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Phenotypic flexibility in nutrition research to quantify human variability: building the bridge to personalised nutrition

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Phenotypic flexibility is a methodology that accurately assesses health in terms of mechanistic understanding of the interrelationship of multiple metabolic and physiological processes. This starts from the perspective that a healthy person is better able to cope with changes in environmental stressors that affect homeostasis compared to people with a compromised health state. The term 'phenotypic flexibility' expresses the cumulative ability of overarching physiological processes to return to homeostatic levels after short-term perturbations. The concept of phenotypic flexibility to define biomarkers for nutrition-related health was introduced in 2009 in the area of health optimisation and prevention and delay of non-communicable disease. The core approach consists of the combination of imposing a challenge test to the body followed by time-resolved analysis of multiple biomarkers. This new approach may better facilitate nutritional health research in intervention studies since it may show effects on early derailed physiological markers and the biomarker response can be extended by perturbing the system, thereby making them more sensitive in detecting health effects from food and nutrition. At the same time, interindividual variation can also be extended and compressed by challenge tests, facilitating the bridge to personalised nutrition. This review will overview where the science is in this research arena and what the phenotypic flexibility potential is for the nutrition field.

Nutritional challenge test: Phenotyping: Tolerance test: Health quantification: Prevention of non-communicable diseases

In recent years the world has been confronted with the Covid-19 pandemic in a world that was already facing the obesity pandemic, its convergence is also referred to as a syndemic[1,2]. Obesity is a condition with abnormal or excessive fat accumulation that may impair health[3]. A relationship between obesity and Covid-19 disease development was clearly shown; persons with obesity or related non-communicable disease (NCD) such as CVD or type 2 diabetes had a higher risk on developing a more severe form of Covid-19[1]. Importantly, eating a healthy diet can help to prevent obesity and the development of NCD[3]. Lifestyle can contribute up to 90 % in the development of diverse NCD[4]. However, this requires early detection of derailment of metabolic health and a (sustained) lifestyle behaviour change in persons at risk.

Phenotypic flexibility is the metabolic and/or physiological adaptation to a disturbance of homeostasis by the sequential response of interconnected adaptive

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response systems that can be followed in space and time in terms of amplitude and duration[5]. This starts from the perspective that persons with an optimal orchestration of metabolic, inflammatory and redox regulatory pathways that are embedded into psycho-neuro-endocrine control mechanisms are healthy, whereas this adaptive response system loses flexibility when chronic metabolic disease develops[5]. In 2009, it was proposed that challenging homeostasis by means of standardised challenge tests could help to define biomarkers for nutrition-related health[6]. These biomarkers would focus on quantification of health and health optimisation, rather than on disease and disease management and would be better suited for nutrition and health-related research. This phenotypic flexibility approach would offer a different approach to quantify health and would result in different type of biomarkers that could help to quantify the effects of foods, diets and nutrition on health. When combined with a new generation of -omics technologies it would provide the potential to measure subtle effects taking place in various tissues and organs and it could monitor through processes instead of (single) biomarkers. This next generation of biomarkers also fitted perfectly in the new proposed definition of health that stated that health equals the capacity to adapt and self-manage in the face of social, physical and emotional challenges[7]. Examples of such daily challenges include eating a meal, being physical active, change in the ambient temperature or stress where the body actively adapts with regulatory and control response mechanisms to keep the body in homeostasis for the core metabolic processes[8].

However, substantiating that a specific diet or dietary product has a beneficial effect on health in terms of health maintenance or NCD delay is not easy. In addition of fuelling our body and being tasty, our diet should provide us with the essential nutrients for optimal physiological functioning in daily life[9,10]. Nutrition is like the oil for a car; it keeps various systems from wearing out and breaking down prematurely[9]. A good example is vitamin D that has a role in multiple mechanisms. This vitamin is essential for the absorption of calcium and phosphorus essential for bone metabolism as well as being a key component for genomic signalling for the production of cell-specific proteins allowing to respond to a wide variety of stimuli such as epithelial proliferation and production of antibacterial peptides by macrophages[9]. A deficiency of vitamin D results in the bone disease rickets, however a sustained inadequate intake of vitamin D can also result in physiological dysfunctioning resulting in NCD such as osteoporosis, hypertension and type 1 diabetes among others[9]. Nutrition has subtle and long-term effects which result from interactions between nutrients within a diet with diverse mechanisms that can target multiple tissues and organs in the body. Furthermore, accurate quantification of effects from food and nutrition requires assessment in a healthy population of free living persons, who will not always comply to the intervention diet, who have large degree of interindividual variation and where the reference or control diet cannot easily be a 'placebo'. To prove a causal relationship of a health benefit by a nutrient, food product or diet requires a relationship of sufficient strength and which is reproducible, ideally a dose–response relationship, a certain time window that the benefit sustains biological plausibility and shown with human studies within the healthy range of the population. Nowadays, the literature describes several human nutritional intervention studies that indicated the added value of evaluating phenotypic flexibility in showing health modulation by nutrition in the healthy range of the population. The aim of this review is to set out the current status of the science, developments and considerations for research focusing on the concept of phenotypic flexibility in the context of nutrition research.

The food–diet–nutrient–physiology context

For the development of a next generation of biomarkers that focus on nutrition that optimise human health and can deliver physiological substantiation by means of phenotypic flexibility it is good to set-out presumed underlying mechanisms where food–diet and nutrition could play a major role for health maintenance or physiology improvement and to translate these into measurable outcomes. A nice overview of biological processes that actively promote health optimisation of an individual within the context of physiological substantiation of effects from food and nutrition has been provided in the recent paper by Witkamp[10]. This was based on the conceptual frameworks of Ayres[11] and López-Otín and Kroemer[12] who describe biological drivers of physiological health and resilience, which are usually distinct from processes that drive disease. Furthermore, this was combined with the health benefits on specific body functions for health and nutrition claims from the European Food Safety Authority. An overview of these body functions and biological drivers of health has been provided in Fig. 1. Health is continuously exposed to multiple external sources of stressors. To achieve homeostasis or biological stability, human physiology uses different strategies to deal with such stressors. These central drivers for the capacity to adapt to stressors are determined by a person's genetics, immune system including both the innate and adaptive system, hormones and metabolism and its endocrine and metabolic circuitries, neural networks or the function of multiple brain circuits to regulate psychobiological response and represent a myriad of neurotransmitters, neuropeptides, receptors and signalling pathways and the repair and regenerative capacity such as DNA damage and repair, and oxidative stress response[12]. In addition to that, also the capacity to synchronise with your biological clock, including the central as well as the peripheral tissue regulated clocks, is an essential driver for health[13]. These central drivers of health are interconnected to the different health domains represented by the organs and/or tissues where beneficial physiological effects from food and nutrition in the maintenance and optimisation of health can be demonstrated. Per health domain, different physiological processes, outcomes or
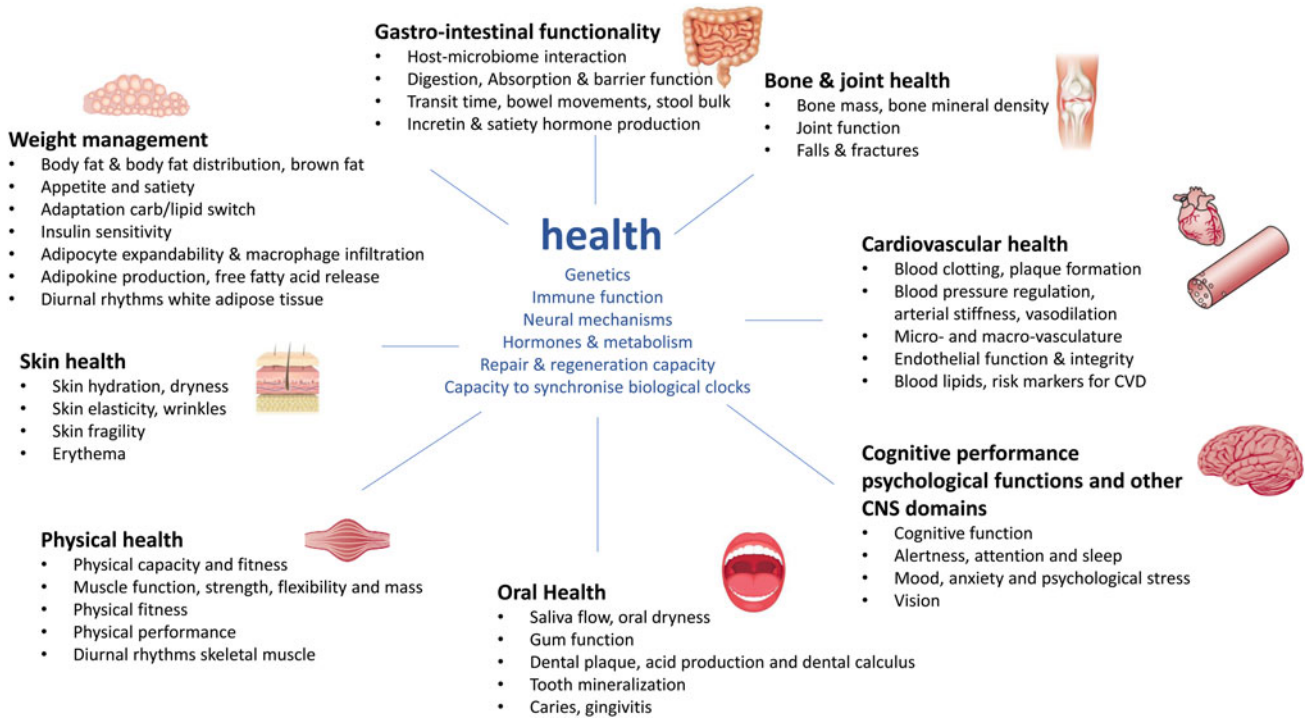
Fig. 1. Overview of drivers of health within the context of phenotypic flexibility and diet-related health. The central drivers of health and health maintenance that are important for the capacity to adapt to daily stressors are depicted in blue[12]. These central drivers are surrounded by the different health domains represented by the body organs and tissues where food–diet and nutrition can have a physiological benefit for health maintenance and optimisation[10].

endpoints have been indicated where many of the nutritional randomised controlled trials focus on[10].

Types of challenge tests used in nutrition and health research

Depending on the health domain and organs and/or tissues involved, as well as on where the health benefit of the food–diet or nutrient is to be expected different types of challenge tests could be used that actively perturb those aspects of the physiology and could serve as a way to demonstrate health improvement in dietary intervention studies. Table 1 overviews the different types of perturbation tests that have been used in nutrition and health research and overviews for what health domains such challenge test could be used. The oral glucose tolerance test and mixed-meal challenge tests (MMCT) have a broad application area modulating some of the key generic drivers of health such as hormones and metabolism, repair and regeneration capacity, immune function and neural mechanisms as well as some specific health domains such as weight management, gastro-intestinal functionality, physical health and cardiovascular health. Both types of challenge tests are evaluating a postprandial or anabolic setting also reflecting how the body deals with the ingestion of meals. Within nutrition research, postprandial studies appear to be an excellent strategy to further determine *nutritional phenotypic flexibility*, where postprandial dynamics relates to the individual's capacity to

handle with the metabolic switch from fasted to fed state[14]. In healthy conditions, the human body orchestrates complex physiological responses in order to manage nutrients from a meal efficiently while avoiding excessive metabolic shifts that would disturb homeostasis[14]. This regular *nutritional phenotypic flexibility* function may be impaired given that the human body functions in postprandial conditions for most of the day. This reduced capacity to handle meals appears to explain the increasing prevalence of obesity and NCD[14]. This is in contrast to a prolonged fasting test, where catabolic metabolism can be studied. This may be one of the simplest metabolic challenges that can be performed in human subjects that affects lipid, protein and carbohydrate handling, connected with the core physiological need to maintain blood glucose levels within the normal range but now from a catabolic perspective[15]. More recently, Ramadan as a model for prolonged fasting was used to study the effects of nutrition focusing on satiety and bowel function[16]. At this stage however, it is unclear how differences in the response to prolonged fasting could be linked to obesity and lifestyle-related diseases in Western societies. Although the use of meal-based or postprandial challenge tests is well established in clinical nutrition research, the compositions and protocols for such postprandial challenge tests remain variable and require further standardisation[17]. The other types of perturbation tests identified seem to have a more focused application area and include the oral lipid tolerance test, diverse immune challenge tests

Table 1. Overview of different types of perturbation tests and what health domains can be modulated by the test used within nutrition and health research

Challenge test and primary function	Health domain	References
Oral glucose tolerance test; glucose metabolism	*Hormones and metabolism*; anabolic carbohydrate metabolism *Weight management*; insulin sensitivity and adaptation carbohydrate/lipid switch and adipokine production, free fatty acid release *Gastro-intestinal functionality*; incretin production *Repair and regeneration capacity*; oxidative, nitrosative and ER stress, muscle injury control, DNA replication (PBMC gene expression) *Physical health*; muscle function (proteolysis) *Cardiovascular health*; blood pressure regulation *Neural mechanisms*; neurotransmitters *Immune function*; PBMC gene expression related to immune system *Capacity to synchronize biological clocks*	[19,20,60]
Oral lipid tolerance test; fat metabolism	*Weight management*; adaptation carbohydrate/lipid switch and adipokine production, free fatty acid release *Gastro-intestinal functionality*; incretin production *Repair and regeneration capacity*; oxidative and nitrosative stress *Cardiovascular health*; blood pressure regulation, endothelial function and integrity, PBMC lipid handling pathways *Immune function*; PBMC expression of inflammation related pathways	[20,61,62]
Mixed meal tolerance test; glucose, fat and protein metabolism	*Hormones and metabolism*; anabolic carbohydrate, fat and protein metabolism *Weight management*; insulin sensitivity and adaptation carbohydrate/lipid switch and adipokine, free fatty acid release *Gastro-intestinal functionality*; incretin and satiety hormone production, transit time, satiety *Repair and regeneration capacity*; oxidative and ER stress, organ and/or tissue injury control (e.g. liver, muscle) *Cardiovascular health*; blood pressure regulation, endothelial function and integrity *Neural mechanisms*; neurotransmitters *Immune function*; inflammation *Capacity to synchronize biological clocks*	[20,49,56,60]
Prolonged fasting (i.e. 36 h of fasting, Ramadan as fasting model); catabolism	*Hormones and metabolism*; catabolic energy metabolism *Weight management*; insulin sensitivity and adaptation carb/lipid switch and adipokine and free fatty acid release *Gastro-intestinal functionality*; satiety, bowel function *Repair and regeneration capacity*; oxidative and ER stress *Physical health*; muscle function (proteolysis)	[15,16]
E. coli, vaccination, endotoxin (LPS), Rhinovirus challenge; immunity, inflammation	*Immune function*; innate and adaptive immune responses, immune cell modulation *Resistance to infection*; infection induced symptoms, such as diarrhoea, gastrointestinal complaints	[31,34,40,63,64]
VO$_2$ max challenge, endurance challenge Physical fitness and cardiovascular function	*Physical health*; physical capacity and fitness, muscle function, physical performance *Immune function*; inflammation *Repair and regeneration capacity*; oxidative stress, muscle injury control *Gut barrier*; intestinal barrier and gut permeability *Immune function*; innate and adaptive immune responses, immune cell modulation *Cognitive performance*; post exercise executive functioning, such as decision making and short term memory	[25,27,65,66]
Cold pressure test; hormonal stress response	*Cardiovascular health*; blood pressure regulation, arterial stiffness, vasodilation *Physical health*; exercise pressor response/metaboreflex *Neural mechanisms*	[28,55]

ER, endoplasmic reticulum; LPS, lipopolysaccharides; PBMC, peripheral blood mononuclear cell.

where the response to vaccination, lipopolysaccharides that are membrane molecules from Gram-negative bacteria or weakened bacterial and/or viruses are being evaluated, as well as the exercise challenge such as the VO$_2$ max test and cold stress test.

Dynamic phenotyping

For most of the perturbation tests that are commonly used within nutrition and health research (Table 1), in particular for the catabolic or postprandial challenge tests, time

course studies have been performed where blood sample collection was done before and at several time points after start of the challenge test. In these studies, blood samples have often been assessed using different types of analysis platforms, such as clinical chemistry, proteomics, metabolomics and transcriptomics measurements, to learn about health and health-related processes reflected by the effect of the challenge tests on the physiology, allowing for dynamic phenotyping[14,18–20]. Depending on the type of the challenge test different aspects of physiology are being modulated as well as the timing and dynamics (i.e. amplitude and duration) of the physiological response. Fig 2 show the dynamic physiological response of an MMCT and how distinct clusters of time responses can be identified on the basis of a total of 132 different biomarkers (metabolites, proteins and clinical chemistry measurements) that have been analysed within blood samples of twenty healthy volunteers as an example of how different aspects of physiology can be modulated over a time-frame of 8 h[21]. Different health conditions/states, i.e. healthy v. diseased, young v. old, lean v. obese, can be compared to learn on how dynamics of physiology change and are being affected by the standardised perturbation. It provides insight in how the physiological capacity to handle such a perturbation test may reach its boundaries, and how activated compensatory mechanisms may cause physiological damages resulting in insulin resistance, plaque formation and low-grade inflammation towards the development of cardiometabolic diseases[8,14,21,22]. For efficacy testing of the health benefits from food and nutrition, the idea is that this challenge test response dynamics will show an improved physiological capacity to deal with such perturbation test with reduced activation of compensatory mechanisms[10].

The added value of using challenge test in detecting and substantiation of effects from food and nutrition

The reason of why the research about phenotypic flexibility as a measurement of health started was based on the idea that it could help defining a new generation of health-focused biomarkers rather than the mainstream disease-focused biomarkers[23]. Table 2 provides an overview of literature and the conclusions deriving from the application of phenotypic flexibility evaluation, where the focus was on medium-term health benefit assessment (interventions ranging from 1 to 12 weeks). The focus was on health benefit substantiation via randomised controlled trials within the healthy range of the population including persons with overweight and/or obesity but not studies focusing on a diseased population using medication(s). Currently, the MMCT and the entero-toxigenic *Escherichia coli (E. coli)* (ETEC) challenge are most commonly used in nutrition research for the evaluation of nutritional benefits. Surprisingly, only one intervention study used an oral glucose tolerance test[19] as well as only one study used an oral lipid tolerance test[24] for detection of nutritional benefits. Most of the nutritional intervention studies including an exercise-based challenge test are being conducted in the area of sports

nutrition and focus on specific subpopulations, or only evaluate the acute effects of supplements on exercise performance and were therefore excluded from the overview. Four exercise-based challenge studies were identified where the application to a broader population or a broader health domain was being considered[25–28]. One study demonstrated an increased exercise capacity with 4 weeks of β-alanine supplementation in a middle-aged population[25]. Interestingly, besides the increased exercise capacity, also an improved cognitive performance was observed in the middle-aged participants that were supplemented with β-alanine immediately after the exercise challenge[25]. Another study successfully demonstrated attenuated blood pressure, arterial stiffness and wave reflection the latter two indicators of vascular ageing[29] in particular when the exercise challenge was combined with a cold pressure test in overweight and obese male volunteers[28]. Two other studies used an exercise challenge to test health effects from the nutritional interventions on the capacity to control for post-exercise-induced inflammatory response[26,27], where one study demonstrated this nutritional immune modulation[26] and the other study did not[27].

The immune system is crucial for health and resistance to infections. Food, diet and nutrition are one of the major factors allowing for the modulation of immunocompetence. In a thorough review focusing on markers to measure immunomodulation in human intervention studies, vaccine-specific serum antibody production, vaccine-specific or total secretory IgA production in saliva were amongst the biomarkers classified with high suitability[30]. However, to date examples using vaccine-based challenge tests to evaluate nutritional immunomodulation were only applied in studies conducted between 2003 and 2006 with no recent intervention studies that included vaccine-based challenge tests for health benefit substantiation for food and nutrition[31,32]. Indeed, the authors of the study published in 2006 concluded that demonstrating immune function improvement in a relatively healthy population with optimal nutritional status and immunity is probably limited[31]. In a follow-up review, providing guidance in biomarkers for the evaluation of immune modulation by nutrition further refined immunomodulation by nutrition into two distinct immune system functions applicable to the general public, which are (1) defence against pathogens and (2) control of low-grade (metabolic) inflammation, where for the first category vaccination challenges were again identified as very useful and for the second category pro-inflammatory (metabolic) challenges with the evaluation of a panel of cytokines, although clinical relevance for such an approach should still be established[33].

In the immunomodulation category 'defence against pathogens' the Rhinovirus and ETEC challenge approaches are quite interesting. One study successfully demonstrated that the probiotic used had a modest local nasal effect attenuating the host inflammatory reaction and virus shedding shown by using the Rhinovirus infection as challenge[34]. The studies that included the ETEC challenge test all had a very similar (and almost standardised approach) allowing for the comparison of
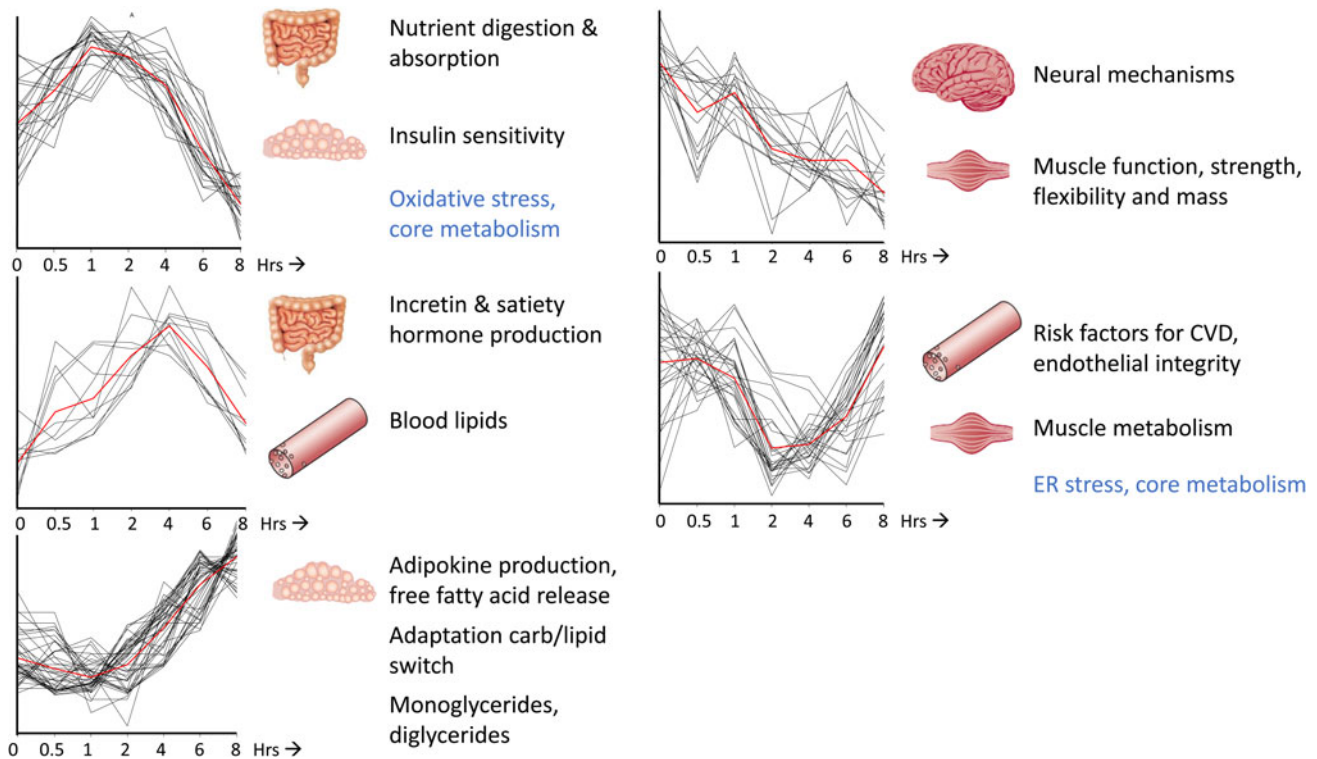
Fig. 2. Time-resolved analysis of biomarkers in response to challenge tests allows for dynamic phenotyping. A mixed-meal challenge test over a time course of 8 h within healthy volunteers resulted in five distinct time-resolved profiles that could be linked to different biological processes important for health within the context of diet-related health. The red line represents the average cluster time profile. The x-axes are expressed as time (in hrs), the y-axes are expressed as relatively scaled concentrations of a total of 132 metabolites, proteins and clinical chemistry concentrations with a significant effect in time upon a mixed-meal challenge test. The processes depicted in blue represent the central drivers of health and health maintenance, whereas the processes in black represent tissue-related processes. This figure is adapted from Wopereis et al.[21].

the impact of several nutrients to increase resistance against *E. coli* infection. The ETEC challenge test studies used a live-attenuated *E. coli* strain (E1392/75-2A) that induces mild, self-limiting diarrhoea, as well as mild gastrointestinal symptoms, which do not require antibiotic treatment[35–39]. The ETEC challenge was used in nutritional intervention studies with dairy milk fat globular membrane[37], dairy calcium-phosphate[35], whey protein concentrate[38], single probiotic[39] and multi probiotic mix[36] compared to a placebo on outcomes such as 24 h faecal wet and dry weight, diarrhoeagenic *E. coli* excretion, self-reported stool frequency and consistency, gastrointestinal complaints and immune responses. This ETEC challenge model successfully demonstrated that dairy calcium-phosphate[35] and milk fat globular membrane[37], but not whey protein concentrate[38] nor the tested probiotics[36,39] could increase resistance to *E. coli*. Only outcomes related to faecal consistency and gastrointestinal complaints were impacted in these interventions, not the immunomodulation-related outputs[35,36]. Recently, a refined and expanded ETEC challenge protocol has been suggested with addition of a second inoculation to study the protective response induced by the primary infection[40].

In contrast to the ETEC challenge tests, the intervention studies that used an MMCT do not yet have a standardised protocol. In three out of the five randomised controlled trial studies from Table 2 the same proposed standardised PhenFlex based liquid MMCT challenge test was used[41–43], which contains 75 g of glucose, 60 g of fat and 20 g of protein[20,21], in contrast to the two other studies that had respectively 52, 46 and 21 g[18] or 121, 57 and 27 g of carbs, fat and protein in the MMCT[44]. Also in terms of statistical evaluation different approaches can be applied, ranging from univariate statistics towards more advanced multivariate approaches, where biological interpretation remains challenging[45]. Recently a computational mixed-meal model was proposed for biological interpretation of the systemic interplay between TAG, free fatty acids, glucose and insulin[46]. Interestingly, it was demonstrated that the outcomes of this computational mixed-meal model was independent of the MMCT macronutrient composition[46]. The application of MMCT in nutrition research is gaining popularity as they allow for a broader probe of the nutritional phenotype, but also allowing for insulin resistance evaluation similar as to an oral glucose tolerance test as well as for evaluation of the clinically relevant non-fasting TAG levels[17]. Recently, the MMCT was validated against a standard 2 h oral glucose tolerance test and resulted in nearly equivalent insulin resistance determinations with similar precision and also

Table 2. Overview of the different types of challenge tests used in nutritional randomised controlled trial studies and what the beneficial health effect was reported

Challenge test	Intervention	Effect	Reference(s)
Mixed-meal tolerance test	5 weeks dietary mixture (resveratrol, tomato extract, n-3 PUFA, green tea extract, α-tocopherol, vitamin C) v. placebo in 36 overweight healthy male with mildly elevated CRP levels; cross-over design	Modulated amino acid and endocrine metabolism, oxidative stress and inflammation. Challenge test response provided additional metabolic changes as a result of the intervention not observed in non-perturbed conditions. Based on plasma metabolic profiling (n 145), clinical chemistry (n 7) and protein profiling (n 79)	[18,47]
	12 weeks of whole grain v. refined wheat provided in 25 overweight and obese participants per arm with mildly elevated total cholesterol levels; parallel design	Beneficial effects of whole grain wheat on inflammatory resilience and liver health, but not metabolism based on evaluation on challenge test response, not observed in fasting conditions. Effects could be linked to improved hepatic fat contents (MRI). Based on clinical chemistry (n 11) and protein profiling (n 16)	[41,67]
	12 weeks of 20 % energy restriction v. weight maintenance in 35 overweight adults per arm, parallel design	Beneficial effects of energy restriction were observed in plasma metabolome (about 300 metabolites) only in persons with reduced phenotypic flexibility. Intervention effects could be linked to body fat distribution (MRI). Postprandial time courses of PBMC gene expression showed inconsistent results	[19,43]
	12 weeks of 25 % energy restriction; one arm with low nutrient quality (n 40), one arm with high nutrient quality (n 40) v. control (maintained = 30, maintenance of habitual dietary intake) in abdominal obese participants, parallel design	High nutrient quality diet reduced more weight (+2·1 kg) as compared to low nutrient quality diet. Postprandial glucose and insulin responses are correlated to postprandial adipose gene expressions and unravelled that the high nutrient quality diet induced additional weight loss only in insulin-sensitive persons by attenuation of postprandial adipose tissue gene expression for lipogenesis. Postprandial evaluation was based on clinical chemistry (n 4), plasma metabolome (n 153) and adipose transcriptome	[42]
	8 weeks weight loss (2·1 MJ/daily for 4–5 weeks followed by MJ/daily) or no weight loss in 29 abdominally obese men. Postprandial responses were compared to postprandial responses of lean persons as a 'healthy' reference. Parallel design	Modest weight loss-induced effect on postprandial plasma metabolome and PBMC gene expression level. Postprandial metabolome analysis revealed differences in oxylipins. Postprandial gene expression changes induced by the weight loss intervention-affected pathways of oxidative phosphorylation and electron transport chain. It was concluded that postprandial assessment was not necessarily superior to fasting metabolic measurements	[44]
Oral lipid tolerance test	4 weeks of dark chocolate enriched in flavanol or with normal flavanol consumption in 29 healthy overweight middle-aged men. Cross-over design	No effects in the endothelial capacity to respond to a high-fat mono-unsaturated fatty acid challenge test as a result of differences in the flavanol concentration of dark chocolate, which was in agreement to results in fasting conditions. Based on plasma biomarkers of endothelial function, leucocyte counts and measurements of vascular function	[24]

OGTT	12 weeks of 20% energy restriction v. weight maintenance on improved dietary quality in 35 overweight adults per study arm. Parallel design	Upon energy restriction the PBMC gene expression in response to an OGTT resulted in a faster and more pronounced down-regulation of pathways related to oxidative phosphorylation, cell adhesion and DNA replication as compared to control	[19]
ETEC challenge At 2 weeks participants were subjected to ETEC challenge unless otherwise stated. In all studies comparison was made to placebo in a parallel study design	4 weeks of dietary milk-fat-globule membrane (MFGM) in n 30 healthy adults	At day 2 after ETEC challenge, stool frequency and ETEC-induced diarrhoea was lower in the MFGM group as compared to control	[37]
	3 weeks of calcium by providing 32 healthy men regular milk products (1100 mg/d Ca). At day 10 ETEC challenge was performed	At day 2 after ETEC challenge, ETEC-induced diarrhoea and faecal mucin excretion was fully normalised in calcium group, whereas in control these endpoints were normalised on day 3	[35]
	4 weeks of whey protein concentrate in 2 doses (low and high whey protein) with 35–38 healthy adults included per arm	No effects were found	[38]
	4 weeks of Lactobacillus acidophilus supplementation with n 20 healthy males per arm and 4 weeks of multi probiotic mix (Lactobacillus helveticus Rosell-52, Lactobacillus rhamnosus Rosell-11, Bifidobacterium longum ssp longum Rosell-175, Saccharomyces cerevisiae var boulardii CNCM I-1079) in n 30 healthy male per arm	Both probiotic treatments were associated with larger rather than lower Bristol stool scores and more rather than less GI complaints	[36,39]
Rhinovirus challenge	4 weeks of probiotic Bifidobacterium animalis subspecies lactis Bl-04 (n 73) on innate and adaptive host responses to Rhinovirus challenge (RV-A39) on n 152 seronegative healthy adult volunteers v. placebo (n 79). At day 28 the Rhinovirus challenge was provided and innate and adaptive immune responses were followed on 5 consecutive study days post challenge; parallel design	Significantly reduced CXCL8 response as well as CXCL10, IL-6 and GCSF, viral titres, exhaled nitric oxide in nasal lavage to Rhinovirus infection in probiotic treated volunteers. No difference in IL-1B and CCL2 nasal lavage concentrations nor respiratory symptom score or infection rate	[34]
Vaccine challenges	10 weeks of (a) bovine colostrum concentrate; (b) micronutrients mix of vitamin E, C, β-carotene, zinc; (c) combination of micronutrient mix with bovine colostrum and (d) placebo on immune health in n 31–35 healthy participants (40–80 years) per arm. Participants underwent at 6 weeks a systemic tetanus and an oral typhoid vaccination. Parallel design	Although the micronutrient mix significantly enhances the DTH responses, no effect was observed in response to the two vaccine challenges conducted	[31]
	12 weeks of (a) 50 % c9,t11 and 50 % t10,c12 conjugated linoleic acid (CLA); (b) 80 % c9,t11 CLA and 20 % t10,c12 CLA; and (c) sunflower-derived fatty acids (control) on immune health in n 22–25 healthy male participants (31–69 years) per arm. Subjects were vaccinated to hepatitis B on days 43, 57 and 71. The intervention arms evaluated concentrations of hepatitis B-specific antibodies and lymphocyte proliferation induced by hepatitis B antigen to determine elicited humoral and cellular responses, besides other aspects of immune function such as DTH response, NK cell activity, production of different cytokines. Parallel design	Although the overall effect in response to the vaccine challenge did not reach statistical significance, twice as many participants on CLA 50:50 reached protective antibody titres following hepatitis B vaccination as compared to control. Also specific lymphocyte proliferation was highest in this group, suggesting that CLA 50:50 may have a biologically relevant enhancing effect on the response to hepatitis B vaccination. No effects were found on the other immune-related parameters	[32]

Table 2. (*Cont.*)

Challenge test	Intervention	Effect	Reference(s)
Exercise-based challenge test	4 weeks of 2·4 g/d β-alanine (BA; n 7) *v.* placebo (microcrystalline; n 5) on exercise capacity in healthy adults >50 years of age. Exercise capacity was tested via bouts on a cycle ergometer at 70 % VO$_2$ peak. Executive function to cognitive function was done before and at 3 time points after the exercise test by means of the Stroop test. Before and after exercise lactate measurements were done as well as continuous heart rate, rating of perceived exertion and VO$_2$. Parallel design	BA supplementation increased exercise capacity as shown by the extended time-to-exhaustion on the exercise test, under conditions of unchanged lactate production, which indicates that BA supplementation enables to extend exercise durations. Furthermore, BA eliminated the endurance exercise challenge-induced declines in executive function seen after recovery	[25]
	1 week of curcumin and piperine supplementation *v.* placebo (cellulose) on exercise performance, exercise-induced inflammatory response and muscle damage in n 16 trained male runners. At the end of the intervention an exercise-based challenge was performed based on a treadmill running training session, where before, directly after and 1 h after the treadmill running session blood was sampled. Cross-over design	Supplementation with curcumin and piperine inhibited the exercise-induced elevation of plasma levels of multiple cytokines (IL-2, TNF-α, interferon, IL-6, IL-10). Performance, immune cell counts and muscle damage were unchanged due to the supplementation	[26]
	A 10 d intervention study on supplementation with bovine colostrum *v.* skim-milk powder (placebo) on exercise impaired immune function in n 9 male athletes. To increase stress on the immune system participants performed a glycogen-depletion trail the evening before the exercise challenge in the form of an endurance trial (90 min at 50 % W_{max}). Blood samples were taken before glycogen-depletion, before and directly after exercise challenge and 22 h after cessation of the exercise challenge. Cross-over design	No differences were observed between colostrum as compared to placebo in plasma cortisol levels, neutrophil count, circulating immunoglobulins, multiple interleukins (IL-6, IL-10, IL-1-receptor agonist, CRP, interferon-ϒ, IL-1α, IL-8, TNF-α), although the exercise protocol affects several of these quantified variables of the immune system	[27]
Combined exercise-based challenge test with cold pressure challenge	A 14 d intervention study on supplementation with L-citrulline (6 g/d) *v.* placebo in n 16 healthy overweight or obese males. After 14 d a combined exercise challenge (isometric handgrip exercise with metaboreflex activation resulting in postexercise ischaemia) with a cold pressure test to stimulate excessive SBP reactivity through increased sympathetic activity to evaluate vascular reactivity before the combined challenge test, during exercise, post-exercise and post-exercise and cold-pressure challenge. Cross-over design	No effects from the citrulline supplementation were observed at rest. During exercise attenuated increases in SBP and wave reflection were observed. Post-exercise attenuated increases in DBP, MBP and wave reflection, whereas the combined post-exercise and cold-pressure challenge showed attenuated SBP, DBP, MBP, wave reflection from aortic as well as brachial-ankle pulse velocity. In particular the combined challenge test conditions demonstrated that citrulline supplementation attenuates the exaggerated blood pressure, wave reflection and arterial stiffness response, suggesting that citrulline has a protective effect against increased cardiac overload during physical stress	[28]

BA, β-alanine; CCL2, C-C motif chemokine ligand 2; CLA, conjugated linoleic acid; CRP, C-reactive protein; CXCL, chemokine ligand; DBP, diastolic blood pressure; DTH, delayed-type hypersensitivity; ETEC, entero-toxigenic *Escherichia coli*; GCSF, granulocyte colony-stimulating factor; GI, gastrointestinal; MBP, mean blood pressure; MFGM, milk-fat-globule membrane; MJ, megajoules; NK cell, natural killer cell; PBMC, peripheral blood mononuclear cell; OGTT, oral glucose tolerance test; RCT, randomised controlled trial; SBP, systolic blood pressure; TNF-α, tumour necrosis factor α; VO$_2$, oxygen volume; W_{max}, maximal work capacity.

demonstrated the added value for postprandial TAG response evaluation by identifying a large proportion of the population within the healthy range with clinically increased levels of TAG not shown for overnight fasting concentrations[17]. The response to the MMCT was successfully modulated by an intervention with a multi-ingredient mixture focusing on control of low-grade (metabolic) inflammation[18,47]. Shifts in MMCT response before and after the whole grain wheat v. refined wheat intervention were evaluated with a health space model[48] against reference groups representing the extremes within the healthy range of the population, allowing for identifying a beneficial effect on inflammatory resilience and liver health, but not on metabolism[41]. Therefore, it seems plausible that MMCT could also be used for evaluation of the capacity to control for low-grade (metabolic) inflammation[49]. The MMCT has been applied multiple times to evaluate the effect of energy restriction[42–44] as being the 'gold standard intervention' for reaching beneficial metabolic health effects and postprandial measurements could indeed demonstrate metabolic benefits in all three studies, although the findings were not always clear or in the expected direction[42–44]. Together, the nutritional intervention studies evaluated that used phenotypic flexibility methodology indeed indicates that there may be a benefit of adding a challenge test in the study design to evaluate health benefits, although it is important to consider the type of challenge test, the type, timing and amplitude for the dynamic read-outs, as well as for the evaluation of the efficacy of a nutritional intervention. This may eventually even lead to a new generation of biomarkers that could be used for health claim substantiation by the European Food Safety Authority, since the scientific committee has accepted phenotypic flexibility or resilience evaluation as a way of demonstrating efficacy of food and nutrition[50,51]. However, before the resilience concept can be used for the authorisation of health claims, it is key to deliver the scientific and clinical argument for quantification and validation of models which reflect this ability to adapt. European Food Safety Authority's experts can only critically review scientific opinions when sufficient well-designed and executed studies are supporting the putative relationship between the consumption of a food or ingredient and their suggested health effects[52]. To date a nutritional health claim was not yet approved (nor declined) based on challenge test-based findings within nutritional intervention studies.

From subtypes, metabotypes, responders and non-responders towards personalised nutrition

In a nutritional intervention study that included an MMCT and focused on demonstrating the health benefits from weight loss it becomes clear that there are responders and non-responders for health benefit evaluation identified in so-called metabotypes that indicate that health benefits are achievable for persons with reduced flexibility, but not for persons who have a large degree of metabolic flexibility at baseline[43].

Interestingly, the degree of weight loss was greater in persons with higher insulin sensitivity, showing fully remitted lipogenesis of white adipose gene transcription, but only on an energy-restricted diet of high nutritional quality[42]. Therefore, for the substantiation of health effects from food and nutrition you may want to aim to include volunteers at the edge of health derailment with 'pre-pre-diabetes' and stratify for inclusion of volunteers at risk of developing insulin resistance[53,54] for demonstrating health effects[42].

Measuring the ability to adapt may also open avenues towards more tailored or personalised nutrition. The authors from a multiple challenge test study to reveal the dynamic range within $n = 15$ very healthy young male Caucasian volunteers refer to this as the 'accordion effect' where interindividual variation can be extended and compressed by the application of a series of metabolic challenge tests[55]. Therefore, the assessment of phenotypic flexibility can be a nice starting point for personalised nutrition also confirmed by the PREDICT landmark study, where the focus was on revealing inter- v. intra-individual variability by using a standardised MMCT in the form of blue muffins provided multiple times to 1002 UK participants including monozygotic and dizygotic twins[56]. Large interindividual variability was observed in postprandial TAG, glucose and insulin concentrations where different underlying factors contributed to this variation. In the meanwhile, the first personalised nutrition intervention study was published that started with postprandial measurements focusing on postprandial glucose, insulin and TAG in combination with anthropometrics, a selected panel of SNP and lifestyle-related questionnaire-based data conducted within the healthy range of the population[57]. In this study, eighty-two healthy male and female from a workforce were extensively phenotyped based on fasting as well as postprandial TAG, insulin and glucose measurements complemented with some SNP with evidence-based gene–nutrient interactions, anthropometrics and behaviour data at baseline. During a 10-week intervention these participants were provided with personalised breakfast and lunches during the workweek as well as engaged with a portal with access to personalised recipes. The personalised algorithms tailored biomarkers with unhealthy values towards substantiated health claims or evidence-based nutritional information from dietary guidelines. The study shows that a personalised system nutrition program promotes lifestyle habits and worker health by reducing body weight and other health-related outcomes[57].

Conclusions and outlook

In conclusion, the methodology focusing on phenotypic flexibility within nutrition research is promising. Fig 3 overviews the potential of phenotypic flexibility methodology for public health. It is increasingly recognised that implementation of health-promoting behaviours as early in life as possible has the most significant impact across the maximal healthspan[58]. Physiological resilience is a
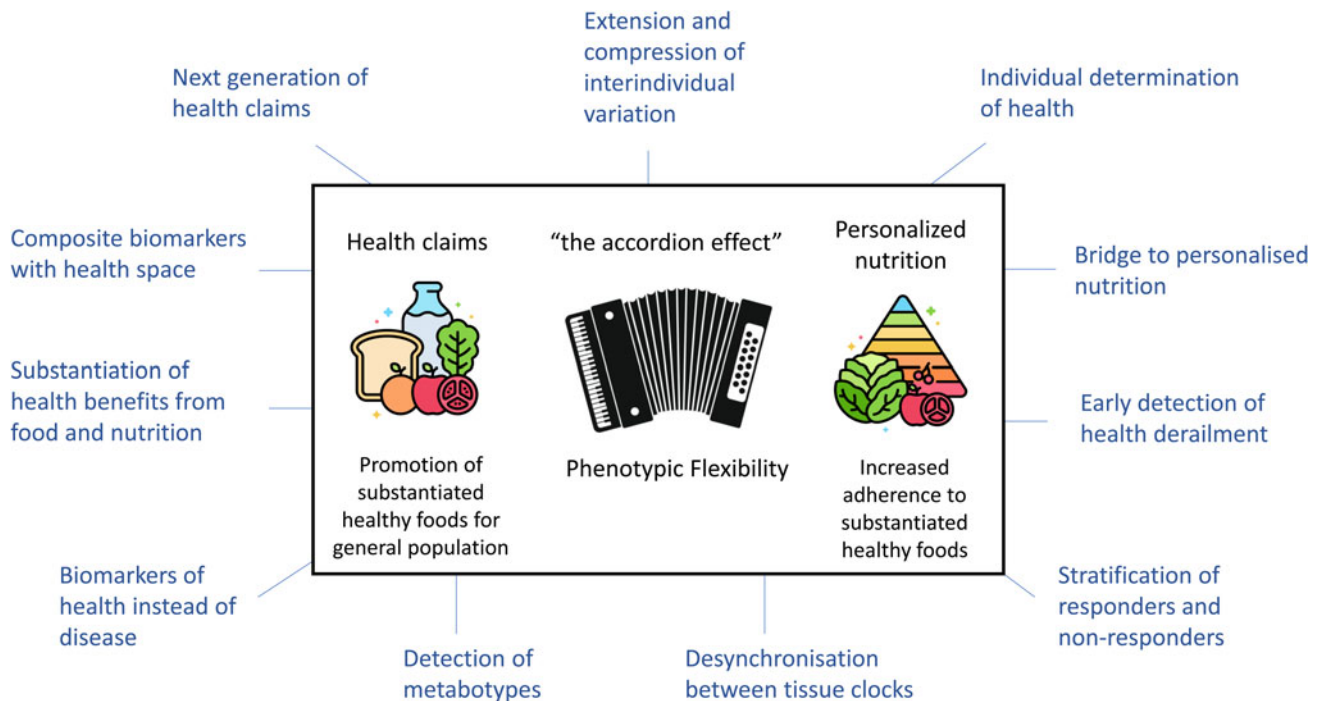
Fig. 3. The potential of phenotypic flexibility for public health. Phenotypic flexibility can extend and compress interindividual variation, also referred to as the accordion effect. On the one side phenotypic flexibility may help for the scientific substantiation of healthy food and nutrition. On the other side phenotypic flexibility may help in the area of personalised nutrition, thereby increasing adherence of individuals to substantiated healthy foods, for example, found in regulatory guidelines. The figure is surrounded by other aspects of phenotypic flexibility that can contribute to nutritional health research. Adapted from Griffiths et al.[58].

very important aspect to maintain health where food, diet and nutrition play a major role to the daily exposures and perturbations of life, but at the same time also offering ways to restore and optimise this coping capacity. The assessment of phenotypic flexibility can contribute in the quantification of this (individual) resilience and how diet, food and nutrition impact our personal coping system by extending and compressing interindividual variation. It may on the one side deliver a new generation of health biomarkers that can help to substantiate benefits from food, diet and nutrition for the promotion of healthy foods for the general population. On the other side it can help in the area of personalised nutrition where increased adherence to evidence-based nutrition and dietary guidelines to the public by providing insight on what is good for individuals considering their personal health and coping system. With the rapid development of digital wearables allowing for passive and continuous measurements in a real-life context of how the body is coping with daily insults, exposures and perturbations will provide even more awareness of personal biological dynamics of resilience, allowing for health maintenance, health optimisation and NCD prevention strategies based on concepts of phenotypic flexibility[59].

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