such as NK-cell activity, which is associated with infection risk in athletes and elderly individuals). Group D provides mechanistic insight into details of the immune function(s) involved, but not necessarily of clinical relevance (e.g. circulating levels of cytokines).

This classification helps in the interpretation of study outcomes and can also be used to further rationalise the selection of markers for inclusion in future studies. To some extent, it may also guide in making decisions in preclinical investigations. Clearly, markers of group A are most useful as they provide evidence for both clinical relevance and involvement of immune function(s) by themselves. However, it may not always be feasible to select markers from this group. In such cases, combining the assessment of markers from group B with that of markers from group C or D will lead to more confidence in the future interpretation of the data regarding clinical relevance and involvement of altered immune function(s). The same argument

applies for a combination of group A markers with group C or D markers, linking clinical relevance with mechanistic insight. Such approaches will also result in new datasets that may be used to establish correlations of particular group C or D markers with clinical endpoints, which may lead to their evolution into group A markers within a reasonable time frame. In contrast, measuring numerous group B markers alone will lead to results that may be very clear regarding the clinical relevance, but remain inconclusive on the involvement of (altered) immune functions. Likewise, studies that focus exclusively on group D markers may very convincingly demonstrate that particular immune functions are altered, but the clinical relevance of these changes will remain largely elusive.

The list of markers given in Table 4 is non-exhaustive and should not be regarded as final since markers may evolve if more data become available. Instead, Table 4 provides the next step in a rational approach towards the design and interpretation of studies that aim to establish effects on immune function in the general population. The markers mentioned serve to illustrate how changes in these markers due to exogenous factors such as nutrition could be interpreted in the context of different scenarios described below. Selection and classification of the markers are based on the authors' expert judgement aided by discussion of an earlier version of this table with a wider group of experts.

It is noteworthy that many of the valuable group A markers indicating both clinical relevance and involvement of the immune system are 'challenge tests' comprising the assessment of *in vivo* responses of the immune system to a relevant (experimental) challenge. For defence against pathogens, these include immune responses to an (experimental) infection, to a vaccination and to a challenge with a recall antigen using a DTH or contact hypersensitivity test. For avoidance or mitigation of allergy, commonly used allergen provocation tests such as prick, intradermal and patch tests and labial, respiratory and oral challenges with specific allergens fall into this category. The corresponding pro-inflammatory challenge tests hold great promise as markers of the resilience of inflammatory control, but their clinical relevance has not been established yet[31]. For the time being, pro-inflammatory challenge tests are, therefore, classified as group C markers. In addition to the challenge tests, several other markers are placed in group A because they are clear indicators of the involvement of the immune system and their clinical relevance in the general population has been established, and modulation in the relevant direction would, therefore, be considered a beneficial health effect. For instance, mucosal IgA is a group A marker for defence against pathogens because it is a marker of immune function and low (salivary) IgA is a risk factor for respiratory infections in children and athletes[103–110]. For avoidance or mitigation of allergy, the basophil activation test[111,112] and tryptase[113] in plasma are considered group A markers because they reflect basophil reactivity in allergic patients and are considered risk factors correlated with the severity of the reaction. Allergen-specific IgE (sIgE) clearly indicates the involvement of immune function; however, in the absence of concurrent clinical assessment, its relevance in the general population is controversial. Some consider it as a clinically relevant marker (risk factor), whereas others (including the European Food Safety Authority) emphasise that not all allergic subjects have sIgE, not all subjects with sIgE have allergic symptoms and changes in sIgE are not always associated with changes in symptoms[114,115]. To acknowledge this controversy, we have indicated sIgE in groups A and C.

Group B markers are clinically relevant markers or endpoints (symptoms) that indicate the relevance of an effect, but in isolation do not prove the involvement of altered immune function as the effects could also be mediated via other mechanisms. For defence against pathogens, these include pathogen load and symptoms of common respiratory tract or gastrointestinal infections. Likewise, for avoidance or mitigation of allergy, symptoms of allergic responses due to undefined natural exposure to allergens indicate clinical relevance, but lack the specificity to prove the involvement of the immune system. This is also true for *in vivo* challenges with whole food products (milk) or substances such as lactose to which subjects may also respond with non-immune system-mediated intolerance. Low-grade inflammation does not lead to overt symptoms in the general population but is associated with reduced sensitivity to insulin and increased systolic blood pressure. However, these symptoms are not specific to low-grade inflammation and can also be modified by other non-immune system-mediated processes.

Group C markers typically reflect critical immune functions involved in the pathophysiological pathways underlying clinically relevant symptoms. Unlike group A markers, these markers are not generally accepted as markers of clinical relevance by themselves (risk factors), but there is ample evidence from relevant subpopulations that (alterations in) these markers are associated with (alterations in) clinical outcomes, and this is supported by a plausible mechanism of action. For instance, the precise predictive value of *ex vivo* NK-cell, phagocyte, or (pathogen-specific) T- or B-cell functions for infection risk is unknown. However, these immune functions are plausibly involved in the defence against pathogens, and a range of studies in elderly individuals, athletes, shift workers and other subpopulations have demonstrated that these *ex vivo* functional markers are inversely associated with the occurrence of symptomatic infections. Likewise, depletion of Langerhans cells in the skin due to UV radiation has been shown to be associated with an increased risk of (skin) infections, which plausibly is related to the reduced ability to present antigens and thus mount a protective immune response. For avoidance or mitigation of allergy, some feel that sIgE falls into this category for reasons discussed above. In addition, the remainder of *ex vivo* allergen-specific Th1- and Th2-cell function, T_{reg} function and antigen-presenting cell function fall into this category. For the time being, *in vivo* responses to a pro-inflammatory challenge are a group C marker for inflammation control, but they may evolve to a group A marker if relevance can be established in prospective cohorts in the general population. Serum markers of the acute-phase response (such as CRP) or (ratios of) pro- and anti-inflammatory mediators do define low-grade asymptomatic inflammation, but are not accepted as proof of clinical

Table 4. Clustering of selected markers according to clinical relevance and involvement of immune function(s)

		Markers of immune function linked to		
Classification		Defence against pathogens	Avoidance or mitigation of hypersensitivity (e.g. allergy)	Inflammation control (reduction of low-grade metabolic inflammation)
Group A marker	Indicative of clinical relevance and involvement of immune function(s)	Pathogen-specific immune response (specific antibodies and specific T-cell response after natural or experimental infection)	Specific response or symptoms after an experimental allergen challenge (skin, labial, respiratory or oral provocation tests)	NA
		Vaccine-specific immune response (seroprotection, seroconversion, specific antibodies and specific T cells)	Basophil activation test	
		Specific DTH or CHS response	Tryptase in plasma	
		Mucosal IgA (in saliva, tears, etc.)	Allergen-specific IgE (sIgE)*	
Group B marker	Indicative of clinical relevance but not necessarily of the involvement of immune function(s) (i.e. clinical symptom)	Symptoms of infection (incidence, duration and severity after natural or experimental infection)	Symptoms of allergy (rhinitis, asthma, urticaria, eczema, GI manifestations, etc.)	Symptoms associated with low-grade inflammation (e.g. insulin resistance and blood pressure)
		Pathogen load	Response to general food provocation	
Group C marker	Indicative of the involvement of immune function(s) and associated with clinical relevance in specific (sub)populations	*Ex vivo* (pathogen-specific) B-cell function	Allergen-specific IgE (sIgE)*	*In vivo* response to a pro-inflammatory challenge
		Ex vivo (pathogen-specific) T-cell function	*Ex vivo* (allergen-specific) Th1/Th2-cell function	Markers of acute-phase response (CRP, TNF, IL-1, IL-6 and blood sedimentation)
		Ex vivo phagocyte function	*Ex vivo* (allergen-induced) production of Th1/Th2 mediators	(Ratio of) pro- and anti-inflammatory mediators
		Ex vivo NK-cell function	*Ex vivo* T_{reg}-cell function	*Ex vivo* Th1/Th17/T_{reg}-cell function
		Migration of Langerhans cells	*Ex vivo* APC function	*Ex vivo* oxidative burst
Group D marker	Provides mechanistic insights but not necessarily into clinical relevance (non-exhaustive list of examples)	Markers of acute-phase response (CRP, TNF, IL-1, IL-6 and blood sedimentation)	Markers of acute-phase response (CRP, TNF, IL-1, IL-6 and blood sedimentation)	Plasma adiponectin and leptin
		(Ratio of) pro- and anti-inflammatory mediators	(Ratio of) pro- and anti-inflammatory mediators	Plasma endotoxin (LPS)
		Percentage of subsets including CD4:CD8 ratio	Total IgE	Plasma and faecal calprotectin
		Circulating or *ex vivo*-produced antibodies (not antigen- or vaccine-specific)	Circulating cytokines	
		Circulating or *ex vivo*-produced cytokines	Plasma and faecal calprotectin	

NA, not applicable; DTH, delayed-type hypersensitivity; CHS, contact hypersensitivity; sIgE, specific IgE; GI, gastrointestinal; Th, T helper; CRP, C-reactive protein; NK, natural killer; T_{reg}, regulatory T cell; APC, antigen-presenting cells; LPS, lipopolysaccharide.

* sIgE clearly indicates the involvement of immune function, but in the absence of concurrent clinical assessment, its relevance is controversial. Some see it as a clinically relevant marker also used to guide therapy, whereas others (including the European Food Safety Authority) emphasise that not all allergic subjects have sIgE, not all subjects with sIgE have allergic symptoms and changes in sIgE are not always associated with changes in symptoms.

relevance in the general population. Nonetheless, there is a wealth of emerging data in various subpopulations including elderly individuals and obese and insulin-insensitive subjects, demonstrating that (alterations in) levels of these markers are associated with an increased risk of cardiovascular events and diabetes[116–119]. To a lesser extent, the same holds true for *ex vivo* oxidative burst and functionality of Th1, Th17 and T_{reg} subsets, all of which are, therefore, also considered group C markers.

Group D markers also indicate the involvement of particular immune functions, but contrary to group C markers, their critical involvement in the pathophysiology is less established and there is no convincing evidence (yet) that changes in these markers are associated with changes in relevant clinical outcomes. This has implications for the ability to interpret changes observed in these markers, as will be discussed below. In essence, group D contains 'all other' markers of immune function. The examples mentioned in Table 4 serve to contrast the categorisation of the same marker for different functional domains, such as markers of acute-phase response and (ratio of) pro- and anti-inflammatory markers as group C markers for inflammation control *v.* group D markers for defence against infection and avoidance or mitigation of allergy. Other examples contrast different markers within one functional domain, such as sIgE (group A or C) *v.* total IgE (group D) for avoidance of allergy or plasma adiponectin and leptin (group D) *v.* (ratio of) pro- and anti-inflammatory mediators (group C) for inflammation control.

It is important to note that for control of low-grade asymptomatic inflammation, there are no specific markers indicating clinical relevance (group A or B). Insulin resistance and blood pressure are very unspecific, and the clinical relevance of the most useful markers in this domain including response to inflammatory challenge, CRP and inflammatory mediators remains to be established in the general population. This lack of markers to establish clinical relevance severely hampers proper interpretation of effects on markers of low-grade inflammation. It is, therefore, of critical importance for progress in this area to establish the clinical relevance of the most promising markers using prospective cohorts in the general population. Such efforts should not only focus on concentrations or ratios of circulating inflammatory mediators, but also include responsiveness to rigorously standardised *in vivo* pro-inflammatory challenge tests as these types of challenge tests have been shown to be most useful in the other domains of immune function.

Scenarios to interpret changes in (sets of) markers

Following the definition of criteria, prioritisation of markers and classification according to conclusiveness for clinical relevance and involvement of immune function, we set out to devise a framework for the interpretation of changes observed in the different types of immune function markers, taking into account the type of marker and the changes observed relative to a defined reference range. Obviously, the proposed approach does not discount the need to critically consider the quality of individual studies and consistency of effects and to base the ultimate conclusions on the totality of evidence. Herein, we propose a theoretical framework to structure the interpretation of changes in immune function markers due to nutrition in the general population. As depicted and explained in more detail in Fig. 3, five scenarios were defined for changes in markers relative to the appropriate reference range.

The first step to interpret immune modulation in a particular setting is to determine whether one or more relevant markers of immune function are statistically significantly modulated by the intervention and, if so, to determine which of the scenarios applies. If the marker by itself indicates clinical relevance but not altered immune function (group B), and the effect is not accompanied by a plausibly linked change in a marker indicating altered immune function, then the effect is clinically relevant but may not necessarily be due to altered immune function and cannot be interpreted as such (e.g. reduced incidence or severity of diarrhoea on its own). If the marker by itself is indicative of immune function(s) and clinical relevance (group A marker), then the result can be interpreted as a beneficial modulation of immune function for scenarios 1, 2, 4 and 5. In scenario 3, this raises some concern and needs to be evaluated in more detail as described below. If the marker by itself is indicative of immune function(s), but in isolation does not indicate clinical relevance (group C), then interpretation as a beneficial modulation of immune function is possible for scenarios 1, 2, 4 and 5, if in the same or a similar study setting (a similar population or the same nutritional intervention), a marker indicating clinical relevance (group A or B) is also significantly changed and the changes are linked via a plausible mechanism. Again, for scenario 3, further evaluation is required to assess the potential for detrimental modulation of immune function, especially after prolonged exposure. For scenario 4, interpretation as a beneficial immune modulation would also be possible, but in this case, the intervention would prevent the negative changes occurring in the reference or control group. Finally, if the change is observed in a marker indicating altered immune function(s) but not necessarily clinical relevance in all populations (group C) and information on the markers of clinical relevance is lacking in the same or a similar study, then interpretation of the immune effects is not possible in scenario 1, but may indicate a beneficial modulation in specific cases (scenarios 2, 4 and 5). This is depicted in Fig. 4. To explore this approach in more detail, it is applied to five scenarios discussed below with some examples for the different functional domains.

Scenario 1: nutrition induces a significant modulation of a marker within the reference range

If a relevant marker of immune function is statistically significantly modulated in the relevant direction and the marker *per se* is also indicative of the clinical relevance of effects observed (group A marker), such as improved responsiveness to an infection or a vaccine or reduced responsiveness to an (oral) allergen provocation test, then the modulation of this response within the reference range would be interpreted as a

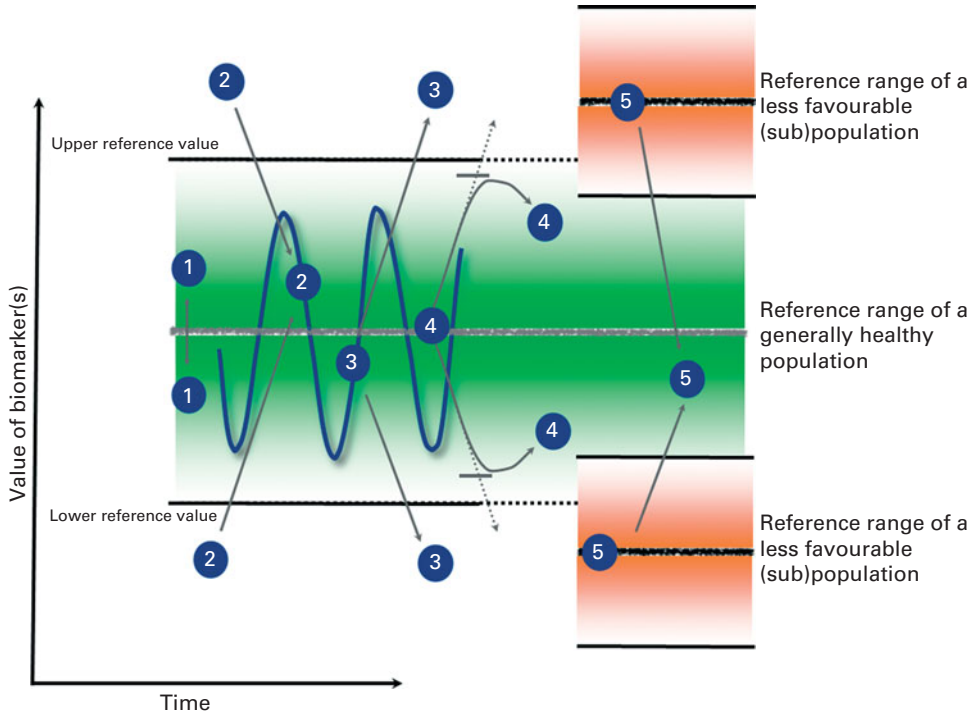

Fig. 3. Graphical representation of the five different scenarios for the modulation of immune function markers relative to the reference range. Scenario 1: statistically significant modulation within the reference range or within the range of a relevant control population, a very common scenario for modulation due to nutrition. Scenario 2: statistically significant modulation from outside the reference or control range of a relevant control population back into the range. Cases are different before intervention and become similar after intervention. Scenario 3: statistically significant modulation from within the reference or control range of a relevant control population out of the range. Cases are similar before intervention and become different after intervention. Scenario 4: nutritional prevention of statistically significant modulation induced by other endogenous or exogenous factors. Markers move out of the reference range of a relevant control population in the reference group, but this is prevented by nutrition in the intervention group (e.g. prevention of negative effects on the immune function of ageing or UV-B exposure or prevention of allergic sensitisation). Scenario 5: statistically significant modulation from a less favourable reference range to the reference range of a comparator group with a more desired immune function (e.g. from bottle-fed infants to breast-fed infants, elderly individuals to healthy adults, strenuous exercise to healthy controls, sleep deprivation to sleep sufficiency, etc.).

beneficial modulation of immune function within the specific domain. It is critical to consider the changes in immune functions within the context of a specific functional domain as for some markers the interpretation may differ depending on the domain. For instance, in the context of defence against pathogens, enhanced DTH to an antigenic challenge would be regarded as an enhanced response to a 'model infection' and would thus be considered beneficial. However, in the context of allergy, enhancement of a (delayed-type) hypersensitive response to an allergen challenge would be undesirable, whereas mitigation of such a response would be considered beneficial.

Similarly, if a statistically significant change in a marker indicative of immune function (group C) is plausibly linked to a statistically significant change in a marker of clinical relevance (group B marker), then the modulation of this marker could be interpreted as beneficial, and one can claim that nutrition improves immune defence against pathogens or helps to avoid or mitigate allergy or inflammation. For example, if consumption of a certain nutrient significantly reduced the duration of gastrointestinal infections during the winter season (group B) compared with a control group and concomitantly phagocyte function (group C) increased within the reference range in the infected subjects and these are plausibly linked based on mechanistic insights, the interpretation would be that this nutrient improves immune defence against pathogens. Another example in the field of allergy would be if consumption of a certain nutrient significantly reduced the severity of rhinitis (group B) compared with a control group and concomitantly Th2-cell function (group C) decreased within the reference range for the general population. In such cases, the interpretation would be that this nutrient mitigates hypersensitivity against allergens because these two markers are plausibly linked.

If the immune function marker belongs to the category of markers that are only indicative of the involvement of the immune system (group C or D) and evidence for concomitant changes in the markers of clinical relevance in the same or similar studies is lacking, then modulation within the reference range cannot be interpreted in terms of its health impact. However, complemented with other studies with acceptable biomarkers, such data could be used as supportive evidence of an immune regulatory effect. Finally, if the marker is indicative of clinical relevance but there is no significant change in plausibly associated markers of immune function (group B alone), clearly this is beneficial to health, but the effect may not necessarily be due to altered immune function and should not be interpreted as such. For instance, if the incidence or duration of diarrhoea is significantly reduced, this is beneficial. However, without additional information on

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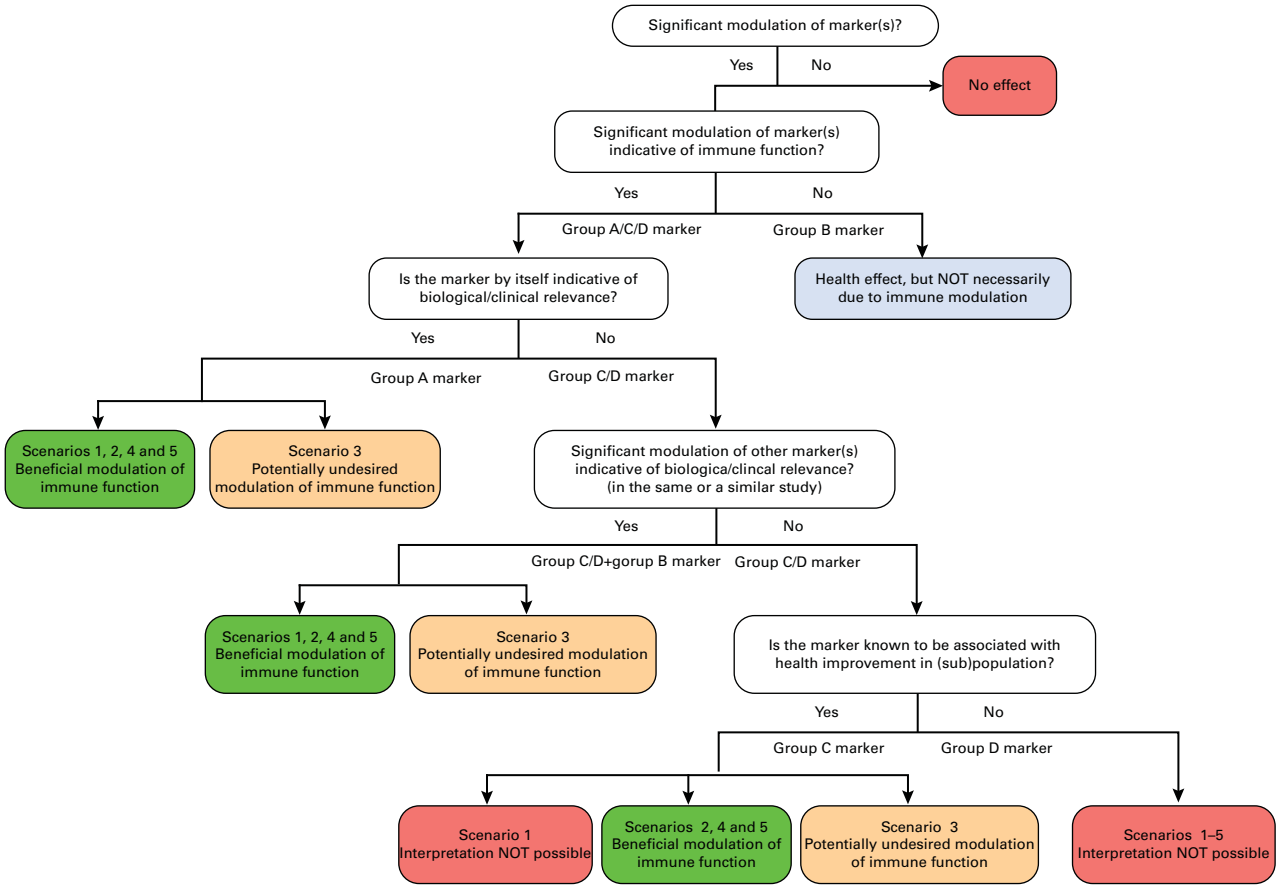

Fig. 4. Flow chart to aid the interpretation of changes in immune function markers in nutrition studies based on information on the type of markers in which significant changes are observed (groups A–D) and the relative change compared with the reference range (scenarios 1–5). Blue indicates a health effect but not necessarily due to immune modulation, green indicates beneficial modulation of immune function, orange indicates potentially undesired modulation of immune function and red indicates no effect or interpretation not possible. Group A, relevance AND the involvement of immune functions (e.g. response to vaccination). Group B, relevance BUT NOT necessarily the involvement of immune functions (e.g. diarrhoea). Group C, involvement of immune function(s) AND associated with clinical relevance in specific (sub)populations (e.g. NK-cell activity in athletes and elderly). Group D, mechanistic insights BUT NOT directly associated with clinical relevance (e.g. cytokines).

changes in plausibly linked markers of immune function, this cannot be interpreted as a beneficial modulation of immune function because the underlying mechanism may be completely different.

Scenario 2: nutrition induces a significant modulation of a marker from outside the reference or control range back into the range

The interpretation for this scenario is essentially identical to that for scenario 1 with one addition. In the absence of data on changes in clinical relevance in the same or a similar study (the same intervention in a similar population), changes in markers indicative of immune function (group C) could potentially also be interpreted using a two-step argumentation if a change in these markers is described in the literature to be strongly associated with health improvement. An example may be if a (nutritional) intervention induced an increase in NK-cell activity back to the reference range and it is known that decreased levels of NK-cell activity have been strongly linked in the literature with decreased defence against pathogens or tumours in particular populations (e.g. athletes and elderly individuals). If there is no such established association with clinically relevant outcomes in the literature, then the interpretation of the relevance of immune function marker modulation back to the reference range is not possible (group D). However, one could argue that modulation back into the reference range could be positively perceived in terms of reassurance against potential negative effects.

Scenario 3: nutrition induces a significant modulation of a marker from within the reference or control range to outside the range

If particular markers of immune function move from within the reference range to outside the reference range, such results should be interpreted within their context. In such situations, it may be useful to take the timing and sequence of events into account. For instance, if this occurs during an acute infection process, allergic manifestation or inflammatory episode, then the out-of-range markers may be more attributable to the ongoing host response to the infection/

allergens/inflammation rather than to the nutritional intervention. In such cases, it is also important to look at the magnitude of the effect and the consistency of changes observed among related markers. If it is not just a (statistical) artifact, but a consistent pattern of substantial modulation of related markers out of the reference range attributable to the intervention, then this is a sign to look more closely at potential unwanted side effects that could develop if this is sustained in the longer term.

Normally, such potentially deleterious effects should surface during animal studies in the preclinical phase. In the event that these emerge at a later stage, it would be recommended to initiate (additional) animal studies to gain a better mechanistic understanding and/or restrict use in humans to populations that are not at risk for the tentative adverse effects while initiating a 'pharmacovigilance'-type study (postmarketing surveillance study). Clearly, a careful consideration of the risks *v.* the benefits of longer-term exposure to such ingredients/products is warranted.

Scenario 4: nutrition prevents a significant modulation of a marker from within the reference or control range to outside the range

If particular markers of immune function in the control or reference group move from within the reference range to outside the range due to other factors and this is prevented by nutrition, this could be interpreted as a beneficial maintenance of normal immune function. In essence, this is the reverse of scenario 3 and would constitute increased resilience of the homeostatic regulatory mechanisms. This could involve the prevention of the negative impact of UV-B exposure or of strenuous exercise on immune function(s) or prevention or delay of ageing-associated immune senescence. Likewise, although allergen-sIgE is normally absent in a population, if followed prospectively, the levels of allergen-sIgE will increase in a subpopulation due to allergic sensitisation. Prevention of such negative effects could be interpreted as a beneficial modulation of immune function if the marker itself signifies clinical relevance as well as immune function (group A) or if evidence of plausibly linked markers of clinical relevance (group B) and contributing immune function (group C) can be combined. In the absence of data on clinical relevance in the same or similar studies, prevention of changes in markers indicative of immune function (group C) could potentially also be interpreted using a two-step argumentation if a change in these markers is described in the literature to be strongly associated with negative health effects. If there is no established association with clinically relevant outcomes, then the interpretation of the prevention of modulation of an immune function marker (group D) out of the reference range is less easy, although it could be positively perceived as an indication of increased resilience of homeostatic control. Finally, if the prevented modulation out of the reference range indicates clinical significance without evidence for the involvement of the immune system (group B by itself), the effect is beneficial for health, but may not necessarily be due to altered immune function.

Scenario 5: nutrition induces a significant modulation of a marker from a less favourable reference range into the reference range of a comparator group with a more favourable immune function

In this scenario, changes in markers are compared with the reference range of a population with a more favourable health profile. This reference range has been linked through a plausible mechanism to one or more immune functions. Typical examples would be to restore markers in elderly individuals to levels in healthy adults, in (overtrained) athletes back to normal ranges, and in more disease-prone shift workers to levels in less disease-prone controls with an undisturbed biorhythm; to move markers from atopic to normal ranges; or to shift markers of bottle-fed infants to normal ranges for breast-fed infants. Again, changes in markers indicative of clinical relevance or clearly established association with a plausible underlying mechanism are key to interpretation. In the case of immune function markers that also indicate clinical relevance (group A) or concomitant changes in plausibly linked group B and C markers, the interpretation is straightforward. For instance, if the responsiveness to vaccination in an elderly population shows increases to the range typically found for younger adults, this will be considered as beneficial. Likewise, reduction of elevated CRP levels in elderly individuals to ranges observed in healthy adults would be considered beneficial because lower levels of CRP have been associated with better survival.

Beyond individual immune function markers

In most nutrition intervention studies, (large) sets of immune function markers are assessed and the interpretation of intervention effects will depend on the consistency of the changes observed. If related immune function markers show consistent changes, the interpretation will be easy and will follow the same logic as for individual markers. For instance, if composite markers such as ratios of pro- and anti-inflammatory cytokines, ratios of distinct T-helper cell subset-related activation markers or cytokines, or a specified immune (risk) profile can be defined before the study, then reference ranges can be determined for these composite markers and nutrition-related changes relative to these ranges can help guide the interpretation as described for individual markers above.

If changes in related markers are not consistent, interpretation is greatly helped by good clinical practices that include the *a priori* definition of the most important 'lead markers' (typically groups A and B). The interpretation would be based on changes in these lead markers combined with mechanistic insights that may help to interpret the differential effects observed on the other markers. This could lead to the formulation of a new hypothesis that could then be tested in a new study specifically designed to test this hypothesis. Patterns or composite markers that are less well defined before the study, including those emerging from the untargeted use of high-content multiplexed or -omics approaches, are more difficult to interpret within this conceptual framework. However, such approaches could lead to the identification

of pathways involved and can help to formulate specific hypotheses that can then be tested in more targeted follow-up studies.

It is thus advisable to define *a priori* a cluster of the most important markers that are predictive of clinical effects or can be used as supportive evidence of clinical outcomes and that will aid in the eventual interpretation of the outcome of the intervention. These may be complemented with secondary parameters that present a profile that is indicative of a certain endpoint that may help in the interpretation of the mechanisms underlying potential changes in primary outcomes.

The same principles as described above for nutritional intervention studies may apply to other types of interventions and, with some caution, to observational studies exploring the relevance of (nutritional) differences between groups.

Conclusions and recommendations

The overall aim of this article is to provide further guidance for the assessment and interpretation of immune modulation by nutrition in the general population. To this end, criteria were defined to evaluate the strengths and weaknesses of symptoms and markers to measure changes in immune function. The markers were evaluated for three distinct domains of immune function: defence against pathogens; avoidance or mitigation of allergy; control of (low-grade) metabolic inflammation. Graded criteria were applied to over seventy-five immune function markers that were rated based on the different scores for their overall usefulness (Tables 2 and 3). Not surprisingly, it was found that markers that involve the standardised assessment of relevant symptoms (e.g. symptoms of common infections or allergies) or *in vivo* responses to a defined challenge with antigens or allergens (e.g. response to vaccination or allergen provocation) provide the most useful indication to interpret the modulation of immune function. Other useful markers include selected *ex vivo* markers of particular immune functions (e.g. NK-cell activity, phagocytosis and responsiveness of specific T-cell subpopulations) and selected basal markers essential in the exertion of critical immune functions, such as mucosal IgA for infection resistance, allergen-sIgE and tryptase for avoidance or mitigation of allergy, and CRP and inflammatory mediators to indicate low-grade inflammation.

A selection of the most useful markers were further classified depending on whether a change in the markers by itself conclusively indicates clinical relevance and/or involvement of altered immune function. Group A markers indicating both clinical relevance and involvement of immune function include pathogen- and vaccine-specific immune responses, DTH and contact hypersensitivity responses and mucosal IgA responses for defence against pathogens and for avoidance or mitigation of allergy-specific responses to allergen provocation, basophil activation and plasma tryptase. No group A markers were identified for control of low-grade metabolic inflammation. The classification of allergen-sIgE was controversial as some regard it to be a group A marker for allergy, whereas others are less convinced of its clinical relevance in the absence of symptoms in the general population. Group B markers demonstrating clinical relevance, but not necessarily the involvement of immune function(s), include symptoms of infections and pathogen load for defence against pathogens as well as symptoms of allergy and response to general food or lactose provocation for allergy and insulin resistance and blood pressure as rather unspecific symptoms associated with low-grade metabolic inflammation. Several *ex vivo* cellular function assays associated with clinically relevant effects in (sub)groups of the general population and indicating the involvement of immune function(s) were clustered as group C. In addition, CRP and inflammatory markers were put into this category for their role in the control of low-grade inflammation, and some argue that sIgE should also fit into this category for avoidance or mitigation of allergy. This group also comprises *in vivo* responses to pro-inflammatory challenges, which are considered promising markers for control of low-grade metabolic inflammation, but direct evidence for their clinical relevance remains to be established. Further optimisation, validation and inclusion of such challenge tests in prospective studies should be a high priority, as there are currently no other markers specifically indicating clinical relevance for the modulation of low-grade metabolic inflammation, hampering possibilities to design studies to demonstrate benefits of improved inflammatory control. Finally, group D contains all other immune function markers that can help to provide mechanistic insights, but for which the clinical relevance is currently unclear.

Clearly, there is no gold standard of immune function that can be recommended for all studies assessing the effects of nutrition on immune function in the general population. It is, therefore, proposed to first define the functional domain of interest and then select (combinations of) markers that indicate clinical relevance and for which a plausible hypothesis explaining how they could be related is available. For instance, to test the effect on resistance to pathogens, one could assess the response to a vaccination or combine the assessment of endpoints with specific responsiveness to an experimental infection. Alternatively, symptoms of natural infections could be assessed alongside with markers of immune function likely to be involved mechanistically. If the aim of the study is to test effects on mitigation of allergy, one could focus on provocation tests using specific allergens or combine the assessment of allergic symptoms with contributing group C or D markers. Finally, for control of low-grade metabolic inflammation, one could select a combination of markers including responsiveness to inflammatory challenge, CRP and pro- and anti-inflammatory mediators. However, it is important to realise that the clinical relevance of these markers remains to be established in the general population.

To aid the interpretation of changes observed in (combinations of) immune function markers, a framework was devised taking into account the type of marker and the changes observed relative to a defined reference range. Within this framework, five different scenarios were identified including (significant) modulation within the reference range (a very common scenario for modulation due to nutrition), modulation from outside the reference range back into the

range, modulation from within the reference range out of the range, prevention of modulation induced by other factors, and modulation into the reference range of a comparator group with a more desired immune function (e.g. from values in bottle-fed infants to those in breast-fed infants). Evidently, this framework does not neglect the need to consider the quality of individual studies and consistency of effects and to consider that ultimate conclusions must be based on the totality of evidence. However, working through the logical steps of the proposed framework as presented in Fig. 4 indicates that selected (combinations of) markers can be used to reach clear conclusions as to whether an observed modulation of immune function could be regarded as beneficial within the functional domains of defence against pathogens and avoidance or mitigation of allergy. For control of low-grade metabolic inflammation, this is more difficult as there is a lack of specific markers linking altered immune function(s) to clinical relevance in this domain. Clearly, it is possible to reach conclusions on beneficial effects in situations of insulin resistance and high blood pressure. However, since these are only very loosely associated with low-grade inflammation, it will be challenging to firmly establish whether a concurrent modulation of (inflammatory) markers is correlated.

Finally, it is important to stipulate that the selection of markers and the complete approach are geared towards the assessment of effects on relevant physiological functions of the immune system or markers indicating benefits or risks at the group level within the general population. This is clearly distinct from the use of markers to diagnose or monitor the progression of disease in individual patients. Moreover, the evaluation and classification of markers indicated herein are based on an expert judgement of the authors, partially validated by discussions at a workshop with a wider group of experts. Scores and classification may be debated to some extent and conclusions for individual markers may change over time as more robust data on the association with relevant clinical outcomes in the general population become available. More importantly, the structured stepwise approach that was followed offers a rationale for selecting markers for future trials and helps to provide a framework for the interpretation of outcomes. In fact, a similar stepwise approach may also be useful to rationalise the selection and interpretation of markers for other physiological processes that are critical to the maintenance of health and well-being in the general population.

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