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Novel insights into the health effects of fruits and vegetables

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Novel insights into the health effects of fruits and vegetables

Challenging the status quo

Hidde van Steenwijk

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Hidde P. van Steenwijk, PharmD

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Novel insights into the health effects of fruits and vegetables

Challenging the status quo

PROEFSCHRIFT

Voor het behalen van de graad van Doctor aan de Universiteit Maastricht,

in opdracht van de Rector Magnificus, prof.dr. Pamela Habibović,

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General introduction

According to the World Health Organization (WHO), non-communicable diseases (NCDs) are the leading cause of death worldwide, accounting for 71% of total deaths each year [1]. Since there are still no optimal treatments or miracle cures for these diseases, more research into the prevention of these conditions is paramount [2]. Unhealthy diets may play a role in the development of NCDs and healthy dietary changes have been suggested to be helpful in preventing such conditions, in particular in NCDs in which inflammation plays a role [3–11]. However, demonstrating causal relationships between nutritional interventions and a reduction in the onset of NCDs in the general population remains difficult through classical methodologies [8,11,20–22,12–19]. Therefore, novel approaches are needed to demonstrate the health-promoting effects of nutrition and potentially support scientifically substantiated health claims to communicate these effects to consumers. The research described in this thesis is therefore aimed at developing an approach to assess the subtle and pleiotropic health effects of fruits and vegetables already in healthy people, in order to better interpret their role in the prevention of inflammation related NCDs.

NCDs are chronic medical conditions associated with a long duration and slow deterioration of health [2]. More than 15 million of these deaths involve relatively young people between the ages of 30 and 69 [1]. The four major NCDs with the highest number of deaths are cardiovascular diseases (CVD) (17.9 million deaths annually), cancers (9.0 million), respiratory diseases (3.9 million), and type II diabetes (1.6 million) [1]. In addition to individuals' health loss, NCDs place a significant and growing burden on the health care system and overall global economy [23]. In the European Union, the four major NCDs claim a substantial share of the total health care budget (at least 25% of health expenditure) and cause a substantial economic loss (2% of gross domestic product) [23]. Most NCDs are non-infectious and are caused by several determinants, including genetic [24,25], physiological [2,26,27], behavioral [28,29] and environmental factors [30]. A better insight into key risk factors for the development of NCDs is essential to reduce the preventable and avoidable burden of morbidity, mortality, and disability due to NCDs – the goal of WHO Global Action Plan (2013-2030) [31].

1.1 MAIN RISK FACTORS OF NCDS

There are modifiable and nonmodifiable risk factors for developing NCDs [2]. Modifiable risk factors are unhealthy diets, the harmful use of alcohol, smoking, physical inactivity, high blood pressure, overweight, obesity, and high blood cholesterol, while the nonmodifiable risk factors include age, gender, genetic factors, race, and ethnicity [32–37]. The modifiable risk factors can be further divided into biological factors (e.g. being overweight, dyslipidemia, hyperinsulinemia, and hypertension) and behavioral factors

(e.g. unhealthy diet, lack of exercise, tobacco smoking, and alcohol consumption) [2,36]. The latter, also known as lifestyle choices, are crucial determinants in global management and prevention strategies for NCDs [2,36,37]. In order for an individual to make healthier lifestyle choices, it is essential to combine modern scientific achievements with innovative decision-making regarding positive impacts on human health [2]. The involvement of an unresolved chronic inflammatory response in the early stages of NCD development is one such discovery that is attracting much interest [3,4,38–42]. Previous research has shown that the onset and progression of many NCDs, including CVD, neurodegenerative diseases, and type II diabetes, are related to or affected by inflammation: chronic low-grade inflammation (CLGI) is central to many different symptoms from which patients suffer in these conditions [43–45]. CLGI is believed to aggravate various mechanisms that reflect poor health, including elevated blood pressure, hyperglycemia, excessive waist circumference, and abnormal cholesterol or triglyceride levels - the so-called “deadly quartet” [44]. In addition, CLGI also appears to affect apparently healthy people as a consequence of poor lifestyle choices e.g. overeating, smoking and excessive alcohol consumption [46–49].

1.2 INFLAMMATION

Inflammation is a central component of innate (non-specific) immunity, triggered by infections (bacteria, viruses) or non-infectious factors (burns, physical injury, chemicals) [3,4]. The function of inflammation is to eliminate the original cause of cell damage, remove necrotic cells and damaged tissue caused by both the injury and the inflammatory response, and initiate tissue repair. This process, clinically referred to as acute inflammation, is a critical survival mechanism used by all higher vertebrates [46]. Active resolution of acute inflammation is a complex process involving (anti-inflammatory) cytokines and other mediators, allowing inflamed tissues to return to homeostasis [3,50,51]. If left unresolved, acute inflammation can lead to chronic inflammation, which is not part of this ancestral healing process and can constitute a harmful process [52]. While the link between inflammation and NCDs is widely recognized, the question of causality and the extent to which inflammation contributes and serves as a risk factor for disease development remain unresolved [4,46,53].

1.2.1 ‘The Good’ - Acute inflammation

The inflammatory response is the coordinated activation of signaling pathways that regulate levels of inflammatory mediators in resident tissue cells and inflammatory cells recruited from the blood [54]. While response processes depend on the precise nature of the initial stimulus e.g., bacterial pathogens trigger Toll-like receptors (TLRs) and viral infections trigger type I interferons (IFN), they share a common mechanism: (1) cell

surface pattern receptors recognize detrimental stimuli; (2) inflammatory pathways are activated; (3) inflammatory markers are released; (4) inflammatory cells are recruited; which results in (5) resolution of inflamed tissues [53,54].

1.2.1.1 Pattern recognition receptor activation

The inflammatory response initiates when Pattern Recognition Receptors (PRRs) on the innate immune cells recognize Pathogen-Associated Molecular Patterns (PAMPs) or Damage-Associated Molecular Patterns (DAMPs) [55–59]. PRRs are proteins mainly expressed by dendritic cells, macrophages, monocytes, neutrophils, and epithelial cells [60,61]. PRRs can be found associated with subcellular compartments, e.g. cellular and endosomal membranes, the cytosol, but also extracellularly, in secreted forms present in the bloodstream and interstitial fluids [56,62]. PAMPs are molecules produced by different classes of microbial pathogens, but importantly, not by the host organisms [62]. The most studied examples include bacterial carbohydrates (e.g. lipopolysaccharide (LPS) [63] and mannose [64]), nucleic acids (e.g. bacterial or viral DNA or RNA [65]), bacterial peptides (flagellin [66]), peptidoglycans [67], lipoteichoic acids [68], N-formyl methionine [69], lipoproteins [70], and fungal beta-glucans [71]. PAMPs function as "molecular signatures" of microbial metabolism and their recognition by the innate immune system signals the presence of an infection [62,72]. Since they are not produced by the host organism, a key aspect of recognition is self-nonself discrimination, allowing immune responses to mount only against antigens and microbial cells [73]. A common criticism of the PAMP hypothesis is that PAMPs are not limited to pathogens but are produced by all microbes, including commensal microorganisms. The mechanisms that enable the innate immune system to distinguish between pathogens and commensals are not well understood and future elucidation of these mechanisms could lead to groundbreaking implications in the field of immunology [74]. Some PRRs also recognize various endogenous molecules that are activated during tissue or cell damage, collectively known as DAMPs [54,56,75]. In this case, the immune system is less concerned with the origin of the antigens (self-nonself) [56,75]. Endogenous molecules are released or activated during tissue stress or damage and initiate or propagate the inflammatory response, which, among other things, empower antigen-presenting cells to activate the adaptive immune response [56,75]. Recent studies have suggested that various DAMPs, e.g. high-mobility group box 1 (HMGB1), S100 proteins, and heat shock proteins, are considered to have a pathogenic role in chronic inflammatory diseases [76]. Together, these two theories advanced the idea that our body is equipped to distinguish “healthy” homeostatic tissue turnover or encounters with foreign “friendly” microorganisms, from potential “danger” that may come from pathogens and/or tissue damage [56].

1.2.1.2 Inflammatory pathway activation

Inflammatory stimuli activate intracellular signaling pathways that subsequently activate the production of inflammatory mediators [77]. Primary inflammatory stimuli, including microbial products and cytokines such as interleukin-1β (IL-1β), interleukin-6 (IL-6), and tumor necrosis factor-α (TNF-α), mediate inflammation through interaction with the TLRs, IL-1 receptor (IL-1R), IL-6 receptor (IL-6R), and the TNF receptor (TNFR) [78]. This receptor activation triggers vital intracellular signaling pathways, including the three most well-known pathways today: mitogen-activated protein kinase (MAPK), nuclear factor kappa-B (NF-κB), and Janus kinase (JAK)-signal transducer and activator of transcription (STAT) pathways [79–81].

NF-κB pathway

The transcription factor NF-κB regulates multiple aspects of innate and adaptive immune functions and serves as a crucial mediator of inflammatory responses [82]. NF-κB activity is induced by a range of stimuli including intercellular inflammatory cytokines, enzymes, and pathogen-derived substances [54,83–86]. Under physiological conditions, IκB proteins (IκB, inhibitor of NF-κB), and the kinase that phosphorylates IκB (the IκB kinase (IKK) complex) inhibit NF-κB activation [87]. Most of the signaling pathways leading to NF-κB activation converge at the IKK complex, which in turn phosphorylates IκB, resulting in degradation by the proteasome and the subsequent release of NF-κB for nuclear translocation and gene transcription activation [54,87]. This, in turn, induces the expression of various pro-inflammatory genes, including genes encoding adhesion molecules (e.g. soluble intercellular adhesion molecule-1 (sICAM-1), soluble vascular adhesion molecule-1 (sVCAM-1)), chemokines (e.g. chemokine (C-C motif) ligand 2 (CCL-2), chemokine (C-X-C motif) ligand 1 (CXCL1) and cytokines (e.g. IL-1, IL-6, TNF-α) [82,85,88,89].

MAPK pathway

Mitogen-activated protein kinase (MAPK) cascades have been shown to play a key role in the transduction of extracellular signals to cellular responses, including mitogens, heat shock, osmotic stress, and cytokines (e.g. IL-1, IL-6, TNF-α) [90–92]. The mammalian MAPKs include extracellular signal-regulated kinase (ERK), p38, and c-Jun NH (2)-terminal kinase (JNK), with each MAPK signaling pathway consisting of at least three components; a MAPK, a MAPK kinase, and a MAPK kinase kinase [91,93]. Extracellular stimuli initiate these pathways through phosphorylation and activation of MAPK kinase by MAPK kinase kinase. Subsequently, MAPK kinase phosphorylates and activates MAPK [91,94,95]. In turn, activated MAPKs phosphorylate various proteins, including transcription factors, resulting in the upregulation of inflammatory responses [91,94,95]. In general, ERKs are activated by mitogens and differentiation signals, while JNK and p38 are activated by stress and inflammatory stimuli [54,94].

JAK-STAT pathway

The JAK/STAT pathway is a highly conserved signal transduction route employed by a range of cytokines, interferons, growth factors and other related molecules to induce gene expression of extracellular factors [96–98]. This pathway provides a straightforward mechanism whereby receptor-associated JAKs are activated by ligands and phosphorylate each other, creating docking sites for STATs, which are latent cytoplasmic transcription factors. Cytoplasmic STATs recruited to these sites undergo phosphorylation and subsequent dimerization before translocation to the nucleus. In the nucleus, STATs are then dephosphorylated, leading to the activation of downstream cytokines [54,96–98].

1.2.1.3 Inflammatory markers

Inflammatory markers, such as CRP and interleukins, are used in clinical settings to differentiate normal versus pathogenic biological conditions, assess response to treatment, and have some prognostic significance for the development of NCDs [54,99–106]. Measurement of inflammatory markers can be used as a general but non-specific test for serious underlying disease [107]. Normal levels of inflammatory markers are valuable in ruling out some specific conditions, notably polymyalgia rheumatica, giant cell arteritis, myeloma, and infection of hip revisions [107]. However, slightly elevated inflammatory markers in the context of non-specific symptoms are found difficult to interpret [108]. Although changes in hematological dynamics, cytokines, acute phase proteins, and other markers are common to virtually all inflammatory conditions, individual markers have yet to be strongly associated with specific pathological events [105]. In other words, while being sensitive indicators of inflammation, these markers generally lack the specificity to identify the offending cause [99,105,107,109]. In general, the profile seen in any given inflammatory condition depends on the severity, chronicity, and mechanisms involved in the inflammatory processes, as well as the ability of the individual's immune system to respond and adapt [105]. The main classes of inflammatory markers, a description of the class, and examples of markers measured in clinical settings are described in Table 1.

Table 1. Types of inflammatory markers: classes, descriptions, and examples

Class	Description of class	Examples of markers
Cytokines	Mainly produced in immune cells during both acute and chronic inflammatory conditions [110,111]; Central in complex networks that involve both synergistic and antagonistic interactions and exhibit both negative and positive regulatory effects on various target cells [112–115]	Interleukins (ILs): IL-1ß, IL-6, IL-8, IL-10 Colony-stimulating factors (CSFs): Granulocyte-CSF Interferons (IFNs): IFN-α Transforming growth factors (TGFs): TGFα, TGFβ Tumor necrosis factors (TNFs): TNF-α Chemokines: Monocyte chemoattractant protein 1 (MCP-1)
Acute-phase proteins	Part of the innate immune response and generally involved in resolution processes [116]; Primarily measured in plasma or serum; Concentrations either increase (+)) or decrease (-) in response to inflammation [109,117–119]	C-reactive protein (CRP) (+) Serum amyloid A (+) Albumin (-) Transferrin (-) Adiponectin (-)
Prostaglandins, leukotrienes and thromboxanes	Eicosanoid lipid mediators derived from arachidonic acid; Found in most tissues and organs; Involved in numerous biological functions and inflammation [111,120–124]; Chemically unstable and rapidly metabolized, their metabolites are used as biomarkers [122]	Tetranor-prostaglandin E metabolite (tetranor-PGEM) Leukotriene E4 (LTE4) 11-dehydro-thromboxane B_2
Oxidative stress products	Associated with the pathogenesis of many NCDs; Indirect markers of the inflammatory response [125–128]	Malondialdehyde Isoprostanes

1.2.1.4 Recruitment of inflammatory cells

Acute inflammation and inflammation resolution are complex coordinated processes involving a number of cell types [129–132]. The first cells attracted to the site of injury are neutrophils, which dominate the early stages of inflammation and set stage for repair of tissue damage by macrophages [133]. Neutrophils recruit, activate and program antigen-presenting cells to activate T cells and release local mediators to attract dendritic cells and monocytes [134,135]. In addition, neutrophils generate signals to determine whether macrophages differentiate into a pro- or anti-inflammatory state and are essential to wound healing and microbial sterilization [134,135]. During inflammation, monocytes migrate from the blood to various tissues and transform into macrophages [136]. At the site of injury, macrophages play a critical role in initiating, maintaining, and resolving inflammation by performing three main functions: phagocytosis, antigen presentation, and immunomodulation through the production of growth factors and cytokines [136]. Mast cells are known for their role in allergic and anaphylactic reactions, as well as their involvement in acquired and innate immunity [137–141]. Inflammatory signals induce mast

cell degranulation and the release of cytokines, chemokines, and vasoactive mediators (e.g. histamine), which enhance vascular permeability and leukocyte recruitment [138,142,143]. Platelets are no longer viewed simply as hemostasis regulators but are now recognized as crucial in coordinating inflammatory and immune responses [144,145]. Platelets contain inflammatory peptide and protein mediators, some of which retain the ability to synthesize *de novo*, while others are stored and secreted by granules [146,147]. The release of these cytokines and chemokines, as well as eicosanoids, upon activation enables platelets to recruit leukocytes to the site of inflammation or injury [148,149].

1.2.1.5 Resolution of inflammation

Previously considered to be passive, resolution of inflammation has now been shown to involve active metabolic and biochemical processes that enable inflamed tissues to return to homeostasis [50,150]. In recent years, biomedical research efforts have focused on discovering new strategies to promote the resolution of inflammation [151]. Since these processes and the mechanisms of action of these lipid mediators appears to be of physiological relevance to the resolution of inflammation, therapeutic approaches targeting this system are likely to have fewer undesirable side effects as they act as agonists, in contrast to the inhibitor approach currently being used in anti-inflammatory therapies [150,151].

1.2.2 'The Bad' – Chronic high-grade inflammation

In contrast to acute inflammation, long-term or chronic inflammation, involves a progressive shift in the type of cells present at the site of inflammation and simultaneous tissue destruction and healing due to the ongoing inflammatory processes [4]. Where this becomes excessive, irreversible damage to host tissues can occur, leading to the development of chronic 'high-grade' inflammatory conditions [4]. Diseases involving high grade chronic inflammation are characterized by markedly elevated levels of inflammatory biomarkers and activated immune cells at the site of injury and in the systemic circulation (e.g. rheumatoid arthritis (RA), Crohn's disease, ulcerative colitis, atopic dermatitis, psoriasis, and asthma) [4,152–155]. In general, itching, pain or aching, swelling, redness, irritation, rashes, fever, fatigue, stiffness, diarrhea, myalgia, weight loss, and shortness of breath are common clinical manifestations seen in these conditions [156–161]. There is currently no cure for these - often autoimmune - conditions, but symptoms are treated on an individual basis to induce remission [160,162]. Since the conventional treatments all have a range of side effects, many new therapies have already been developed that selectively target specific parts of the immunological pathway [110,163–166]. The introduction of drugs targeting a specific part of inflammatory pathways, e.g. TNF-α blockers Infliximab, Adalimumab, and Golimumab and IL-1 receptor antagonist Anakinra, is a revolutionary achievement, enabling long-term remission and change of RA, Crohn's disease, and ulcerative colitis course in a significant proportion of patients [166–172].

Despite its novel role in Crohn's disease and ulcerative colitis treatment, up to 40% of patients fail to respond to TNF-α inhibitors and a quarter of patients experience a secondary loss of response within a year [173]. In conclusion, specific inhibitors do not appear to be a panacea to date, so subtle, multiple target approaches could be more beneficial.

1.2.3 'The Ugly' – Chronic low-grade inflammation

Dysregulation of processes involved in the inflammatory response or failure to resolve inflammation or injury can also lead to chronic low-grade inflammation (CLGI), which is not part of the body's healing process and involved in the pathogenesis of many NCDs (e.g. type II diabetes and CVD) [4,46,53,155,174]. In the state of CLGI, a typical inflammatory stimulator or pathogen can no longer be determined, and inflammatory stimuli and pathways remain activated on the back burner [3]. In contrast to the 'high grade' inflammatory conditions described above, in CLGI overt clinical manifestations may be minimal or absent [4]. Since the same biomarkers are involved in both, these biomarkers alone cannot be used to differentiate between high-grade versus low-grade inflammatory conditions [4]. Furthermore, conditions involving CLGI, such as diabetes and cardiovascular disease, are usually not treated with anti-inflammatory drugs, although recent evidence shows that some medication (e.g. statins) may attribute their protective effects by reducing inflammation [4,5,152,175,176]. Since CLGI can also affect apparently healthy people as a result of poor lifestyle choices, a large portion of the general population is unaware that their health is slowly deteriorating [46–49]. To illustrate, asymptomatic inflammation can be present in adipose tissue as a feature of obesity [49,152]. Under overweight conditions, the adipocyte itself becomes the source of inflammation-related adipokines, although there is also some infiltration of adipose tissue by macrophages [4,177]. The adipokines and cytokines released from adipose tissue are at least partly responsible for obesity-induced insulin resistance [178]. In smokers, the low-grade inflammatory state triggered by smoking likely leads to increased serum cholesterol and triglyceride levels, impaired glucose tolerance, and decreased insulin sensitivity [179,180]. Smoking cessation significantly improves these processes and reverses biomarkers of systemic inflammation [181,182]. The pro-inflammatory effects of chronic alcohol abuse play an important role in the pathogenesis of alcoholic fatty liver disease and pancreatitis, but also affect numerous other organs and tissues [183]. Chronic alcohol abuse also disrupts the normal functioning of the adaptive immune response, leading to an increased susceptibility to viral and bacterial infections [183–185]. Aging is accompanied by an increase in inflammation, and it is possible that these associations are at least in part due to an increased susceptibility to chronic diseases and frailty that are more prevalent with age rather than to biological aging itself [186–189].

1.3 PREVENTION OF INFLAMMATION

As described in the previous paragraphs, inflammation comprises a complex interaction between integrated networks and mechanisms [53,54]. Drugs targeting a specific part of inflammatory pathways do not appear to be a panacea to date. Drug discovery is still dominated by the "one disease - one target - one drug" paradigm [190,191]. This approach oversimplifies disease mechanisms, which are actually complex subnetworks within the interactome [191–193]. In addition, disease definitions are mostly based on symptoms rather than mechanisms, which is why so are the therapies, e.g. in high-grade inflammatory conditions, the symptoms are treated to achieve remission [160,162]. It is therefore not surprising that drug discovery has only limited success as a result [191,194]. Although several drugs are applied in high-grade inflammatory diseases, the efficiency, specificity, and side effects are still important issues to be solved [195]. On the other hand, in CLGI conditions hardly any anti-inflammatory drugs are used because the side effects do not outweigh the benefits. Moreover, signs of CLGI are present in apparently healthy people as a consequence of poor lifestyle choices including smoking, stress, or alcohol consumption and reflect their increased risk of developing disease [46]. Given the relationship between CLGI and NCDs, prevention is a much better way to fight disease than therapy [3–5].

Many lifestyle factors are believed to influence different aspects of inflammation, with the four major behavioral factors: unhealthy diets, lack of exercise, tobacco smoking and alcohol consumption possibly having the greatest influence [2,36]. In the Global Action Plan, the WHO has set global targets for the prevention and control of NCDs [31]. For the lifestyle factors, the targets are the following: (I) At least 10% relative reduction in the harmful use of alcohol, as appropriate, within the national context, (II) a 30% relative reduction in the prevalence of current tobacco use in persons aged 15+ years, and (III) a 10% relative reduction in prevalence of insufficient physical activity [31]. It is astonishing and rather worrying that no targets have been set for nutrition, which has a direct impact on overweight, obesity and health in general, other than a 30% relative reduction in mean population intake of sodium/salt [31]. Most global discussions concern the risk factors of self–management and focus on the role of individual responsibility to manage the risk factors of NCDs [2,36,37]. Salt-reduction interventions can have a positive effect on health, but for most people, "hidden" salt in processed foods disconnects salt intake from discretionary control [196]. Therefore, this target primarily concerns governments, manufacturers, and the food industry, rather than an individual's lifestyle choice [196–198].

Nutrition plays a role in predisposition to conditions with an inflammatory component and dietary changes may be helpful in preventing or treating such conditions [3–11]. A wealth of observational data indicates that diets rich in fruits and vegetables have a particularly positive effect on inflammatory status and prevents development of various NCDs [14,199–204]. Surprisingly, only 10 out of 136 countries (7%) have an adequate intake and adequate supply of vegetables, whereas in 119 of the 136 countries (88%) vegetable intake is below the recommendations [205]. Public health campaigns are required to encourage vegetable consumption worldwide, but in vain no goals were set in Global Action Plan [31,205,206].

1.4 THE NUTRITIONAL COMPOSITION OF FRUITS AND VEGETABLES

Large observational studies show that consuming fruit and vegetables can make a positive contribution to health, but so far, it has not been clarified to what extent specific fresh produce and nutrients in these products are responsible for particular health effects [14,199,210,200–204,207–209]. Consumers are becoming increasingly aware of the influence of food on their health, but because it is still difficult for companies to explain exactly what is in their product and why it is healthy, the potential of fruit and vegetables is insufficiently exploited. A wide variety of fruits and vegetables provides a range of nutrients and different bioactive compounds including fibers, vitamins, minerals, and phytochemicals [211].

1.4.1 Dietary fibers

As a category of carbohydrates in the constitution of plants, dietary fibers are not completely digested in the human intestine [212]. The WHO recommends an average daily intake of 25 grams of fiber for adults [213,214]. Although the exact mechanisms of action of dietary fiber in the human body have not yet been fully deciphered, health professionals unanimously recognize its therapeutic benefits [212]. The health effects of dietary fibers depend on the type of fiber consumed [215]. Bulking fibers, e.g. cellulose, absorb water and can significantly increase stool weight and regularity [215]. Viscous fibers, e.g., beta-glucans and pectins, thicken the fecal mass and reduce postprandial glucose and insulin concentrations [216,217]. Fermentable fibers, e.g. resistant starch, and inulin, feed the anaerobic intestinal microbiota and are metabolized to yield short-chain fatty acids (SCFA), which have several roles in gastrointestinal health [218–221].

1.4.2 Vitamins and minerals

Vitamins are defined as essential organic constituents of the diet that are insufficiently synthesized by humans [222]. Vitamins have diverse biochemical functions. Vitamin A is a general term that encompasses several fat-soluble substances such as retinol, retinyl palmitate, and beta-carotene. The various molecules are essential for vision, cellular differentiation, and immune function [223]. The B complex is a group of eight water-soluble vitamins. In general, their function can be divided into catabolic metabolism, leading to energy production, and anabolic metabolism, resulting in bioactive molecules [224]. Vitamins C and E function as essential antioxidants but have more pleiotropic properties [225]. Vitamin D performs a more hormone-like function and plays an essential role in the maintenance of bones, teeth, and optimal immune function [226,227]. Vitamin K has important functions in the body, e.g. anti-calcification, bone formation and blood clotting, some of which are still being discovered [228]. Minerals are inorganic elements that come from soil and water and are absorbed by plants or eaten by animals. Minerals play a key role in the regulation of metabolic and physiological pathways. Calcium, copper, iron, selenium, and zinc are considered particularly important for their physiological roles and their participation in a variety of biological processes [229].

1.4.3 Phytochemicals

Phytochemicals are bioactive non-nutrient metabolites that enable plants and fungi to overcome transient or continuous threats integral to their environment while also controlling essential functions of growth and reproduction [211,230,231]. These functions are generally beneficial to the producing organisms, but the inherent biological activity of such constituents often causes adverse consequences on other organisms that may be exposed to them [230]. A notorious example is cyanide poisoning caused by amygdalin, a cyanogenic glycoside found in the seeds of bitter almonds and peaches [232,233]. Nevertheless, such effects can be the essential indicator of desirable properties and therapeutic potential, e.g. Taxol [230,234]. More than 5000 phytochemicals have been identified in fruits, vegetables, nuts and legumes [235]. The most studied phytochemicals can be divided into six categories: organosulfur compounds, phenolics, alkaloids, nitrogen-containing compounds, phytosterols, and carotenoids [211,235]. Organosulfur compounds are molecules that contain sulfur and are therefore often associated with the characteristic pungent odors, but also play a vital role in plant physiology and protection against various environmental stressors [236–238]. During research into potential health-promoting effects in humans, they have become particularly known for their antioxidant, anti-inflammatory, anti-carcinogenic and anti-angiogenic properties [236–238]. Phenolics are a group of compounds with one or more aromatic rings containing at least one hydroxyl group. Phenolics are generally classified as subgroups of phenolic acids, flavonoids, stilbenes, coumarins and tannins [239]. Phenolics are essential in plant reproduction and growth, give plants their color, and act as a defense mechanism against pathological virus

and fungal infections, parasites, and predators [211]. In addition to their functions in plants, observational studies indicate that intake of phenolic compounds may reduce the risk of CVD [240,241], lung cancer [242], dementia [243] and stroke [244] in humans [245,246]. Alkaloids are a class of compounds containing at least one nitrogen atom. In plants, they protect against predators and regulate their growth [247,248]. Therapeutically, alkaloids are particularly well known as anesthetics, cardioprotective, and anti-inflammatory agents, e.g. morphine, strychnine, quinine, and ephedrine [249]. Moreover, alkaloids are among the most common stimulants used in society, e.g. caffeine, nicotine, cocaine [250]. Nitrogen-containing compounds are nutritious for all kinds of plants and thus increase the nutritional value [251]. Plant sterols and plant stanols, commonly known as phytosterols, are compounds that help support the structure of cell membranes [252]. Phytosterols help lower cholesterol in humans, which could reduce the risk of heart disease [253]. Carotenoids are classified into hydrocarbons (carotenes) and their oxygenated derivatives (xanthophylls), with a 40-carbon skeleton of isoprene units [211]. In plants, carotenoids play essential functions in photosynthesis and photoprotection due to their ability to quench reactive oxygen species [211,254]. In non-photosynthetic tissue, carotenoids are responsible for the characteristic coloration of fruits such as red tomatoes, orange carrots, and red flesh in watermelon [255,256]. In terms of health benefits, carotenoids have received considerable attention due to their unique physiological functions as provitamins and antioxidant effects [211].

1.5 THE HEALTH-PROMOTING EFFECTS OF FRUITS AND VEGETABLES

Over the past few decades, research has increasingly focused on antioxidants as the main health-promoting compounds in fruits and vegetables, leading to a gigantic array of antioxidant supplements on the market today [6,199–203,257]. However, initial excitement regarding the potential health benefits of antioxidants has diminished [6]. There is quite a bit of debate about whether supplementing with antioxidants is healthy, ineffective, or even harmful [3,6,260–264,7,8,40,199–201,258,259]. Antioxidant supplementation is based on the belief that oxygen radicals and other reactive oxygen species are involved in the pathogenesis of many NCDs by causing oxidative damage, and that reducing that damage will slow or prevent the development of diseases [258]. The term "antioxidant paradox" is often used to refer to the observation that supplementing antioxidants to counteract oxidative damage has had little or no preventive effect in most human trials [258,264–266], and high-dose supplementation (pharmacological rather than nutritional) may even have harmful consequences [267,268,277–280,269–276]. One of the other hypotheses about the health benefits of fruits and vegetables are attributed to the wide variety of bioactive substances in the food matrices with the synergy or interactions

between the different mechanisms of action of these compounds in the body, rather than just antioxidants [6,8,11,211,235,239]. Thus, the action of the nutrient matrix (the composition of naturally occurring food components) on human biological systems might be greater than or different from the corresponding actions of the individual nutrients [281]. One aspect of synergy may be a buffering effect [281,282]. The effect of a large intake of a given nutrient may vary depending on whether it is taken in concentrated form or as part of a food matrix, e.g. the matrix may slow down the absorption of the nutrient, which lowers the likelihood of a bolus effect [282]. Another aspect of synergy can be the influence of nutrients on each other's absorption, e.g. copper and zinc, manganese and iron, and alcohol and ferritin [282–285]. The buffering and competition effects reflect forms of control over the entry of food components into the human system [281]. In addition, providing weak pro-oxidants to manipulate endogenous antioxidant levels may be a more useful approach for prevention of NCDs compared to the consumption of large doses of dietary antioxidants [258]. To illustrate, it is well-known that physical activity increases the level of oxidative stress, but regular exercise appears to be beneficial to health. This same stimulus is in fact necessary to allow upregulation of endogenous antioxidant defenses, a phenomenon known as hormesis [258,263,294,295,286–293].

Hormesis has been defined as a dose-response relationship in which there is a stimulatory response at low doses, but an inhibitory response at high doses, resulting in a U- or inverted U-shaped dose response [296]. Hormesis should be considered an adaptive response characterized by biphasic dose responses of generally similar quantitative features with respect to amplitude and range of the stimulatory response that are either directly induced or the result of compensatory biological processes following an initial disruption in homeostasis [289,297–299]. A critical element of the homeodynamic property of living systems is their ability to respond to stress [300]. In this context, the term ‘stressor’ is defined as a chemical or biological agent, environmental condition, external stimulus, or psychological factor, which initiates a series of events in a living system to counter, adapt and survive [300]. A well-known phenomenon in experimental studies is that exposure to low levels of one type of hormetic agent can protect living systems against multiple types of stress, e.g. initial exposure to mild heat stress or herbicides can protect cells from oxidative stress, toxins, and ischemia [289,301,302]. This “cross-modal” aspect of hormesis in biological systems, may explain the broad benefits of lifestyle and environmental factors [289,303,304]. Exercise is the best documented lifestyle factor exerting health effects through hormetic principles [289]. Exercise triggers a robust inflammatory response mainly characterized by the mobilization of leukocytes and an increase in circulating inflammatory mediators produced by immune cells and directly from the active muscle tissue [204]. While moderate activity can boost immune function above sedentary levels, excessive amounts of prolonged, high-intensity exercise can impair immune function [204]. In addition, the improvement of other tissues after exercise, including the

nervous and digestive systems, demonstrates the cross-modal effects of physical activity [305–308]. Worth mentioning, also detrimental lifestyle factors e.g. nicotine and alcohol, appear to exert their effects through hormetic responses, where small amounts may have positive effects, while higher concentrations are toxic [309–313]. Recent findings suggest that health benefits of many phytochemicals may also be conferred through cross-modal hormetic mechanisms [303]. In this process, a phytochemical (hormetin) activates one or more adaptive cellular stress response pathways [303]. In addition to vitamins, minerals, and fibers, hormetins may also be partly responsible for the health effects of fruits and vegetables. Most dietary hormetins are known to induce the expression of antioxidant enzymes by triggering a pro-oxidant response via activation of the nuclear factor E2-related factor 2 (Nrf2)-pathway [314–322].

1.5.1 Nrf2 pathway

Nrf2 is a transcription factor that is blocked when bound to the Neh2 domain by the protein Kelch-like ECH-associated protein 1 (Keap1) in the cytosol [323]. Keap1 functions as an adaptor for Cul3-based ubiquitin E3 ligase to regulate proteasomal degradation of Nrf2 [323–325]. Conformational changes and disruption of Keap1-mediated Nrf2 ubiquitination results in the release and activation of Nrf2 [326,327]. Nrf2 is translocated to the nucleus and binds to the antioxidant response element (ARE) located in the promoters of genes coding for antioxidant and detoxifying enzymes. Nrf2/ARE-dependent genes code for several mediators of the antioxidant response and NF-κB inhibitors, including glutathione S-transferases (GSTs), thioredoxin, NAD(*p*)H quinone oxidoreductase 1 (NQO-1), and heme oxygenase 1 (HO-1) [315]. Nrf2 appears to participate in a complex regulatory network and plays a pleiotropic role in the regulation of metabolism, inflammation, autophagy, mitochondrial physiology, and immune responses [328]. While *in vitro* studies have elucidated the involvement of many phytochemicals (sulforaphane, curcumin, resveratrol, diallyl disulfide) in the Nrf2-pathway, the available clinical evidence in humans is more limited [316,329–332]. Moreover, it remains challenging to demonstrate the health-promoting effects of phytochemicals in the general population using classical methodologies adopted from pharmaceutical research [8,11,20–22,12–19]. Therefore, new approaches to evaluation of the effects of nutrients are needed.

1.6 NOVEL EXPERIMENTAL METHODOLOGIES FOR NUTRITIONAL STUDIES

With an increasing understanding of disease, health is no longer seen as simply a fixed entity of complete physical, mental, and social well-being, but as our body's ability to cope with everyday challenges [6,8,9,11,333]. The concept of this phenotypic flexibility or resilience implies that health can be measured by the ability to maintain homeostasis

through a highly energy-dependent, rapid, and orchestrated adaptation to continuous environmental changes and challenges [3,334,335]. While this progressive shift in thinking about health is increasingly recognized, this shift has not yet been reflected in the methods for the scientific substantiation of health effects, e.g. in the EU under the Nutrition and Health Claim Regulation (NHCR), Regulation (EC) No 1924/2006. The current methods advised and used in scientific dossiers to support such claims are still quite pharmaceutical, in other words: they focus on one disease - one target - one substance [336,337]. As described in previous sections, this can be considered not the best approach to study inflammation, given the complex interaction between integrated networks and mechanisms [53,54]. To study the effects of compounds targeting inflammatory pathways, a number of inflammatory challenges have been described to induce inflammation in healthy subjects [3]. These include caloric overload (e.g. oral glucose tolerance test (OGTT), oral fat load) [338–344], strenuous exercise [345,346], exposure to UV irradiation [347,348], and administration of bacterial endotoxins [349,350]. While each of these challenges has been used in nutritional studies, many are poorly standardized, limiting the comparisons that can be made [3]. In addition, there is no consensus as to which biomarkers best represent CLGI. The multi-component approach to monitor CLGI, measuring a range of biomarkers, may be superior to measuring single biomarkers and is increasingly used in studies examining the pleiotropic effects of nutrition (e.g. blood cellular biomarkers, soluble mediators, adhesion molecules, adipokines and acute-phase proteins) [3]. Finally, there is a need to take a multifaceted approach and consider the impact of whole foods over individual bioactive compounds, with likely greater overall benefit than every single component might have on its own. Altogether this would allow for more accurate quantification of the effects of fruits and vegetables in relation to inflammation and metabolic processes and could reveal the true effects of phytonutrients on NCDs.

1.7 OUTLINE OF THE THESIS

The research in this thesis aims to further elucidate the role of fruit and vegetables in the development and prevention of NCDs and to investigate ways to measure the health-promoting effects of phytonutrients related to inflammation. As indicated in this introduction, one group of phytonutrients that has been suggested to elicit anti-inflammatory effects are carotenoids [211]. **Chapter 2** provides a systematic overview of the role of the carotenoid lycopene in inflammation and examines the mechanism of action of this antioxidant in the human body. In **Chapter 3**, the effects of fungal beta-glucans on the inflammatory response and the immune system are evaluated. As described earlier, this group of polysaccharides acts as PAMPs and initiates the inflammatory response. Fungal beta-glucans have, however, been valued for centuries by traditional Chinese

medicine for their beneficial effects [351]. The potential application of fungal beta-glucans in nutrition and Western medicine is described in this chapter.

Research has shown that sulforaphane, a hormetin found in abundance in cruciferous vegetables, shows promise as a potent anti-inflammatory substance [352,353]. However, its potential in the settings of a calorie-induced inflammatory response has not been tested. The following **Chapters 4, 5, and 6,** are related to the PROtective effects of SulforAphaNe on chronic low-grade Inflammation (PRO SANI) study. The PRO SANI study is a crossover, double-blind RCT investigating the efficacy of broccoli sprouts, as a source of sulforaphane, on biomarkers of inflammation and other markers of phenotypic flexibility in healthy participants subjected to a standardized caloric load.

In **Chapter 4** the effects of broccoli sprouts and caloric overload on circulating inflammatory biomarkers and metabolic parameters and the relationships between them are assessed. In addition, it is investigated whether integrative outcome measures provide a better approach to study the subtle and pleiotropic effects of phytonutrients.

As introduced in previous sections, platelets play an essential role in coordinating inflammatory responses, and targeting thromboxanes via antiplatelet agents has become a cornerstone of cardiovascular disease treatment. In **Chapter 5**, the acute effects of sulforaphane on urinary 11-dehydro-thromboxane B_2 are examined and the potential of this novel non-invasive biomarker to investigate the effects of phytonutrients on platelet functionality is evaluated. Moreover, the influence of interindividual genetic variability on sulforaphane metabolism and its potential antithrombotic effects are studied.

The inflammatory status has been associated with autonomic activity, but heart rate variability (HRV) monitoring for the assessment of inflammation in humans has rarely been used. In **Chapter 6**, the potential of HRV as a non-invasive tool to monitor inflammation induced by caloric overload is explored and the effects of sulforaphane in the setting of this metabolic challenge are assessed.

Finally, in **Chapter 7**, the main findings of the individual chapters are discussed and suggestions for future research are provided.

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2

The Role of Circulating Lycopene in Low-Grade Chronic Inflammation: A Systematic Review of the Literature

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// ABSTRACT

Background and aims: In recent years, it has become clear that low-grade chronic inflammation is involved in the onset and progression of many non-communicable diseases. Many studies have investigated the association between inflammation and lycopene, however, results have been inconsistent. This systematic review aims to determine the impact of circulating lycopene on inflammation and to investigate the effect of consuming tomato products and/or lycopene supplements on markers of inflammation.

Methods: Eligible studies, published before March 2020, were identified from PubMed, EBSCOhost and ScienceDirect. Human studies published in English, that evaluated the effect of circulating lycopene in relation to inflammation biomarkers were screened and included. Studies assessing lycopene intake or general intake of carotenoids/antioxidants without measuring circulating lycopene, as well as those not reporting inflammation biomarkers as outcomes, were excluded.

Results: Out of 80 publications identified and screened, 35 met the inclusion criteria. Results from 18 cross-sectional studies suggest that lycopene levels are adversely affected during inflammation and homeostatic imbalance. Most of the 17 included intervention studies reported increased circulating lycopene levels after tomato/lycopene supplementation, but almost no changes in inflammation biomarkers were observed.

Conclusion: There is little evidence that increasing tomato intake or lycopene supplementation diminuates this inflammation. However, depletion of lycopene may be one of the first signs of low-grade inflammation. The available data thereby imply that it is beneficial to consume lycopene-rich foods occasionally to stay healthy and keep circulating lycopene at a basal level.

2.1 INTRODUCTION

The understanding of health has changed in recent years: in addition to medicine and pharmacology, there has been an increasing interest in lifestyle medicine in which nutrition plays a pivotal role [1]. In addition to conventional drug therapies, lifestyle adjustments, such as dietary changes, are also advised to reduce disease. Diets with a high proportion of fruits and vegetables seem to have a particularly positive effect on nutritional status as well as different non-communicable diseases, such as heart diseases, neurodegenerative diseases, and diabetes type II. As most non-communicable diseases are partially affected by inflammation, more research is being conducted on potential anti-inflammatory substances derived from fruits and vegetables [2–6].

2.1.1 Low-Grade Chronic Inflammation

Previous research has shown that the onset and progression of many non-communicable diseases, including heart diseases, neurodegenerative diseases, and diabetes type II, are (partly) related to, or affected by inflammation: low-grade chronic inflammation is central to many different symptoms from which patients suffer in these conditions. Chronic inflammation is believed to aggravate various mechanisms that reflect poor health, including elevated blood pressure, high blood sugar, excessive waist circumference, and abnormal cholesterol or triglyceride levels (the so-called “deadly quartet”) [7]. In normal homeostasis, the function of inflammation is to eliminate the initial cause of cell injury, dispose of necrotic cells and damaged tissue caused by both the injury and the inflammation, and to initiate tissue repair. This natural response, acute inflammation, is a critical survival mechanism used by all higher vertebrates [8]. However, if acute inflammation is not resolved, it can lead to chronic inflammation, which is not part of the body’s natural healing process and can constitute a damaging process. Damaged tissues release pro-inflammatory cytokines and other biological inflammatory mediators into the circulation, converting tissue-based low-grade inflammation into a systemic inflammatory condition. Moreover, autoimmune disorders and long-term exposure to irritants can also lead to a systemic inflammatory condition [8–10].

The inflammatory response is the coordinated activation of signaling pathways that regulate inflammatory mediator levels in resident tissue cells and inflammatory cells recruited from the blood. Although inflammatory response processes depend on the precise nature of the initial stimulus and its location in the body, for example, bacterial pathogens trigger Toll-like receptors (TLRs) and viral infections trigger type I interferons (IFN), they all share a common mechanism, which can be summarized as follows: 1) Cell surface pattern receptors recognize detrimental stimuli; 2) inflammatory pathways are activated; 3) inflammatory markers are released; and 4) inflammatory cells are recruited [9,11]. Inflammatory stimuli activate intracellular signaling pathways that subsequently

activate the production of inflammatory mediators. Primary inflammatory stimuli, including microbial products and cytokines such as interleukin-1β (IL-1β), interleukin-6 (IL-6), and tumor necrosis factor-α (TNF-α), mediate inflammation through interaction with the TLRs, IL-1 receptor (IL-1R), IL-6 receptor (IL-6R), and the TNF receptor (TNFR). This receptor activation triggers important intracellular signaling pathways, including the mitogen-activated protein kinase (MAPK), nuclear factor kappa-B (NF-κB), NF-E2 p45-related factor 2 (Nrf2), and Janus kinase (JAK)- signal transducer, and activator of transcription (STAT) pathways [11]. In the state of low-grade chronic inflammation, a typical inflammatory stimulator or pathogen can no longer be determined, and inflammatory stimuli and pathways remain activated. Inflammatory stimuli, such as IL-6 and C-reactive protein (CRP), can then be used as biomarkers to measure inflammation [12].

Low grade inflammation is involved in the progression of many non-communicable diseases, but also seems to affect apparently healthy people as a consequence of smoking, stress, or alcohol consumption [8]. A wealth of epidemiological evidence indicates that overall health is strongly influenced by diets with a high proportion of fruits and vegetables [2–4,13,14]. Phytochemicals with anti-inflammatory activity present in fruits and vegetables are believed to be largely responsible for overall health. Therefore, new possibilities may exist in the reduction and prevention of non-communicable diseases by increasing the intake of anti-inflammatory food (ingredients) in both healthy and diseased individuals [15–17].

2.1.2 Lycopene

One group of nutritional compounds that has been suggested to elicit anti-inflammatory effects are carotenoids. As carotenoids are pigments in photosynthetic tissue, they are ubiquitous in leafy green vegetables. In non-photosynthetic tissue, carotenoids are responsible for the characteristic coloration of fruits such as red tomatoes, orange carrots, and red flesh in watermelon [18,19]. Of all carotenoids, a substantial amount of research has been conducted on the acyclic lycopene, present in e.g.; tomatoes.

2.1.2.1 Physicochemical Properties of Lycopene

Lycopene has a chemical formula of C40H56 and like all carotenoids, is a tetraterpene; assembled from eight isoprene units that are solely composed of hydrogen and carbon [20]. Lycopene is an acyclic isomer of β-carotene, however, unlike β-carotene lycopene lacks the β-ionic ring structure. Therefore, it lacks provitamin A activity [20,21]. However, lycopene is one of the most potent antioxidants, with a singlet-oxygen-quenching ability twice as high as that of β-carotene and ten times higher than that of α-tocopherol (Vitamin E) [22]. Lycopene is a highly unsaturated, open-chain hydrocarbon containing eleven conjugated and two non-conjugated double bonds arranged in a linear array. The double bonds in lycopene can undergo isomerization from *trans* to *cis* isomers by thermal energy,

chemical reactions, and light [20,21]. The all-*trans* isomeric form is primarily present in nature, followed by the 5-*cis*, 9-*cis*, 13-*cis*, and 15-*cis* isomeric forms. Several methods for analysis of circulating lycopene are described. Methods differ in that (i) either plasma or serum lycopene is measured, (ii) multiple isomers, *trans*-lycopene or total lycopene are measured, (iii) circulating lycopene is adjusted for total cholesterol. The correction for total cholesterol has been made in more recent intervention studies because there is a risk of carotenoid status being misinterpreted in subjects on cholesterol-lowering therapy if they rely on crude serum or plasma levels.

2.1.2.2 Lycopene Kinetics after Oral Administration: Absorption, Distribution, Metabolism, Excretion

Absorption of lycopene is similar to that of other lipid soluble compounds. Ingested lycopene is incorporated into dietary lipid micelles and absorbed across the gastrointestinal tract via passive diffusion into the intestinal mucosal lining. Then they are incorporated into chylomicrons and released into the lymphatic system for transport to the liver. Lycopene is transported by lipoproteins in the blood for distribution to the different organs [23]. Because of its lipophilic nature, the primary carrier of lycopene is LDL and not HDL [24]. Generally, 10–30% of dietary lycopene is absorbed with the remainder being excreted. The bioavailability of lycopene is greater from tomato paste than from fresh tomatoes. The increased absorption of lycopene from processed products is attributed to the presence of cis isomeric forms [25]. The absorption of lycopene in humans is influenced by several biological and lifestyle factors including gender, age, body mass index and composition, hormonal status, blood lipids concentrations, alcohol consumption, smoking, and the presence of other carotenoids in the consumed products [20]. When lycopene is administered as the all-*trans* isomer it rapidly isomerizes to a mixture containing more than 50% *cis*-isomers during absorption in the bloodstream and tissues. Moreover, a study showed that administration of all-trans lycopene in tomato sauce to human subjects for three weeks resulted in 77.3% *cis* isomers in prostate tissue and thus only 22.7% all-*trans* lycopene [26]. Liver, seminal vesicles, and prostate tissue are the primary sites of lycopene accumulation in humans [27]. Recent studies indicate that the accumulation in these sites may be due to the involvement of an active process for the uptake of carotenoids via the scavenger receptor class B type 1 protein (SR-B1) transporter, in addition to passive diffusion [28]. The full metabolic routes of lycopene in humans is still unclear. Only a few metabolites, such as 5,6-dihydroxy-5,6-dihydro-lycopene, have been detected in human plasma. It is suggested that lycopene may undergo in vivo oxidation to form epoxides which then may be converted to the polar 5,6-dihydoxy-5,6-dihydro-lycopene through metabolic reduction [29].

2.1.2.3 Mechanism of Action (In Vitro)

Lycopene has been shown to inhibit the binding abilities of NF-κB and stimulatory protein-1 (SP1), and decreased expression of insulin-like growth factor-1 receptor (IGF-1R) and intracellular ROS concentrations in human SK-Hep-1 cells [30]. Recently, Fenni et al. [31] confirmed the potential involvement of lycopene in decreasing the binding abilities of NF-κB. They demonstrated the ability of lycopene supplementation to inhibit high-fat diet-induced obesity, inflammatory response, and associated metabolic disorder in mice. They evaluated the effect of lycopene on the phosphorylation of p65 and IκB, which are involved as modulators in the NF-κB pathway. Lycopene was able to strongly reduce phosphorylation of p65 and IκB, resulting in the deactivation of the NF-κB pathway, that previously was induced by the consumption of a high-fat diet. This effect can thus be seen as the induction of an anti-inflammatory effect. These results have also been observed in SW480 human colorectal cancer cells, where lycopene restrained NF-κB and JNK activation, resulting in a suppression of TNF-α, IL-1β, IL-6, COX-2, and iNOS expression. However, relatively high concentrations of lycopene were used (10–30 μM) compared to usual detectable plasma levels (1–2 μM) [32].

While *in vitro* and animal studies show promise for the potential health effects of lycopene, the relationship between lycopene and low-grade chronic inflammation in itself has so far been inconclusive in humans. Various systematic reviews have already been conducted on lycopene and how it affects different diseases and their symptoms, such as prostate and bladder cancer, cardiovascular. risk and metabolic syndrome [33–36]. The cross-sectional and intervention studies assessed in these reviews were often inconclusive, and the inconsistency among studies and the type of lycopene tested makes comparison difficult. The different lycopene measurements (self-reported FFQ, measurement of product, circulating lycopene) are a possible reason for the inconsistent results. Circulating measures are preferred for assessing relations, because self-reported measures of lycopene intake are subject to recall bias or memory error and intake measurements do not provide insight in the absorption, distribution, metabolism, and excretion of lycopene in the body. For in vivo studies, however, it is necessary to not just focus on lycopene intake but to actually measure the circulating lycopene concentrations in plasma or serum, in order to understand the health effects on humans [21]. C-reactive protein (CRP) and interleukin-6 (IL-6) are most commonly used to measure inflammation, but some studies have reviewed other inflammatory biomarkers (hyaluronic acid (HA), malondialdehyde (MDA), adiponectin, monocyte chemoattractant protein 1 (MCP-1), thiobarbituric acid reactive substances (TBARS), serum amyloid A (SAA), tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β)). These will also be included in this study [37,38]. As such, this is the first systematic review of the literature to investigate the relationship between circulating lycopene and inflammation.

2.2 METHODS

2.2.1 Literature Search

This systematic review was conducted following the Cochrane and the Centre for Reviews and Dissemination guidelines on systematic reviews and is reported according to PRISMA guidelines [39,40]. This systematic review of the literature was conducted to investigate the relationship between circulating lycopene and inflammation in order to understand the health effects of lycopene in humans. To identify relevant human studies in which the relationship between lycopene and inflammatory markers was measured, a systematic search was conducted in Pubmed, EBSCOhost and ScienceDirect as databases. Using the Boolean search terms "serum lycopene" and "inflammation," articles published in peer-reviewed journals in the English language were flagged for further review. As this review focuses on the effect of lycopene intake on inflammation in vivo, only human studies were considered for inclusion. The date of publication did not serve as an exclusion criterion. The search was conducted in duplicate by the first and last author and all potentially relevant publications up to March 2020 were included in the search.

The following search terms yielded 42 articles in Pubmed and 13 articles in EBSCOhost: (("serum"[MeSH Terms] OR "serum"[All Fields]) AND ("lycopene"[MeSH Terms] OR "lycopene"[All Fields]) AND ("inflammation"[MeSH Terms] OR "inflammation"[All Fields])).

In ScienceDirect, the search terms "lycopene" and "inflammation" in "Find articles with these terms" and in "Title, abstract or author-specified keywords," 79 articles published in English from peer-reviewed journals were flagged for further review in ScienceDirect. After removing duplicates (62), a total of 72 articles were therefore screened for inclusion in this systematic review. Additionally, the reference list of each included article was examined to identify any additional studies for inclusion that might not have appeared in the search results. This led to the identification of eight more articles for further review.

2.2.2 Application of Inclusion/Exclusion Criteria

As this systematic review focusses on circulating lycopene, only studies that report serum or plasma lycopene levels as independent measures and their relation to inflammation (inflammatory biomarkers) were considered for inclusion in this review. Although human studies were selected in the search terms, the search still identified a few in vitro and animal studies, which were subsequently excluded (3). Only full text, original human research studies were included. This led to the exclusion of two additional studies that presented research only as an abstract or in the form of a presentation.

Various studies were seen to report merely the intake of lycopene and its relationship with inflammation biomarkers, whereas the main interest of this study is to identify actual levels of lycopene in plasma/serum detected after/following consumption. Therefore, when analyzing full text versions of all studies, studies were excluded from this assessment based on the following exclusion criteria: (i) Assessment of lycopene intake or general intake of carotenoids/antioxidants without measuring circulating lycopene; and (ii) no inflammation biomarkers reported as outcomes. In total, the screening and eligibility process of this literature search led to the identification of 35 studies that were included and subjected to critical analysis (Figure 1).

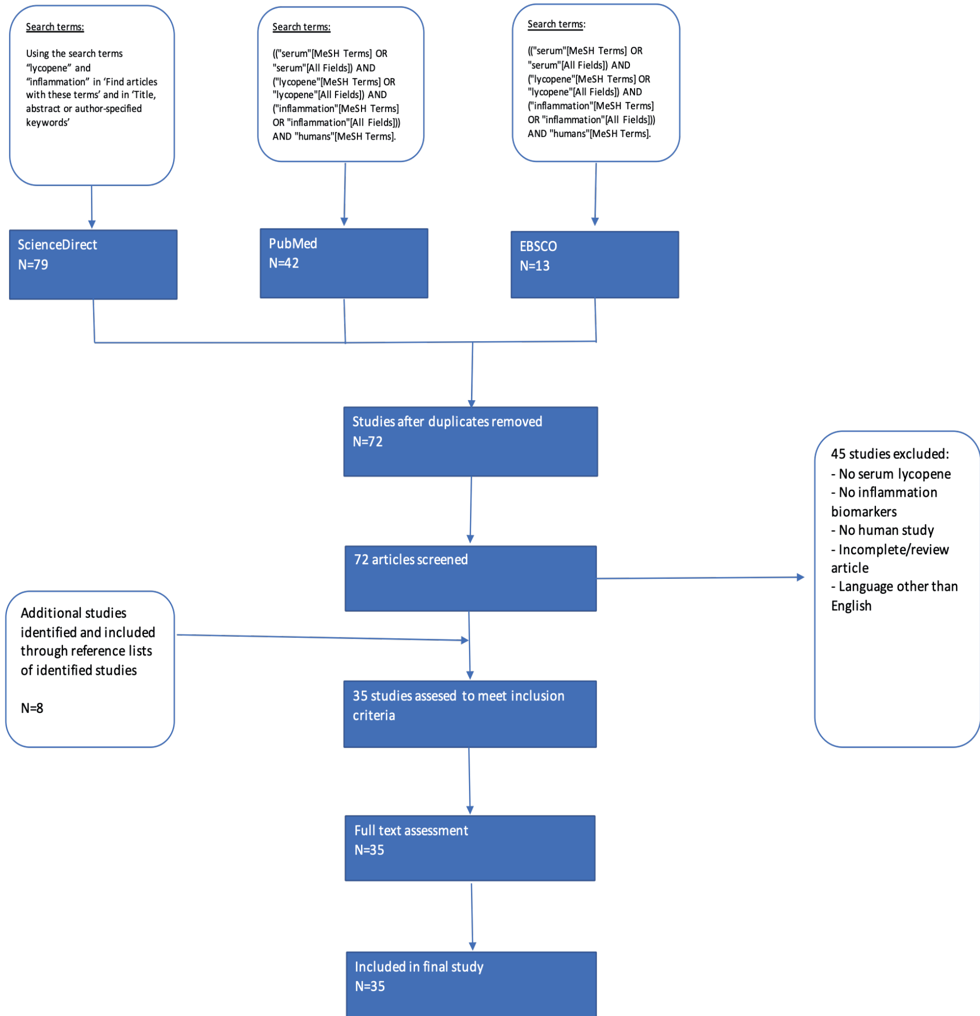

Figure 1. Flowchart of systematic search strategy

2.2.3 Data Extraction and Analysis

To assess the quality and to minimize the risk of reporting bias, each author independently analyzed the articles. The authors discussed the extracted data and thoroughly considered differing interpretations before establishing consensus. Extracted information included: study design, randomization, duration and length of follow-up, methods of analysis, participants characteristics (population, settings of intervention, baseline characteristics), outcome measures (biomarkers), intervention details (i.e.; tomato or lycopene), and conclusions. Brief descriptions and summaries of results for the final articles included in this review are presented in Table 1: Cross-sectional studies assessing the relation between circulating lycopene and inflammation; and Table 2: Intervention studies assessing the influence of lycopene on inflammation.

Table 1. *Cross-sectional studies assessing the relation between circulating lycopene and inflammation.*

Study (Ref)	Study Population	Final *n*	Lycopene Measurement	Inflammation Biomarkers	Conclusions
Mazidi et al. [41]	Participants divided in quartiles depending on CRP and Fibrinogen	Q1: (n = 193) Q2: (n = 190) Q3: (n = 183) Q4: (n = 199)	**Serum *trans*-Lycopene (µmol/L)** Q1: 0.431 ± 0.007 Q2: 0.425 ± 0.007 Q3: 0.421 ± 0.005 Q4: 0.387 ± 0.009	**CRP (mg/dL)** Q1: 0.03 ± 0.01 Q2: 0.14 ± 0.04 Q3: 0.33 ± 0.07 Q4: 1.2 ± 0.89	A higher trans-lycopene level for each µmol/L correlated with 0.067 mg/dL lower CRP and 0.048 mg/dL Fibrinogen
Crespo-Sanjuán et al. [42]	Control subjects (n = 14) Patients with intestinal polyps (n = 39) Patients with colorectal adenocarcinoma (CRC) (n = 128)	Control (n = 14) Patients (n = 167)	**Plasma Lycopene (µg/L)** Control: 194.33 ± 66.17 Carc. in Situ: 138.57 ± 106.62 Cancer IV: 100.42 ± 71.20	**Plasma CRP (mg/L)** Control: 2.05 ± 2.33 Carc. in Situ: 13.93 ± 26.53 Cancer IV: 41.83 ± 62.01	Levels of lycopene were higher in the control group and low in the stage-IV group (p = 0.03) and were inversely correlated with CRP (p = 0.005, R = −0.215). We found a consistent relationship between high lycopene and absence of atherosclerosis (p = 0.002).
Kim et al. [43]	Healthy women (31–75 yrs) classified into tertiles according to serum lycopene concentration (n = 264)	T1 (n = 88) T2 (n = 88) T3 (n = 88)	**Serum Lycopene (mmol/L)** T1: 0.029 ± 0.000 T2: 0.039 ± 0.000 T3: 0.052 ± 0.001	**hs-CRP (mg/dL)** T1: 1.27 ± 0.24 T2: Data not shown T3: 0.80 ± 0.25	Subjects in T3 showed lower C-reactive protein (hs-CRP) (0.80 ± 0.25 mg/dL vs. 1.27 ± 0.24 mg/dL, p = 0.015), compared with those in T1.
Riccioni et al. [44]	Participants asymptomatic with respect to carotid artery disease divided over 3 groups based on Carotid intima-media thickness (n = 640)	C1 (n = 291) C2 (n = 232) C3 (n = 117)	**Plasma Lycopene (µmol/L)** C1: 0.82 ± 0.33 C2: 0.33 ± 0.63 C3: 0.34 ± 0.21	**CRP (g/dL)** C1: 2.90 ± 1.30 C2: 3.84 ± 1.75 C3: 4.86 ± 2.20	Elevated CIMT was significantly associated with having a low concentration of all antioxidants evaluated (vitamin A, vitamin E, lycopene, and b-carotene) and a higher concentration of inflammatory factors including serum uric acid, CRP, and fibrinogen.

Cont. Table 1 *Cross-sectional studies assessing the relation between circulating lycopene and inflammation*

Study (Ref)	**Study Population**	**Final *n***	**Lycopene Measurement**	**Inflammation Biomarkers**	**Conclusions**
Hozawa et al. [45]	Men and women in the Coronary Artery Risk Development in Young Adults study (18–30 years) divided in quartiles depending on Lycopene levels (*n* = 4580)	Q1: (*n* = 1144) Q2: (*n* = 1144) Q3: (*n* = 1144) Q4: (*n* = 1148)	**Serum Lycopene (nmol/L)** Q1: 24.2 Q2: 44.1 Q3: 62.0 Q4: 91.8	**CRP (mg/L)** Q1: 1.04 Q2: 1.11 Q3: 0.99 Q4: 1.11	Serum total and individual carotenoids, with the exception of lycopene, were inversely associated with markers of inflammation
Walston et al. [46]	Subjects were disabled women aged >65 years (*n* = 619)	(*n* = 619)	**Serum Lycopene (µmol/L)** 0.56 ± 0.31	**IL-6 (pg/mL)** 5.51 ± 12.69	Persons with the highest levels of b-carotene, lycopene, lutein/zeaxanthin, b-cryptoxanthin, and retinol were also significantly less likely to be in the highest interleukin-6 tertile.
Eboumbou et al. [47]	Sudanese subjects exposed and not exposed to Schistosoma infection and French control subjects	Rural Sudan: (*n* = 35) Urban Sudan: (*n* = 27) French: (*n* = 34)	**Serum Lycopene (µM)/ Lycopene:B-carotene ratio** RS: 0.21 (0.04)/1.10 US: 0.68 (0.10)/5.11 F: 1.10 (0.25)/4.52	**Hyaluronic acid (HA)/ Malondialdehyde (MDA)** around 60 **µ**g/L/200 nM	Drastic decrease of lycopene levels in the subjects exposed to schistosomiasis in comparison with non-exposed Sudanese and French control subjects
van Herpen-Broekmans et al. [48]	Healthy men and women (*n* = 379)	Men: (*n* = 178) Women: (*n* = 201) Total: (*n* = 379)	**Serum Lycopene (µmol/L)** Men: 0.35 ± 0.18 Women: 0.37 ± 0.18 Total: 0.36 ± 0.18	**CRP (mg/L)** Men: 0.9 (0.2–5.9) Women: 1.4 (0.2–7) Total: 1.1 (0.2–6.7)	An inverse relation between lycopene and CRP (−1.14 ± 0.54 per umol/l; $p = 0.04$) was found in men and not in women (0.50 ± 0.50 per umol/l; $p = 0.32$)
Jonasson et al. [49]	Men with stable angina and angiographically verified CAD and healthy controls (*n* = 113)	Patients: (*n* = 44) Controls: (*n* = 69)	**Serum Lycopene (nmol/L)** Patients: 177 (115–242) Controls: 298 (212–408)	**CRP (mg/L)** Patients: 2.30 (1.35–4.41) Controls: 1.22 (0.66–2.16)	Compared with controls, patients had signs of an enhanced inflammatory activity assessed by significantly increased levels of CRP. Patients also had significantly lower B-carotene and lycopene levels.

Cont. Table 1 *Cross-sectional studies assessing the relation between circulating lycopene and inflammation*

Study (Ref)	Study Population	Final *n*	Lycopene Measurement	Inflammation Biomarkers	Conclusions
Dhiraj et al. [50]	Patients with Chronic Hepatitis C and controls (*n* = 42)	Patients: (*n* = 20) Controls: (*n* = 22)	**Serum Lycopene (µg/dL)** Patients: 6.2 ± 3 Controls: 59 ± 28	**MDA (µM)** Patients: 1.62 ± 0.57 Controls: 0.23 ± 0.15	Serum MDA levels were significantly higher in CHC patients compared with controls (1.62 ± 0.57 vs. 0.23 ± 0.15 µmol/L) Serum levels of lycopene were significantly decreased in CHC patients.
Kritchevsky et al. [51]	Nonsmoking participants aged 25–55 years (*n* = 4557) divided in tertiles depending on CRP levels	C1 (*n* = 3180) C2 (*n* = 924) C3 (*n* = 453)	**Serum Lycopene (µmol/L)** C1: 0.46 ± 0.004 C2: 0.45 ± 0.006 C3: 0.41 ± 0.010	**CRP (mg/dL)** C1: < 0.21 C2: 0.22–0.88 C3: >0.88–12.8	Lycopene is significantly lower in higher CRP tertile
McMillan et al. [52]	Healthy control subjects and patients with gastrointestinal cancer (*n* = 24)	Patients: (*n* = 12) Controls: (*n* = 12)	**Plasma Lycopene (µmol/L)** Patients: <0.02 (<0.02–0.10) Controls: 0.37 (0.15–0.76)	**CRP (mg/L)** Patients: 91 (5–182) Controls: <5 (<5–10)	The cancer group had significantly higher C-reactive protein concentrations ($p < 0.001$) and concentrations of lycopene were significantly lower ($p < 0.001$)
Boosalis et al. [53]	Catholic sisters (nuns) age 77–99 years (*n* = 85) divided in 2 groups depending on CRP levels	Elevated CRP: (*n* = 10) Normal CRP: (*n* = 75)	**Plasma Lycopene (µg/dL)** Elevated CRP: 9.0 ± 4.0 Normal CRP: 16.6 ± 10.6	**Serum CRP (mg/dL)** Elevated CRP: > 1.5 mg/dL Normal CRP: < 1.5 mg/dL	Results showed that the presence of elevated CRP resulted in a significant decrease of lycopene concentrations ($p = 0.03$)
Almushatat et al. *[54]*	Healthy subjects (C) Patients with benign prostate hyperplasia (B) Localized (L) Metastatic prostate cancer (M) (*n* = 112)	C: (*n* = 14) B: (*n* = 20) L: (*n* = 40) M: (*n* = 38)	**Plasma Lycopene (µg/L)** C: 127 (17–320) B: 128 (18–223) L: 83 (14–687) M: 42 (<10–226)	**MDA (µmol/L)** C: 0.73 (0.50–1.40) B: 0.74 (0.35–1.48) L: 0.93 (0.47–2.93) M: 1.01 (0.44–4.67)	Prostate cancer patients had higher concentrations of malondialdehyde ($p < 0.05$) and lower circulating concentrations of lycopene ($p < 0.001$). There was a negative correlation between MDA concentrations and lycopene

Cont. Table 1 *Cross-sectional studies assessing the relation between circulating lycopene and inflammation*

Study (Ref)	Study Population	Final *n*	Lycopene Measurement	Inflammation Biomarkers	Conclusions
McMillan et al. [55]	Healthy subjects (C) Breast cancer patients (B) Prostate (*p*) Colorectal (R) (*n* = 71)	C: (*n* = 30) B: (*n* = 15) *p*: (*n* = 15) R: (*n* = 11)	**Plasma Lycopene (µg/100 mL)** C: 18.0 (6.0–41.0) B: 1.8 (<1.0–14.6) *p*: 6.7 (1.5–47.1) R: <1.0 (<1.0–5.6)	**CRP (mg/L)** C: 2.0 (0.2–8.5) B: 3.9 (0.29–14.0) *p*: 8.0 (4.0–123) R: 70 (5.0–182)	Concentrations of CRP were higher and vitamin antioxidants lower in the cancer patients. In normal subjects and cancer patients, CRP concentrations were inversely correlated with circulating concentrations of lycopene.
Chang et al. [56]	Healthy controls (H) Ischemic stroke patients, small (S) or large artery (L) (*n* = 109)	H: (*n* = 41) S: (*n* = 35) L: (*n* = 33)	**Plasma Lycopene (µmol/L)** H: 0.13 ± 0.09 S: 0.10 ± 0.07 L: 0.09 ± 0.07	**hs-CRP (mg/L)** H: 1.6 ± 1.7 S: 6.0 ± 7.0 L: 8.4 ± 15.4	hs-CRP concentrations are significantly higher in patients with acute ischemic stroke than in healthy controls. Plasma lycopene, was inversely and significantly correlated with CRP.
Chung et al. [57]	Patients with stable angina (SA) or acute coronary syndrome (ACS) (*n* = 193)	SA: (*n* = 134) ACS: (*n* = 59)	**Plasma Lycopene (µM)** SA: 0.41 (0.25–0.65) ACS: 0.37 (0.26–0.58)	**IL-6 (pg/mL)** SA: 2.21 (1.45–3.03) ACS: 5.01 (2.68–9.36)	Only lutein + zeaxanthin was inversely correlated with IL-6 in SA patients at baseline
Quasim et al. [58]	Healthy controls (H) and critically-ill patients (C) (*n* = 67)	H: (*n* = 24) C: (*n* = 43)	**Plasma Lycopene (µg/L)** H: 189.0 (62.0–465.0) C: 15.5 (<10.0–137.0)	**CRP (mg/L)** H: <5 C: 204 (6–345)	Systemic inflammatory response is associated with low carotenoid concentrations

Table 2. *Intervention studies assessing the influence of lycopene supplementation on inflammation.*

Study [Ref]	Study Population	Intervention	Final *n*	Lycopene Measurement	Inflammation Biomarkers	Conclusions
Nieman et al. [59]	Healthy runners (n = 20)	Lycopene capsule (5 mg/d) or placebo for 4 weeks	(n = 20)	**Plasma Lycopene (ng/mL)** Pre-supplement: around 500 Post-supplement: around 750	**CRP (mg/L)** Pre-supplement: 1.21 ± 1.2 Post-supplement: 1.28 ± 1.0	Plasma lycopene increased significantly in intervention group compared to placebo ($p < 0.001$). No alterations in post-exercise measures of oxidative stress and inflammation were found.
Li et al. [60]	Healthy young Taiwanese females (n = 25)	100% pure tomato juice, containing 11.6 mg of lycopene per 100 mL 280 mL/day for 56 days	(n = 25)	**Serum Lycopene (µM)** Pre-supplement: 0.72 ± 0.36 Post-supplement: 1.94 ± 0.74	**Adiponectin (µg/mL)** Pre-supplement: 11.5 ± 5.8 Post-supplement: 14.4 ± 5.2 **MCP-1 (pg/mL)** Pre-supplement: 126 ± 36 Post-supplement: 97.3 ± 17.9 **TBARS (nM)** Pre-supplement: 2.35 ± 1.11 Post-supplement: 1.84 ± 0.89	Tomato juice supplementation resulted in a decrease in levels of the inflammatory adipokine MCP-1, and an increase in levels of the anti-inflammatory adipokine adiponectin.
Biddle et al. [61]	Patients NYHA class II or III (n = 40)	V8 juice containing 29.4 mg of lycopene/ day for 30 days	Control (n = 18) Intervention (n = 22)	**Plasma Lycopene (µmol/L)** Control, pre-supl: 0.56 Control, post-supl: 0.58 Intervention, pre-supl: 0.51 Intervention, post-supl: 0.76	**Serum CRP (mg/L)** Control, pre-supl: 4.8 ± 3.4 Control, post-supl: 4.5 ± 3.8 Intervention, pre-supl: 3.4 ± 3.1 Intervention, post-supl: 3.1 ± 2.8	C-reactive protein levels decreased significantly in the intervention group in women and but not in men (p = 0.04).

Cont. Table 2 *Intervention studies assessing the influence of lycopene supplementation on inflammation.*

Study [Ref]	Study Population	Intervention	Final *n*	Lycopene Measurement	Inflammation Biomarkers	Conclusions
McEneny et al. [62]	Moderately overweight, middle-aged individuals (*n* = 54)	Control diet (<10 mg lycopene/ week) lycopene-rich diet (224–350 mg/week) lycopene supplement (70 mg/week) for 12 weeks	Control diet (*n* = 18) Lycopene diet (*n* = 18) Lycopene supl (*n* = 18)	**Serum Lycopene (mmol/L)** Baseline Control: 0.26 (0.03) Lycopene diet: 0.41 (0.04) Lycopene supl: 0.29 (0.03) Week 12 Control: 0.27 (0.03) Lycopene diet: 1.14 (0.05) Lycopene supl: 0.87 (0.06)	**Serum Amyloid A (SAA) (µg/L)** Baseline Control: 16,269 Lycopene diet: 15,566 Lycopene supl: 16,899 Week 12 Control: 18,882 Lycopene diet: 17,038 Lycopene supl: 12,070	Lycopene supplement tended to produce a greater response in reducing SAA concentrations and in influencing HDL's function compared to the high-tomato diet.
Petyaev et al. [63]	Patients with coronary vascular disease (*n* = 142)	7 mg of lycopene/day for 1 month, two different lycopene supplements	Lactolycopene (L1) (*n* = 68) Lycosome GA (L2) (*n* = 74)	**Serum Lycopene (ng/mg cholesterol)** Baseline L1: 58.0 L2: 55.0 Week 4 L1: 87.0 L2: 237.0	**CRP (mg/L)/MDA (µM)** Baseline L1: 6.0/141.0 L2: 6.8/154.0 Week 4 L1: 6.2/156.0 L2: 6.1/51.0	Lycopene supplementation had no impact on serum CRP level. Lactolycopene did not affect inflammatory markers by the end of the interventional period, whereas lycosome-formulated lycopene significantly reduced MDA
Gajendragadkar et al. [64]	Statin treated CVD patients and healthy controls (*n* = 72)	7 mg lycopene (1) or placebo (2)/ day for 2 months Patients (*p*) and Healthy (H)	P1: (*n* = 24) P2: (*n* = 12) H1: (*n* = 24) H2: (*n* = 12)	**Serum Lycopene (µg/L)** Baseline/Day 56 P1: 146/275 P2: 128/178 H1: 170/267 H2: 182/160	**hsCRP (mg/L)/IL-6 (pg/mL)/ TNF-a (pg/mL)** Baseline P1: 2.13/1.54/2.13 P2: 1.45/1.20/5.55 H1: 1.15/1.32/5.39 H2: 2.83/0.92/5.55 Day 56 P1: 2.37/1.51/2.37 P2: 1.68/0.92/5.65 H1: 1.87/1.02/4.92 H2: 1.65/0.84/5.32	hsCRP, IL-6 and TNF-a levels were unchanged for lycopene vs. placebo treatment groups in the CVD arm as well as the HV arm

Cont. Table 2 *Intervention studies assessing the influence of lycopene supplementation on inflammation.*

Study [Ref]	Study Population	Intervention	Final *n*	Lycopene Measurement	Inflammation Biomarkers	Conclusions
Kim et al. [65]	Healthy men (n = 126)	Placebo (*p*) Low lycopene, 6 mg/d (L) High lycopene, 15 mg/d (H) For 8 weeks	*p*: (n = 38) L: (n = 41) H: (n = 37)	**Serum Lycopene (µg/mL)** Baseline/8 weeks *p*: 0.2/0.2 L: 0.2/0.26 H: 0.2/0.33	**hsCRP (mg/dL)** Baseline/8 weeks *p*: 1.14 ± 0.22/1.10 ± 0.27 L: 1.39 ± 0.33/1.40 ± 0.37 H: 1.25 ± 0.44/0.54 ± 0.10	A reduction in hs-CRP in the 15-mg lycopene/day group and the inverse correlation between changes in lycopene and changes in hs-CRP in this study, suggest that lycopene may play a role in inflammatory processes by interfering the action of cytokines.
Markovits et al. [66]	Obese patients (*p*) and healthy controls (C) (n = 16)	Patients received Lyc-o-mato, 30 mg/d for 4 weeks	*p*: (n = 8) C: (n = 8)	**Serum Lycopene (µg/mL)** C: 0.14 ± 0.07 *p*;baseline: 0.23 ± 0.22 *p*;supple: 1.15 ± 0.21	**CRP (mg/L)/IL-6 (pg/mL)/ TNF-a (pg/mL)** Baseline C: 1.1/1.0/1.4 *p*: 6.5/3.6/1.4 Week 4 *p*; placebo: 5.5/3.5/1.4 *p*; supple: 5.6/4.7/1.5	CRP and IL-6 levels were significantly higher in obese vs. controls. Following lycopene treatment, a significant elevation of lycopene (1.15 vs. 0.23 µg/mL) ($p < 0.001$) occurred in the treatment vs. the placebo group. Markers of inflammation were not altered by lycopene.

Cont. Table 2 *Intervention studies assessing the influence of lycopene supplementation on inflammation.*

Study [Ref]	Study Population	Intervention	Final *n*	Lycopene Measurement	Inflammation Biomarkers	Conclusions
Thies et al. [67]	Moderately overweight, disease-free, middle-aged adults (*n* = 225)	Control diet (C) High-tomato diet (H) Lycopene capsules (10 mg/d) (L) for 12 weeks	C: (*n* = 76) H: (*n* = 81) L: (*n* = 68)	**Plasma Lycopene (µg/mL)** Baseline/12 weeks C: 0.4/0.4 H: 0.4/1.1 L: 0.4/0.85	**hsCRP (mg/L)** Baseline/12 weeks C: 3.18/2.08 H: 1.51/1.37 L: 2.27/2.16 IL-6 (pg/L) Baseline/12 weeks C: 1.37/1.38 H: 1.21/1.15 L: 1.44/1.31	None of the inflammatory markers changed significantly after the dietary intervention. These data indicate that a relatively high daily consumption of tomato-based products (equivalent to 32–50 mg lycopene/d) or lycopene supplements (10 mg/d) is ineffective at reducing conventional CVD risk markers in moderately overweight, healthy, middle-aged individuals.
Upritchard et al. [68]	Patients with well-controlled type 2 diabetes aged <75 years (*n* = 57)	Placebo (C) Tomato juice 500 mL/d (T) for 4 weeks	C: (*n* = 13) T: (*n* = 15)	**Plasma Lycopene (µmol/L)** Baseline/4 weeks C: 0.31/0.28 T: 0.39/1.08	**Plasma CRP (mg/L)** Baseline/4 weeks C: 3.1/3.1 T: 3.8/4.1	Plasma lycopene levels increased nearly three-fold ($p = 0.001$) and no significant decreases in plasma levels of CRP
Jacob et al. [69]	Healthy subjects (*n* = 24)	2 weeks depletion followed by 2 weeks tomato juice 500 mL/d (41 mg/L lycopene, 90 mg/L Vitamin C) (L) or enriched with Vitamin C (870 mg/L) (LC)	T-2: baseline T0: after depl. T + 2: after inter. L: (*n* = 12) LC: (*n* = 12)	**Plasma Lycopene (µmol/L)** L/LC T-2: 0.72/0.71 T0: 0.42/0.34 T + 2: 1.05/0.91	**L/LC** **CRP (ug/L)** T-2: 336.2/349.5,T0: 315.6/319.2,T + 2: 262.3/247.1 **IL-1 B (ng/L)** T-2: 3.45/12.59,T0: 3.87/10.68,T + 2: 4.39/6.40 **TNF-a (ng/L)** T-2: 6.97/2.93,T0: 6.01/3.35,T + 2: 3.45/3.28 **MDA (µmol/L)** T-2: 0.55/0.60, T0: 0.54/0.56, T + 2: 0.53/0.50	The consumption of tomato juice led to a reduction of CRP in both groups. All other markers were affected to a lesser extent or remained unchanged.

Cont. Table 2 *Intervention studies assessing the influence of lycopene supplementation on inflammation.*

Study [Ref]	Study Population	Intervention	Final *n*	Lycopene Measurement	Inflammation Biomarkers	Conclusions
Williams et al. [70]	COPD patients (*n* = 11)	Rosuvastatin (20 mg/day) for 4 weeks then a combination of rosuvastatin (20 mg/day), DHA and EPA (1.5 g/day) and lycopene (45 mg/day) for 8 weeks.	T1: baseline T2: rosuvastatin T3: lycopene	**Plasma Lycopene (mg/L)** T1: 0.30 (0.13–0.54) T2: 0.56 (0.14–0.77) T3: 0.50 (0.22–0.96)	**CRP (mg/L)** T1: 3.9 (1.9–7.9) T2: 3.3 (0.7–7.6) T3: 3.8 (1.3–8.9) **IL-6 (pg/mL)** T1: 2.2 (1.6–3.0) T2: 3.2 (2.3–5.1) T3: 3.1 (1.6–4.8)	Treatment interventions did not significantly change plasma carotenoid levels. However, there was a trend for increased lycopene concentration at visit 2 and 3. Following the interventions, plasma IL-6 and CRP were unchanged.
Rydén et al. [71]	Middle-aged men with mild to moderate hypercholesterolemia (*n* = 76)	Placebo (*p*) Simvastatin 40 mg (S) for 6 weeks	*p*: (*n* = 39) S: (*n* = 37)	**Plasma Lycopene (nmol/L/cholesterol)** Baseline *p*: 116 (89–149) S: 100 (75–142) Week 6 *p*: 125 (98–160) S: 147 (104–182)	**CRP (mg/L)/IL-6 (pg/mL)** Baseline *p*: 1.1/1.2 S: 1.3/1.5 Week 6 *p*: 1.0/1.3 S: 0.9/1.4	Simvastatin use was associated with significant reductions in CRP and reduced plasma levels of lycopene. However, when adjusted for lipids, lycopene showed significant increases after simvastatin therapy.
Hurtado-Barroso et al. [72]	Healthy male subjects (*n* = 22)	Single dose of sofrito (240 g/70 kg)	T1: baseline T2: intervention	**Plasma Lycopene (µmol/L)** Baseline/After consumption trans-lycopene: 2.15 ± 0.30/6.33 ± 1.53 5-cis-lycopene: 1.87 ± 0.28/7.93 ± 2.73 13-cis-lycopene: 0.21 ± 0.11/2.08 ± 0.78 9-cis-lycopene: *n*.d./0.90 ± 0.58	**CRP (mg/dL)** T1: 0.1 T2: 0.08 **IL-6 (pg/mL)** T1: 1.4 T2: 1.0 **TNF-a (pg/mL)** T1: 1.0 T2: 0.8	After the sofrito intake, a significant decrease in CRP (*p* = 0.010) and TNF-α (*p* = 0.011) was observed.

Cont. Table 2 *Intervention studies assessing the influence of lycopene supplementation on inflammation.*

Study [Ref]	Study Population	Intervention	Final *n*	Lycopene Measurement	Inflammation Biomarkers	Conclusions
Colmán-Martínez et al. [73]	Subject at high Cardiovasc. risk (n = 28)	Tomato Juice HD 400 mL/d LD 200 mL/d Control: Water for 4 weeks	C: (n = 28) LD: (n = 28) HD: (n = 28)	**Plasma Lycopene (µmol/L)** trans-lycopene C: 0.70 ± 0.44 LD: 4.04 ± 0.39 HD: 6.67 ± 0.38 5-cis-lycopene C: 1.13 ± 0.28 LD: 2.38 ± 0.27 HD: 4.08 ± 0.26 13-*cis*-lycopene C: 1.07 ± 0.39 LD: 1.90 ± 0.30 HD: 4.01 ± 0.29 9-*cis*-lycopene C: 0.42 ± 0.43 LD: 1.05 ± 0.29 HD: 1.92 ± 0.21	**CRP (ng/mL)** C: 546 ± 46 LD: 442 ± 44 HD: 530 ± 43 **IL-8 (pg/mL)** C: 40 ± 17 LD: 23 ± 16 HD: 24 ± 15	Plasma lycopene increased significantly in intervention group compared to placebo (p < 0.001). No significant alterations in CRP and IL-8 were found.
Wood et al. [74]	Asthmatic adults (n = 137)	High-antioxidant diet (HAO) or a low-antioxidant diet (LAO) for 14 d Subjects who consumed the low-antioxidant diet received placebo or tomato extract (45 mg lycopene/d).	HAO: (n = 46) LAO: (n = 91)	**Plasma Lycopene (mg/L)** Baseline/day 14 HAO: 0.15/0.18 LAO: 0.20/0.13	**hsCRP (mg/L)/IL-6 (pg/mL)/ TNF-a (pg/L)** HAO baseline: 4.2/1.9/1.3 HAO day 14: 3.0/1.9/1.3 LAO baseline: 2.5/1.9/1.4 LAO day 14: 3.3/2.0/1.5	After 14 d of dietary modification, a significant decrease from baseline in plasma lycopene concentrations was observed in the LAO diet group, which was significantly different from the increase in the HAO. No effect of the lycopene-rich supplement compared with placebo was observed. Subjects in the low-antioxidant diet group had increased plasma C-reactive protein at week 14.

2

Cont. Table 2 *Intervention studies assessing the influence of lycopene supplementation on inflammation.*

Study [Ref]	Study Population	Intervention	Final *n*	Lycopene Measurement	Inflammation Biomarkers	Conclusions
Yeon et al. [75]	Overweight women (*n* = 22)	High-Vegetable/ Fruit (VF) diet (12 servings of VF/ day) or low-VF diet (2 servings of VF/day) for 2 weeks, 2 weeks wash-out, 2 weeks	Low base (LB): (*n* = 22) Low post (LP): (*n* = 22) High base (HB): (*n* = 22) High post (HP): (*n* = 22)	**Plasma Lycopene (µmol/L)** LB: 0.39 ± 0.18 LP: 0.32 ± 0.14 HB: 0.31 ± 0.19 HP: 0.38 ± 0.32	**CRP (µg/mL)/IL-6 (pg/mL)** LB: 0.54 ± 0.44/3.65 ± 1.51 LP: 0.75 ± 0.70/3.08 ± 0.35 HB: 0.56 ± 0.61/3.52 ± 1.08 HP: 0.40 ± 0.40/3.44 ± 0.83	Results from this study showed that the low-VF diet decreased the average plasma carotenoids by 26%, and the high-VF diet increased the average plasma carotenoids by 32% compared to the baseline values. Changes in plasma lycopene were inversely correlated with changes in plasma IL-6 concentrations when the subjects consumed the low-VF diet.

2.3 RESULTS

2.3.1 Study Characteristics

Of the 80 articles identified, screened, and considered for systematic review, 35 articles met the inclusion criteria for critical analysis. All papers were published between 1996 and 2018. Eighteen of the 35 studies used a cross-sectional study design and the remaining 17 were intervention trials. Studies varied widely not only in design and lycopene measurements, but also in the assessment of inflammation biomarkers. While some studies have identified multiple outcome measures, only the measurements of circulating lycopene and inflammatory biomarkers are highlighted in this review. Furthermore, studies that examined possible correlations or made conclusions regarding the relationship between circulating lycopene and inflammatory biomarkers, were also included in this systematic review. In various studies, the reported sample size for the outcomes of interest differed from the total number of participants. As such, these unique sample sizes have been reported with the corresponding measurement (Tables 1 and 2).

2.3.2 Cross-Sectional Studies

As shown in Tables 1 and 2, the discussion of the 35 articles is separated by study design. As displayed in Table 1, the results reported in the 18 included cross-sectional studies reviewed were grouped according to the following categories: type of lycopene measurement, assessment of inflammation biomarkers, and conclusions drawn from the study.

Five of these studies classified participants based on CRP concentrations or lycopene levels [41,43,45,51,53]. A study by Mazidi et al. [41] divided participants in quartiles depending on CRP concentrations and concluded that a higher lycopene level for each µmol/l correlated with 0.067 mg/dl lower CRP levels. Kritchevsky et al. [51] divided participants in tertiles depending on CRP levels and concluded that participants in the higher tertile CRP had significantly lower circulating lycopene levels. Furthermore, Boosalis et al. [53] divided elderly women (77–99 years) into two groups, based on either normal or elevated CRP levels, and showed that the presence of elevated CRP resulted in a significant decrease of lycopene concentrations. In addition, Kim et al. [43] divided healthy women (31–75 years) into tertiles according to serum lycopene concentrations and reported that subjects in the highest tertile showed significantly lower CRP levels compared to those individuals in the lowest tertile. On the contrary, this association was not found in a study [45] in which young adults were divided into quartiles depending on the lycopene concentrations.

Thirteen other studies assessed the relationship between circulating lycopene and inflammation in healthy participants or patients. These studies report lower circulating

lycopene concentrations and higher inflammation biomarker levels in patients with colorectal adenocarcinoma [42,55], carotid artery disease [44], stable angina pectoris [49,57], ischemic stroke [56], chronic hepatitis C [50], gastrointestinal cancer [52], benign prostate hyperplasia, localized and metastatic prostate cancer [54,55] and breast cancer [55] compared to healthy controls [48]. In addition, this relationship was also observed in critically ill patients [58], elderly disabled women [46], and people exposed to Schistosoma [47].

In general, these results suggest that lycopene levels are adversely affected during inflammation and homeostatic imbalance. These cross-sectional data do not clarify the biological relationship between lycopene and inflammation biomarkers. However, they do indicate the extent to which lycopene is associated with inflammation. They also indicate that the depletion of lycopene may be, in part, the first signs of low-grade inflammation.

2.3.3 Intervention Studies

As displayed in Table 2, the results reported in the 17 included intervention studies reviewed were grouped according to the following categories: type of lycopene measurement, assessment of inflammation biomarkers, type of intervention and conclusions drawn from the study.

Each of the included 17 intervention studies assessed lycopene levels and inflammatory biomarkers pre- and postintervention. All studies, except one, reported increased circulating lycopene levels following tomato/lycopene supplementation. In the exceptional study, supplementation with Lactolycopene capsules (supplements with lycopene entrapped with whey proteins) did not lead to a significant increase, but supplementation with Lycosome GA capsules (supplements with microencapsulated lycopene) did [63]. In a second study, supplementation with a combination of lycopene and rosuvastatin also did not significantly change plasma lycopene levels [70].

In ten intervention studies, biomarkers of inflammation were not reported to change after tomato/lycopene supplementation [59,62,64,66–68,70,73–75]. On the contrary, in a study by Li et al. [60], tomato juice supplementation led to a decrease of inflammatory adipokine MCP-1, and an increase in anti-inflammatory adiponectin levels in healthy Taiwanese females (20–30 years). Additionally, Biddle et al. [61] reported that tomato juice supplementation significantly decreased CRP levels in female heart failure patients, but not in male patients. Conversely, a decrease in hs-CRP was observed in healthy men following high lycopene (15 mg/day) supplementation [65] and after a single dose of tomato sauce (sofrito) [72].

Petyaev et al. [63] investigated the effect of supplementation with Lactolycopene or Lycosome GA capsules in patients with coronary artery disease. Serum lycopene levels of participants receiving Lactolycopene did not increase, and CRP and MDA levels did not change after one month of supplementation. Nevertheless, in the group that received Lycosome GA capsules, circulating lycopene increased after one month, but only MDA was significantly reduced. In addition, opposite results were observed in healthy subjects in a study by Jacob et al. [69] in which CRP levels decreased following tomato juice supplementation, but IL-1β, TNF-α, and MDA levels remained stable.

Four of the seventeen selected studies conducted intervention studies in moderately overweight or obese individuals. Biomarkers for inflammation are often elevated in obese individuals compared to healthy individuals. One study [66] also used a healthy control group and concluded that pre-intervention CRP and IL-6 levels were significantly higher in obesity versus controls. All four concluded that markers of inflammation were not altered by lycopene, despite the significant increase in circulating lycopene after supplementation [62,66,67,75].

Four of the selected intervention studies investigated the effect of lycopene supplementation on inflammatory markers in patients with Cardiovasc. diseases [61,63,64,73]. These studies did not show consistent results. In one study, only MDA decreased [63]. In the next study, only CRP decreased in women (not in men) [61]. In the other two studies no alterations in inflammation biomarkers were observed after supplementation with lycopene [64,73]. The latter results were also observed in a study conducted in patients with type 2 diabetes, in which plasma lycopene levels increased nearly three-fold ($p = 0.001$), but no significant decreases in plasma levels of CRP were observed [68].

Six included intervention studies evaluated possible associations between lycopene/ tomato supplementation and inflammation in healthy participants. In three of these studies, markers of inflammation did not change after supplementation, although circulating lycopene had increased by about 50 percent [59,64,69]. However, in another study the lycopene concentration also increased 1.5 times, and a significant decrease in hs-CRP was observed [65]. Furthermore, Hurtado-Barroso et al. [72] observed a three-fold increase in circulating lycopene and a significant decrease in CRP after a single dose of tomato sauce (sofrito). It is worth mentioning that CRP values in both studies were already below standard values before the start of the intervention. Li et al. [60] demonstrated that tomato juice supplementation led to a decrease of inflammatory adipokine MCP-1, and an increase in anti-inflammatory adiponectin levels in healthy young Taiwanese females. Compared to the other studies in which no or minor effects were seen on CRP, MCP-1 and

adiponectin may be more sensitive biomarkers and therefore more suitable for studying inflammation in healthy individuals.

Rydén et al. [71] investigated the effect of simvastatin therapy on plasma lycopene levels and inflammatory markers in middle-aged men with mild to moderate hypercholesterolemia. Lycopene levels per total cholesterol (expressed as lycopene/ total cholesterol) were significantly increased by simvastatin treatment. The findings may indicate that atherogenic lipoprotein particles have improved their antioxidant status through enrichment of carotenoids during simvastatin therapy.

Overall, most studies reported increased circulating lycopene levels after tomato/lycopene supplementation, but less than half of them observed alterations in inflammation biomarkers. In addition, two studies examined the effects of a low antioxidant diet in overweight women and asthmatic adults and observed a decrease in circulating lycopene and an increase in CRP [74,75]. Compared to supplementation, lycopene depletion appears to increase inflammation.

2.4 DISCUSSION

This is, to our knowledge, the first systematic review to assess the correlation and causation between circulating lycopene (the bioavailable lycopene following consumption) and low-grade chronic inflammation. This review reveals that there is strong evidence indicating that lower circulating lycopene concentrations are related with higher inflammation biomarkers in patients with various diseases. In addition, this systematic review shows that there is little evidence that tomato intake or lycopene supplementation diminishes this inflammation.

In only one of the five studies in which CRP or lycopene levels were arranged into tertiles/quartiles, no association was found between circulating lycopene and CRP [41,43,45,51,53]. This could be attributable to the low CRP levels of the studied young adults (18–30); all mean CRP levels measured were between 0.99 and 1.11 mg/L [45]. On the contrary, the results from another study [43] showed a significant association and measured high-sensitivity CRP (hs-CRP) ranging from 0.80 and 1.27 mg/L. Moreover, when comparing the corresponding lycopene levels, it is striking that the values of Hozawa et al. [45] lie between 0.0242 and 0.0918 µmol/L, whereas most lycopene levels measured in all studies are between 0.1 and 1 µmol/L. It is therefore also possible that a non-reliable lycopene measurement has been carried out, so that no association could be found. The other three studies [41,51,53] did confirm the findings of Kim et al. [43], so there is strong evidence to suggest an association between circulating lycopene and CRP.

The eighteen studies evaluating the relationship between circulating lycopene and inflammation in healthy participants and patients gave similar results. These studies found lower circulating lycopene concentrations coincide with higher inflammation biomarkers in patients suffering from various diseases. These comparable results suggest that lycopene levels are adversely affected during inflammation and disturbed homeostasis. One possible explanation is that the development of oxidative stress during inflammation is responsible for the decreased lycopene levels. The prooxidant–antioxidant imbalance that ensues during oxidative stress may result in the increased utilization of endogenous and exogenous antioxidants, depleting circulating antioxidant concentrations. For that reason, any protective association that exists between serum lycopene and inflammation in patients may be attenuated [76–79]. Although the mechanisms underpinning reduced lycopene levels during inflammation are not fully elucidated, depletion of lycopene may be in part the first sign of low-grade inflammation.

Seventeen intervention studies were identified which better elucidate this carotenoid's causal effect on inflammation and outcomes. Results from cross-sectional studies preclude the ability to ascribe causality because of both potential confounding and a lack of knowledge about the temporal relation between variables of interest. Most studies successfully increased lycopene levels through supplementation or tomato intake. In one study, supplementation with Lactolycopene capsules did not significantly increase lycopene levels. The authors emphasized the importance of proper supplement development, as another supplement increased circulating lycopene. In addition, supplementation with a combination of lycopene and rosuvastatin did not increase lycopene levels either [70]. The latter result could be explained by another study, in which supplementation with simvastatin, a comparable statin, led to a decrease in circulating lycopene. However, lycopene levels per total cholesterol were significantly increased following simvastatin treatment. The observed change in carotenoid status during simvastatin treatment was mainly attributed to the decrease in cholesterol, emphasizing the importance of cholesterol adjustment for expressing carotenoid levels [71].

This review found that the effect of lycopene supplementation or tomato intake on inflammation is incongruent: no changes in inflammation biomarkers were observed in half of the studies, and in the other half not all results were in line. Inflammatory markers were not altered by lycopene in moderately overweight or obese people, despite the significant increase in circulating lycopene after supplementation [62,66,67]. Intervention studies in patients with cardiovascular disease or type 2 diabetes also showed minimal reduction of inflammatory markers [61,63,64,68]. In some intervention studies, it was stated that the intervention period was too short to observe a decrease in inflammatory biomarkers in patients. However, previous research has shown that treatment with non-steroidal anti-inflammatory drugs (NSAIDs) for a short period (two weeks) may reduce

inflammatory biomarkers in patients, so these inflammatory biomarkers are unlikely to take longer to decrease [80,81]. Likewise, the results of lycopene supplementation in healthy participants were also inconsistent. Only two studies observed a significant decrease in hs-CRP after high lycopene supplementation (15 mg/day) or tomato sauce (sofrito) intake [72], but no effects were found after low lycopene supplementation (6 mg/day) [65] nor 7 mg/day [64]. The hs-CRP test accurately measures low CRP levels to identify low but persistent inflammatory levels. Therefore, it is more suitable for studying low-grade chronic inflammation in healthy participants in further research. However, it is debatable whether such a significant reduction in CRP below the standard values of 1–3 mg/L is clinically relevant and shows an actual anti-inflammatory effect, as these low CRP values already demonstrate that there is hardly any inflammation present. The other studies evaluating CRP report no significant changes in CRP levels following lycopene intake, probably because of the already low basal value in healthy participants. In addition, it would be of interest to evaluate new, more sensitive biomarkers in subsequent studies, as MCP-1 and adiponectin prove to be suitable biomarkers to study inflammation in healthy subjects [60].

Two intervention studies investigated the potential beneficial effects of lycopene in its isolated form (supplement) and via a lycopene-rich diet. These particular studies showed that both methods were successful in increasing circulating lycopene, but not in changing inflammation biomarkers [62,67]. These results suggest that the form in which lycopene is administered is of less importance than the absorption per se. For example, the absorption of lycopene can be improved by method of preparation such as adding olive oil [82]. Current literature indicates that the incorporation of a functional food with the compound of interest could potentially enhance these protective properties through the provision of an intact food matrix. However, more research is needed to elucidate these speculations. The matrix may provide a synergistic environment to promote the bioactivity of phytonutrients. However, this matrix also presents a challenge, since the direct effects of lycopene cannot be separated from other bioactive compounds within the food [83,84].

2.4.1 Molecular Mechanisms of Action

The incongruent results observed between the cross-sectional and intervention studies may be attributed to the different mechanisms of action of lycopene. Many in vitro studies elucidated the protective properties of carotenoids. As free radical scavengers, carotenoids react with reactive oxygen species (ROS) by three distinct mechanisms: (i) radical addition/adduct formation, (ii) electron transfer, and (iii) allylic hydrogen abstraction [85]. However, it is difficult to extrapolate the results of such studies because processes in the human body are more complex. It is probable that a number of factors may serve to decrease the antioxidant effectiveness of carotenoids in vivo, making

them ineffective against certain ROS [86]. Furthermore, recent findings have shown that the participation of phytochemicals in redox metabolism is far more complicated than simply scavenging free radicals and avoiding oxidation of molecules. The cellular redox homeostasis is sustained by an overall and well-adjusted network of subcellular redox circuits that oscillate constantly depending on nutrients and energy supplies, genetic and epigenetic codes, and interactions with the external environment [87].

Barros et al. [87] suggest the hypothetical existence of the l NAD(*p*) +/NAD(*p*)H-responsive redox switch of eukaryotic cells that triggers distinct phenotypic fates depending upon cellular redox balance. This theory may explain the reduced lycopene levels in impaired situations as well as the paradoxical phenomenon where depletion of lycopene appears to increase inflammation, but lycopene supplementation does not decrease inflammation.

This theory suggests that an increase of the cellular antioxidant capacity (from dietary intake or generated endogenously) slides the antioxidant "seesaw" pivot point to the right, attenuating the magnitude of ROS/RNS production in the cell. However, an excessive antioxidant load in cells (sliding further to the right) could prevent beneficial processes mediated by the Nrf2−Keap1−EpRE system (Figure 2) [87,88]. Halliwell [89] describes the "antioxidant paradox" that supports this theory. The term "antioxidant paradox" is often used to refer to the observation that oxygen radicals and other ROS are implicated in several human diseases, but giving large doses of dietary antioxidants to human subjects has, in most studies, little or no preventative or therapeutic effect on inflammation. In addition, providing weak pro-oxidants to manipulate endogenous antioxidant levels may be a more useful approach for prevention of non-communicable diseases than is consumption of large doses of dietary antioxidants [89]. For example, it is well-known that physical activity increases the level of oxidative stress, but this appears to be beneficial to health. This same stimulus is in fact necessary to allow upregulation of endogenous antioxidant defenses, a phenomenon known as hormesis [90–92]. In addition to physical activity, various phytochemicals present in fruits and vegetables also can increase the level of oxidative stress and may exert health effects in other ways than lycopene.

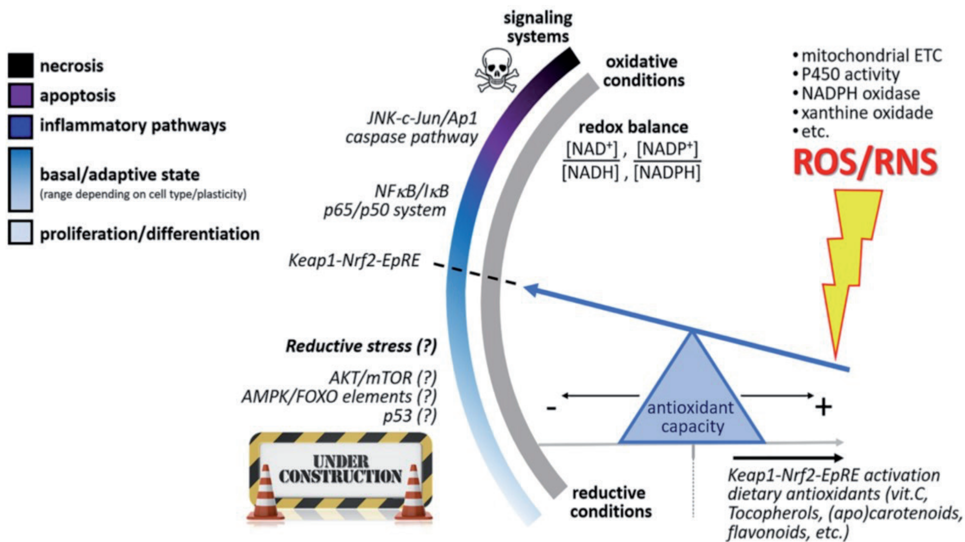

Figure 2. *Hypothetical NAD(p) +/NAD(p)H-responsive redox switch of eukaryotic cells that triggers distinct phenotypic fates depending upon cellular redox balance. From a basal condition (optimum redox balance), the redox switch elicits inflammatory pathways, apoptosis, or necrosis, following increasing oxidative conditions, whereas unclear "reductive stress" mechanisms are triggered when NAD(p)H coenzymes prevail in cellular compartments. An increase of the cellular antioxidant capacity (from diet intake or generated endogenously) slides the antioxidant "seesaw" pivot point to the right, attenuating the magnitude of ROS/RNS production in the cell. However, an excessive antioxidant load in cells (sliding further to the right) could prevent beneficial processes mediated by the Nrf2−Keap1−EpRE system. This figure was adapted from reference [87].*

Isothiocyanates from cruciferous vegetables can react directly with sulfhydryl residues of Keap1, causing the release of Nrf2. The ROS scavenging capacity of curcumin from turmeric is mainly attributed to its structure as a bis-α, β-unsaturated β-diketone of the two ferulic acid units, connected through a methylene group, which in addition can modify the thiol groups of Keap1, causing the release of Nrf2 [93]. The semi-synthetic flavonoid 7-mono-O-(β-hydroxyethyl)-rutoside (monoHER) acts as a double-edged sword in cells subjected to oxidative stress; the antioxidant offers direct protection by scavenging ROS and the oxidized monoHER adducts Keap1, causing the release of Nrf2 [94]. Alternatively, epigallocatechin gallate (from tea), cinnamaldehyde (from cinnamon), and resveratrol (from grapes) act on upstream kinases such as Akt, ERK, PI3K, PKC, and JNK causing the indirect release of Nrf2 from Keap1 [93]. Nrf2 is translocated to the nucleus and binds to the antioxidant response element (ARE) located in the promoters of genes coding for antioxidant and detoxifying enzymes. Nrf2/ARE-dependent genes code for several mediators of the antioxidant response, including glutathione S-transferases (GSTs), thioredoxin, NAD(*p*)H quinone oxidoreductase 1 (NQO-1), and heme oxygenase 1 (HO-1) [95]. Paradoxically, this reaction is considered weakly pro-oxidant [96]. The resulting oxidative stress supports the hormetic feedback and therefore leads to an

endogenous increase in antioxidant defenses. It is possible that an excess of exogenous antioxidants may have detrimental effects on health by blocking the hormetic process [90,91]. In addition, various phytochemicals, such as the flavonoid quercetin from onions, can increase the endogenous antioxidant defenses in multiple ways. Similar to monoHER, oxidation products of quercetin are able to modify the thiol groups of Keap1, causing the release of Nrf2 and by up-regulation of Nrf2 through the regulation of both transcription and posttranscription sites and repression of Keap1 by affecting the posttranscription site [97,98].

2.4.2 Dietary Recommendations

A wealth of epidemiological evidence indicates that diets rich in plant products (grains, fruits and vegetables) contribute to overall health [2–4,99,100]. It is not clear whether this health-promoting effect is mainly attributable to the antioxidants in these plant products. However, the evidence suggests that antioxidants do play an important role in maintaining health: two studies included in this review examined a low antioxidant diet, during which a decrease in circulating lycopene and a subsequent increase in CRP was observed [74,75]. The available data thereby imply that it is beneficial to consume lycopene-rich foods occasionally to stay healthy and keep circulating lycopene at a basal level. It is preferable to consume lycopene through whole food sources such as tomatoes, rather than to ingest it through supplementation. This is because (i) lycopene is stable during preparation methods, (ii) other phytonutrients are also present in e.g., tomatoes, (iii) the potential benefits of the food matrix, and (iv) the costs. But as this study shows, it is unlikely that taking additional lycopene will help restore health if inflammation is already present. Nevertheless, additional research is needed to determine evidence-based recommendations on the effect of long-term lycopene intake or supplementation and reduction of inflammation. In today's society, antioxidants are considered healthy, partly because of results from in vitro studies. It is possible that the health effects of fruit and vegetables are due to the wide variety of bioactive substances in the food matrices and the synergy between the different mechanisms of action of these phytochemicals in the body. Nevertheless, the riddle of the "antioxidant paradox," as described in Section 4.1, is yet to be fully deciphered. Phytochemicals in fruits and vegetables, both anti- and pro-inflammatory, appear to play a key role in this. In further research, it is important to consider the complexity of the endogenous antioxidant defense system [90]. Epidemiological evidence indicates that a multifactorial strategy of exercise, a healthy weight, no smoking, and a balanced diet that includes plenty of fruits, grains, and vegetables, is optimal to prevent low-grade chronic inflammation and maintain health overall [89].

2.4.3 Strengths and Limitations

This systematic review is one of the first studies that focusses on studying circulating lycopene measurements and its effect on inflammation, instead of merely the intake of lycopene. Self-reported measures of lycopene intake are subject to recall bias or memory errors, and do not provide insights into the body's absorption, distribution, metabolism, and excretion of lycopene. Another strength of this study is that in this review, a clear distinction is made between effects reported in observational studies versus intervention studies. These reported effects are furthermore explained by incorporating details on the molecular mechanism of action of lycopene. The results of the cross-sectional studies are consistent with the findings of previous systematic reviews assessing the relationship between lycopene and vascular risk, metabolic syndrome, prostate, and bladder cancer [33–36]. However, suggestions made in these reviews about the effect of lycopene supplementation to reduce the risk of these diseases differ from the findings in this review, as this review highlights that there is little evidence that lycopene supplementation reduces inflammation. Furthermore, this study is not without limitations. Overall, intervention studies were characterized by small sample sizes and short duration. In follow-up research, it would be of interest to investigate the effects of long-term lycopene supplementation on inflammation. Additionally, it is important to acknowledge that there might be publication bias in the intervention studies. It is known that positive results are published in scientific literature more often than negative or inconclusive ones. This study has also not been able to perform a meta-analysis to quantify the potential effects of lycopene because studies differed widely in lycopene and inflammatory biomarkers measurements. Lastly, even though all authors were involved in conducting the systematic search, setting inclusion criteria, and reviewing the inclusion of publications, selection bias may have affected the in- and exclusion of certain studies.

2.5 CONCLUSION

The available evidence indicates that lycopene levels are adversely affected during inflammation and homeostatic imbalance. Although the mechanisms underpinning these reduced lycopene levels are not fully elucidated, depletion of lycopene may be one of the first signs of low-grade inflammation. Even though supplementation with lycopene or an increased intake of tomatoes does result in an increase in circulating lycopene, there is little evidence that the lycopene increase also results in relieving this inflammation. This phenomenon, also known as the "antioxidant paradox," limits the added value of lycopene supplementation in both patients and healthy individuals.

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3

Immunomodulating effects of fungal beta-glucans: From traditional use to medicine

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ABSTRACT

The importance of a well-functioning and balanced immune system has become more apparent in recent decades. Various elements have however not yet been uncovered as shown, for example, in the uncertainty on immune system responses to COVID-19. Fungal beta-glucans are bioactive molecules with immunomodulating properties. Insights into the effects and function of beta-glucans, which have been used in traditional Chinese medicine for centuries, advances with the help of modern immunological and biotechnological methods. However, it is still unclear into which area beta-glucans fit best: supplements or medicine? This review has highlighted the potential application of fungal beta-glucans in nutrition and medicine, reviewing their formulation, efficacy, safety profile, and immunomodulating effects. The current status of dietary fungal glucans with respect to the European scientific requirements for health claims related to the immune system and defense against pathogens has been reviewed. Comparing the evidence base of the putative health effects of fungal beta-glucan supplements with the published guidance documents by EFSA on substantiating immune stimulation and pathogen defense by food products shows that fungal beta-glucans could play a role in supporting and maintaining health and, thus, can be seen as a good health-promoting substance from food, which could mean that this effect may also be claimed if approved. In addition to these developments related to food uses of beta-glucan-containing supplements, beta-glucans could also hold a novel position in Western medicine as the concept of trained immunity is relatively new and has not been investigated to a large extent. These innovative concepts, together with the emerging success of modern immunological and biotechnological methods, suggest that fungal glucans may play a promising role in both perspectives, and that there are possibilities for traditional medicine to provide an immunological application in both medicine and nutrition.

3.1 INTRODUCTION

Many chronic diseases can be explained by an underlying chronic inflammation; more appealing to the imagination is the recent COVID-19 pandemic, which has presented the modern world with a challenge that global health care has not faced in more than a century since the Spanish flu pandemic in 1918 [1]. A characteristic feature of an infection with COVID-19 is a pro-inflammatory status characterized by high levels of different cytokines, including interleukin (IL)-1β, IL-1Rα, IL-2, IL-10, fibroblast growth factor (FGF), granulocyte-macrophage colony stimulating factor (GM-CSF), granulocyte-colony stimulating factor (G-CSF), interferon-γ-inducible protein (IP10), monocyte chemoattractant protein (MCP1), macrophage inflammatory protein 1 alpha (MIP1A), platelet-derived growth factor (PDGF), tumor necrosis factor (TNF-α), and vascular endothelial growth factor (VEGF) [2]. These changes in cytokine levels are associated with various changes in cellular components of the immune response [3]. It becomes more evident that there is a close interaction between the virus and an individual's immune system, resulting in different clinical manifestations of the disease [4]. Moreover, with the aid of modern immunological and biotechnological methods, the importance of a well-functioning and balanced immune system in maintaining overall health has become more apparent in recent decades [5]. In anticipation of the global immunization of the population through vaccines developed for the adaptive immune system, innate immune-based strategies for therapeutic purposes are also being investigated [2]. The innate immune system constitutes the host's first line of defense during infection and therefore plays a critical role in the early recognition and subsequent activation of a pro-inflammatory response to invading pathogens (for review, see [6]). Nonspecific immunostimulants (NSIs) are natural, synthetic, or recombinant molecules that stimulate the innate immune system by inducing activation or increasing activity of any of its components. In contrast to specific immunostimulants such as vaccines, NSIs act irrespective of antigenic specificity to augment immune response of other antigen or stimulate components of the immune system without antigenic specificity. Despite the tremendous advances in this field of immunology over the years, many areas of uncertainty remain. For example, one question that remains is what is the precise mechanism of action of cell activation, immunomodulation, and tumor reduction for NSIs used in cancer therapy (e.g., mifamurtide, BCG vaccine). In addition to increasing our understanding of these drugs, there is an increasing interest in the development of NSIs that can be used in infectious and inflammatory diseases [6]. Recently, immunomodulators used in traditional Chinese medicine (TCM) for centuries, such as the shiitake and the pearl oyster mushroom, have gained interest for these new developments [7]. Many of the traditionally used substances, however, are substantiated by only limited scientific studies. An exception to this is the fungal beta-glucans which, with more than 20,000 published studies, are the most studied mushroom-derived molecules with potential immunomodulating properties [8,9].

3.1.1 Structure, Chemical Properties, and Natural Sources of Beta-Glucans

Beta-glucans are groups of polysaccharides or dietary fibers composed of D-glucose monomers, linked by (1 → 3), (1 → 4) or (1 → 6) glycosidic bonds. Beta-glucans are naturally found in the cell wall of bacteria, fungi, algae, and cereals such as oat and barley [10]. The different sources of beta-glucans, however, also differ in the linkages between the D-glucose monomers. The beta-glucans present in cereals include a mixture of (1 → 3) and (1 → 4) glycosidic bonds. Beta-glucans in mushrooms mostly contain a linear (1 → 3) backbone with (1 → 6)-linked glucose branches attached. Furthermore, beta-glucans found in yeasts, seaweeds, and bacteria display different structural forms and branching, of which curdlan, extracted from *Agrobacterium*, is the simplest structure; it is only composed of unbranched (1 → 3) glycosidic bonds [11]. These differences in the shape, structure, and molecular weight of beta-glucans determine their biological activity. For cereal beta-glucans (dietary fibers), mainly physicochemical properties are reported, including reactive oxygen species (ROS) scavenging activity, and their ability to lower serum cholesterol and improve gut microbiome [11]. These properties have been attributed to the mixture of only the (1 → 3) and (1 → 4) glycosidic bonds, making them resistant to absorption and digestion in the small intestine of humans [12,13]. The effects of beta-glucans containing (1 → 6) branching, such as fungal or bacterial glucans, are related to the activation/inactivation of specific receptors such as dectin-1 (mostly insoluble beta-glucans), complement receptor 3 (CR3), or toll-like receptor 2 (TLR2) (mainly water-soluble glucans) [11,14]. Individual fungi contain specific beta-glucans, which differ from each other by the amount of (1 → 6) linked side chains (Figure 1). Moreover, the content and proportions of beta-glucans in fungi is mainly determined by their genetic profile and differs between species and even cultivars. Upon ingestion, fungal glucans affect the mucosal immune system in the gastrointestinal tract. Similar to antigens, the uptake of beta-glucans occurs via microfold cells (M cells) localized within Peyer's patches in the small intestine. M cells subsequently present the antigen or beta-glucan at their basal surfaces to immune cells, such as macrophages and dendritic cells. Here, beta-glucan particles bind with macrophages with the help of dectin-1, the primary receptor for most insoluble beta-glucans. Subsequently, dectin-1 induces the secretion of pro-inflammatory cytokines via nuclear factor kappa-B (NF-κB) and various interconnected inflammatory and immunoregulatory processes such as chemokinesis and chemotaxis (for review, see [15]). Given these immunomodulatory effects, the use of fungal glucans as pharmaceutical agents, which act as (adjuvant) immunomodulators, has been authorized in several countries, including the United States of America, Canada, Finland, Sweden, China, Japan, and Korea [16]. Lentinan, isolated from shiitake mushroom, is an example of a pharmaceutically formulated polysaccharide approved as an intravenous immunostimulant in the treatment of multiple cancers in China and Japan [17]. In addition, fungal glucans are widely used in the nutritional field as dietary supplements. Unlike pharmaceutical drugs that are supposed to suppress or stimulate

the immune system in patients, dietary supplements are primarily intended as a daily oral dose to support the immune system in healthy people. Pleuran, isolated from pearl oyster mushrooms, is an example of a polysaccharide developed as a dietary supplement to support the immune system and overcome the first signs of exhaustion and fatigue in adults and children [18]. In addition to these formulations, the edible mushrooms shiitake (*Lentinula edodes*) and the pearl oyster mushroom (*Pleurotus ostreatus*) are some of the main dietary sources of beta-glucans (Figure 1) [19].

Modern immunological and biotechnological methods provide us with increasing insight into the effects and function of beta-glucans [8]. However, there are still many unresolved questions, for example, one of the biggest challenges remains the standardization and correct characterization of the molecules themselves. Furthermore, it is still unclear into which area beta-glucans fit best: supplements or medicine? In this review, we shed light on both perspectives to discover which area the beta-glucans, in addition to TCM, could fit, and what evidence is needed for this. In the field of dietary supplements, we highlight the evidence required to use health claims on products according to regulatory authorities in Europe. In the field of medicine, the potential pharmaceutical application of beta-glucans within a novel concept in immunology, namely trained immunity, is discussed.

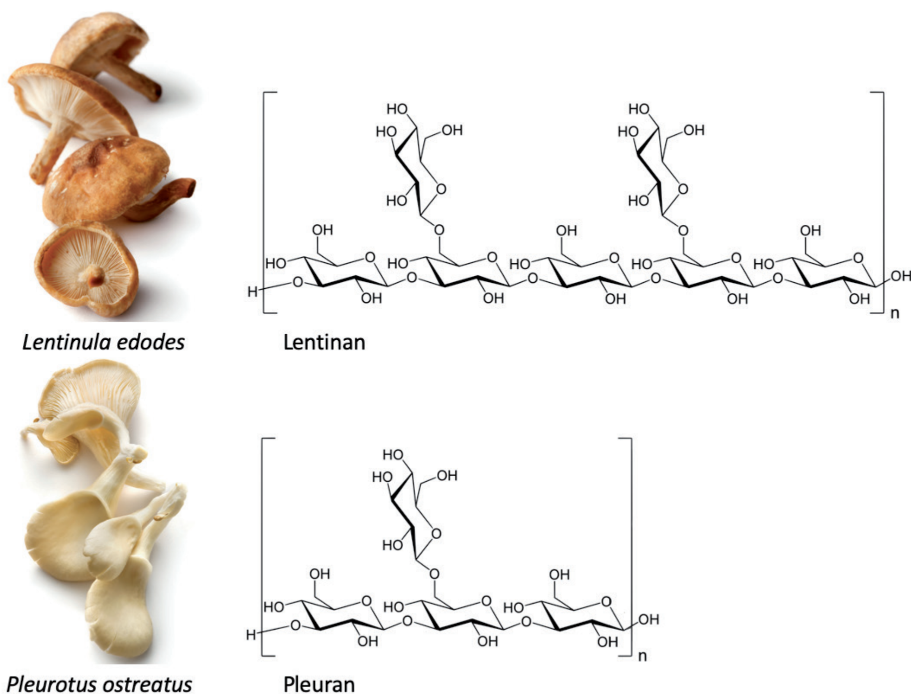

Figure 1. *Shiitake (Lentinula edodes) and pearl oyster mushroom (Pleurotus ostreatus) and their specific beta-glucan structures.*

3.2 BETA-GLUCANS IN IMMUNOMODULATING DIETARY SUPPLEMENTS

3.2.1 Health Claims in Europe

Supplement manufacturers are interested in using health claims on their product to show the health benefits of consuming these products [20–22]. In Europe, the use of voluntary statements related to either the nutritional content (nutrition claims) or health benefits of a food product (health claims) is regulated under the Nutrition and Health Claim Regulation (NHCR). Before such claims can be used on foods, they need to be authorized by the European Commission [23]. Scientific evidence is key in the authorization decision of the European Commission to allow new claims in the EU market [24]. A health claim is defined as any voluntary statement that refers to the relationship between food and health. Four categories of claims are known in the EU: two types of function claims that are based on generally accepted (general function claims, in Art. 13.1) or newly developed scientific evidence (new function claims, Art. 13.5), reduction of disease risk claims (Art. 14.1a) and, finally, claims referring to children's development and health (Art. 14.1b) [23]. The food business operator needs to submit a scientific dossier along with its request for authoring a newly proposed claim. The European Commission subsequently asks the European Food Safety Authority (EFSA) to evaluate the evidence on the proposed claims [25]. This evaluation involves a critical review of three main criteria: (1) the bioactive substance is sufficiently characterized, (2) the proposed claim is well characterized and should comprise a beneficial physiological effect, and (3) the cause-and-effect relationship between the bioactive substance and the beneficial physiological effect should be established [23,24]. A beneficial effect shown in at least two independently conducted intervention trials increases the chance of receiving a positive opinion of EFSA [20,24]. Since the assessment procedure follows this specific order of evaluation of these criteria, the assessment will be discontinued if the evidence is insufficiently supporting a criterion [26].

3.2.2 Immune Functioning Health Claims

An effective functioning immune system is crucial for maintaining physiological integrity and, thus, for maintaining health. The immune system provides defense against infections caused by pathogenic microorganisms [27,28]. In recent years, food companies have continued to develop innovative foods in this specific field [5]. Despite its positive aim of fostering innovation, the Nutrition and Health Claims Regulation (EC) No. 1924/2006 (NHCR) may present several compliance challenges which might affect innovation in the EU food sector [29,30]. In order to provide stakeholders with greater clarity on which health effects related to immunology could be studied to support health claims, in 2011, a guidance was published by EFSA's Panel on Dietetic Products, Nutrition and Allergies

(NDA Panel) that provides more detailed guidelines for the evaluation of Articles 13.1, 13.5, and 14 health claims in this area [31]. According to the NDA Panel, maintaining a well-functioning immune function can be considered to be a beneficial physiological effect. However, given the multiple roles of the immune system, the specific aspect of immune function to which the claim relates should be noted. This means that changes in multiple biomarkers can indicate a well-functioning immune system. Markers of immune system functioning that are proposed as suitable outcomes for substantiating claims on immune function effects are listed in Table 1 [31]. In addition to a positive influence on these markers, changes should be accompanied by a favorable physiological or clinical outcome, preferably demonstrated in the same study [31].

Table 1. *Changes in biomarkers that are proposed as outcomes for substantiating health claims related to immune function [31].*

Described Changes	Examples
· Immune biomarkers	- Numbers of various lymphoid subpopulations in the circulation - Proliferative responses of lymphocytes - Phagocytic activity of phagocytes - Lytic activity of NK cells and cytolytic T cells - Production of cellular mediators - Serum and secretory immunoglobulin levels - Delayed-type hypersensitivity responses
· Inflammation biomarkers	- C-reactive protein (CRP) - Interleukins (e.g., IL-6, IL-8, IL-10) - Tumor necrosis factor-α (TNF-α)
· Short-chain fatty acid production in the gut	- Acetate - Propionate - Butyrate
· Structure of the intestinal epithelium	- Composition of cells (e.g., enterocytes, Paneth cells, M cells)
· Composition of gut microbiota	- Phyla (e.g., Actinobacteria, Firmicutes, Bacteroidetes, Proteobacteria)

So far, ten proposed health claims have been considered to be substantiated with sufficient scientific evidence according to the NDA Panel and have subsequently been authorized by the European Commission. The following six vitamins are reported to play a role in maintaining a well-functioning immune system: A, B6, folate (B9), B12, C, and D [32–34]. Meanwhile, a similar assessment was made for four essential trace elements: zinc, copper, iron, and selenium, which are considered by EFSA as necessary for the optimal functioning of the immune system [35–39]. Therefore, these ten micronutrients may be labeled with the health claim 'contributes to the normal functioning of the immune system'. Moreover, foods containing 200 mg or more vitamin C may be labeled with an additional health claim: 'Vitamin C contributes to maintain the normal function

of the immune system during and after intense physical exercise' [40]. The evidence for vitamin C's additional health claim comes from three systematic reviews examining the role of vitamin C supplementation in the prevention, severity, and treatment of the common cold [41–43]. The results of the reviews showed that there is some evidence suggesting that individuals who are exposed to short periods of vigorous exercise and/or cold environments benefit from regular vitamin C intake above 200 mg/day based on the duration and severity of the common cold [40]. Next to this second substantiated claim for the effect of vitamin C, the NDA Panel considered that the role of vitamin D in the functioning of the immune system applies to all ages, including children. Therefore, vitamin D containing products may also use an additional Article 14.1(b) claim: 'Vitamin D contributes to the normal function of the immune system in children' [40]. This does not mean that other nutrients and foods are not also relevant; so far, however, insufficient scientific evidence has been gathered to demonstrate this [44]. In addition to the 2011 guidelines for claims related to support of the immune system, the guidelines were extended 5 years later to include claims related to stimulation of the immune system and defense against pathogenic microorganisms [31,40]. The scientific evidence to substantiate a claim related to the body's defense against pathogens can be obtained by studying effects on clinical outcomes related to infections (e.g., incidence, severity, and/or duration of symptoms). As put forward in EFSA's guideline, the infectious nature of the disease should be established, e.g., by clinical differential diagnosis in itself or combining this with microbiological data and/or the use of validated questionnaires, depending on the study context and type of infection [40]. Donabedian et al. (2006) concluded that the ten micronutrients which have received a positive opinion for supporting the immune system do not play a supporting role in the treatment of certain ongoing infections [45]. So far, all applications for putative health claims related to stimulation of the immune system and defense against pathogenic microorganisms have been rejected by the NDA Panel. Most of the rejected claims focused on the effects of probiotic bacteria such as *Lactobacillus* and *Bifidobacterium* strains. Claims on probiotics, however, have often been rejected because of a lack of specifying the active ingredient itself, the first step in the scientific assessment of the claim [46–49]. Other unapproved applications for products claiming immune stimulation and defense against pathogenic microorganisms mainly involved amino acids, antioxidants, oligosaccharides, and fungal compounds, including beta-glucans. The unapproved immune related applications for products containing fungal beta-glucans are listed in Table 2.

Table 2. *Claims regarding fungal beta-glucan applications related to the immune system.*

Claim Type	Nutrient, Substance, Food or Food Category	Claim	Non-Authorization/Discontinuation Based on Criteria	Health Relationship	EFSA Opinion/ Journal Reference	Entry ID
Art. 13(1)	Beta-glucan (WGP)	For immunity. Strengthens immunity.	(2) Rejected on the basis of an unclear health relationship or no clear association with health.	Immune system	2011;9(6):2228	1792
Art. 13(1)	Beta-glucan + olive leaf extract	Supports the body's own defense mechanism/immunity. Maintains natural defense mechanism/immunity. Helps strengthen natural immunity.	(2) Rejected on the basis of an unclear health relationship or no clear association with health.	Immune function/ immune system	2011;9(4):2061	1793
Art. 13(1)	Beta-glucan of *Saccharomyces cerevisiae*	Beta-glucan from yeast as immunomodulators. Beta-glucan from yeast support of natural defenses.	(2) Rejected on the basis of an unclear health relationship or no clear association with health.	Immune system	2011;9(6):2228	847
Art. 13(1)	Beta-glucan of *Saccharomyces cerevisiae*	Beta-glucan from yeast as immunomodulators. Beta-glucan from yeast support of natural defenses.	(2) Rejected on the basis of an unclear health relationship or no clear association with health.	Increasing nonspecific serum IgA secretion	2011;9(6):2228	1944
Art. 13(1)	WGP beta-glucan; (WGP® (1,3)-b-D-glucan) ;(from *Saccharomyces cerevisiae*)	WGP beta-glucan contributes to the normal function to the immune system. WGP beta-glucan naturally contributes to adequate immune responses. The daily dietary supplementation with WGP beta-glucan promotes the normal function of the immune system. WGP beta-glucan enhances the production and activity of macrophages and neutrophils. Thus, it plays an important role in the adequate function of the immune system. WGP beta-glucan contributes to maintain the normal function of the upper respiratory tract.	(3) Rejected on the basis of an unproven cause and effect relationship: no evidence (yet) for a relationship between intake and effect.	Maintenance of the upper respiratory tract defense against pathogens by maintaining immune defenses.	2011;9(6):2248	1910

Cont. Table 2 *Claims regarding fungal beta-glucan applications related to the immune system.*

Claim Type	Nutrient, Substance, Food or Food Category	Claim	Non-Authorization/Discontinuation Based on Criteria	Health Relationship	EFSA Opinion/ Journal Reference	Entry ID
Art. 13(5)	Yestimun®	Daily administration of Yestimun® helps to maintain the body's defense against pathogens.	(3) Rejected on the basis of an unproven cause and effect relationship: no evidence (yet) for a relationship between intake and effect.	N/A	Q-2012-00761 Commission Regulation (EU) No 1154/2014 of 29/10/2014	N/A
Art. 13(5)	Yestimun®, consisting of (1,3)-(1,6)-β-D-glucans of brewer's yeast cell wall (100% *Saccharomyces cerevisiae*)	Daily administration of Yestimun® strengthens the body's defense during the cold season.	(3) Rejected on the basis of an unproven cause and effect relationship: no evidence (yet) for a relationship between intake and effect.	N/A	Q-2008-667 Commission Regulation (EU) 432/2011 of 04/05/2011	N/A
Art. 13(1)	*Lentinula edodes* (common name: Shiitake)	Contributes to natural immunological defenses.	(3) Rejected on the basis of an unproven cause and effect relationship: no evidence (yet) for a relationship between intake and effect.	Immune function/ immune system	2011;9(4):2061	3774
Art. 13(1)	*Lentinula edodes* (common name: shiitake)	Contributes to natural immunological defenses.	(2) Rejected on the basis of an unclear health relationship or no clear association with health.	Stimulation of immunological responses	2011;9(4):2061	2075
Art. 13(1)	*Pleurotus ostreatus* (oyster mushroom)	Contributes to natural immunological defenses.	(2) Rejected on the basis of an unclear health relationship or no clear association with health.	Immune function/ immune system	2011;9(4):2061	3521
Art. 13(1)	Brewer's yeast	Strengthens immunity	(2) Rejected on the basis of an unclear health relationship or no clear association with health.	Immune function/ immune system	2010;8(10):1799	1384

Cont. Table 2 *Claims regarding fungal beta-glucan applications related to the immune system.*

Claim Type	Nutrient, Substance, Food or Food Category	Claim	Non-Authorization/Discontinuation Based on Criteria	Health Relationship	EFSA Opinion/ Journal Reference	Entry ID
Art. 13(5)	Immune balance drink, containing vitamin C, green tea, grape skin, grape seed, and shiitake mushroom extract	The immune balance drink activates body's defense.	(3) Rejected on the basis of an unproven cause and effect relationship: no evidence (yet) for a relationship between intake and effect.	N/A	Q-2009-517 Commission Regulation (EU) No 958/2010 of 22/10/2010	N/A
Art. 13(1)	Active hexose correlated compound (AHCC)	Activates immune system, exert potential effects on the immune system—stimulating immunity.	(2) Rejected on the basis of an unclear health relationship or no clear association with health.	Stimulation of immunological responses	2011;9(4):2061	3139
Art. 13(1)	Herbal yeast plasmolysate (*Saccharomyces cerevisiae*)	Strengthens the body's defense system. Increases immunity.	(2) Rejected on the basis of an unclear health relationship or no clear association with health.	Immune function/ immune system	2011;9(4):2061	1817

3

These negative evaluations of putative health claims have not stopped research into the effects of specific beta-glucan-containing supplements, such as Yestimun® and pleuran. Since these two supplements are interesting cases of a growing body of evidence, the following sections discuss, in detail, the current state of the evidence in light of the requirements for substantiating health claims.

3.2.3 Yestimun®

Yestimun® is an insoluble, highly purified, well-characterized β-glucan from spent brewer's yeast (*Saccharomyces cerevisiae*) [50]. Brewer's yeast is grown exclusively on malt and clean spring water and is a natural byproduct of the fermentation process used for beer production. During various possessing steps, these β-glucans are further purified, and soluble compounds are removed. This results in a relative β-1,6 glucan side chain binding percentage of 22% with a minimum purity of 85% [50]. Animal studies showed that orally ingested Yestimun® in rats increased the phagocytic activity of granulocytes and monocytes, the percentage of phagocytic cells and nonspecific humoral immune parameters lysozyme, ceruloplasmin and serum γ-globulin [51–53]. Phagocytes derived from the β-glucan fed group showed higher respiratory burst and phagocytic activity. When stimulated by LPS, the proliferation rate of lymphocytes was higher in the β-glucan group [53]. Moreover, a study in dogs with inflammatory bowel disease (IBD) showed that animals treated with β-glucan had a decreased level of IL-6 and an increased level of anti-inflammatory IL-10 as compared to untreated control animals [54]. In addition to these animal studies, clinical trials have examined the ability of Yestimun® to increase the body's defense against invading pathogens. Auinger et al. (2013) performed a placebo-controlled, double-blind, randomized clinical trial in 162 healthy participants with recurring infections and concluded that supplementation with Yestimun® (900 mg/day) for 16 weeks reduced the number of symptomatic common cold infections by 25% compared to placebo ($p = 0.041$) [55]. Another trial with 100 participants confirmed these results by reporting significantly more subjects without a cold episode and its typical symptoms in the β-glucan group compared to the placebo group [56]. Although these clinical studies with Yestimun® showed positive effects on the immune system, the inclusion of these studies into the scientific dossier to support an immune claim was not considered to provide sufficient evidence for substantiating the claim [50,57,58]. The NDA Panel concluded that a cause-and-effect relationship had not been established, mainly because of study design issues: a non-validated questionnaire on common cold was used and limitations of statistical analyses were identified [50]. Recently, another intervention study was conducted that used validated questionnaires on upper respiratory tract infections (URTI) episodes as a primary endpoint [59,60]. Dharsono et al. (2019) concluded that supplementation with Yestimun® reduced the severity of physical URTI symptoms during the first week of an episode, even though the incidence and overall severity of common colds was not shown to be altered in comparison to placebo. Furthermore, accompanying health benefits in

terms of lowering blood pressure and improved mood were reported [59]. However, no research has been done on the viral load and the type of viruses/bacteria causing the symptoms. This is an important limitation of the study in substantiating a health claim related to pathogen defense, as the infectious nature of the disease must be established [40]. The nature of the virus might have an impact on incidence, severity, and duration of URTI episodes [59]. In practice, however, it is uncommon to perform routine laboratory tests for the diagnosis of the common cold as it can be caused by many different agents (adenovirus, coronavirus, influenza virus, rhinovirus, etc.) [60,61]. The viral pathogens associated with the common cold may be detected by culture, antigen detection, PCR, or serologic methods. These studies are generally not indicated in patients with the common cold, because a specific etiological diagnosis is only meaningful when considering treatment with an antiviral agent [60,61].

3.2.4 Pleuran

Pleuran is an insoluble polysaccharide (β-(1,3/1,6)-d-glucan), isolated from the fruiting bodies of the edible mushroom *Pleurotus ostreatus*. Pleuran was developed as a dietary supplement to support the immune system and overcome the first signs of exhaustion and fatigue in adults and children [18]. The effect of Imunoglukan P4H®, a formulation of pleuran and vitamin C, on respiratory tract infections (RTI) and recurrent respiratory tract infections (RRTI) in children has been investigated in several clinical studies. In an open-label study, a decrease in the frequency of RRTI was observed in 153 children (71.2%). The mean annual incidence of respiratory tract infections in children with a positive response to Imunoglukan P4H® was significantly lower compared to that in unresponsive patients (3.6 vs. 8.9, $p < 0.001$) [62]. Another study examined the effect of Imunoglukan P4H® supplementation on the frequency of RTI in a group of 151 children with RRTI. A comparison between the number and type of RTI during the previous October period was compared to those observed during the intervention period and 6-month follow-up. Supplementation with Imunoglukan P4H® reduced the RRTI rate from 8.88 ± 3.35 episodes in the previous year to 4.27 ± 2.21 episodes in the study year ($p < 0.001$) [63]. Similar efficacy was observed in another prospective open-label study in 194 children in Poland, where a significant decrease in total RTI was reported during the intervention and follow-up periods (4.18 ± 2.132 vs. 8.71 ± 1.89, $p < 0.001$) [64].

However, the design of these studies also shows some weaknesses when considering their usage for substantiating an immune function health claim. Firstly, the prospective open-label design is a weakness. Secondly, the lack of information on viral load and the type of virus/bacteria causing symptoms are limitations. The major weakness in substantiating immune claims for beta-glucans, however, is the fact that Imunoglukan P4H® also contains vitamin C (15% of the recommended dietary allowance). The recommended daily dose contains sufficient vitamin C to make use of the health claim 'contributes to the

normal function of the immune system'. However, when looking specifically at respiratory infections, randomized, placebo-controlled studies have not clearly shown that vitamin C on its own has the potential to prevent them [45,65]. Interestingly, a double-blind, placebo-controlled, randomized trial in children with RRTI examined the effect of the *Pleurotus* extract in itself. Next to self-reporting RRTI symptoms, a validated health questionnaire was used to examine general health status and RRTI symptoms. Vitamin C was used as an 'active placebo' to investigate whether the immunomodulatory action, which is clinically manifested in the reduction of RTI, can primarily be attributed to the highly purified *Pleurotus ostreatus* extract [66]. In the *Pleurotus* extract group, 36% of the children did not suffer from any respiratory infections throughout the treatment, compared to 21% in the vitamin C group. *Pleurotus* extract also significantly decreased the frequency of flu and flu-like symptoms, as well as the frequency of lower respiratory tract infections compared to the vitamin C group (0.20 ± 0.55 per 12 months vs. 0.42 ± 0.78 per 12 months, $p < 0.05$). The results of this RCT, that uses validated questionnaires, are promising but need to be confirmed in more studies as multiple intervention studies conducted by independent institutions increase the likelihood of receiving a positive EFSA opinion [20,24]. In addition, with the target population being children with (recurring) respiratory infections instead of the general population, this evidence mainly substantiates an Article 14.1(b) claim. Should such studies yield positive results again, pleuran could apply for an additional health claim, such as the authorized claim for vitamin D discussed in Section 3.2.2. Few studies have been conducted with Imunoglukan P4H® in the general population, but several clinical studies have been conducted in other populations susceptible to RTI, e.g., elite athletes [67,68]. Epidemiological evidence suggests that heavy acute or chronic exercise is related to an increased incidence of upper respiratory tract infections in athletes [69]. In a placebo-controlled study, the daily consumption of 100 mg Imunoglukan P4H® for two months prevented post-exercise immune suppression in elite athletes, and in another study, supplementation reduced RTIs (even though this was measured by a non-validated questionnaire) [67,68]. These studies demonstrate the potential for the use of Imunoglukan P4H® as an immunostimulant in elite athletes. Still, follow-up studies will have to be conducted following the guidance documents to qualify as supportive evidence for a possible health claim. When such studies would again yield positive results, Imunoglukan P4H® could apply for an additional health claim, such as the authorized claim for vitamin C: 'Vitamin C contributes to the maintenance of the normal function of the immune system during and after intense physical exercise'.

3.3 TRAINED IMMUNITY

When smallpox vaccination was introduced about 200 years ago and up to its discontinuation in 1980, positive side effects were noted by physicians, such as protection against measles, scarlet fever, and whooping cough [70]. Investigation of these observations led to evidence in 1956 of the 'ring zone phenomenon', i.e., the production of soluble antivirals in infected chicken embryos and cell cultures. With the help of modern immunological and bioengineering methods, it was later possible to demonstrate that these effects are based on the activation of lymphoreticular cells and the regulatory effect of certain cytokines within the context of the nonspecific immune system [70]. In recent years, it has become evident that cells of the innate immunity may be primed upon encounter with certain pathogens or molecular patterns associated to pathogens, thereby acquiring a higher resistance to a second infection against the same or unrelated pathogens [71–73]. This concept, known as 'trained immunity', gives rise to the development of trained immunity-based vaccines (TIbV), defined as vaccine formulations that induce training in innate immune cells, and the use of nonspecific immunostimulants as 'trained immunity' inducers [71,73]. TIbV could be used during viral outbreaks to confer nonspecific protection as well as to enhance adaptive specific immune responses. Moreover, the ability of TIbV to promote responses beyond their nominal antigens may be useful when conventional vaccines are not available or when multiple co-infections and/or recurrent infections occur in susceptible individuals [71]. Several drugs used in cancer therapy (e.g., BCG-vaccine, mifamurtide) have recently been shown to be able to elicit an enhanced immune response in monocytes after nonspecific restimulation [74]. Interestingly, in vitro and animal studies showed that fungal beta-glucans are also able to elicit trained immunity through activation of the pattern recognition receptor (PRR) dectin-1 [71,73,75–77]. It has been speculated that the increased expression of certain PRRs in innate trained cells, as well as the release of typical innate immunity cytokines, such as IL-1β, contribute to enhance adaptive T-cell responses [71]. The pharmaceutical application of beta-glucans has been carried out within TCM for decades, but until now, this concept is largely unknown in Western medicine. The new insights and developments in trained immunity may lead to the possible application of these drugs and fungal beta-glucans as NSIs and/or adjuvants in TIbV in Western medicine. The following sections discuss, in detail, the currently available evidence from clinical intervention studies in humans investigating nonspecific immunity. Subsequently, the pharmaceutical application of fungal glucans in Western medicine and the substantiation of the required evidence for this are discussed.

3.3.1 BCG Vaccine

The bacillus Calmette–Guérin (BCG) vaccine contains live-attenuated *Mycobacterium bovis* bacilli that protect against tuberculosis, one of the world's deadliest infectious diseases [78]. In countries where tuberculosis is common, one dose is recommended in healthy babies as close to birth as possible. In addition, it is sometimes used in cancer therapy, such as in the treatment of bladder cancer [79,80]. For example, the BCG vaccine is indicated as curative treatment of carcinoma in situ of the urothelium of the bladder and as an adjuvant after transurethral resection of a primary or recurrent superficial papillary carcinoma of the urothelium of the bladder. Upon instillation, the BCG vaccine locally stimulates the immune system (with an increase in granulocytes, monocytes/macrophages and T-lymphocytes). Early in vitro and animal studies indicated that BCG vaccine immunization could induce nonspecific, protective effects against other pathogens. For example, it was observed that mice vaccinated against tuberculosis were also found to be protected when secondary infections with *Staphylococcus aureus*, *Listeria monocytogenes*, *Salmonella typhimurium*, or *Schistosoma mansoni* had occurred [76,81–84]. Only in recent years, attempts have been made to identify mechanistic events responsible for the nonspecific effects in humans. These efforts resulted in the identification of a plethora of methods to identify human biomarkers, from cytokine responses to epigenetic changes [85]. A Dutch study on a small group of BCG-vaccinated young adults demonstrated that non-mycobacterial stimuli were able to induce heterologous Th1 and Th17 cytokines, such as IFN-γ and IL- 17, up to 1 year after receiving the vaccine. The vaccination only induced a primed status of the cells to respond more strongly to secondary microbial stimulation. Without stimulation, no higher production of these cytokines was seen [86]. The beneficial effects of BCG vaccination are therefore suggested to be the induction of the innate immunity reprogramming expressed as the long-term sustained changes in the nonspecific resistance against infections [76]. As described in Table 3, following the identification of these beneficial effects, more clinical studies on BCG-induced trained immunity have been conducted in healthy subjects, neonates, infants, as well as the elderly [86–93].

Table 3. *Clinical trials on trained immunity.*

Population	Intervention	Conclusions	Ref.
20 Healthy individuals (age: 20–36 years)	Participants would receive a BCG vaccination from the public health agency for traveling to or working in countries where tuberculosis is prevalent.	The production of TNF-α and IL-1β to mycobacteria or unrelated pathogens was higher after 2 weeks and 3 months post-vaccination, but these effects were less pronounced 1 year after vaccination. However, monocytes recovered 1 year after vaccination had an increased expression of pattern recognition receptors such as CD14, toll-like receptor 4 (TLR4) and mannose receptor, and this correlated with an increase in proinflammatory cytokine production after stimulation with the TLR4 ligand lipopolysaccharide.	[86]
30 Healthy Dutch male participants (age: 19–37 years)	Participants received either BCG (n = 15) or placebo (the diluent used to dissolve BCG) (n = 15). One month after placebo or BCG vaccination, all volunteers received a single dose of yellow fever vaccine.	BCG-vaccinated volunteers displayed a significant reduction of viremia compared to the placebo group, which highly correlated with enhanced IL-1β production.	[87]
20 Healthy, BCG-naive volunteers (age: 18–35 years)	Ten subjects received standard dose (0.1 mL of the reconstituted vaccine) of intradermal BCG vaccination 5 weeks prior to challenge infection. Ten controls received no vaccination. Five weeks after BCG vaccination, both groups were exposed to bites of five *Plasmodium falciparum* NF54 strain infected *Anopheles stephensi* mosquitoes (sporozoite challenge).	BCG vaccination altered some of the clinical, immunological, and parasitological outcomes of malaria infection in a subset of volunteers. Earlier NK cell and monocyte activation in this subset of vaccinated volunteers is consistent with the possibility that induction of trained innate immunity in vivo may have functional activity against a heterologous pathogen in humans.	[88]
212 Neonates; BCG vaccinated (n = 119) BCG naïve (n = 93)	Participants were randomized 1:1 to undergo vaccination with BCG (0.05 mL) intradermally within 10 days of birth or to receive no BCG vaccine.	BCG-vaccinated infants had increased production of IL-6 in unstimulated samples and decreased production of interleukin 1 receptor antagonist, IL-6, and IL-10 and the chemokines macrophage inflammatory protein 1α (MIP-1α), MIP-1β, and monocyte chemoattractant protein 1 (MCP-1) following stimulation with peptidoglycan (TLR2) and R848 (TLR7/8). BCG-vaccinated infants also had decreased MCP-1 responses following stimulation with heterologous pathogens.	[89]

40 Healthy volunteers (age: 20–25 years)	Participants received either live attenuated BCG vaccine (*n* = 20) or placebo (*n* = 20), followed by intramuscular injection of trivalent influenza vaccine 14 days later.	In BCG-vaccinated subjects, HI antibody responses against the 2009 pandemic influenza A(H1N1) vaccine strain were significantly enhanced compared with the placebo group. Additionally, apart from enhanced proinflammatory leukocyte responses following BCG vaccination, nonspecific effects of influenza vaccination were also observed, with modulation of cytokine responses against unrelated pathogens.	[90]
15 Healthy individuals (age: 20–34 years)	Participants received inactivated gamma-irradiated BCG (γBCG). The inactivated BCG was cultured for 6 weeks to confirm inactivation.	γBCG vaccination in volunteers had only minimal effects on innate immunity. The results indicate that γBCG induces long-term training of innate immunity in vitro. In vivo, γBCG induces effects on innate cytokine production are limited.	[91]
198 Elderly patients (age >65 years)	Participants received BCG (*n* = 100) or placebo (*n* = 98) vaccine and were followed for 12 months for new infections.	At interim analysis, BCG vaccination significantly increased the time to first infection. The incidence of new infections was 42.3% after placebo vaccination and 25.0% after BCG vaccination; most of the protection was against respiratory tract infections of probable viral origin.	[92]
158 Infants	Infants received BCG within 7 days of birth (*n* = 80). Controls (*n* = 78) were bled 4 days post-randomization, and at age 3 and 13 months.	BCG vaccination of Danish newborns did not induce nonspecific in vitro cytokine responses.	[93]
21 Healthy participants (age: 21–59 years)	Participants received a single 4 mg i.v. infusion of L-MTP-PE over 30 min.	Serum concentrations of IL-6, TNF-α, and CRP increased following L-MTP-PE infusion. Maximum observed increases in IL-6 and TNF-α occurred at 4 and 2 h, respectively, returning toward baseline by 8 h post-dose.	[94]
15 Healthy male participants (age: 19–24 years)	Beta-glucan (*n* = 10) or the control group (*n* = 5). Subjects in the glucan group ingested beta-glucan 1000 mg once daily for 7 days. Water-insoluble beta-glucan derived from baker's yeast (*S. cerevisiae*) sold as a dietary supplement (Glucan #300, BG). This preparation has a purity of at least 83% guaranteed by the manufacturer.	Beta-glucan was barely detectable in serum of volunteers at all time points. Neither cytokine production nor microbicidal activity of leukocytes were affected by orally administered beta-glucan.	[95]

Trained immunity responses induced by BCG vaccination were shown to be dependent on the engagement of the intracellular receptor nucleotide-binding oligomerization domain 2 (NOD2) by the peptidoglycan component muramyl dipeptide (MDP) [84,96]. MDP is the smallest naturally occurring nonspecific immunostimulating component of the cell wall of Mycobacterium. MDP is derived from various sources and is present in human peripheral blood [97,98].

3.3.2 Mifamurtide

Mifamurtide (muramyl tripeptide phosphatidylethanolamine, MTP-PE) is a fully synthetic derivative of MDP. Mifamurtide has the same immunostimulating effect as natural MDP. As mifamurtide is lipophilic, it may be incorporated into liposomal lipid bilayers and subsequently phagocytosed by macrophages and monocytes. These activated cells may then selectively target and destroy tumor cells without affecting normal cells [99]. Both mifamurtide and MDP stimulate immune responses by binding to nucleotide-binding oligomerization domain-containing protein 2 (NOD2), an intracellular pattern-recognition receptor molecule expressed mainly in monocytes, macrophages, and dendritic cells [99]. Mutations in NOD2 are frequently observed in patients with Crohn's disease, an autoimmune disorder, suggesting the significance of the MDP–NOD2 pathway in activating immunity. As more became known about the MDP–NOD2 pathway, structural modifications of MDP and its derivatives have been extensively studied in an attempt to increase adjuvant activity and boost the immune response effectively for clinical use in the treatment of cancer and other diseases [100]. By binding to NOD2, mifamurtide activates the NF-κB pathway that leads to an increased production of proinflammatory cytokines such as TNF-α, IL-1, IL-6, IL-8, interferon gamma (IFN-gamma), and increased levels of immune stimulation markers plasma neopterin and serum C-reactive protein [99,100]. There are two formulations of mifamurtide, the free-drug form (MTP-PE) and the liposomal-encapsulated form (L-MTP-PE). The maximum tolerated dose (MTD) of mifamurtide is 6 mg/m2, with a moderate toxicity that has some dose-limiting side effects such as chills, fever, malaise, and nausea. The precise mechanism of action of cell activation, immunomodulation, and tumor reduction by mifamurtide in humans is unknown [99]. A recent study by Mourits et al. (2020) investigated whether variation in circulating levels of MDP can modulate trained immune responses induced by BCG vaccination in vivo and explain the variability of response between individuals. They concluded that circulating pre-vaccination MDP concentrations correlated with systemic inflammation and induction of trained immunity after BCG vaccination, but not with specific T-cell cytokine responses. In addition, BCG vaccination was shown to result in a sustained increase in circulating levels of MDP, but this change in MDP did not affect trained immune responses or specific memory immune responses [96]. As described in Table 3, a pharmacokinetic and pharmacodynamic study in healthy adults showed an increase of serum concentrations of IL-6, TNF-α, and CRP after infusion of L-MTP-PE [94]. However, in

this study, no restimulation with a second infection was investigated to see the effects of mifamurtide on trained immunity. For follow-up studies, it would be interesting to further unravel our understanding of trained immunity by conducting clinical trials with MDP or mifamurtide and restimulating with a second infection, as previously done with BCG.

3.3.3 Fungal Glucans and Trained Immunity

In addition to the above-described approved medicinal products that induce trained immunity, in vitro and animal studies show that beta-glucans from 'medicinal' mushrooms may also play an interesting role in this new field of immunology. For example, initial stimulation of myeloid cells by fungal β-glucan has been shown to promote control of subsequent infection with bacterial pathogens [73,75–77]. Furthermore, as beta-glucans are inexpensive and well-tolerated compared to most pharmaceutical products, and can be taken orally, beta-glucan appears to be a promising candidate to enhance the immune response [101]. Compared to the BCG vaccine, hardly any RCTs have been performed to study putative immunostimulating effects of orally administered beta-glucans in humans. In a randomized open-label intervention pilot study, the potential immunostimulating effects of commercially available orally administered water-insoluble beta-glucan in healthy participants were investigated [95]. This supplement, Glucan 300®, derived from baker's yeast, was found to be the most active compared to other beta-glucans in a mouse study and its use was considered safe for human consumption [73]. Leentjens et al. (2014) concluded that beta-glucan was barely detectable in the serum of volunteers at all studied time points. In addition, neither the production of cytokines nor the microbicidal activity of leukocytes was affected by orally administered beta-glucan, as described in Table 3 [95]. This lack of reported effects may be attributed to the selected commercial dietary supplement, which may have degraded the beta-glucans before absorption could occur. A highly purified pharmaceutical composition optimized for oral administration may have immunostimulating effects in humans. It was further recommended that intravenous administration of a pharmaceutical preparation may exert immunostimulating effects [95]. The most studied intravenously administered beta-glucan, lentinan, is clinically approved in several countries in Asia [102].

3.3.3.1 Lentinan

Lentinan is a polysaccharide isolated from the fruiting body of the shiitake mushroom and has been used in Asia for thousands of years to improve health [102]. Lentinan is approved for treating multiple types of cancer, hepatitis, and other diseases in China and as an adjuvant for stomach cancer therapy in Japan [102]. Intravenous injection of lentinan is clinically approved with an average dose of 1–1.5 mg/day. Furthermore, lentinan is available in capsules and tablets and taken orally as a traditional medicine. The primary structure of β-glucan in lentinan is composed of a β-(1–3)-glucose backbone with two (1–6)-β-glucose branches of every five glucose units [17]. Recently, a systematic review

including 38 RCTs (3117 patients), investigated the clinical effectiveness of intravenously administered lentinan as an adjuvant therapeutic drug in the treatment of patients with lung cancer [102]. It was concluded that lentinan had a favorable safety profile compared to chemotherapy, hormone therapy and immunotherapy. In addition, lentinan was considered effective not only for improving quality of life, but also for promoting the efficacy of chemotherapy in the treatment of lung cancer [102]. Early in vitro and animal studies indicated that lentinan could induce nonspecific, protective effects against pathogens [103–106]. Moreover, clinical data from TCM indicate that lentinan is a nonspecific immunostimulant and a biological response modifier with proven efficacy in treating viral infections, such as hepatitis and HIV [107–109]. In view of these results, it appears that lentinan is a promising candidate for follow-up studies into trained immunity in healthy subjects, as previously performed with BCG. However, the question remains how this routine Eastern practice can be translated into Western medicine or novel food [110,111]. For novel foods, the scientific dossier must provide evidence that no adverse effects are elicited by consuming the product and consequently, kinetics, toxicology, nutritional information and allergenicity must be analyzed [112]. Information from nonclinical studies required by Western standards of evidence is often lacking for these medicinal mushrooms. During the early preclinical development process, a drug candidate must go through several steps, such as determination of bioavailability, pharmacokinetics, pharmacodynamics, absorption, distribution, metabolism, and elimination (ADME), and preliminary studies aimed at investigating the candidate's safety including genotoxicity, mutagenicity, safety pharmacology, and general toxicology [113]. Although side effects of lentinan are rare, anaphylaxis after intravenous administration has been reported in some clinical cases [114]. Anaphylaxis could be caused by a toxic reaction to lentinan as there are also case reports of contact dermatitis, asthma, rhinitis, and hypersensitivity pneumonitis in shiitake workers [115]. These cases indicate the need to conduct additional (preclinical) studies, as regulators take the safety of novel food and new agents as their primary consideration [112]. In addition to safety studies, another issue for purified or crude medicinal mushroom extracts is ensuring good manufacturing practice (GMP) standards with batch-to-batch consistency, end-product stability, and consistent analytical profiles of products [116,117]. In the absence of suitable methods for standardization and characterization of fungal glucans, guaranteeing GMP is particularly difficult. The potential molecular mechanisms underlying the induction of innate immune memory by medicinal mushrooms is a complex interaction between immunological, metabolic, and epigenetic changes through many as yet unknown pathways [102]. Bringing traditional Eastern practices into Western practice does not require a full understanding of the mechanism of action, but requires clinical studies that provide data that the clinical community will accept [110]. An example of a pharmaceutical beta-glucan preparation currently in clinical development for cancer according to Western regulations is Imprime PGG.

3.3.3.2 Imprime PGG

Imprime PGG is an intravenous formulation of a yeast-derived, uncharged, water-soluble, 1,3–1,6 beta glucan purified from the cell wall of a proprietary, non-recombinant, strain of *Saccharomyces cerevisiae*. Initial in vitro and animal studies provided insights into bioavailability, pharmacokinetics, pharmacodynamics, ADME, genotoxicity, mutagenicity, safety pharmacology, and general toxicology, information necessary for initiating later clinical trials [116–132]. Moreover, ex vivo human whole blood studies demonstrated that Imprime-induced responses were consistent with the innate immune activation elicited by a pathogen. Imprime binding and functional activation of these innate effector cells was critically dependent on Imprime first forming an immune complex with endogenous IgG anti-beta-glucan Ab (ABA). The formation of Imprime–ABA complexes induced significant activation of complement proteins as well as phenotypic activation and chemokine production by innate immune cells [133]. As a follow-up to these studies, two randomized, double-blind, placebo-controlled phase 1 dose escalation studies evaluating Imprime in healthy participants were conducted [134]. Recently, Bose et al. (2019) conducted a phase 1 study in healthy participants, examining the relationship between ABA levels and Imprime-mediated innate immune activation. Consistent with ex vivo results, Imprime–ABA complexes induced significant activation of complement proteins as well as phenotypic activation and chemokine production by innate immune cells, as described in Table 3 [135]. These results demonstrate that intravenously administered beta-glucan has immunostimulating effects in vivo and thus may be of significance in trained immunity. Based on the favorable safety and tolerability results observed in these studies, phase 2 studies in which Imprime was administered in combination with antitumor monoclonal antibodies have been initiated in cancer patients [136,137].

3.4 CONCLUSION

This review has highlighted the potential application of fungal beta-glucans—immunomodulators that have been used in traditional Chinese medicine for centuries—in nutrition and medicine. From this review, it can be concluded that fungal glucans may play a promising role within both perspectives, and that there are possibilities to give traditional medicine an immunological application in both medicinal products and foods. Depending on the dosage, formulation, efficacy, safety profile, and route of administration, the immunomodulating effects that can be expected from fungal beta-glucans can either be considered a pharmaceutical effect (treating or curing a disease) or as a health effect originating from foods, focusing on the prevention of negative health effects.

In Europe, claims on health benefits are strictly regulated, with EFSA reviewing the scientific evidence that supports putative statements about health effects. As shown in this paper, all applications for putative health claims related to stimulation of the immune system and defense against pathogenic microorganisms have so far been rejected. Since EFSA has only approved immune claims for six vitamins and four essential trace elements, it can only be speculated that the temptation to add these ingredients to products is growing, rather than stimulating research into innovative foods. Comparing the evidence base of the putative health effects of fungal beta-glucan supplements with the guidance documents on immune support health claims, but even more importantly, the guidance documents on substantiating immune stimulation and pathogen defense by food products, it is shown that fungal glucans could play a role in supporting and maintaining health and, thus, can be seen as a good health-promoting substance from food—which could mean that this effect may also be claimed if approved.

In addition to these developments related to food uses of beta-glucan-containing supplements, beta-glucans could also hold a novel position in Western medicine, as the concept of trained immunity is relatively new and has not been investigated to a larger extent. The new insights and developments in trained immunity may lead to the possible application of fungal beta-glucans as NSIs in Western medicine. Due to the experience from Asian medicine and the relatively favorable safety profile, lentinan (i.v.) could potentially be a suitable fungal glucan within this new field of immunity. However, additional (preclinical) safety studies must first be performed to be eligible as a medicine in Europe. Imprime PGG, which is currently going through the stages of drug development, is another fungal beta-glucan worth investigating. Finally, given the different ways to purify and process beta-glucans, one of the biggest challenges remains the standardization and proper characterization of the active compounds themselves. However, with the help of modern immunological and biotechnological methods, increasing insights are gained into immunomodulating fungal beta-glucans, with potential applications both in foods and pharmaceutical products.

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4

Sulforaphane as a potential modifier of calorie-induced inflammation: a double-blind, placebo-controlled, crossover trial

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ABSTRACT

Background & Aims: Observational data indicate that diets rich in fruits and vegetables have a positive effect on inflammatory status, improve metabolic resilience and may protect against the development of non-communicable diseases. Nevertheless, experimental evidence demonstrating a causal relationship between nutrient intake (especially whole foods) and changes in metabolic health is scarce. This study investigated the pleiotropic effects of sulforaphane from broccoli sprouts, compared to pea sprouts, on biomarkers of endothelial function, inflammation and metabolic stress in healthy participants subjected to a standardized caloric challenge.

Methods: In this double-blind, crossover, randomized, placebo-controlled trial twelve healthy participants were administered 16 g broccoli sprouts, or pea sprouts (placebo) followed by the standardized high-caloric drink PhenFlex given to disturb healthy homeostasis. Levels of inflammatory biomarkers and metabolic parameters were measured in plasma before and two hours after the caloric overload.

Results: Administration of broccoli sprouts promoted an increase in levels of CCL-2 induced by caloric load ($p = 0.017$). Other biomarkers (sICAM-1, sVCAM-1, hs-CRP, and IL-10) individually showed insignificant tendencies towards increase with administration of sulforaphane. Combining all studied biomarkers into the systemic low-grade inflammation score further confirmed upregulation of the inflammatory activity ($p=0.087$) after sulforaphane. No significant effects on biomarkers of metabolic stress were detected.

Conclusion: This study has demonstrated that sulforaphane facilitated development of a mild pro-inflammatory state during the caloric challenge, which could be suggestive of the onset of the hormetic response induced by this phytonutrient. The use of integrative outcomes measures such as the systemic low-grade inflammation score can be viewed as a more robust approach to study the subtle and pleiotropic effects of phytonutrients.

4.1 INTRODUCTION

Non-communicable diseases (NCDs) are the leading cause of death worldwide, accounting for 71% of total deaths each year [1]. Chronic low-grade inflammation (CLGI) plays a crucial role in the pathology of NCDs, but also appears to affect apparently healthy people as a consequence of poor lifestyle choices e.g. overeating, smoking and excessive alcohol consumption [2–4]. A wealth of observational data indicates that healthy lifestyle choices, such as moderate exercise and diets rich in fruits and vegetables, have a particularly positive effect on inflammatory status and the development of various NCDs [5–7]. Nevertheless, randomized placebo-controlled trials frequently fail to demonstrate causal relationships between nutrient intake (especially whole foods) and changes in metabolic health [8–11].

With an increasing understanding of disease, health is no longer seen as simply a fixed entity of complete physical, mental, and social well-being, but redefined as our body's ability to cope with everyday challenges [11–15]. The concept of this phenotypic flexibility implies that health can be measured by the ability to adapt to conditions of temporary stress. Challenge testing, which may involve exercise or caloric overload, is often used in practice to assess phenotypic flexibility. This may be a more sensitive way of assessing the effects of fruits and vegetables on the health status of the healthy low-risk population [14–21]. Unlike drugs, food-derived compounds exert subtle effects in the general population rather than treating specific disease states in patients [12,13]. Whilst pharmacology is still dominated by the "one disease - one target - one drug" paradigm, nutritional interventions frequently work on many pathways involved in the development of chronic diseases, with hormetic principles at its heart [11– 14,22,23]. Moreover, nutritional science (and within claims substantiation) often still focuses on this more pharmacological approach, so that it is only considered 'effective' if one nutrient affects one target [24]. All things considered, it is challenging to measure beneficial effects in healthy people, and especially when you're trying to see the effect of one nutrient on one target. It is assumed that any intervention works via a hormetic mechanism if the final beneficial effect on phenotypic flexibility is in fact achieved through initial structural damage or functional overstrain, which is ultimately responsible for the activation of the protective mechanisms [25–37]. For example, physical activity and mild stress-inducing phytonutrients called hormetins are known to increase levels of oxidative stress, but this appears to be beneficial for health [11,22,38–41]. Most dietary hormetins are known to induce the expression of antioxidant enzymes by triggering a pro-oxidant response via activation of the nuclear factor E2-related factor 2 (Nrf2)-pathway [42–50]. While the degree of immediate hormetic effects following exposure to a particular stress may be only moderate, the chain of events following the initial phase leads to biologically amplified effects that are much larger, synergistic, and pleiotropic and therefore require

integrative approach to assessment of the outcomes [12,13,16,37,38,40]. Norde et al. [51] proposed a novel approach to measure CLGI by combining multiple biomarkers into a systemic low-grade inflammation score.

Glucoraphanin, the biogenic precursor of sulforaphane, is present in large amounts in broccoli sprouts [52,53]. After damage to plant tissue, e.g. through chewing, glucoraphanin comes into contact with the enzyme myrosinase, which is separated from its substrate in the intact vegetable, and subsequently is converted to sulforaphane [53–55]. Sulforaphane is the most potent naturally occurring inducer of Nrf2 [42–48]. Previous studies showed that long-term consumption of broccoli sprouts improved fasting blood glucose levels and stabilization of insulin response in type 2 diabetic patients, particularly obese patients [56,57]. To our knowledge, no experimental study has been conducted to investigate the effects of broccoli sprouts on integrative outcome measures. In the current study, we investigate the pleiotropic effects of broccoli sprouts, compared to pea sprouts, on biomarkers of endothelial function, inflammation and metabolic stress in healthy participants subjected to a standardized caloric challenge.

4.2 METHODS

We have conducted a randomized, placebo-controlled, double-blind study with a cross-over design. The study protocol (NL77272.068.21) was approved by the Medical Ethics Review Committee of Maastricht University Medical Centre+ (MUMC+) and Maastricht University, Maastricht, the Netherlands, and performed in full accordance with the declaration of Helsinki of 1975 as revised in 2013, Fortaleza, Brazil [58]. The trial registration number within ClinicalTrials.gov is NCT05146804. All subjects provided written informed consent.

4.2.1 Subjects

Twelve healthy participants (eleven males and one female) were recruited by local and social media advertisements. Inclusion criteria were that participants were between 18 and 50 years old, had a body mass index (BMI) between 18.5 and 30 kg/m2, with a stable weight (< 5% body weight change) and constant eating habits over the past three months. Exclusion criteria were the previous diagnosis of an inflammatory condition or disease or a history of hypothyroidism, chronic kidney or/and liver disorders, coronary artery disease, malignant hypertension, seizures, involved in intensive sports activities more than four times a week or at top sport level, regular intake of medication that may affect inflammatory response including NSAIDs, psychotic, addictive, or other mental disorders, aversion, intolerance or allergy to cruciferous vegetables and/or palm olein, dextrose, protein supplement, vanilla aroma, the use of dietary supplements with potential effects on antioxidant or inflammatory status and/or viral or bacterial infections requiring

the use of antibiotics, laxatives and anti-diarrheal drugs four weeks prior to inclusion, excessive alcohol consumption (≥ 28 consumptions approx. 250 g alcohol per week), pregnancy and/or breastfeeding, reported slimming or medically prescribed diet, as well as adhering to a vegetarian or vegan lifestyle. The sample size calculation is based on a crossover study by Meijer et al. [59] [Trial NL3290 (NTR3435)] in which they examined whether broccoli seedlings could reduce glucose-induced postprandial inflammation in healthy male participants. Meijer et al. measured plasma concentrations of sVCAM-1 and sICAM-1 (primary outcomes) at different timepoints in healthy men after consumption of broccoli seedlings or lettuce (placebo). The detectable difference used for the sample size calculation is calculated based on the mean concentrations of sVCAM-1 (ng/ml). Variance = Mean difference/SD Variance = (26.7-2.4)/14.20 = 1.711

'Variance explained by special effect' was set to 0.5. We calculated an effect size of 0.54. A power of 80% was implemented, the chance of having a type I error was 5% and an effect size of 0.54 (medium effect size). In present study, we have two groups (two repeated measures, within-between interaction) yielding 10 participants, and considering a 20% dropout, which results in the final sample size of 12 participants.

4

4.2.2 Study design and procedures

Commercially available broccoli sprouts BroccoCress®,a rich source of glucoraphanin (the parental glucosinolate of sulforaphane), were used as the experimental product. In total, 16 grams of sprouts were utilized, equivalent to one serving (portion) of microgreens. Sulforaphane (BroccoCress®) and the placebo (Affilla Cress®) were randomly administered to each participant on separate testing days (as detailed in paragraph 2.3). The period between two visits was 7±3 days. Information about demographics, alcohol consumption, and anthropometric data were assessed on the first visit. Body mass index (BMI), total body fat and visceral fat were measured using the Omron BF511R® monitor. The same testing scheme was applied during two visits (Figure 1), i.e. each participant received a single serving of intervention/placebo, which after 90 min was followed by oral administration of the PhenFlex challenge. Blood samples were collected twice, just before intake of PhenFlex and 120 min after. All participants were instructed to come fasted to each visit, to avoid consumption of broccoli or other cruciferous vegetables two days before the visit and to restrain from intense physical activity on the day of the visit. During the visit, participants remained in the testing location and were allowed to drink water ad libitum. No food intake was permitted during the visit.

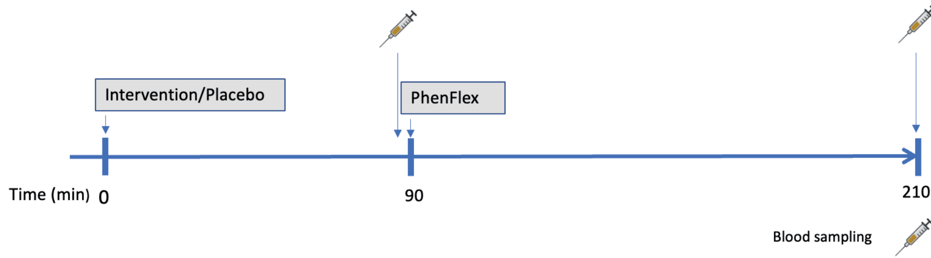

Figure 1. *Schematic presentation of a study visit.*

Note: Administration of intervention (sulforaphane/placebo) was followed in 90 min by administration of standardized caloric challenge PhenFlex. Blood samples were obtained before (90 min) and 2 hours after (210 min) PhenFlex challenge.

4.2.3 Intervention and caloric challenge (PhenFlex)

The broccoli sprouts were cut approximately 1 cm below the leaves (with the hypocotyl being cut below the cotyledons), weighed, and mashed with a small amount of tap water (approximately 13°C) in a kitchen blender for 30s at room temperature immediately before administration (Premium Impuls Blender Smoothiemaker; Impuls; 180 W). Subsequently, tap water (approximately 13°C) was added to the mixture to bring the total amount to 250mL and participants were instructed to drink the entire mixture immediately. Commercially available pea sprouts (Affilla Cress®) were used as a placebo in this study since pea sprouts do not contain glucoraphanin/sulforaphane. Affilla Cress (16 g) was prepared and administered in a similar fashion. Blinding of participants was ensured by the even appearance of both drinks and the use of nasal plugs during the consumption of the investigational products. The placebo or intervention product was prepared by a researcher who was not involved in any other study procedures and data analysis. Ninety minutes after administration of the investigational products, participants were asked to drink the high-fat, high-glucose, high-caloric product (PhenFlex) [60]. For the preparation of the PhenFlex (400 mL, 950 kcal) 60 g palm olein, 75 g dextrose, 20 g protein, 0.5 g artificial vanilla aroma and 320 mL tap water were used [60]. In all cases, PhenFlex mixtures were freshly prepared, and the participants were instructed to consume the drink within 5 min.

4.2.4 Blood sampling and assessment of biomarkers

Samples of venous blood were taken twice per visit from the antecubital vein for measurement of inflammatory and metabolic biomarkers. Samples were collected in 4 mL BD tubes containing K2EDTA as anticoagulant, and centrifuged for 5 min (at 3000 g, 4 °C) within 30 min after collection. Plasma was stored at ≤ −80 °C until the day of analysis. Plasma samples were analyzed for inflammatory biomarkers, sVCAM-1, sICAM-1, IL-1β, IL-6, TNF-α, CCL-2, IL-8, IL-10, adiponectin, Hs-CRP, and IL-12 p70 using Enzyme-linked

immunosorbent assays (R&D Systems Netherlands; Supplementary Table 1. A systemic low-grade inflammation score was generated summing the z-score log-transformed inflammatory biomarkers plasma concentration (sICAM-1, sVCAM-1, IL-6, TNF-α, CRP, IL-12, CCL-2, IL-1β, and IL-8). The z-score log-transformed plasma adiponectin and IL-10 levels were subtracted from the systemic low-grade inflammation score due to their anti-inflammatory properties [66]. Plasma samples were analyzed for glucose via colorimetric assay (Cayman Chemical; Glucose Colorimetric Assay Kit, Item No. 10009582) and lipoprotein A using Enzyme-linked immunosorbent assay (Abcam®; Human Lipoprotein A SimpleStep ELISA® Kit, ab212165).

4.2.5 Statistical analysis

All normally distributed data are presented as mean ± standard deviation (SD). The non-normally distributed data are shown as median (interquartile range). For categorical variables, frequency and/or percentages are presented. Differences between the groups were assessed by paired sample T-tests for normally distributed parameters, or Wilcoxon signed-rank tests for the data that was not normally distributed. Wilcoxon signed-rank tests were performed to check for potential carryover effects. Associations between clinical features and biomarkers were assessed using Pearson (normally distributed parameters) or Spearman's rank (non-normally distributed) correlation coefficient. All analyses were performed, two-tailed with a $p \leq 0.05$ considered statistically significant.

4.3 RESULTS

4.3.1 Subject characteristics

Between November 2021 and January 2022, a total of 12 subjects were enrolled into the study and randomly allocated to either initial administration of sulforaphane or placebo. Baseline characteristics of the study population (n=12) are summarized in Table 1. All participants completed the study and were included in the data analysis for biochemical testing (Figure 2). The pre-test (Wilcoxon rank sum test) revealed no differences between treatment allocations for all parameters (all $z < 0.00$, $p > 0.14$). Absence of sulforaphane in placebo was verified by testing urine samples which showed significantly higher concentration of the total metabolites of sulforaphane after intake of broccoli sprouts (8.2 vs. 0.4 µmol, $p < 0.001$).

Table 1. *Characteristics of the study participants*

Characteristics	Population (n=12)
Sex (n, %)	
Female	1 (8.3)
Male	11 (91.7)
Age (years, mean (SD))	26.9 (3.6)
BMI (kg/m^2)	23.1 (1.6)
Body Fat (%)	
Female	28.9 (n/a)
Male	21.4 (3.1)
All	22.0 (3.6)
Visceral Fat Level, (mean (SD))	5.17 (1.57)
Alcohol Consumption, n (%)	
Moderate	0 (0)
Heavy	9 (75)
Very Heavy	3 (25)
Smoking Status, n (%)	
Smoker	5 (42)
Non-smoker	7 (58)

4.3.2 The effect of sulforaphane on endothelial activation

The single serving of sulforaphane or placebo induced no significant changes in concentrations of sICAM-1 and sVCAM-1 in healthy participants before and two hours after the PhenFlex challenge. A general trend was seen in the enhancement of endothelial activation in the sulforaphane group, however not significant; sICAM-1 (sulforaphane 1.5 ± 10.1 vs. placebo 3.1 ± 7.8 ng/mL, p = 0.696) and sVCAM-1 (sulforaphane 3.1 ± 5.2 vs. placebo 0.9 ± 4.5 ng/mL, p = 0.431) (Table 2, Figure 3).

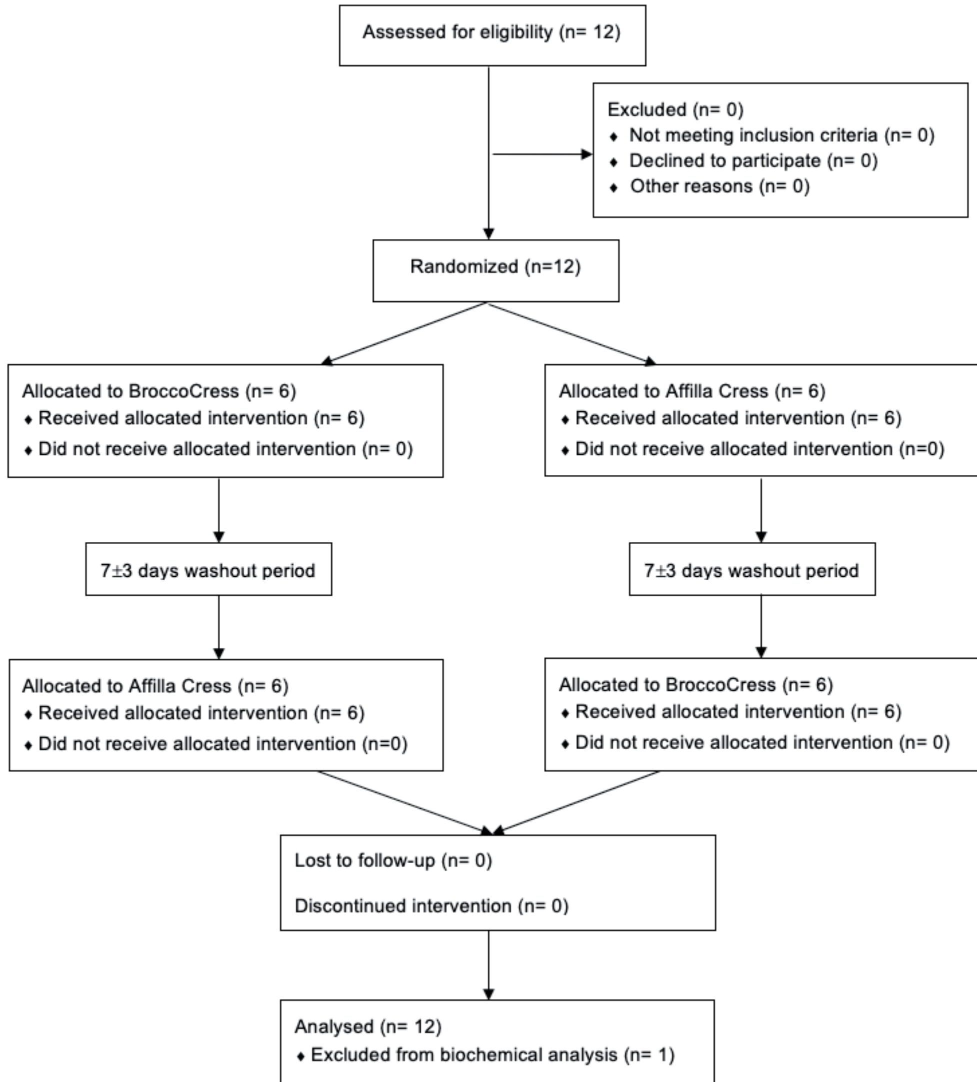

Figure 2. *CONSORT flow diagram.*

***Table 2**. The plasma concentrations of sICAM-1, sVCAM-1, hs-CRP, Adiponectin, CCL-2, and IL-10 before (min 90) and after (min 210) the PhenFlex challenge, Median (IQR)*

Inflammatory biomarker		Sulforaphane		Placebo	
sICAM-1* (ng/mL)	before	56.6 ± 25.6	*#p=0.428*	63.9 ± 23.3	*#p=0.513*
	after	59.0 ± 22.3		65.8 ± 20.0	
Δ sICAM-1* (ng/mL)		1.5 ± 10.1		3.1 ± 7.8	##p=0.696
sVCAM-1* (ng/mL)	before	50.5 ± 5.6	*#p=0.128*	51.5 ± 7.9	*#p=0.659*
	after	54.5 ± 10.2		52.1 ± 7.9	
Δ sVCAM-1* (ng/mL)		3.1 ± 5.2		0.9 ± 4.5	##p=0.431
hs-CRP* (ng/mL)	before	50.9 ± 9.0	*#p=0.277*	53.4 ± 10.8	*#p=0.466*
	after	52.4 ± 8.7		52.7 ± 10.7	
Δ hs-CRP* (ng/mL)		2.2 ± 4.3		-0.5 ± 2.9	##p=0.275
Adiponectin^ (ng/mL)	before	50.4 (6.9)	*#p=0.799*	51.1 (0.8)	*#p=0.767*
	after	50.9 (3.7)		52.5 (3.8)	
Δ Adiponectin^ (ng/mL)		0.5 (4.1)		0.5 (3.7)	##p=0.779
CCL-2 (pg/mL)	before	16.9 (25.6)	*#p=0.314*	18.2 (24.2)	*#p=0.374*
	after	19.1 (24.1)		18.3 (23.5)	
Δ CCL-2 (pg/mL)		1.9 (3.3)		0.0 (4.8)	*##p=0.017*
IL-10^ (pg/mL)	before	55.7 (213.4)	*#p=0.893*	51.4 (178.8)	*#p=0.225*
	after	51.2 (231.9)		56.1 (184.1)	
Δ IL-10^ (pg/mL)		-0.6 (26.3)		4.4 (5.1)	##p=0.715

*Note: significance for #within and ##between group comparison; ^Anti-inflammatory biomarker; *Normally distributed (mean ± SD)*

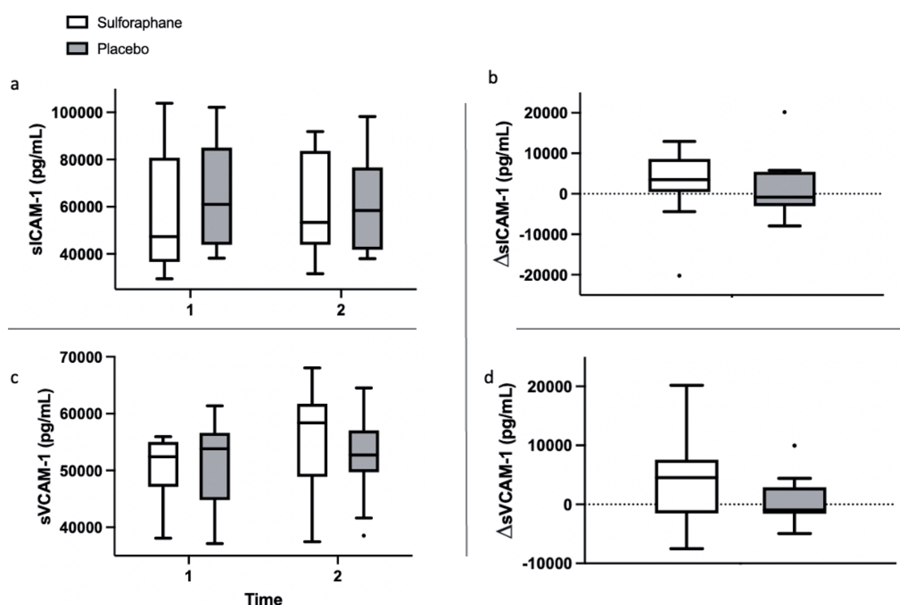

***Figure 3.** Plasma concentrations of sICAM-1 (pg/mL) and sVCAM-1 (pg/mL) in the sulforaphane and placebo groups before, after, and the changes during the PhenFlex challenge. Data are presented as boxplots (median, interquartile range, outliers (circles). Timepoints: 1 - before administration of PhenFlex (90 min); 2 - two hours after PhenFlex (210 min). Comparison between timepoints in sulforaphane/placebo.*

4.3.3 The effect of sulforaphane on inflammatory biomarkers

Eleven inflammatory biomarkers, sICAM-1, sVCAM-1, IL-6, TNF-α, hs-CRP, adiponectin, IL-12 p70, CCL-2, IL-10, IL-1β, and IL-8, were measured in plasma before and two hours after the PhenFlex challenge. Levels of sICAM-1, sVCAM-1, hs-CRP, adiponectin, CCL-2, and IL-10 and changes are listed in Table 2. Changes in CCL-2, measured as differential concentrations before and two hours after caloric load, showed a significant change between groups, with sulforaphane causing a significant increase in this biomarker compared to placebo (1.9 (3.3) vs. 0.0 (4.8) pg/mL, p = 0.017) (Figure 4). Changes in sICAM-1, sVCAM-1, hs-CRP (sulforaphane 2.2 ± 4.3 vs. placebo -0.5 ± 2.9 pg/mL, p = 0.275), and IL-10 (sulforaphane -0.6 (26.3) vs. placebo 4.4 (5.1) pg/mL, p = 0.715) revealed an overall slight and statistically non-significant pro-inflammatory effect of sulforaphane (Table 2). No detectable levels of IL-6, TNF-α, IL-12, IL-1β, and IL-8 were quantified.

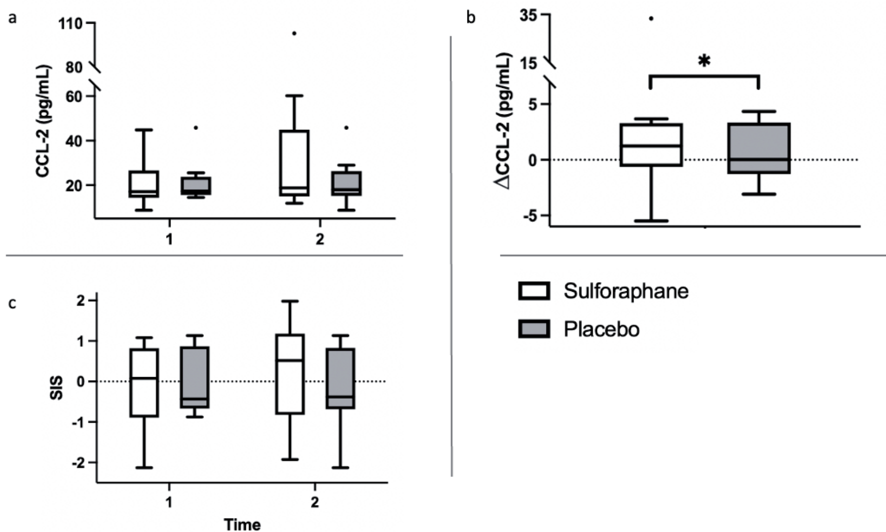

*Figure 4. Plasma concentrations of CCL-2 (pg/mL) in the sulforaphane and placebo groups before, after, and the changes during the PhenFlex challenge (a, b). The systemic low-grade inflammation score (SIS) in the sulforaphane and placebo before and after the PhenFlex challenge (c). Data are presented as boxplots (median, interquartile range, outliers (circles). Timepoints: 1 - before administration of PhenFlex (90 min); 2 - two hours after PhenFlex (210 min). Comparison between timepoints in sulforaphane/placebo, *p<0.05.*

4.3.4 The effect of sulforaphane on the systemic low-grade inflammation score

Aside from CCL-2, a more robust change was observed with the integration of the individual biomarkers in the composite systemic low-grade inflammation score (Table 3, Figure 4). In the sulforaphane group the composite score revealed a pro-inflammatory trend after caloric challenge (-0.092 (1.06) before versus after 0.018 (1.06), p=0.087) which was less prominent in the placebo group (-0.001 (0.81) before versus after 0.014 (0.81), p=0.251).

Table 3. *The systemic low-grade inflammation score (SIS) before (min 90) and after (min 210) the PhenFlex challenge, Mean ± SD.*

Systemic low-grade Inflammation Score (SIS)		**Sulforaphane**		**Placebo**	
SIS	before	-0.092 ± 1.06	#*p*=0.087	-0.001 ± 0.81	#*p*=0.251
	after	0.018 ± 1.06		0.014 ± 0.81	

Note: #significance for within group comparison

Before the PhenFlex challenge, no significant correlations between inflammation biomarkers and demographic, anthropometric, and lifestyle data were observed, with the exemption of IL-10 and smoking (p = 0.042), and the number of cigarettes per day (p = 0.042). The integration of the separate biomarkers into the composite systemic low-grade-inflammation score revealed strong significant correlations between the score and sex (p = 0.030) (Table 4). Moreover, moderate strength correlations between the systemic low-grade inflammation score and fat percentage (r_s = -0.551, p = 0.079) and the number of cigarettes per day (r_s = 0.557, p = 0.075) were observed.

***Table 4**. Univariate correlates of demographic parameters with fasting metabolic and inflammatory parameters. Variables were correlated using Pearson (normally distributed parameters) or Spearman's rank (non-normally distributed) correlation. Only significant correlations are shown*

Parameter		**Demographic**					**Inflammatory**		
		Age	BMI	FP	Smo	Cig	Adi	CCL-2	IL-10
Metabolic	Glucose	–0.81	–0.61	–0.64	-	-	0.71	-	-
	Lp(a)	-	-	-	-	-	-	0.61	0.94
Inflammatory	IL-10	-	-	-	–0.83	–0.83			

Note: FP – Fat percentage; Smo – Smoking; Cig – Cigarettes per day; Lp(a) – Lipoprotein A; Adi – Adiponectin; IL-10 – Interleukin-10; CCL-2 – Chemokine ligand 2

4.3.5 The effect of sulforaphane on glucose and lipid metabolism during caloric overload

The single serving of sulforaphane or placebo induced no significant changes in concentrations of glucose and lipoprotein A in healthy participants before and two hours after the PhenFlex challenge (Table 5). Before the PhenFlex challenge, fasting glucose levels correlated negatively with age ($p = 0.003$), BMI ($p = 0.046$) and fat percentage ($p = 0.034$), and positively with adiponectin concentrations ($p = 0.015$). Fasting lipoprotein A concentrations correlated positively with CCL-2 ($p = 0.049$) and IL-10 levels ($p = 0.005$) (Table 4). Moreover, administration of sulforaphane caused changes in glucose levels in response to the caloric load which correlated positively with changes in sICAM-1 ($p = 0.006$), adiponectin ($p = 0.048$) and lipoprotein A ($p = 0.005$). Changes in lipoprotein A concentrations in response to the challenge after sulforaphane administration correlated positively with changes in sICAM-1 levels ($p = 0.004$). In the placebo group, changes in glucose levels in response to caloric loading only negatively correlated with changes in IL-10 ($p < 0.001$) (Table 6).

Table 5. *The plasma concentrations of glucose and lipoprotein A before (min 90) and after (min 210) the PhenFlex challenge, Mean ± SD.*

Parameter		Sulforaphane		Placebo	
Glucose* (mg/dL)	before	73.8 ± 7.8	#*p=0.363*	76.8 ± 5.2	#*p=0.133*
	after	66.8 ± 11.5		67.0 ± 16.7	
Δ Glucose* (mg/dL)		-6.9 ± 17.8		-9.8 ± 18.5	##*p*=0.589
Lipoprotein A* (µg/mL)	before	166 ± 128	#*p=0.214*	189 ± 144	#*p=0.269*
	after	172 ± 132		199 ± 130	
Δ Lipoprotein A* (µg/mL)		3.8 ± 14.6		10.7 ± 26.3	##*p*=0.580

*Note: significance for #within and ##between group comparison; *Normally distributed*

Table 6. *Univariate correlates of changes in metabolic parameters (glucose and lipoprotein A) with inflammatory parameters during the PhenFlex challenge. Variables were correlated using Pearson (normally distributed parameters) or Spearman's rank (non-normally distributed) correlation. Only significant correlations are shown*

Parameter	Sulforaphane			Placebo
	sICAM-1	Adi	Lp(a)	IL-10
Glucose	0.79	0.64	0.81	–1.00
Lipoprotein A	0.82	-	n/a	-

Note: Lp(a) – Lipoprotein A; Adi – Adiponectin

4.4 DISCUSSION

4.4.1 The PhenFlex challenge did not unbalance endothelial homeostasis in young healthy participants

In the present study, metabolic overload did not significantly affect plasma adhesion marker levels in healthy participants, as measured two hours after caloric overload. In contrast, previous research has shown that a single administration of the PhenFlex challenge increased the levels of sVCAM-1 and sICAM-1 after two hours in healthy volunteers [18]. Additionally, Derosa et al. (2010) demonstrated significant increases in these plasma adhesion markers within two hours after an oral glucose tolerance test (OGTT) [61]. A possible explanation for the lack of effect found on these markers in our study is the fact that the subjects in the current study were given whole food products (sprouts of broccoli or pea) before undergoing the challenge. Both products contain retinol, vitamin E and ascorbic acid, which could have counteracted the expected transient disruption of post-exposure endothelial homeostasis observed in other studies. This hypothesis is supported by findings of Nappo et al. (2002), who showed that supplementation with vitamin C and E prevented an increase in sICAM-1 and sVCAM-1 in healthy middle-aged subjects after a high-fat meal [62]. Furthermore, Rubin et al. (2008) found no changes in plasma adhesion markers in young participants (25 years on average), after a standardized lipid-rich meal which contained retinol [63]. Thus, in this study, the effect of sulforaphane on endothelial homeostasis may have been influenced by the other nutrients present in the whole food product. Nonetheless, the associations between metabolic parameters and inflammatory biomarkers during the PhenFlex challenge, particularly between plasma sICAM-1, and glucose and lipoprotein A concentrations in the sulforaphane group, provided relevant information on the modulation of endothelial function and metabolic homeostasis by sulforaphane in response to a high-glucose, high-fat product. Consistent with our findings, Chen et al. (2000) demonstrated significant relationships between the plasma glucose and insulin responses to an OGTT and plasma sICAM-1 concentrations in healthy participants [64]. The fact that these correlations were not observed in the pea sprouts group (placebo) supports the hypothesis that the other bioactive compounds in the whole food products may have blunted the transient disruption of post-exposure homeostasis expected after caloric load. This is also revealed in part by the only correlation between circulating IL-10 and plasma glucose. The presence of other nutrients in broccoli sprouts may have interfered with the strong effects of sulforaphane, which, however, were still evident as more correlations were shown in this group.

4.4.2 An integrative measure to investigate the pleiotropic effects of phytonutrients is superior to single biomarkers

In this study, sulforaphane facilitated the development of a mild pro-inflammatory state during caloric challenge, as evidenced by a moderate increase in sICAM-1, sVCAM-1, hs-CRP, CCL-2 and decrease in IL-10. The effects of dietary intervention on chronic inflammation in other studies that used the PhenFlex challenge are inconsistent [60,65]. Kim et al. (2018) examined the effect of a single-intake microencapsulated garlic powder and/or tomato extract in healthy male smokers during the metabolic challenge. Consumption of tomato extract elicited a differential response, increasing CCL-2 and decreasing sVCAM-1 six hours after PhenFlex compared to placebo. When garlic powder was consumed, IL-13 levels decreased after two hours and IL-1α increased six hours after the challenge, indicating a pro- inflammatory effect of the food product. The combination of interventions elicited a mixed response, with IL-10 and CCL-7 being reduced six hours after the metabolic challenge [65]. Hoevenaars et al. (2019) investigated the effects of a 12-week whole grain wheat (WGW) intervention compared to refined wheat (RW) and observed increased CRP, IL-6, IL-8, and decreased IL-1β in RW and decreased CRP, serum amyloid A, IL-8, and IL-10 in WGW, indicating pro- and anti-inflammatory effects in respective groups [60]. These inconclusive results highlight the importance of implementing integrative outcome measures to unravel the subtle, pleiotropic effects of phytonutrients. As an illustration, the study of Weseler et al. (2011) testing the effects of grape seed extract on multiple biomarkers reflecting vascular health integrated them into a vascular health index, which unveiled an improvement in overall vascular health from flavanols, which was less clear from the analysis of individual outcomes [12,16]. In addition, previous cross-sectional studies evaluated CLGI through an index that pools multiple indicators to provide a better overall picture of the synergistic changes of inflammatory biomarkers [51,66–70].

To the best of our knowledge, this experimental study is the first to examine the effects of phytonutrients on calorie-induced inflammation as measured by a composite scoring system. Intriguingly, the score more accurately reflected the pro-inflammatory effect of broccoli sprouts than single biomarkers during phasic response. In addition, the relationships between risk factors for the development of NCDs such as high visceral fat and smoking, and inflammation became more apparent through the use of the score. Specifically, fat percentage and smoking showed a moderate inverse relationship with the score. Using the systemic low- grade inflammation score also led to another finding; five of the eleven inflammatory biomarkers were undetectable in the blood of our young and healthy population. These findings suggest that even a well-characterized scoring system may show limitations. For future research, assessing the health status or risk profile of the test population and adjusting the scoring system could be beneficial. The six biomarkers detectable under basal conditions in our study may be more suitable for challenge testing in young healthy subjects [19,28,71– 74].

4.4.3 Metabolic challenge studies may reveal the beneficial effects of phytonutrients in multiple ways depending on mechanism of action in the body

Over the past few decades, research has increasingly focused on antioxidants as the main health-promoting compounds in fruits and vegetables, leading to a gigantic array of antioxidant supplements on the market today [5–7,13,75–77]. However, initial excitement regarding the potential health benefits of antioxidants failed to be confirmed by clinical evidence [13]. There is quite a bit of debate about whether supplementing with antioxidants is healthy, ineffective, or even harmful [5,11,13,22,28,31,75,76,78–84]. So far, whole foods and fresh produce have not shown a clear protective effect against the PhenFlex challenge [60,65]. In fact, dietary interventions high in hormetins facilitated the development of a mild pro-inflammatory state during caloric overload, e.g. broccoli sprouts and garlic extract [65]. We hypothesize that this moderate pro-inflammatory state in the sprouts containing sulforaphane may be due to the initial pro-oxidative action (to activate Nrf2) of hormetins present in fresh produce. As a result, the exogenous antioxidant capacity of the direct antioxidants present in both sprouts is blunted in the broccoli sprouts compared to the pea sprouts. This, in turn, led to a reduced initial integrative anti-inflammatory capacity against the caloric overload demonstrated by the broccoli sprouts compared to the placebo. However, we speculate that because sulforaphane also enhances endogenous antioxidant defenses via Nrf2 activation at a later stage, whole foods that increase both exogenous and endogenous antioxidants may have more significant effects on phenotypic flexibility (Figure 5). This may explain why supplementation of direct antioxidants such as ascorbic acid and vitamin E attenuates the metabolic stress of caloric loads [62,63], while hormetins in fresh produce, e.g. sulforaphane, diallyl sulphide, withaferin A and rutin, induce a mild pro-inflammatory effect via activation of the Nrf2-pathway [38,49,50,65,85]. We hypothesize that the health effects of fruit and vegetable consumption are due to the wide variety of bioactive compounds in the food matrices and the synergy between the different mechanisms of action of these phytonutrients in the body, rather than just antioxidants [28]. One aspect of synergy may be a buffering effect [86,87]. The effect of a large intake of a given nutrient may vary depending on whether it is taken in concentrated form or as part of a food matrix, e.g. the matrix may slow down the absorption of the nutrient, which lowers the likelihood of a bolus effect [87].

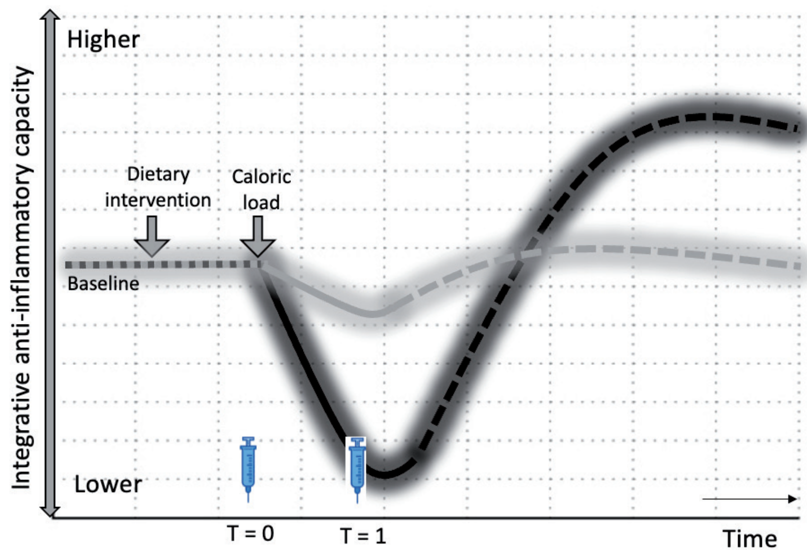

Figure 5. *Hormesis hypothesis on health effects of fruits and vegetables. Changes in integrative anti-inflammatory capacity in response to intervention, followed by caloric load. The dotted lines represent the expected sustained effects on integrative anti-inflammatory potential through an increase in endogenous antioxidants via Nrf2 activation by hormetins. Black: Broccoli sprouts (with sulforaphane); Grey: Pea sprouts (without sulforaphane); T0 and T1 - time points of blood sampling*

4

The limitations of our study include a small sample size and a short observation period. A longer time of observation (6, 8, 12, or even 24h) could have helped to demonstrate that the increase in inflammatory activity caused by sulforaphane represents the initial part of the hormetic response. In fact, previous research, conducted with larger sample sizes, has demonstrated the sustained anti-inflammatory effects of sulforaphane [56,88]. At the same time, biotechnological advancements that allow continuous monitoring of certain functions (e.g. glucose) and innovative designs (e.g. n-of-1 trials) may enable more accurate research into personalized nutrition strategies in the future [89–92]. Learning more about the interplay between phytonutrients may eventually reveal whether "an apple a day can keep the doctor away"—at least for a while.

4.5 CONCLUSION

This study has shown that the subtle and pleiotropic effects of phytonutrients can be studied in a short time by challenging the resilience and efficacy of adaptive mechanisms of healthy participants. Broccoli sprouts containing sulforaphane facilitated the development of a mild pro-inflammatory state during the caloric challenge, which suggests the onset of a hormetic response and became more evident when applying integrative outcome measures. The multifaceted approach allowed for more accurate quantification of the effects of phytonutrients in relation to inflammation and metabolic processes. Considering innovative integrative research approaches (e.g. composite scores, wearables, n-of-1 designs) would enhance our understanding of the hormetic principles of phytonutrients and stimulate research into the health effects of food.

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***Supplementary Table 1**: DuoSet ELISA kits from R&D systems used for detection of biomarkers in the plasma samples*

sVCAM-1	Human VCAM-1/CD106 DuoSet ELISA (R&D systems, #DY809-05)
sICAM-1	Human ICAM-1/CD54 DuoSet ELISA (R&D systems, #DY720-05)
IL-1β	Human IL-1 beta/IL-1F2 DuoSet ELISA (R&D systems, #DY201-05)
IL-6	Human IL-6 DuoSet ELISA (R&D systems, #DY206-05)
TNF-α	Human TNF-alpha DuoSet ELISA (R&D systems, #DY210-05)
CCL-2	Human CCL2/MCP-1 DuoSet ELISA (R&D systems, #DY279-05)
IL-8	Human IL-8/CXCL8 DuoSet ELISA (R&D systems, #DY208-05)
IL-10	Human IL-10 DuoSet ELISA (R&D systems, #DY217B-05)
Adiponectin	Human Adiponectin/Acrp30 DuoSet ELISA (R&D systems, #DY1065-05)
Hs-CRP	Human C-Reactive Protein/CRP DuoSet ELISA (R&D systems, #DY1707)
IL-12 p70	Human IL-12 p70 DuoSet ELISA (R&D systems, #DY1270-05)

5

The beneficial effect of sulforaphane on platelet responsiveness during caloric load: A single-intake, double-blind, placebo-controlled, crossover trial in healthy participants

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ABSTRACT

Background & Aims: As our understanding of platelet activation in response to infections and/or inflammatory conditions is growing, it is becoming clearer that safe, yet efficacious, platelet-targeted phytochemicals could improve public health beyond the field of cardiovascular diseases. The phytonutrient sulforaphane shows promise for clinical use due to its effect on inflammatory pathways, favorable pharmacokinetic profile, and high bioavailability. The potential of sulforaphane to improve platelet functionality in impaired metabolic processes has however hardly been studied in humans. This study investigated the effects of broccoli sprout consumption, as a source of sulforaphane, on urinary 11-dehydro-thromboxane B_2 (TXB_2), a stable thromboxane metabolite used to monitor eicosanoid biosynthesis and response to antithrombotic therapy, in healthy participants exposed to caloric overload.

Methods: In this double-blind, placebo-controlled, crossover trial twelve healthy participants were administered 16 g of broccoli sprouts, or pea sprouts (placebo) followed by the standardized high-caloric drink PhenFlex given to challenge healthy homeostasis. Urine samples were collected during the study visits and analyzed for 11-dehydro-TXB_2, sulforaphane and its metabolites. Genotyping was performed using Illumina GSA v3.0 DTCBooster.

Results: Administration of broccoli sprouts before the caloric load reduced urinary 11-dehydro-TXB_2 levels by 50% ($p = 0.018$). The amount of sulforaphane excreted in the urine during the study visits correlated negatively with 11-dehydro-TXB_2 ($r_s = -0.377$, $p = 0.025$). Participants carrying the polymorphic variant NAD(P)H dehydrogenase quinone 1 (NQO1*2) showed decreased excretion of sulforaphane ($p = 0.035$).

Conclusion: Sulforaphane was shown to be effective in targeting platelet responsiveness after a single intake. Our results indicate an inverse causal relationship between sulforaphane and 11-dehydro-TXB_2, which is unaffected by the concomitant intake of the metabolic challenge. 11-dehydro-TXB_2 shows promise as a non-invasive, sensitive, and suitable biomarker to investigate the effects of phytonutrients on platelet aggregation within hours.

5.1 INTRODUCTION

Our understanding of platelet functionality has changed dramatically over the past decade. Platelets are no longer viewed simply as hemostasis regulators; they are now recognized as crucial in coordinating inflammatory and immune responses [1,2]. Impaired platelet function has been observed in several chronic inflammatory conditions, including thrombosis and thrombotic disorders [3], asthma [4], myocardial infarction [5], unstable angina pectoris [6], atherosclerosis [7], and type 2 diabetes [8,9]. Interestingly, also postprandial hyperglycemia has been shown to cause platelet activation [10] and promote inflammatory state in healthy individuals [11–13]. In the clinical setting, administering caloric loads to humans has been used as a tool to pressurize adaptive mechanisms and trigger inflammation which may also be associated with an increase in platelet reactivity [14]. Thromboxanes are arachidonic acid metabolites with significant biological activity, including regulation of platelet functionality [15–17]. Increased production of thromboxanes was recognized to contribute to vasculopathy by adversely affecting endothelial function and promoting vascular inflammation [18]. By activating the thromboxane receptor, those metabolites cause significant alterations of platelet shape, inside-out activation of integrins, and degranulation [15–17], which might subsequently lead to increased platelet aggregation and thrombosis. On the other hand, increased production of thromboxane A and activation of platelets are recognized to be crucial modulators of the functionality of multiple inflammatory pathways involved in the progression of the cardiovascular diseases [19]. 11-dehydro-thromboxane B_2 (TXB_2) is produced from the breakdown of thromboxane A and can be measured in urine as a relevant marker of platelet reactivity, e.g. to monitor the response to aspirin (ASA) therapy when used to prevent heart disease [20,21]. In fact, targeting thromboxane production via antiplatelet agents has become a cornerstone of cardiovascular disease treatment [1,22]. Antiplatelet drugs (i.e., ASA, clopidogrel, prasugrel and abciximab) typically irreversibly suppress a specific pathway of platelet aggregation [1,22]. Therefore, the main limitations of current antiplatelet agents include the risk of bleeding and prolonged duration of action that cannot be reversed if the need for hemostasis or emergency surgery arises [22,23], which limits their use in many clinical situations e.g. prophylaxis of cardiovascular events in high-risk groups [1]. Phytonutrients have also been shown to affect platelet functionality with water-soluble tomato concentrate (WSTC) being the first to be recognized by the European Food Safety Agency (EFSA) as functional food that helps to maintain normal platelet aggregation, which contributes to healthy blood flow [26]. Unlike drug therapy, the antiplatelet effect of most phytochemicals is reversible, making them potentially safe for use in a variety of clinical scenarios, including primary prevention of cardiovascular diseases (17.9 million deaths annually) in the general population [1,24,25]. Therefore, it is paramount to search for novel nutritional strategies that support platelet functionality. Of the various plant-derived bioactive nutrients, sulforaphane holds the most promise for clinical testing due to superior absorption and pharmacokinetic profile [27–46]. Glucoraphanin, the biogenic precursor of

sulforaphane, is present in large amounts in broccoli and to a lesser extent in other Brassica species [47,48]. Since glucoraphanin is biologically inert, factors controlling the biosynthesis of glucoraphanin and conversion to sulforaphane by the enzyme myrosinase are of great importance to the potential health effects of these vegetables [28,39,48]. The antithrombotic properties of sulforaphane have been demonstrated in animals and *in vitro* studies [49,50]. Sulforaphane exerts antiplatelet activity which may initially activate adenylate cyclase/cAMP, followed by reversible inhibition of multiple intracellular signals such as the PI3-kinase/Akt and PLCγ2-PKC-p47 cascades [49,50]. Although promising, demonstrating an antithrombotic effect *in vivo* after oral administration of the compound has been scarcely explored in clinical trials, hence sulforaphane's ability to modulate platelet functionality in humans is still poorly studied [51]. These findings motivated us to investigate the effects of broccoli sprout consumption, as a source of sulforaphane, on urinary 11-dehydro-TXB_2 in healthy participants exposed to a standardized caloric load and to investigate interindividual genetic variability.

5.2 METHODS

A randomized, placebo-controlled, double-blind study was conducted with a cross-over design. The study protocol (NL77272.068.21) was approved by the Medical Ethics Review Committee of Maastricht University Medical Centre+ (MUMC+) and Maastricht University, Maastricht, the Netherlands, and performed in full accordance with the declaration of Helsinki of 1975 as revised in 2013, Fortaleza, Brazil [52]. The trial registration number within ClinicalTrials.gov is NCT05146804. All subjects provided written informed consent to participate.

5.2.1 Subjects

Healthy men and women were recruited by local and social media advertisements. Inclusion criteria were that participants were between 18 and 50 years old, had a body mass index (BMI) between 18.5 and 30 kg/m^2, with a stable weight (< 5% body weight change) and constant eating habits over the past three months. Exclusion criteria were the previous diagnosis of an inflammatory condition or disease or a history of hypothyroidism, chronic kidney or/and liver disorders, coronary artery disease, malignant hypertension, seizures, involved in intensive sports activities more than four times a week or at top sport level, regular intake of medication that may affect inflammatory response including NSAIDs, psychotic, addictive, or other mental disorders, aversion, intolerance or allergy to cruciferous vegetables and/or palm olein, dextrose, protein supplement, vanilla aroma, the use of dietary supplements with potential effects on antioxidant or inflammatory status and/or viral or bacterial infections requiring the use of antibiotics, laxatives and anti-diarrheal drugs four weeks prior to inclusion, excessive alcohol consumption (≥ 28 consumptions, approx.

250 g alcohol per week), pregnancy and/or breastfeeding, reported slimming or medically prescribed diet, as well as adhering to a vegetarian or vegan lifestyle.

5.2.2 Study design and procedures

Commercially available broccoli sprouts BroccoCress®, a rich source of sulforaphane, were used as the experimental product. In total, 16 g of sprouts were used per serving. Sulforaphane (BroccoCress®) and placebo (Affilla Cress®) were administered to each participant in the randomized fashion on different testing days. The period between two visits was 7±3 days. Information about demographics, alcohol consumption, and anthropometric data were assessed on the first visit. BMI, total body fat and visceral fat were measured using the Omron BF511R® monitor. The same testing scheme was applied during two visits (Figure 1), i.e. each participant received a single serving of intervention/placebo, which after 90 min was followed by oral administration of the PhenFlex challenge. Urine samples were collected throughout the day of the visit, preferably before the intervention/placebo, between intervention/placebo and the PhenFlex challenge, and after the PhenFlex administration. All participants were instructed to come fasted to each visit, to avoid consumption of broccoli or other cruciferous vegetables two days before each visit and to refrain from intense physical activity on the day of the visit. During the visit, participants remained in the testing location and were allowed to drink water *ad libitum*. No food intake was permitted during the visit.

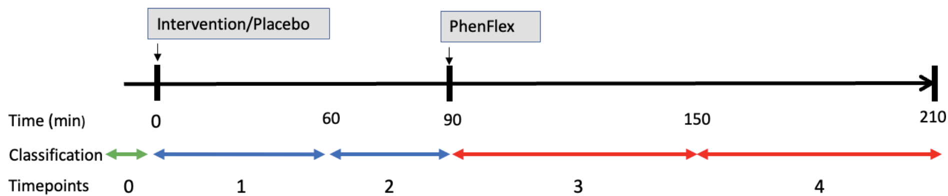

Figure 1. *Schematic presentation of a study visit.*

Administration of intervention (sulforaphane/placebo) was followed in 90 min by administration of standardized caloric challenge PhenFlex. Urine samples were classified into three groups: A (baseline, green line), B (after intervention or placebo, blue lines) and C (after PhenFlex challenge, red lines). In addition, samples were also divided into 5 timepoints: 0 (baseline), 1 (<60 min after intervention or placebo), 2 (>60 min after intervention or placebo), 3 (<60 min after PhenFlex challenge) and 4 (>60 min after PhenFlex challenge)

5.2.3 Intervention and caloric challenge (PhenFlex)

Shortly (maximum of 3 minutes) before administration, the sprouts were cut approximately 1 cm below the leaves, weighed, and mashed with a small amount of tap water (approximately 13°C) in a kitchen blender for 30s at room temperature (Premium Impuls Blender Smoothiemaker; Impuls; 180 W). Subsequently, tap water (approximately 13°C) was added to a total amount of 250 mL and participants were instructed to drink the entire

mixture. Commercially available pea sprouts (Affilla Cress®) were used as placebo in this study since pea sprouts do not contain glucoraphanin/sulforaphane. Affilla Cress (16 g) was prepared and administered in a similar fashion. Blinding of participants was ensured by the even appearance of both drinks and the use of nasal plugs during consumption of the investigational products. The placebo or intervention product was prepared by a researcher who was not involved in any other study procedures and data analysis. Ninety minutes after administration of the investigational products, participants were asked to drink a high-fat, high-glucose, high-caloric product (PhenFlex) [53]. For the preparation of the PhenFlex (400 mL, 950 kcal) 60 g palm olein, 75 g dextrose, 20 g protein, 0.5 g artificial vanilla aroma and 320 mL tap water were used [53]. In all cases, PhenFlex mixtures were freshly prepared, and the participants were instructed to consume the drink within 5 min.

5.2.4 Urine sampling and assessment of 11-dehydro-TXB_2

Urine samples were collected throughout the days of the visits in pre-labelled containers. The total volume and time of collection of each sample was recorded. Samples were aliquoted and stored at ≤ −80 °C until the day of analysis. Urine samples were analyzed for 11-dehydro-TXB_2 using an enzyme-linked immunoassay kit (UTxB2: assay #519510, Cayman Chemical, Ann Arbor, MI) following the manufacturer's instructions. The manufacturer recommended standardizing urinary 11-dehydro-TXB_2 values for creatinine levels using a colorimetric assay kit (Creatinine (urinary) Colorimetric Assay Kit: assay #500701, Cayman Chemical, Ann Arbor, MI). 11-dehydro-TXB_2 concentrations were normalized to urinary creatinine concentrations expressed as µg/mg Cr to account for inter-individual variability in urine dilution. Urine samples were classified into three groups for analysis: A (baseline), B (after intervention or placebo) and C (after PhenFlex challenge). In addition, samples were also divided into five timepoints: 0 (baseline), 1 (<60 min after intervention or placebo), 2 (>60 min after intervention or placebo), 3 (<60 min after PhenFlex challenge) and 4 (>60 min after PhenFlex challenge).

5.2.5 Measurement of urinary sulforaphane and metabolites

The determination of sulforaphane (SFN) and sulforaphane-glutathione (SFN-GSH), sulforaphane-cysteine (SFN-Cys), sulforaphane-cysteine-glycine (SFN-CG) and sulforaphane-N-acetylcysteine (SFN-NAC) in human urine was based on an HPLC-MS/MS method from Egner et al. [54].

5.2.5.1 Materials

Trc Canada deuterated stable Isotope solutions of sulforaphane-d8 and sulforaphane-d8-N-acetyl-L-cysteine were purchased from LGC standards (Wesel, Germany). J.T. Baker ethanol and Biosolve acetonitrile were purchased from Boom (Meppel, the Netherlands). Formic acid (FA) was purchased from VWR (Amsterdam, the Netherlands). Fresh water

was obtained from an inhouse MilliQ Advantage A10 system containing a LC-MS filter pack (Millipore, Burlington, MA). All chemicals were of analytical quality or higher.

5.2.5.2 Internal standard preparation

The stock solutions of SFN-d8-NAC and SFN-d8 were diluted with ethanol containing 0.2% FA by a factor of 10 and 100, respectively. From both dilutions 40µL was taken and combined in a new tube. The mixture was further diluted with ethanol containing 0.2% FA to give final concentrations of 2µg/mL for SFN-d8-NAC and 0.2µg/mL for SFN-d8 (IS mix). Stability of IS mix was verified by repeated analysis over a time period of several days.

5.2.5.3 Sample preparation

Urine samples were taken from the -80°C storage and thawed on ice. The samples were vortexed briefly and centrifuged at 21000 *g* and 4 °C for 5 minutes. From the supernatant 20 µL was transferred to a HPLC vial with a 200 µl insert. IS mix (20 µL) was added and mixed with the urine sample. Thereafter, the samples (5 µl) were directly injected into the analytical system.

5.2.5.4 Instrumental and data processing

For chromatographic separation an ExionLC UHPLC system (AB Sciex Framingham, MA) equipped with a binary pump, a thermostated autosampler and a column oven was used to maintain a constant temperature (40°C) during the analytical run. For detection a X500R qToF MS system (AB Sciex Framingham, MA) was used. Data acquisition was performed using SciexOS V2.1.6.59781 (AB Sciex). Data processing and identification of sulforaphane and its metabolites was performed by MS-DIAL (version 4.92).

5.2.6 Genotyping

The participants were requested to take a DNA sample using cheek swabs. Subsequently, all samples were processed at the Human Genomics Facility (HuGe-F) of the Genetic Laboratory of the Department of Internal Medicine at Erasmus MC using the Illumina GSA v3.0 DTC array consisting of 703,320 unique Single Nucleotide Polymorphisms (SNPs). The HuGe-F applied genotyping using the GenomeStudio v2 software with in-house cluster files for reference genome GRCh37. The resulting genotype files are subjected to in-house quality control pipelines which includes correcting for missing data and removing SNPs violating Hardy-Weinberg Equilibrium [55]. Finally, imputation is applied to the corrected and filtered SNPs with Beagle software [56] using a reference panel from the 1.000 Genomes project consisting of 2,141 samples [57]. Seven gene regions were selected for the analysis based on literature review [36,58–64]. For these genes, their genomic location according to the GRCh37 reference genome was determined from the NCBI Gene database and can be viewed in Table 1 [72]. The preprocessed SNPs were filtered on overlap with these gene regions. The resulting subset of potentially interesting SNPs was annotated with information from the NCBI dbSNP database [72].

Table 1. Selected genes based on sulforaphane (SFN) metabolism and effects

Gene name	Chromosome	Start position (bp)	End position (bp)	Influence on sulforaphane metabolism and/or effects of sulforaphane in humans
GSTM1	1	110,230,439	110,236,367	GSTM1-null mutations may benefit more from SFN, due to decreased metabolism [58,59,65].
GSTP1	11	67,351,283	67,354,124	GSTP1 Ile105Val genotypes may benefit more from SFN, due to decreased metabolism [66,67].
GSTT1	22	24,376,133	24,384,311	GSTT1-null mutations may benefit more from SFN, due to decreased metabolism [58,59,65].
NQO1	16	69,743,304	69,760,463	NQO1 polymorphisms may indirectly affect SFN metabolism [58].
CYP1A2	15	75,041,186	75,048,948	CYP1A2 polymorphisms may influence the effects of SFN [59].
UGT1A1	2	234,668,916	234,681,946	UGT1A1 polymorphisms may influence the effects of SFN [62,68–70].
NAT2	8	18,248,792	18,258,728	NAT2 polymorphisms may influence the effects of SFN [44,71].

Note: The genomic locations are listed according to the GRCh37 reference genome and the NCBI gene database

5.2.7 Statistical analysis

All normally distributed data are presented as mean ± standard deviation (SD). The non-normally distributed data are shown as median (interquartile range). For categorical variables, frequency and/or percentages are presented. Differences between the groups were assessed by repeated measures ANOVA for normally distributed parameters, or Skillings-Mack tests for the data that was not normally distributed. In addition, paired sample t-tests and Wilcoxon rank-sum tests were performed as post-hoc tests for the normally distributed samples and the non-normally distributed samples, respectively. Mann-Whitney U tests were performed to compare the change in urinary 11-dehydro-TXB_2 within sample groups between subjects. To study the association between 11-dehydro-TXB_2 and sulforaphane (metabolites), Spearman correlation was performed. The tool Stargazer [73] was used for phenotyping the metabolizer type of the participants for the NAT1 and NAT2 pharmacogenes. Participants were subdivided into low versus high excretion of sulforaphane. An adaptation of Fisher's Exact test within the plink genomics software was applied on the groups to test for significantly different genotypes [74]. Additionally, adaptive permutation testing within plink was executed to test the validity of the resulting p-values. All analyses were performed two-tailed with $p \leq 0.05$ considered statistically significant.

5.3 RESULTS

5

5.3.1 Subject characteristics

Between November 2021 and January 2022, a total of 12 subjects were found to be eligible to participate in the present study and were randomly allocated to either initial administration of sulforaphane or placebo. Baseline characteristics of the study population (n=12) are summarized in Table 2. All participants completed the study and were included in the data analysis for biochemical testing (Figure 2). A total of 54 urine samples were collected and assessed. Urine samples were classified into three groups: A (baseline), B (after intervention or placebo) and C (after PhenFlex challenge). In addition, samples were also divided into five timepoints: 0 (baseline), 1 (<60 min after intervention or placebo), 2 (>60 min after intervention or placebo), 3 (<60 min after PhenFlex challenge) and 4 (>60 min after PhenFlex challenge). Given the crossover design of the study, a pre-test was first performed to verify whether the time of the washout period was sufficient and to exclude any carry-over effects. The pre-test (Wilcoxon rank sum test) revealed no differences between treatment allocations for 11-dehydro-TXB_2 ($z < -4.47$, $p > 0.655$). A significant difference between the time of sampling in each sample group was checked by Kruskal-Wallis test $H(5) = 63.647$, $p < 0.001$.

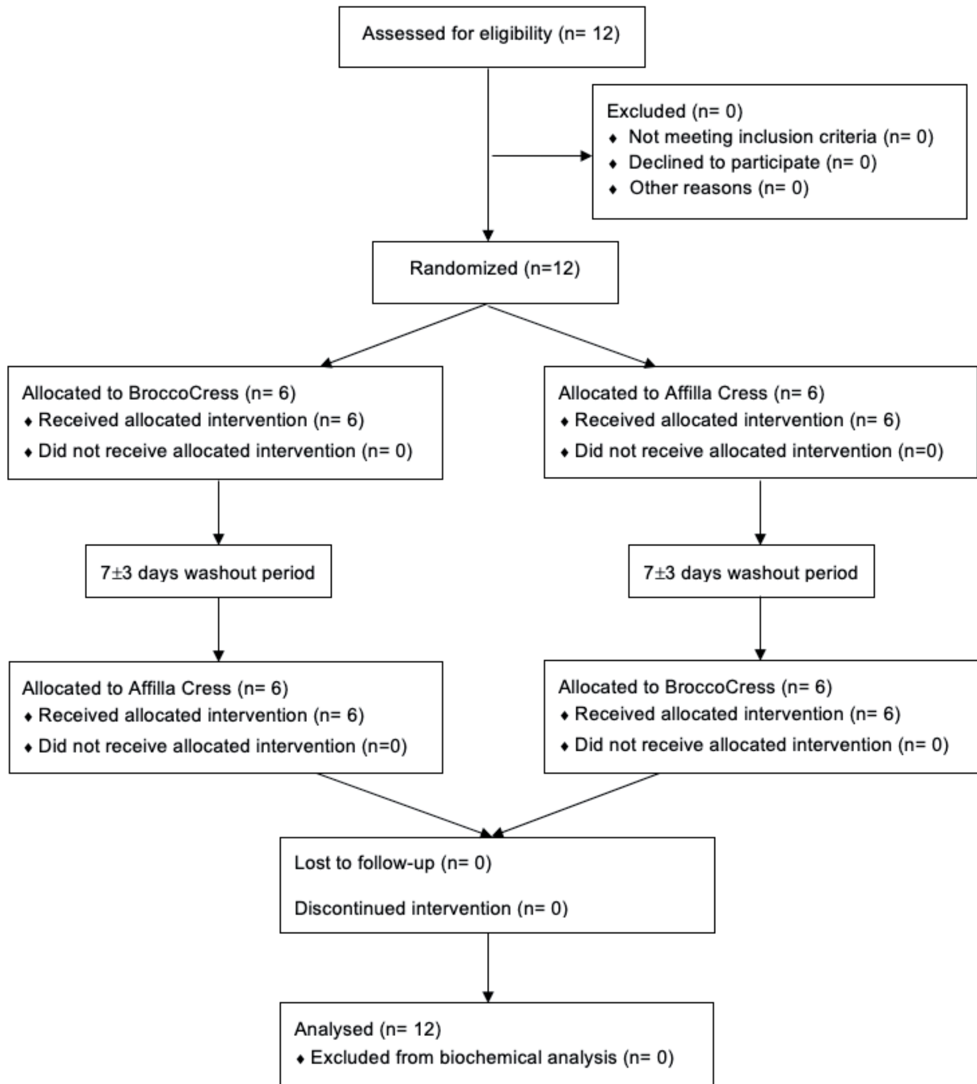

Figure 2. *CONSORT flow diagram.*

Table 2. *Characteristics of the study participants, (mean (SD))*

Characteristics	Population (n=12)
Sex (n, %)	
Female	1 (8.3)
Male	11 (91.7)
Age (years)	26.9 (3.6)
BMI (kg/m^2)	23.1 (1.6)
Body Fat (%)	
Female	28.9 (n/a)
Male	21.4 (3.1)
All	22.0 (3.6)
Visceral Fat Level	5.17 (1.57)
Alcohol Consumption, n (%)	
Moderate	0 (0)
Heavy	9 (75)
Very Heavy	3 (25)
Smoking Status, n (%)	
Smoker	5 (42)
Non-smoker	7 (58)

5.3.2 The effect of sulforaphane on 11-dehydro-TXB_2

Differences between groups were assessed for uncorrected and creatinine-corrected 11-dehydro-TXB_2. For the uncorrected data, a significant difference was observed in urinary 11-dehydro-TXB_2 concentrations between all groups in the sulforaphane group ($X^2(2) = 11.60$, $p = 0.003$) and in the placebo group ($X^2(2) = 7.58$, $p = 0.023$). Post hoc analysis showed significant differences for the sulforaphane group between samples A and B, and between samples A and C ($p = 0.028$ and $p = 0.018$), and between samples A and B for placebo ($p = 0.028$) (Figure 3). Analysis of the corrected data showed a significant difference in urinary 11-dehydro-TXB_2 concentration between all groups in the sulforaphane group ($X^2(2) = 9.27$, $p = 0.010$), no significant differences were observed in the placebo group ($X^2(2) = 3.53$, $p = 0.171$). Post hoc analysis showed a significant difference in the sulforaphane group between samples A and C for corrected data ($p = 0.018$) (Figure 4). Analysis between treatment allocations within samples showed no significant results (Table 3).

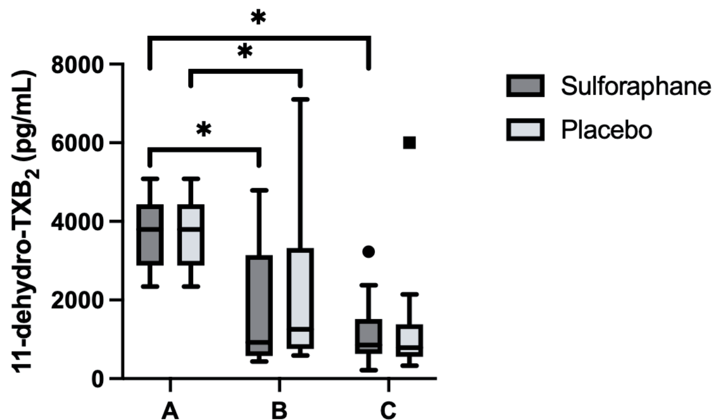

Figure 3. *Changes in urinary 11-dehydro-TXB$_2$ concentration in sulforaphane and placebo groups. Uncorrected data are presented as boxplots (median, interquartile range, outliers (circles) and far outliers (squares)). Urine samples were clustered into three timepoints: A (baseline), B (after intervention or placebo) and C (after PhenFlex challenge) * $p < 0.05$.*

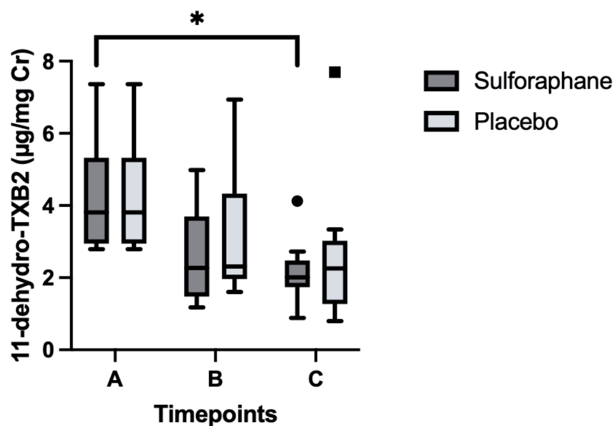

Figure 4. *Changes in urinary 11-dehydro-TXB$_2$ concentration in sulforaphane and placebo groups corrected for creatinine. Data are presented as boxplots (median, interquartile range, outliers (circles) and far outliers (squares)). Urine samples were clustered into three timepoints: A (baseline), B (after intervention or placebo) and C (after PhenFlex challenge). * $p < 0.05$.*

Table 3. *Uncorrected and corrected urinary 11-dehydro-TXB$_2$ concentrations (mean ± SD) per timepoint per treatment allocation*

		Population (n=12)				
Treatment	**Timepoints^**	**0 (A)**	**1 (B)**	**2 (B)**	**3 (C)**	**4 (C)**
Sulforaphane	Samples (n)	7	6	5	10	7
	Time of sampling (median (IQR))	n/a	45 (28)	75 (43)	120 (38)	180 (40)
	Sample volumes (median (IQR))	90 (93)	383 (485)	240 (330)	418 (380)	380 (490)
	Uncorrected TXB$_2$ (pg/mL)	3671 ± 860	4115 ± 7247	2076 ± 1637	1131 ± 1132	1106 ± 843
	Corrected TXB$_2$ (µg/mg Cr)	4.21 ± 1.51	3.74 ± 4.38	3.21 ± 1.08	2.07 ± 1.07	2.12 ± 1.02
Placebo	Samples (n)	7	7	7	6	11
	Time of sampling (median (IQR))	n/a	45 (20)	85 (30)	118 (16)	200 (35)
	Sample volumes (median (IQR))	90 (93)	240 (320)	260 (310)	400 (278)	350 (150)
	Uncorrected TXB$_2$ (pg/mL)	3671 ± 860	2908 ± 2927	1839 ± 1540	618 ± 154	1357 ± 1558
	Corrected TXB$_2$ (µg/mg Cr)	4.21 ± 1.51	3.74 ± 2.44	2.78 ± 1.26	1.48 ± 0.55	2.67 ± 1.85

Note: Cr – corrected for creatinine (mg/mL). ^ Timepoints clustered in three groups: 0 = A (baseline), 1+2 = B (after intervention or placebo) and 3+4 = C (after PhenFlex challenge)

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5.3.3 Sulforaphane excretion

The presence of sulforaphane after consuming fresh broccoli sprouts, and absence after placebo, was determined in urine samples collected during the study visits, indicating an adequate period of abstinence (mean total amount of sulforaphane and metabolites: 8.2 vs. 0.4 µmol, $p < 0.001$). Sulforaphane, SFN-N-acetylcysteine (SFN-NAC), SFN-cysteine, and total amount of metabolites were determined in all urine samples. No detectable levels of SFN-glutathione and SFN-cysteine-glycine were quantified. In the intervention group, the amount of sulforaphane excreted in urine significantly increased 120 min after its administration ($p = 0.028$) and decreased during the subsequent hour ($p = 0.043$) (Figure 5a). The amount of SFN-NAC excreted in urine significantly increased 120 min after the intervention ($p = 0.028$), and from the first 45 min to 1.5 hours afterwards ($p = 0.028$) decreased during the subsequent hour ($p = 0.043$) (Figure 5b). The amount of SFN-cysteine excreted in urine significantly increased two hours after the intervention ($p = 0.028$) (Figure 5c). The total amount of metabolites excreted in urine significantly increased 120 min after the intervention ($p = 0.028$) and from the first 45 min to an hour and a half afterwards ($p = 0.046$) and decreased during the subsequent hour ($p = 0.043$) (Figure 5d). In addition, the amount of sulforaphane excreted in the urine during the study visits correlated negatively with 11-dehydro-TXB$_2$ ($r_s = -0.377$, $p = 0.025$). Moderate

strength, inverse correlations between other metabolites SFN-NAC (r_s = -0.210, p = 0.225), SFN-Cys (r_s = -0.131, p = 0.452) and total metabolites (r_s = -0.196, p = 0.258) were observed.

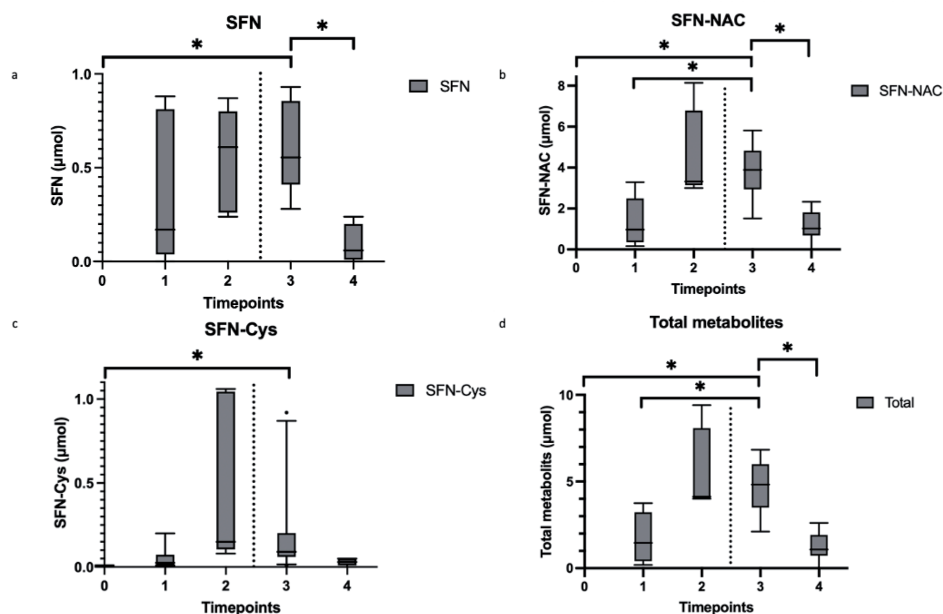

Figure 5. *Urinary sulforaphane (SFN), sulforaphane-cysteine (SFN-Cys), sulforaphane-N-acetylcysteine (SFN-NAC), and total metabolites excretion after participants consumed a single intake (16 g) of broccoli sprouts. Data are presented as boxplots (median, interquartile range, and outliers (circles)). The dashed lines indicate when the PhenFlex challenge was administered. Urine samples were clustered into five timepoints: 0 (baseline), 1 (<60 min after intervention), 2 (>60 min after intervention), 3 (<60 min after PhenFlex challenge) and 4 (>60 min after PhenFlex challenge).The amount of SFN (a) excreted in the urine correlated negatively with 11-dehydro-TXB$_2$ (r_s = -0.377, p = 0.025). Moderate strength, inverse correlations between other metabolites were observed, i.e. SFN-NAC (b) (r_s = -0.210, p = 0.225), SFN-Cys (c) (r_s = -0.131, p = 0.452) and total metabolites (d) (r_s = -0.196, p = 0.258). * p < 0.05.*

5.3.4 The influence of single nucleotide polymorphisms on sulforaphane and 11-dehydro-TXB$_2$

The 703,320 SNPs were subjected to the quality control pipeline, after which 624,240 SNPs were left. The subsequent imputation with Beagle resulted in 24,232,949 SNPs available for downstream analysis. Seven genes were selected for further analysis based on previous literature. Filtering the imputed SNPs using the gene regions as defined in Table 1 resulted in 77 SNPs that were further annotated and tested. Based on dbSNP annotations these included seven missense, 63 intronic, three synonymous, three 3’ untranslated regions (UTR), and one 5’ prime UTR variants. Testing the 77 relevant SNPs using Plink’s adoption of Fisher’s Exact test resulted in six SNPs associated with sulforaphane excretion

(Table 4). All SNPs were found in the NQO1 gene and all, but one were intronic variants, while the other SNP was a missense variant causing a change from proline to serine. Participants carrying this polymorphic variant NQO1*2, which is characterized by a C609T (rs1800566, Pro187Ser) polymorphism of the NQO1 gene, showed decreased excretion of sulforaphane during the study visits (6.2 (1.0) vs. 9.2 (1.6) µmol total metabolites, p = 0.035). No influence of these SNPs on 11-dehydro-TXB_2 levels was observed. No influence of SNPs in GSTM1, GSTP1, GSTT1, CYP1A2, UGT1A1 and NAT2 genes, or type of NAT1 and NAT2 metabolizer on sulforaphane excretion and 11-dehydro-TXB_2 levels was observed (Supplementary Table 1 and 2).

Table 4*: Single nucleotide polymorphisms (SNPs) associated with sulforaphane excretion*

Gene name	Reference SNP ID (RsID)	Chromosome	Base pair (bp) position	Type	Consequence (missense only)	Fisher's exact test p-value	Permutation p-value
NQO1	rs1800566	16	69,745,145	Missense	Proline→Serine	0.087	0.035
NQO1	rs57964521	16	69,750,092	Intron	-	0.087	0.035
NQO1	rs7186002	16	69,751,065	Intron	-	0.087	0.035
NQO1	rs1437135	16	69,757,828	Intron	-	0.087	0.035
NQO1	rs2196574	16	69,758,076	Intron	-	0.087	0.035
NQO1	rs2361839	16	69,758,395	Intron	-	0.087	0.035

Note: The genomic locations are listed according to the GRCh37 reference genome and the NCBI gene database

5.4 DISCUSSION

The aim of this work was to investigate the effects of sulforaphane on the arachidonic acid metabolite, as well as the relationship between 11-dehydro-TXB_2 and sulforaphane and its mercapturic conjugates in urine. Therefore, we assessed the effect of a single intake of fresh broccoli sprouts on urinary 11-dehydro-TXB_2 in healthy participants exposed to a standardized caloric load.

5.4.1 Sulforaphane decreased urinary 11-dehydro-TXB_2 in young healthy subjects exposed to caloric overload

This study showed that consumption of 16 g of broccoli sprouts before a caloric overload reduced the amount of urinary 11-dehydro-TXB_2 in healthy participants by 50%. These results are consistent with previous research examining the effects of sulforaphane on urinary biomarkers associated with inflammation. Medina et al. [51] showed that higher doses (30 g and 60 g) of broccoli sprouts significantly reduced urinary 11-dehydro-TXB_2 levels by 91% and 94%, respectively, within 12 hours in healthy subjects. The higher reduction found by Medina et al. [51] is probably due to the combination of the higher amount of broccoli sprouts given, the lack of a caloric challenge, and the longer time of observation. These results, additionally indicate that urinary 11-dehydro-TXB_2 shows

promise as a sensitive and suitable biomarker to investigate the effects of phytonutrients on platelet aggregation, at least in young healthy participants who are metabolically challenged in a short period of time. For example, in this study, the amount of urinary inflammatory biomarker high-sensitivity C-reactive protein (hs-CRP) remained unchanged during the study visits and was only detectable in 33/54 samples (data not shown).

5.4.2 The amount of sulforaphane and 11-dehydro TXB_2 excreted in the urine showed an inverse relationship during the caloric challenge

Our data on sulforaphane excretion and its relationship to urinary thromboxane metabolites shows a significant inverse correlation between sulforaphane and urinary 11-dehydro-TXB_2 (r_s = -0.377, p = 0.025) during the metabolic challenge. In contrast to previous studies, this inverse relationship was only significant for sulforaphane and not for other metabolites nor total metabolites, which may have been influenced by the co-ingestion of the caloric load and the shorter duration of urine collection [51]. It is striking however, that from these results it can be concluded that the metabolic state apparently has no effect on the effect of sulforaphane on 11-dehydro-TXB_2 levels.

Previous research has shown that a single administration of a standardized caloric load, the PhenFlex challenge, impacts relevant metabolic processes involved in maintaining or regaining homeostasis of metabolic health in healthy volunteers [12]. Contrary to our expectations, the caloric overload did not increase urinary 11-dehydro-TXB_2 measured two hours after administration of placebo (pea sprouts). Although pea sprouts have a similar nutrient profile to broccoli sprouts, except presence of sulforaphane, the increased amount of retinol and beta-carotene may have counteracted the expected post-challenge increase in 11-dehydro-TXB_2. Retinol and beta-carotene are transformed to retinoic acid (RA) by retinaldehyde dehydrogenases via numerous conversions [75]. RA is mediated by two nuclear receptor families, the retinoic acid receptors, and the retinoid X receptors (RXR) [76]. These receptors interact with retinoic acid response elements on the promoter regions of target genes, resulting in the activation of nuclear transcription factors [77,78]. The main antithrombotic effect of retinol and beta-carotene is based on the interference of RXR with the NF-κB pathway [79,80]. Inhibition of NF-κB disrupts platelet function by reducing its thrombogenic potential and shows promise when compounds that block NF-κB activation, such as sulforaphane and RA, are considered for prophylaxis of various thrombo-inflammatory diseases [80].

5.4.3 The NQO1*2 polymorphism may decrease sulforaphane excretion

Previous reports have shown that genetic predisposition may influence the bioavailability and excretion rate of sulforaphane, however results are incongruent [48,58,86,87,59,61,64,81–85]. Partially consistent with Boddupalli et al. [58], we found

lower excretion of sulforaphane in participants carrying the polymorphic variant NQO1*2, but no effect of SNPs in other phase II detoxification enzyme genes.

When cruciferous vegetables are damaged, such as when preparing or chewing, myrosinase is released. This enzyme is normally stored separately from glucosinolates, such as glucoraphanin, in different cells or in different intracellular compartments, depending on the plant species [34]. Myrosinase catalyzes the hydrolysis of glucoraphanin to liberate the glucose group, resulting in an unstable aglucone that spontaneously rearranges to give rise to a range of products, of which sulforaphane is the most reactive (Figure 6) [34]. Cooking cruciferous vegetables inactivates myrosinase, resulting in less sulforaphane formation [34]. Bacterial populations of the gut microbiota are also able to converse glucoraphanin to sulforaphane; however, it is estimated to be approximately six-fold less effective than plant myrosinase [88]. After this critical first step, sulforaphane is conjugated with glutathione (GSH) upon entry into the mammalian cell, catalyzed by glutathione S-transferases (GSTs), in the liver, entering the mercapturic acid pathway [89]. The glutathione conjugate is subjected to a series of sequential conversions, resulting in the *N*-acetylcysteine conjugate (mercapturic acid) as the main metabolite, which is excreted in the urine (Figure 6) [34]. Consistent with other studies, this study found the *N*-acetylcysteine conjugate as the primary metabolite in the urine samples [34,48]. However, compared to studies that examined timed urine samples over a longer period of time (8 h and longer), we found lower ratios of SFN-cysteine/total metabolites and no detectable levels of SFN-glutathione and SFN-cysteine-glycine [34,48]. We hypothesize that the longer period of observation and timed collection of urine samples could explain the differences in ratios. The results of Medina et al. [51], which showed that the SFN-cysteine/total metabolites ratio was twice as high in the first 12 hours as in the subsequent 12 hours, are in line with this hypothesis. Future research into the relationship between different metabolite ratios and time differences could provide an explanation for these inconsistencies.

Polymorphisms in phase II enzymes involved in the mercapturic acid pathway may help explain the individual outcomes of sulforaphane interventions (Table 1, Figure 6) [58,59]. In the current study, we found that not the enzymes directly involved, but an antioxidant enzyme, NQO1, indirectly involved in this metabolic route influenced sulforaphane secretion. Previous studies showed that the null NQO1*2 polymorphism results in a lack of functional NQO1 protein due to reduced stability, the ability to bind flavin adenine dinucleotide, and a dramatically reduced half-life due to rapid polyubiquitination and proteasomal degradation [81,90]. The lack of functional NQO1 would then in turn reduce the pool of other enzymes that catalyze detoxification of electrophiles, e.g. GSTs [91,92]. We hypothesize that because sulforaphane is metabolized by GSTs, the reduced glutathione pool will result in longer half-lives of circulating sulforaphane and

potentially greater systemic effects of cruciferous vegetables. Paradoxically, the increased activation of Nrf2/ARE-dependent genes by sulforaphane, including GSTs, thioredoxin and heme oxygenase 1, would also result in more non-functional NQO1 in individuals with the NQO1*2 polymorphism [31]. This positive feedback loop could explain the lower excretion of sulforaphane in participants carrying the polymorphic variant NQO1*2, while no initial differences in antithrombotic effect were observed after a single administration of broccoli sprouts. Repeated-dose studies could reveal whether individuals with genetic polymorphisms that reduce the activity of detoxification enzymes could benefit even more from consumption of cruciferous vegetables. Further clarification of the interactions between polymorphisms and the downstream effectors of sulforaphane are promising new lines of research to elucidate the relationship between the consumption of cruciferous vegetables and human health and well-being [58].

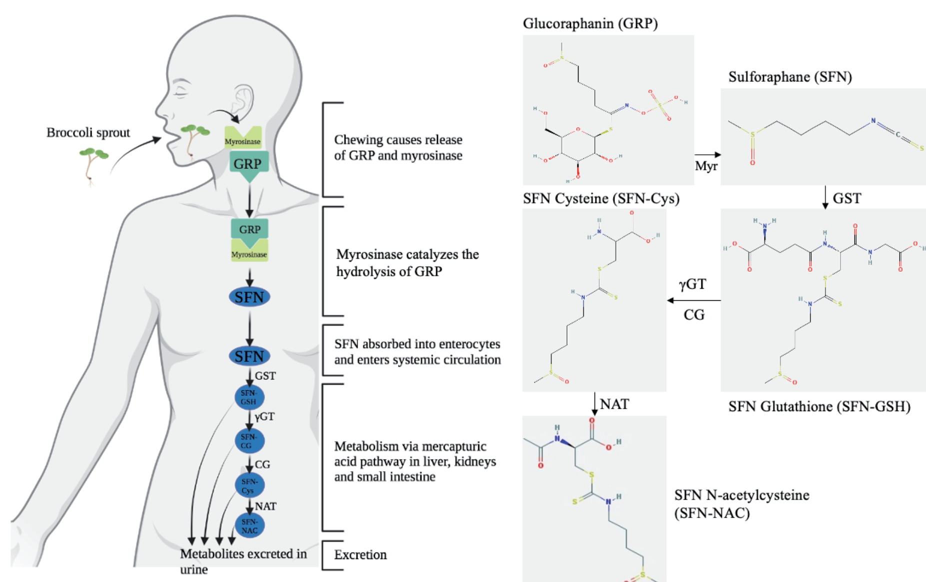

Figure 6. *The enzymatic conversion of glucoraphanin (GRP) to sulforaphane (SFN) by myrosinase (Myr), followed by the absorption, distribution, metabolism, and excretion of SFN via the mercapturic acid pathway. The enzymatic conversion of GRP to SFN is considered to be the rate-limiting step affecting bioavailability within this cascade, as the efficiency of conversion of SFN to metabolites in humans is very high (70-90%) [48].*

GRP, glucoraphanin; GST, glutathione S-transferase; Myr, myrosinase; γGT, γ-glutamyl transferase; CG, cysteinylglycinase; NAT, N-acetyl transferase; SFN-Cys, sulforaphane-cysteine; SFN-NAC, sulforaphane-N-acetylcysteine; SFN-GSH, sulforaphane glutathione conjugate.

5.4.4 Food-derived sulforaphane has implications for functional food innovation

Overall, only a limited number of food-derived compounds have been clinically investigated for their antiplatelet effects. Some success has been achieved in developing antiplatelet nutraceuticals, with WSTC to date being the only functional food proven to function as a natural cardio-protective functional ingredient, as assessed by the EFSA [25]. Therefore, Fruitflow®, the trademarked name of WSTC, is authorized by the European Commission to use the health claim (new function claims, Art. 13.5): "water-soluble tomato concentrate (WSTC) I and II helps maintain normal platelet aggregation, which contributes to healthy blood flow" [93,94]. To provide stakeholders with greater clarity on which effects related to cardiovascular health could be studied to support health claims, in 2018, a guidance was published by EFSA's Panel on Dietetic Products, Nutrition and Allergies (NDA Panel). This guidance document provides more detailed guidelines for the evaluation of Articles 13.1, 13.5, and 14 health claims in this area [95]. According to the Panel, a reduction in platelet aggregation (i.e., the percentage of inhibition of platelet aggregation using light transmission aggregometry (LTA) according to well-accepted and standardized protocols) in subjects with platelet activation during sustained exposure to the food/constituent (at least 4 weeks) is a beneficial physiological effect. Other outcome variables, such as thromboxane A_2 (TXA_2) or plasma soluble P-selectin, are not considered established markers of platelet aggregation, but can be used as supporting evidence for the scientific substantiation of these claims [95,96].

Although LTA has been considered the gold standard to assess platelet function for over 40 years, poor standardization and the required manipulation by a skilled technician limit its use to specialized laboratories [20,97–100]. Numerous platelet function tests are currently available; however, their methodologies are diverse, and little is known about the comparability or interchangeability of these tests [20,97]. O'Kennedy et al. examined TXA_2 levels (via thromboxane B_2, the stable metabolite of TXA_2) after a single dose of Fruitflow® (65 mg tomato total active fraction), or 75 mg aspirin (ASA) and found a reduction of approximately 25% at 3 hours and 37% at 5 hours for WSTC and 66% for ASA after 5 hours [26,94]. Our results indicate that sulforaphane seems more effective in reducing overall platelet aggregation at lower doses. In addition, the correlations between sulforaphane and thromboxane demonstrate causation, which is the last criterion in the evaluation of a scientific health claim dossier by EFSA [51,101,102]. A logical next step for future research would therefore be to investigate the effects of sulforaphane on inhibition of platelet aggregation using LTA in subjects with platelet activation.

5.4.5 Limitations and future directions

This study is the first to demonstrate that urinary 11-dehydro-TXB_2 is a sensitive, easy-to-use, and suitable biomarker to investigate the effects of phytonutrients on platelet aggregation in young healthy participants who are metabolically challenged in a short period of time. This study is not without limitations. First, the glucoraphanin content of the active sprout material and placebo may have differed from product labels and may have been adversely affected by cultivation conditions, batch-to-batch variation, and preparation methods. Second, it is challenging to find an impeccable placebo in nutritional intervention studies, especially for whole foods in a double-blinded setting. In current study, the placebo had to match the broccoli sprouts in terms of nutrient value, except for sulforaphane content. Despite our efforts, the increased amount of β-carotene and some vitamins in the pea sprouts may have counteracted the expected post-challenge increase in 11-dehydro-TXB_2 [79,80]. In addition, follow-up studies with a larger sample size will shed more light on the effects of interindividual genetic variability with respect to cruciferous vegetable consumption. Nevertheless, this study has increased our understanding of the antithrombotic effects of sulforaphane and can be used as supporting evidence for the scientific substantiation of claims on the reduction of platelet aggregation [95,96]. When follow-up studies on the effects of sulforaphane using LTA yield positive results, fresh broccoli sprouts or other produce containing enough sulforaphane per serving, could apply for the same authorized claim as WSTC.

5.5 CONCLUSION

This study demonstrated that a single administration of broccoli sprouts reduced urinary 11-dehydro-TXB_2 levels by clinically relevant amounts in healthy participants exposed to a standardized caloric load. In addition, the correlations between sulforaphane and thromboxane indicate a causal relationship, which is not influenced by the co-ingestion of the metabolic challenge. 11-dehydro-TXB_2 shows promise as a non-invasive, sensitive, and suitable biomarker to investigate the acute effects of phytonutrients on platelet aggregation within hours. Genetic predisposition may influence the health effects of cruciferous vegetable consumption, but more research is needed to ultimately provide personalized dietary advice for consumers. This study forms the basis for a scientific substantiation of claims on the reduction of platelet aggregation for fresh produce containing sulforaphane in the future.

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Supplementary Table 1. *Stargazer results for NAT1.*

Participant number	Haplotype 1 main * allele	Haplotype 2 main * allele	Haplotype 1 candidate * alleles	Haplotype 2 candidate * alleles	Haplotype 1 activity score	Haplotype 2 activity score	Combined activity score	Phenotype
230	*1	*1	*1	*1	1	1	2	normal metabolizer
204	*1	*1	*1	*1	1	1	2	normal metabolizer
303	*1	*11	*1	*11, *30	1	unknown	unknown	unknown[#]
454	*1	*1	*1	*1	1	1	2	normal metabolizer
422	*1	*1	*1	*1	1	1	2	normal metabolizer
919	*1	*1	*1	*1	1	1	2	normal metabolizer
084	*1	*1	*1	*1	1	1	2	normal metabolizer
740	*1	*1	*1	*1	1	1	2	normal metabolizer
323	*1	*14	*1	*14	1	0.5	1.5	normal metabolizer
771	*1	*1	*1	*1	1	1	2	normal metabolizer
178	*1	*1	*1	*1	1	1	2	normal metabolizer
029	*1	*1	*1	*1	1	1	2	normal metabolizer

*Note: * alleles are determined for both haplotypes and corresponding activity scores are added. The combined activity score translates to the metabolizer phenotype where poor metabolizers have a combined activity score of 0, intermediate metabolizers between 0 and 1 normal metabolizers between 1 and 2, rapid metabolizers between 2 and 2.5 and ultrarapid metabolizers larger than 2.5. [#] Stargazer phenotyped all but one participant as normal metabolizers by the pharmacogene NAT1 and was unable to determine the metabolism phenotype for one participant due to both NAT1*11 and NAT1*30 having an unknown activity score.*

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Supplementary Table 2. *Stargazer results for NAT2.*

Participant number	Haplotype 1 main * allele	Haplotype 2 main * allele	Haplotype 1 candidate * alleles	Haplotype 2 candidate * alleles	Haplotype 1 activity score	Haplotype 2 activity score	Combined activity score	Phenotype
230	*1	*1	*1	*1	1	1	2	normal metabolizer
204	*1	*6	*1	*6, *13	1	0.5	1.5	normal metabolizer
303	*1	*6	*1	*6, *13	1	0.5	1.5	normal metabolizer
454	*1	*6	*1	*6, *13	1	0.5	1.5	normal metabolizer
422	*1	*6	*1	*6, *13	1	0.5	1.5	normal metabolizer
919	*1	*1	*1	*1	1	1	2	normal metabolizer
084	*1	*1	*1	*1	1	1	2	normal metabolizer
740	*5	*6	*5, *11	*6, *13	0.5	0.5	1	intermediate metabolizer
323	*1	*5	*1	*5, *11	1	0.5	1.5	normal metabolizer
771	*1	*5	*1	*5, *11	1	0.5	1.5	normal metabolizer
178	*5	*6	*5, *11	*6, *13	0.5	0.5	1	intermediate metabolizer
029	*6	*6	*6, *13	*6, *13	0.5	0.5	1	intermediate metabolizer

*Note: * alleles are determined for both haplotypes and corresponding activity scores are added. The combined activity score translates to the metabolizer phenotype where poor metabolizers have a combined activity score of 0, intermediate metabolizers between 0 and 1 normal metabolizers between 1 and 2, rapid metabolizers between 2 and 2.5 and ultrarapid metabolizers larger than 2.5.*

6

Heart rate variability correlates with the effect of sulforaphane on calorie-induced inflammation in healthy participants: a randomized placebo-controlled study

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ABSTRACT

Background & Aims: The role of nutrition in modulating the inflammatory response is increasingly recognized. The phytonutrient sulforaphane shows promise for clinical use due to its effect on inflammatory pathways, favorable pharmacokinetic profile, and high bioavailability. The inflammatory status has been linked to autonomic activity, which can be assessed by the study of heart rate variability (HRV). However, monitoring of HRV for assessment of inflammation in humans has hardly been used. We investigated the potential of HRV as a non-invasive tool to monitor inflammation induced by the caloric load and assessed the effects of sulforaphane on caloric load-induced inflammation in healthy participants.

Methods: In this double-blind, crossover, randomized, placebo-controlled trial twelve healthy participants (26.9 (3.6) years) were administered 16 g of broccoli sprouts, or pea sprouts (placebo) followed over 90 min by the standardized high-calorie drink PhenFlex given to induce an inflammatory response. Levels of high-sensitivity C-reactive protein (hs-CRP) and interleukin (IL)-6 were measured in plasma before and two hours after the PhenFlex challenge. Changes in the autonomic function were assessed by HRV on four timepoints.

Results: The caloric challenge triggered a significant increase in total power (TP) (p = 0.028) and very low frequency (VLF) component (p = 0.013) 30 min after its administration. Those changes were followed by reduction of TP (p = 0.028) and low frequency (LF) component (p = 0.005), suggesting marked decrease in the sympathetic activity two hours after the caloric load. When sulforaphane was given prior to the caloric challenge, decreased parasympathetic activity was observed via a reduction of RMSSD (p = 0.007), pNN50 (p = 0.013) and HF (p = 0.047). In addition, sulforaphane elicited a pro-inflammatory response as measured by the change of hs-CRP with caloric exposure (sulforaphane 2.7 (4.2) vs. placebo -1.8 (3.1) ng/mL, p = 0.048). The pro-inflammatory effect of sulforaphane was associated with vagal withdrawal and sympathetic dominance as suggested by correlations between the changes in hs-CRP and HF (r_s = -0.68, p = 0.029) as well as LF/HF (r_s = 0.56, p = 0.093) components assessed before and two hours after the PhenFlex challenge.

Conclusion: Monitoring of HRV might be a sensitive tool to follow activity of the inflammatory response in various clinical conditions. The standardized caloric PhenFlex challenge induced significant changes in the autonomic regulation in healthy young individuals. Administration of sulforaphane prior to the caloric challenge caused a pro-inflammatory effect which was accompanied by vagal withdrawal and sympathetic dominance.

6.1 INTRODUCTION

Chronic low-grade inflammation (CLGI) plays an important role in the pathobiology of non-communicable diseases (NCDs), which are predicted to be among the leading causes of death by 2030 [1–3]. Inflammatory activity is generally assessed by certain biomarkers, such as interleukin 6 (IL-6) and high-sensitivity C-reactive protein (hs-CRP), and their levels in blood were linked to the severity and prognosis of CLGI-related diseases, such as metabolic syndrome and cardiovascular disease [4,5]. Elevated levels of inflammatory biomarkers have been reported in healthy people thought to reflect a risk of developing NCDs due to poor lifestyle choices [6–9]. However, it remains unclear which specific biomarkers truly reflect the activity of CLGI in the human body while their measurement in body fluids often requires invasive sampling and complex laboratory analysis, making their use impractical [10].

High caloric challenge tests, involving oral administration of glucose, fats, or even whole meals, are used to artificially induce inflammation in healthy individuals, which shows similarities to CLGI [11–13]. The strain imposed on adaptive mechanisms by a caloric challenge allows to show the ability to withstand homeostatic disturbances, also known as phenotypic flexibility or resilience. The latter is also believed to be a valid health biomarker, particularly in healthy or at-risk populations [14]. Among the caloric loads used in practice, the "PhenFlex" test with its standardized macronutrient composition, appears to be the most suitable to mimic the CLGI state due to its well-characterized effects on a wide range of metabolic parameters in humans [11,15].

HRV reflects the temporal variation between consecutive heartbeats (RR intervals) monitored by means of electrocardiogram (ECG). The analysis of HRV generally involves assessment of the time and frequency domain parameters, which are closely linked to the functional activity of the different regulatory systems in the human body [16,17]. For example, the high frequency (HF) component has been linked to the activity of the parasympathetic nervous system (PNS), the low frequency (LF) component was related to activity of the sympathetic nervous system (SNS) and baroreflex sensitivity [17]. Evidence from clinical studies shows that reduction of HRV associated with an increase of the LF component is linked to increased inflammatory activity [18]. At the same time, vagal stimulation was proposed to be used in treatment of CLGI-associated disease due to its ability to trigger anti-inflammatory response [19]. These findings demonstrate the functionality of the vagal anti-inflammatory pathway [20] and also provide grounds for assessing inflammation through HRV.

Epidemiological studies indicate that diets with a high proportion of fruits and vegetables might have a positive effect on inflammatory status and thereby can protect against

various NCDs [21–25]. Of the various plant-derived bioactive nutrients, sulforaphane which is present in high amounts in cruciferous vegetables, such as broccoli, Brussels sprouts, cabbage, cauliflower, and kale [26], holds the most promise for clinical testing due to superior absorption and pharmacokinetic profile [27–46]. The anti-inflammatory properties of sulforaphane have been demonstrated in animals and *in vitro* studies [27,28,39–44,47,48]. At the cellular level, sulforaphane is known to induce a pro-oxidant response which stimulates the expression of endogenous antioxidant enzymes via the activation of the nuclear factor E2-related factor 2 (Nrf2) pathway responsible for the ultimate anti-inflammatory effect of this phytonutrient [40,41,49–53].

These findings motivated us to explore the potential of HRV parameters as a non-invasive tool to monitor inflammation induced by the PhenFlex challenge and to test the effect of sulforaphane on calorie-induced inflammation in healthy participants.

6.2 METHODS

We have conducted a randomized, placebo-controlled, double-blind study with a cross-over design. The study protocol (NL77272.068.21) was approved by the Medical Ethics Review Committee of Maastricht University Medical Centre+ (MUMC+) and Maastricht University, Maastricht, the Netherlands, and performed in full accordance with the declaration of Helsinki of 1975 as revised in 2013, Fortaleza, Brazil [54]. The trial registration number within ClinicalTrials.gov is NCT05146804. All subjects provided written informed consent.

6.2.1 Subjects

Healthy men and women were recruited by local and social media advertisements. Inclusion criteria were that participants were between 18 and 50 years old, had a body mass index (BMI) between 18.5 and 30 kg/m^2, with a stable weight (< 5% body weight change) and constant eating habits over the past three months. Exclusion criteria were the previous diagnosis of an inflammatory condition or disease or a history of hypothyroidism, chronic kidney or/and liver disorders, coronary artery disease, malignant hypertension, seizures, involved in intensive sports activities more than four times a week or at top sport level, regular intake of medication that may affect inflammatory response including NSAIDs, psychotic, addictive, or other mental disorders, aversion, intolerance or allergy to cruciferous vegetables and/or palm olein, dextrose, protein supplement, vanilla aroma, the use of dietary supplements with potential effects on antioxidant or inflammatory status and/or viral or bacterial infections requiring the use of antibiotics, laxatives and anti-diarrheal drugs four weeks prior to inclusion, excessive alcohol consumption (≥ 28 consumptions approx. 250 g alcohol per week), pregnancy and/or breastfeeding, reported slimming or medically prescribed diet, as well as adhering to a vegetarian or vegan lifestyle.

6.2.2 Study design and procedures

Sulforaphane (BroccoCress®) and placebo (Affilla Cress®) were administered to each participant in the randomized fashion on different testing days. The period between two visits was 7±3 days. Information about demographics, alcohol consumption [55] and anthropometric data were assessed on the first visit. Body mass index (BMI), total body fat and visceral fat were measured using the Omron BF511R® monitor. The same testing scheme was applied during two visits (Figure 1), i.e. each participant received a single serving of intervention/placebo, which after 90 min was followed by oral administration of the PhenFlex challenge.

During each visit heart rate variability (HRV) was assessed four times: (i) prior to administration of intervention/placebo (HRV1), (ii) just before the intake of PhenFlex (HRV2), (iii) 30 min after PhenFlex (HRV3), (iv) 120 min after PhenFlex (HRV4, Figure 1). Blood samples were collected twice, just before intake of PhenFlex and 120 min after. All participants were instructed to come fasted to each visit, to avoid consumption of broccoli or other cruciferous vegetables two days before the visit and to restrain from intense physical activity on the day of the visit. During the visit, participants remained in the testing location and were allowed to drink water ad libitum. No food intake was permitted during the testing period.

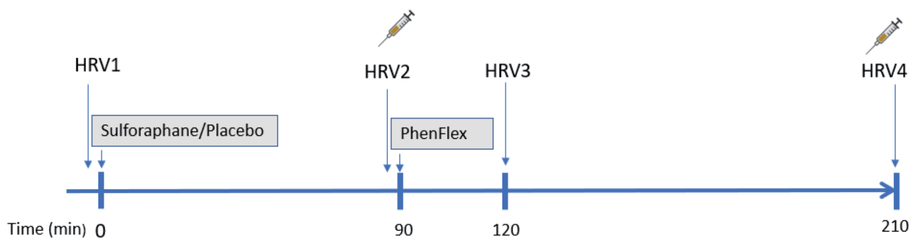

***Figure 1.** Schematic presentation of a study visit.*

Note: Administration of intervention (sulforaphane/placebo) was followed in 90 min by administration of standardized caloric challenge PhenFlex. Heart rate variability (HRV) was assessed in short-term ECG recording obtained before the intervention (0 min), before administration of PhenFlex (90 min) as well as 30 min (120 min) and 2 hours (210 min) after the PhenFlex load. Blood samples were obtained before (90 min) and 2 hours after (210 min) PhenFlex challenge.

6.2.3 Intervention and caloric challenge (PhenFlex)

Commercially available broccoli sprouts BroccoCress®, a rich source of sulforaphane, were used as the experimental product. In total, 16 g of sprouts were used per serving, which is equivalent to approximately 25 mg of sulforaphane [28]. Shortly before administration, the sprouts were cut approximately 1 cm below the leaves, weighed, and mashed with a small amount of water to ensure complete conversion of glucoraphanin to sulforaphane.

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Subsequently, water was added to a total amount of 250 mL, which was consumed by the participants. Commercially available pea sprouts (Affilla Cress®) were used as placebo in this study since pea sprouts do not contain glucoraphanin/sulforaphane. Affilla Cress (16 g) was prepared and administered in a similar fashion. Blinding of participants was ensured by the even appearance of both drinks and the use of nasal plugs during consumption of the investigational products. The placebo or intervention product was prepared by a researcher who was not involved in any other study procedures and data analysis.

Ninety minutes after administration of the investigational products, participants were asked to drink the high-fat, high-glucose, high-caloric product (PhenFlex) [56]. For the preparation of the PhenFlex (400 mL, 950 kcal) 60 g palm olein, 75 g dextrose, 20 g protein, 0.5 g artificial vanilla aroma and 320 mL tap water were used [56]. In all cases, PhenFlex mixtures were freshly prepared, and the participants were instructed to consume the drink within 5 min.

6.2.4 Heart rate variability

HRV was assessed in short term (5 min) ECG recordings obtained in supine position with a sampling frequency of 2 kHz using a computer electrocardiograph 'Poly-Spectrum-8/E. For the analysis, Neurosoft® software version 5.3.1.0 was used. The recordings were performed in a quiet room with a temperature of 20-22°C. Participants were instructed to relax, breathe normally, avoid coughing, and close their eyes during the ECG recording. Prior to the recording, the ECG signal was monitored for approximately 5 min.

The time- and spectral-domain indexes were analysed based on the recommended standards [57]. Next to the changes in heart rate, the following time domain indices were assessed: (i) standard deviation of normal RR intervals (SDNN, ms); (ii) square root of the mean squared differences of successive RR intervals (RMSSD, ms); percentage of differences between adjacent normal RR intervals exceeding 50 milliseconds (pNN50, %). All time-domain parameters largely reflect the activity of parasympathetic nervous system [17]. The spectral HRV analysis included assessment of total power (TP, 0.01-0.4 Hz), which reflects total activity of regulatory components; very low frequency power (VLF, 0.01-0.04 Hz), which characterizes mainly the activity of the neurohumoral regulation and neurohormonal balance; low frequency power (LF, 0.04-0.15 Hz), which characterizes predominantly sympathetic activity with some input of baroreflex sensitivity; high frequency power (HF, 0.15-0.4 Hz), which reflects vagal component of the heart rate regulation. The LF/HF ratio was used to characterize the sympathovagal balance. The normalized LF component (LFnorm) and the normalized high-frequency component (HFnorm) were analyzed. Like their non-normalized counterparts, the former has been shown to correlate with baroreflex sensitivity, while the latter mainly represents respiratory variations [17].

6.2.5 Blood sampling and assessment of inflammatory biomarkers

Samples of venous blood were taken twice per visit from the antecubital vein for measurement of inflammatory biomarkers. Samples were collected in 4 mL BD tubes containing K2EDTA as anticoagulant, and centrifuged for 5 min (at 3000 g, 4 °C) within 30 min after collection. Plasma was stored at ≤ −80 °C until the day of analysis. Plasma samples were analyzed for hs-CRP and IL-6 using Enzyme-linked immunosorbent assays (R&D Systems Netherlands; Human C-Reactive Protein DuoSet (DY1707), R&D Systems Netherlands; Human IL-6 DuoSet (DY206)).

6.2.6 Statistical analysis

All normally distributed data are presented as mean ± standard deviation (SD). The non-normally distributed data are shown as median (interquartile range). For categorical variables, frequency and/or percentages are presented. Differences between the groups were assessed by repeated measures ANOVA for normally distributed parameters, or Friedman's test for the data that was not normally distributed. In addition, paired sample t-tests and the Wilcoxon rank-sum tests were performed as post-hoc tests for the normally distributed samples and the non-normally distributed samples, respectively. Wilcoxon rank sum tests were performed to check for potential carryover effects. To study the association between the inflammatory biomarkers and HRV parameters, Spearman's rank correlation was performed. All analyses were performed two-tailed with $p \leq 0.05$ considered statistically significant.

6.3 RESULTS

6.3.1 Subject characteristics

Between November 2021 and January 2022, a total of 12 subjects were found to be eligible and were randomly allocated to either initial administration of sulforaphane or placebo. Baseline characteristics of the study population (n=12) are summarized in Table 1. All participants completed the study and were included in the data analysis for biochemical testing. For the HRV analysis data from two participants was excluded due to major editing of ECG recording required (Figure 2). No adverse events were reported; however, ECG analysis revealed the occurrence of multiple extrasystole after caloric overload in one participant which was not accompanied by any symptoms. Given the crossover design of the present study, a pre-test was first performed to verify whether the time of the washout period was sufficient and to exclude any carry-over effects. The pre-test (Wilcoxon rank sum test) revealed no differences between treatment allocations for all parameters (all $z < -0.153$, $p > 0.2$).

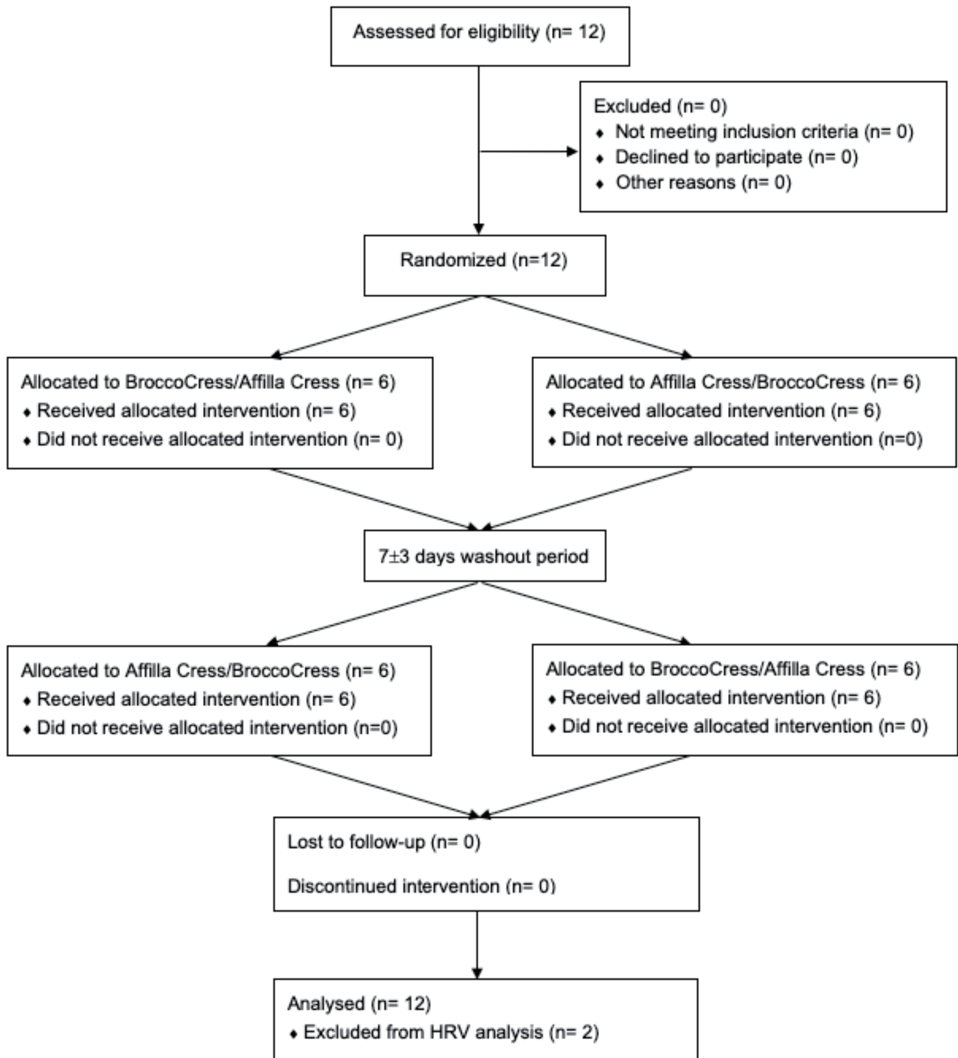

Figure 2. *CONSORT flow diagram.*

Table 1. Characteristics of the study participants, (mean (SD))

Characteristics	Population (n=12)
Sex (n, %)	
Female	1 (8.3)
Male	11 (91.7)
Age (years)	26.9 (3.6)
BMI (kg/m^2)	23.1 (1.6)
Body Fat (%)	
Female	28.9 (n/a)
Male	21.4 (3.1)
All	22.0 (3.6)
Visceral Fat Level	5.17 (1.57)
Alcohol Consumption, n (%)	
Moderate	0 (0)
Heavy	9 (75)
Very Heavy	3 (25)
Smoking Status, n (%)	
Smoker	5 (42)
Non-smoker	7 (58)

6.3.2 The effect of sulforaphane on HRV

The single serving of sulforaphane (25 mg) induced no significant changes in HRV parameters tested 90 min after its administration. In contrast, we have found that placebo caused a reduction in, VLF ($p = 0.037$), LF ($p = 0.047$) and TP ($p = 0.037$) assessed on the same timepoint (Table 2, Figure 4a, 4b, 4c).

Table 2. Changes of HRV in response to Sulforaphane/Placebo administered after min 0 followed by the PhenFlex challenge (after min 90), Median (IQR)

HRV parameter		Sulforaphane	Placebo
HR* (bpm),	0 min	61.5 (7.7)	60.5 (8.9)
	90 min	59.4 (7.7)	58.7 (9.3)
	120 min	68.2 (10.5)	68.1 (10.8)
	210 min	66.9 (8.8)	65.3 (10.0)
SDNN (ms),	0 min	68.0 (48.3)	56.0 (51.8)
	90 min	63.0 (34.0)	59.0 (39.3)
	120 min	50.0 (56.3)	80.5 (78.5)
	210 min	49.5 (66.8)	60.5 (39.8)
RMSSD (ms),	0 min	62.0 (52.8)	54.0 (51.5)
	90 min	57.0 (52.3)	61.0 (51.5)
	120 min	46.0 (51.5)	68.5 (43.5)
	210 min	48.5 (37.5)	55.0 (40.3)
pNN50 (%),	0 min	37.6 (39.3)	38.4 (35.7)
	90 min	37.2 (39.0)	44.4 (36.4)

Table 2. Continued

HRV parameter		Sulforaphane	Placebo
	120 min	27.6 (35.6)	36.1 (38.6)
	210 min	27.6 (38.4)	30.6 (38.9)
TP (ms^2),	0 min	4452 (7061)	3325 (7595)
	90 min	3830 (6621)	3218 (4845)
	120 min	2551 (7147)	6169 (12022)
	210 min	2319 (8988)	3839 (5020)
VLF (ms^2),	0 min	1843 (1325)	1510 (2807)
	90 min	1323 (1489)	968 (1155)
	120 min	800 (2566)	2294 (6328)
	210 min	787 (2933)	1312 (1695)
LF (ms^2),	0 min	1457 (2206)	1014 (1640)
	90 min	1317 (2338)	906 (1383)
	120 min	819 (2517)	1462 (2529)
	210 min	766 (2305)	793 (1255)
HF (ms^2),	0 min	1382 (2781)	1222 (3618)
	90 min	1211 (4292)	1441 (2850)
	120 min	827 (2102)	1595 (2208)
	210 min	881 (1903)	1296 (2201)
LFnorm* (n.u.),	0 min	49.6 (17.1)	45.0 (13.6)
	90 min	50.4 (18.9)	42.7 (10.4)
	120 min	51.7 (20.3)	54.2 (18.9)
	210 min	52.4 (19.9)	42.0 (20.5)
HFnorm* (n.u.),	0 min	50.4 (17.1)	55.1 (13.6)
	90 min	49.6 (18.9)	57.3 (10.4)
	120 min	48.3 (20.3)	45.8 (18.9)
	210 min	47.6 (19.9)	58.0 (20.5)
LF/HF*,	0 min	1.21 (0.75)	0.93 (0.51)
	90 min	1.34 (0.97)	0.80 (0.36)
	120 min	1.50 (1.20)	1.64 (1.35)
	210 min	1.62 (1.48)	1.00 (0.88)

**Normally distributed (mean (SD))*

6.3.3 The effect of caloric overload on HRV

Administration of the caloric challenge in the placebo group caused an increase in VLF ($p = 0.013$), LF ($p = 0.241$), and TP ($p = 0.028$) during the first 30 min, indicating respectively higher activity of regulatory systems predominantly on account of sympathetic component. During the subsequent 90 min of observation, the caloric challenge significantly reduced LF ($p = 0.005$), SDNN ($p = 0.018$) and TP ($p = 0.028$). The heart rate showed a significant increase over time following the PhenFlex challenge ($F(2,18) = 20.42$, $p < 0.001$) (Figure 3a, 3b, 4a, 4b, 4c).

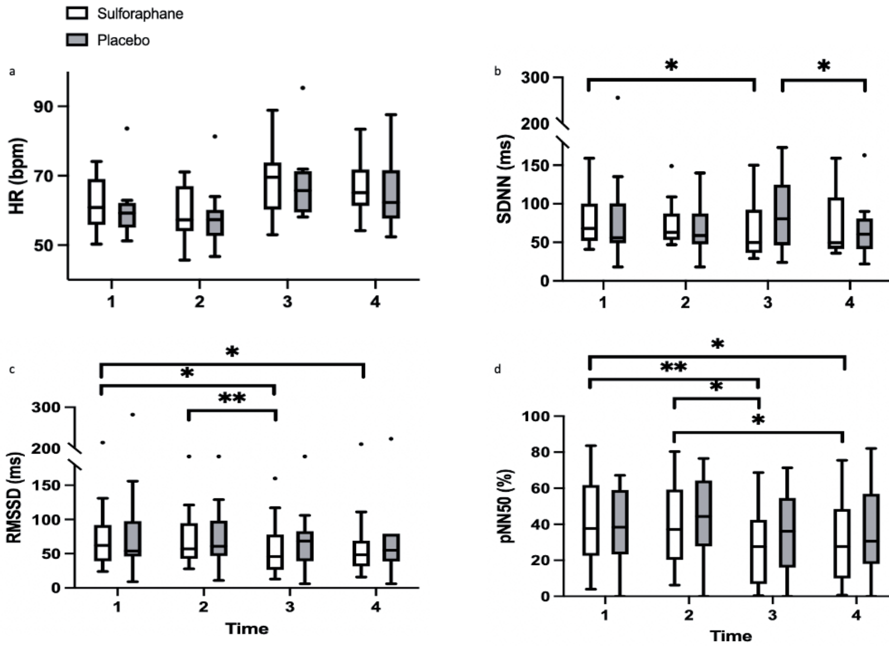

Figure 3. *Box plots of time-domain HRV parameters during the PhenFlex challenge. Timepoints: 1 - before intervention (0 min); 2 - before administration of PhenFlex (90 min); 3 - 30 min after PhenFlex (120 min); 4 - 2 hours after PhenFlex (210 min). Comparison between timepoints in sulforaphane/placebo *p<0.05, **p<0.01*

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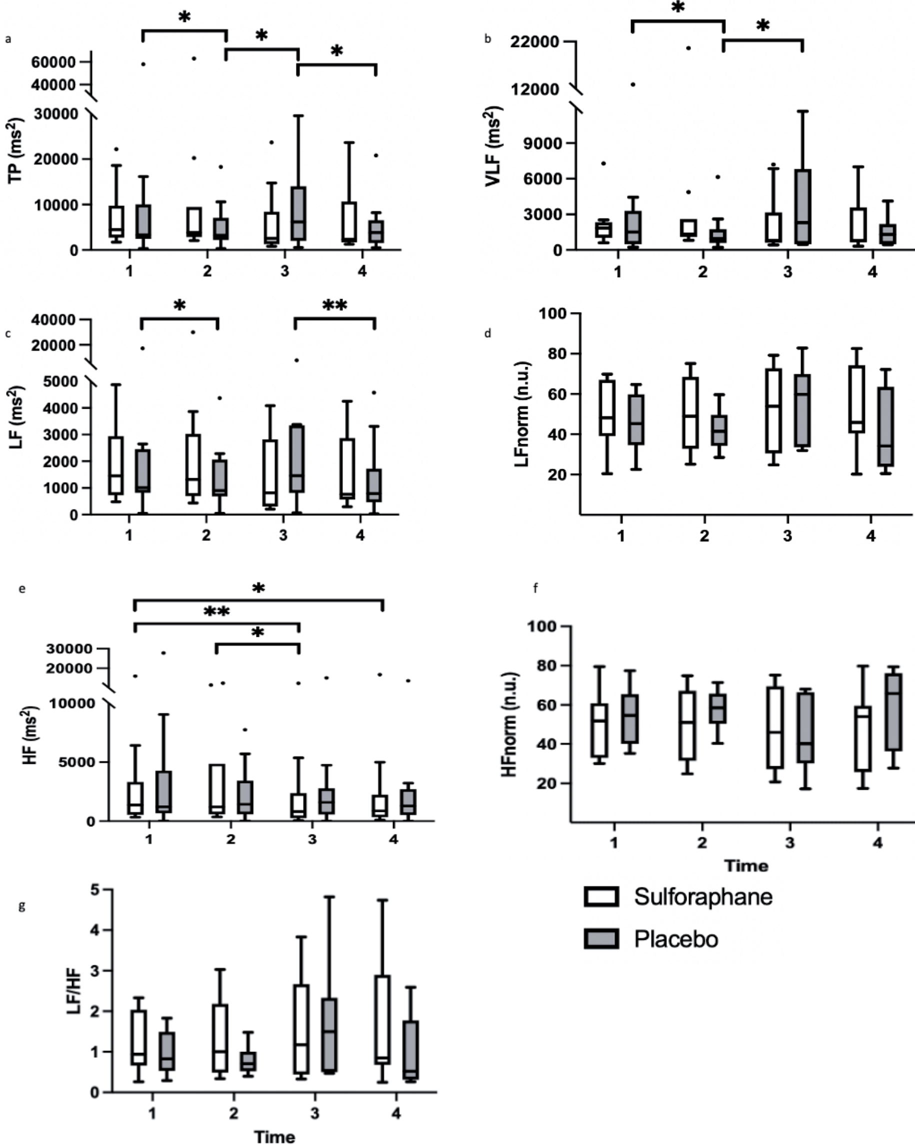

Figure 4. *Box plots of frequency-domain HRV parameters during the PhenFlex challenge. Timepoints: 1 - before intervention (0 min); 2 - before administration of PhenFlex (90 min); 3 - 30 min after PhenFlex (120 min); 4 - 2 hours after PhenFlex (210 min). Comparison between timepoints in sulforaphane/placebo *p<0.05, **p<0.01*

6.3.4 The effect of sulforaphane on HRV during caloric overload

During the PhenFlex challenge, sulforaphane induced differences in the following HRV parameters: lower SDNN from 0 to 120 min (p = 0.037); lower RMSSD from 0 to 120 min (p = 0.015), 0 to 210 min (p = 0.021) and 90 to 120 min (p = 0.007); lower pNN50 from 0 to 120 min (p = 0.007), 0 to 210 min (p = 0.017), 90 to 120 min (p = 0.013) and 90 to 210 min (p = 0.028); lower HF from 0 to 120 min (p = 0.009), from 0 to 210 min (p = 0.047), and from 90 to 120 min (p = 0.047) (Table 2, Figure 3b, 3c, 3d, 4e).

Furthermore, compared to placebo, sulforaphane significantly increased the LF/HF ratio (p = 0.014) and LFnorm (p = 0.016), and decreased HFnorm (p = 0.016) from 120 to 210 min. Repeated measures ANOVA showed a significant difference between treatments in LF/HF ratio following the caloric overload (F(2,18) = 3.482, p = 0.05).

6.3.5 Inflammatory biomarkers

Two inflammatory markers, hs-CRP and IL-6 were measured in plasma before and two hours after the PhenFlex challenge (Figure 1). Changes in hs-CRP (Table 3) showed completely different dynamics between groups with sulforaphane causing a significant increase and with placebo a decrease in this biomarker (sulforaphane 2.7 (4.2) vs. placebo -1.8 (3.1) ng/mL, p = 0.048). Before the PhenFlex challenge, significant correlations between HRV parameters and hs-CRP were observed for sulforaphane, but not for placebo: HFnorm (r_s = -0.67, p = 0.033), and LF/HF (r_s = 0.67, p = 0.033) (Figure 5a). Moreover, administration of sulforaphane caused an increase in hs-CRP in response to the caloric load which negatively correlated with changes in HFnorm (r_s = -0.68, p = 0.029) and positively with LF/HF (r_s = 0.56, p = 0.093) assessed at 90 min and 210 min (Figure 5b). For the placebo group, moderate strength, inverse correlations between changes of HRV and hs-CRP were observed, i.e. for HFnorm (r_s = 0.405, p = 0.32), and LF/HF (r_s = -0.548, p = 0.16).

Table 3. *The concentration of high sensitivity C-reactive protein (hs-CRP) before (min 90) and after (min 210) PhenFlex challenge, Mean (SD)*

Inflammatory biomarker		Sulforaphane		Placebo	
hs-CRP (ng/mL)	before	68.0 (17.3)	#*p=0.073*	73.5 (15.7)	#*p=0.350*
	after	70.7 (16.8)		71.7 (18.8)	
Δ hs-CRP (ng/mL)		2.7 (4.2)		-1.8 (3.1)	
		##*p=0.048*			

Note: significance for #within and ##between group comparison

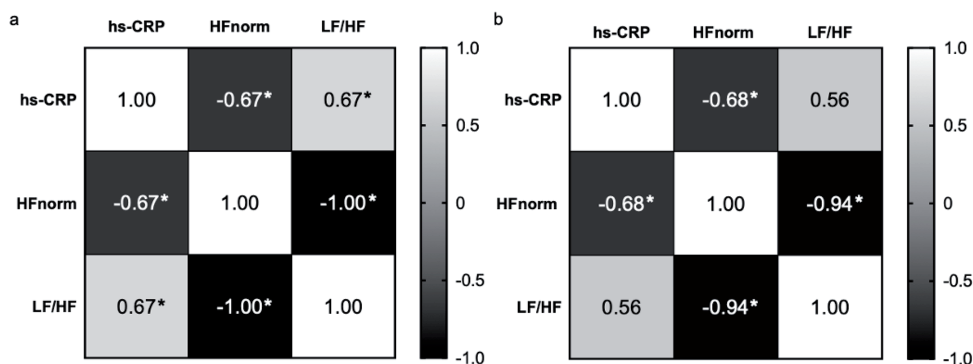

Figure 5. *Correlation matrix (r_s) of values of high-sensitivity C-reactive protein (hs-CRP) (ng/mL), HFnorm (n.u.), and LF/HF after intervention with sulforaphane (a). Correlation matrix (r_s) of changes in hs-CRP, HFnorm, and LF/HF during the PhenFlex challenge assessed at 90 min and 210 min in the sulforaphane group (b). Variables were correlated using Spearman's rank correlation *p<0.05*

6.4 DISCUSSION

6.4.1 The PhenFlex challenge affected HRV parameters in young healthy subjects

The intake of calorie-dense meals or large quantities of macronutrients challenges the physiological and metabolic systems of the human body and can promote inflammation, contributing in the long term to the development of NCDs. Previous research has shown that single administration of this standardized caloric load PhenFlex triggers a sustained inflammatory response similar to that seen in CLGI [11,12,15]. Therefore, the PhenFlex challenge can be viewed as an inflammation model that may be useful to test the effects of various interventions, including phytochemicals, on the inflammatory response in humans [56,58].

In the present study plasma concentrations of hs-CRP slightly decreased in two hours following the PhenFlex intake. Absence of a significant increase of this pro-inflammatory biomarker can be contributed to the preserved compensatory response and healthy phenotypic flexibility in our study population, which, in general, was younger and healthier compared to the populations in which PhenFlex has been characterized [11,12,15]. The dynamics of HRV parameters monitored at 30 min and 2 hours after the challenge provided additional insights into the homeostatic mechanisms activated by mixed caloric load. During the first 30 min of the postprandial phase, elevation in TP on account of VLF and some raise in the LF component was observed. Over the next 90 minutes, VLF

returned to baseline and HRV reciprocated through a significant decrease in LF and TP, suggesting lowering of SNS activity at this time point.

Nagai et al. (2005) also reported an increase of the VLF band after a high-fat meal (20% of energy as carbohydrate, 70% fat and 10% as protein) in healthy men [59]. The VLF component is less well understood and has been associated with the activity of the neurohumoral regulation and sympathovagal balance [17,60]. VLF has been shown to be dependent on the sympathetic regulation as well as on various neurohormonal mechanisms while increase in this component has been reported in response to stressors (e.g., physical activity, emotional stress) [61]. At the same time, it might reflect the increased parasympathetic activity following a caloric overload. Taylor et al. (1998) examined the effect of a parasympathetic blockade induced by atropine on HRV and found a 92% reduction in the VLF band in healthy participants [62]. Feeding increases the parasympathetic neural activity to the pancreas and stimulates insulin secretion [63,64]. For example, insulin levels significantly rise 30 minutes after ingestion of a carbohydrate-rich meal and return to baseline within two hours in healthy subjects [12,65], as did VLF in our study.

In addition to being linked to neurohormonal regulation, VLF is also influenced by the central regulatory mechanism and mental strain [60,66]. Usui et al. (2017) observed that immediately after a mental stress task (Stroop color and word test), the VLF and HF bands decreased, and the LF/HF ratio increased in healthy men [60]. Increased alertness due to psychological stress and activation of the prefrontal cortex may explain the observed decrease in VLF. In the present study most of the participants reported postprandial somnolence shortly after the caloric load, popularly known as the 'after-dinner dip' [67–69]. This state of a non-pathological lassitude following a meal was accompanied by VLF upregulation possibly reflecting inhibition of the prefrontal cortex, resulting in upregulation of the autonomic regulation in the postprandial state [70]. However, further research is needed to investigate this and other physiological processes underlying VLF activation.

In general, the observed dynamic oscillation of HRV may be explained by a well-functioning response of the ANS and various homeostatic mechanisms to the caloric challenge, which is able to compensate for the imposed metabolic stress and indicates a healthy phenotypic flexibility.

6.4.2 Sulforaphane induced an inflammatory state which correlated with HRV

Sulforaphane facilitated the development of a pro-inflammatory status after caloric challenge as evidenced by an increase in hs-CRP levels. This metabolic change was accompanied by a vagal withdrawal and sympathetic dominance, as showed by reduction of HFnorm and several time-domain parameters with simultaneous increase in LFnorm and LF/HF ratio (Table 2, Figure 4). Moreover, the increase in hs-CRP was correlated negatively with HFnorm and positively with LFnorm values. Interestingly, after the administration of sulforaphane alone a negative correlation between hs-CRP and HFnorm and a positive correlation between hs-CRP and LFnorm and LF/HF was observed, indicating a pro-inflammatory effect of sulforaphane even in absence of caloric challenge. This effect may have led to an increase in CRP levels that was not observed until after the challenge, as de novo synthesis of C-reactive protein occurs within hours upon stimulation [71]. Overall, our results support the notion that sympathetic activation and reduction of vagal regulation are associated with an increase in the pro-inflammatory metabolic response and HRV can be regarded as a useful tool to monitor inflammation.

6.4.3 Hormesis could explain the pro-inflammatory effect of sulforaphane

We believe that the observed pro-inflammatory effect of sulforaphane is part of the hormetic response induced by this nutrient. Sulforaphane is known to induce the expression of antioxidant enzymes by triggering a pro-oxidant response via activation of the nuclear factor E2-related factor 2 (Nrf2)-pathway [40,41,49–53]. Likewise, exercise is known to increase levels of oxidative stress in order to improve resilience towards oxidative damage - a phenomenon known as hormesis [72–83]. For example, an increase in pro-inflammatory biomarkers was seen in young men immediately after strenuous exercise, which was later offset by cytokine inhibitors and anti-inflammatory biomarkers [84,85]. Sulforaphane might also trigger a pro-inflammatory response preceding its anti-inflammatory effect. In fact, the sustained anti-inflammatory effects of sulforaphane has been shown in previous research [86,87]. In the current study, longer time of observation (6, 8, 12, 24h) could have helped to demonstrate that increase in inflammatory activity caused by sulforaphane administration represents the initial part of the hormetic response. Compared to other phytochemicals, like curcumin, silymarin, alpha-lipoic acid, and resveratrol, sulforaphane is the most potent activator of Nrf2, which is known to be associated with hormetic responses [40,41,78,88–94]. The strong modulatory effect of sulforaphane on inflammatory status, along with its beneficial pharmacokinetic profile and high bioavailability, contribute to the therapeutic potential of this phytonutrient in the prevention and treatment of diseases associated with CLGI [27,28,37–46,29–36]. The organization of follow-up studies of longer duration including the testing of biomarkers of Nrf-2 pathway activation could shed more light on the mechanism of action of sulforaphane

in humans and would help to further characterize its long-term effects in people with, or those at risk of developing, CLGI [72].

6.4.4 Limitations and future directions

The present study is the first to combine one-time dietary intervention and a caloric challenge with continuous monitoring of multiple biomarkers over time (phasic response and their relationship with the integrative biomarker HRV). The innovation of this work lies in the use of HRV as a sensitive non-invasive method to monitor metabolic effects of nutritional interventions and caloric overload. An intriguing finding is that in a healthy young population, administration of sulforaphane potentiated the inflammatory response to caloric load, which may represent a phase of the hormetic response. A longer observation period would have been required to show that the expected anti-inflammatory effect would follow. Another limitation of the current study is that IL-6 concentrations remained undetectable in study participants even after the PhenFlex challenge, which may be attributed to the fact that younger and more fit subjects have been enrolled [11,12,15].

Nonetheless, this study increased our understanding of the inflammatory and metabolic effects of sulforaphane and showed correlations with non-invasive HRV measurements even in a short period of time. Although the biological significance of some HRV parameters is still not fully understood, this non-invasive tool is increasingly utilized to monitor the coping capacity of lifestyle interventions and daily challenges. Modern biotechnological methods (e.g. wearables) further promote gathering insight on HRV as an important indicator of health [95–98]. At the same time, standardization of the approaches to study design and data reporting in studies involving HRV is needed.

6.5 CONCLUSION

This study has shown that the correlations between changes in spectral parameters (LFnorm and HFnorm) and hs-CRP in blood further prove that HRV is a sensitive tool for investigating inflammatory response and disorders associated with CLGI. The standardized PhenFlex challenge, which mimics a daily heavy meal, induced significant changes in the regulation of the autonomic nervous system in healthy young individuals. The caloric overload mainly affected SNS activity, observed by the increase in VLF and LF during the first 30 minutes of the postprandial phase, followed by the reciprocation of these parameters in the subsequent 90 minutes. Administration of sulforaphane showed a pro-inflammatory effect over the 3.5 h period, which suggests the onset of a hormetic response. The increased inflammatory activity was accompanied by vagal withdrawal and sympathetic dominance. Finally, the marked differences in HRV dynamics between groups during caloric overload demonstrate that HRV has promise as a non-invasive tool to

investigate the effects of phytochemicals and their role in the prevention of inflammation over a short period of time.

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General discussion

7.1 MOTIVATION OF THIS THESIS

With the emerging global rise in non-communicable diseases (NCDs), the number one cause of death worldwide, more research into the prevention of these conditions is warranted [1]. The involvement of an unresolved chronic inflammatory response has been attracting much interest recently because this distortion of healthy homeostasis seems to occur before most NCDs manifest themselves, making it a potential target for preventive strategies [2–12]. Since chronic low-grade inflammation (CLGI) is suggested to affect apparently healthy people as a result of unhealthy lifestyles, healthy lifestyle modifications logically may be more effective than medication in the fight against this 'silent killer' [4,10–12]. Nutrition may play a role in predisposition to conditions with an inflammatory component and dietary changes may be helpful in preventing or treating such conditions [2,5,13–19]. Nevertheless, it remains difficult to demonstrate the health-promoting effects of nutrition in the general population using classical methodologies adopted from pharmaceutical research [16,19–31]. Whilst pharmacology is still dominated by the "one disease - one target - one drug" paradigm, nutritional interventions frequently exert subtle effects on many pathways involved in the development of chronic diseases [14–19,23]. All things considered, it is challenging to measure beneficial effects in healthy people, especially when trying to establish the effect of one nutrient on one target. Therefore, novel methodologies and integrative approaches are needed to demonstrate the health-promoting effects of nutrition and potentially support scientifically substantiated health claims to communicate these effects to the general population.

7.2 MAIN FINDINGS

7.2.1 Phytonutrients can influence chronic low-grade inflammation through multiple mechanisms of action

Observational data show that consuming fruit and vegetables can make a positive contribution to overall health [26,32–41]. In addition to the differences in macronutrient composition, fruits and vegetables are distinguished from other food products (e.g. animal-based produce) by their high antioxidant content [42]. In **Chapter 2** the influence of lycopene, a well-studied antioxidant, on inflammation and the effect of consuming tomato products and/or lycopene supplements on markers of inflammation is described. The review unveiled that lycopene levels were adversely affected during inflammation and homeostatic imbalance. To illustrate, lower circulating lycopene concentrations and higher inflammatory biomarker levels were found in several studies included in the systematic review, such as in patients with colorectal adenocarcinoma, carotid artery disease, stable angina pectoris, ischemic stroke, chronic hepatitis C, benign prostatic

hyperplasia, and various cancers compared to healthy controls. Although intervention studies showed that supplementation with lycopene or an increased intake of tomato products led to an increase in circulating lycopene, little evidence was found that the increase in lycopene also led to inflammation relief. The results of the cross-sectional studies in the systematic review were consistent with the findings of previous systematic reviews assessing the relationship between lycopene intake and/or levels and vascular risk, metabolic syndrome, and prostate and bladder cancer [43–46]. Lower intakes or levels of lycopene were associated with a higher risk of developing these diseases [43–46]. However, suggestions in these reviews about increasing circulating lycopene through supplementation or increased tomato intake to reduce the risk of these diseases differed from our findings. In fact, our results were consistent with studies where the antioxidant paradox was observed [47–64]. Halliwell is the originator of this theory, which entails the involvement of oxygen radicals and other reactive oxygen species in various human diseases, but giving large doses of antioxidant supplements to human subjects has, in most studies, demonstrated little or no preventive or therapeutic effect [47,48]. The results of the Alpha-Tocopherol, Beta Carotene Cancer Prevention Study Group are a notorious example of detrimental effects seen with antioxidant supplementation [59]. In this clinical study, which involved nearly 30,000 Finnish male smokers, the research group found a higher incidence of lung cancer in the men who received beta-carotene than in those who did not [59]. Halliwell describes that manipulating our own defense systems, resulting in enhanced protection against subsequent stressors, may be a more useful approach for the prevention of NCDs [48].

One way in which bioactive nutrients can manipulate our body's defenses is through activation of the innate immune system, our first line of defense during infection [65]. The innate immune system plays a critical role in the early recognition and subsequent activation of a pro-inflammatory response to invading pathogens [65]. Similar to antigens, nonspecific immunostimulants (NSIs) can stimulate the innate immune system by inducing activation or increasing activity of any of its components. Well-known clinical examples of NSIs are the bacillus Calmette-Guérin (BCG) vaccine and mifamurtide [66,67]. The beneficial health effects of NSIs are suggested to be exerted through the induction of the innate immunity reprogramming expressed as the long-term sustained changes in the nonspecific resistance against infections [68]. In addition to the clinical use of NSIs for specific disease conditions, NSIs can also be ingested through our diet. With more than 20,000 published studies, fungal beta-glucans are the most studied bioactive nutrients with potential immunomodulatory properties [69,70]. In **Chapter 3** the potential application of fungal beta-glucans in nutrition and medicine is described, reviewing their formulation, efficacy, safety profile, and immunomodulating effects. The review showed that fungal beta-glucans could play a role in supporting and maintaining a healthy immune system. As an illustration, beta-glucan supplementation reduced the number of symptomatic

common cold infections in healthy participants [71,72] and respiratory tract infections in children [73–76] and athletes [77,78] by activating the innate immune system. While interesting, the design of these studies had some shortcomings when considering their use to support a health claim about immune function. In addition to these developments related to food uses of fungal beta-glucans, the new insights and developments in trained immunity discussed in the review may lead to the possible pharmaceutical application of fungal beta-glucans as NSIs in Western medicine. Although the reviewed studies demonstrated a clear protective effect of beta-glucans against subsequent pathogens, the potential molecular mechanisms underlying the induction of innate immune memory by beta-glucans is a complex interaction between immunological, metabolic, and epigenetic changes through many to hitherto unknown routes [79].

Recent findings suggest that another way many phytonutrients provide health benefits may be through cross-modal hormetic mechanisms [80]. In this process, a phytonutrient (hormetin) activates one or more adaptive cellular stress response pathways [80]. In **Chapter 4**, the hormetin sulforaphane was shown to enable the development of a mild pro-inflammatory state during a caloric challenge in healthy participants. We hypothesize that this state is temporary and may be due to the initial pro-oxidative action (to activate Nrf2) of hormetins present in fresh produce. We speculate that because sulforaphane also enhances our endogenous antioxidant defenses via Nrf2 activation at a later stage, whole foods that contain direct antioxidants and phytonutrients that stimulate endogenous antioxidant systems may have more anti-inflammatory effects on phenotypic flexibility than foods that do not.

Taken together, these results show that phytonutrients can influence CLGI through multiple mechanisms of action. While depletion of circulating lycopene and possibly other exogenous antioxidants may be one of the first signs of CLGI, there is little evidence that increasing circulating lycopene attenuates CLGI. Manipulation of cellular stress response pathways, for example via NSIs or Nrf2 activators, might be a more effective approach to increase resilience and prevent CLGI. The protective effects of fruits and vegetables on inflammation can be attributed to the wide variety of bioactive substances in the food matrices and the synergy between the different mechanisms of action of these phytochemicals in the body.

7.2.2 Integrative and novel outcome measures represent an effective approach to study the subtle and pleiotropic effects of phytonutrients on inflammation

Human epidemiological and interventional outcome measures have so far relied heavily on the measurement of circulatory inflammation markers, and in particular fasting cytokines in blood, which are recognized as an insensitive and highly variable

index of tissue inflammation [2]. There is ongoing debate whether static, single-point measurements of biomarkers are truly informative about health status, reasoning from the concept that health is defined by the ability to adapt adequately to everyday stressors [81]. New approaches to investigate the effects of diet on our body's adaptability, also referred to as phenotypic flexibility, use challenge tests and examine integrated outcome measures during these challenges for a kinetic quantification of the effect [2,5,82–94]. Such approaches are likely to provide a more effective way to quantify the ability of diet to positively modulate inflammation [2,5]. The PRO SANI study (**Chapters 4-6**) investigated markers of phenotypic flexibility in healthy participants challenged with a standardized caloric load and examined the effect of broccoli sprouts against this stressor. In **Chapter 4** it was shown that the use of integrative outcomes measures such as the systemic low-grade inflammation score (SIS), first coined by Norde et al. (2020) [95], could be viewed as an efficient approach to study the subtle and pleiotropic effects of compounds that affect inflammation. In this study, sulforaphane facilitated the development of a mild pro-inflammatory state after the caloric challenge, as evidenced by an increase in sICAM-1, sVCAM-1, hs-CRP, CCL-2 and decrease in IL-10. Intriguingly, the score more accurately reflected the pro-inflammatory effect of broccoli sprouts than the individual biomarkers. In addition, the relationships between risk factors for the development of NCDs such as high visceral fat and smoking, and inflammation became more apparent through the use of the score. These results seem to confirm that integrated outcome measures be more suitable as markers of inflammation. For example, the effects of dietary intervention on inflammatory biomarkers in other studies that used the standardized caloric load (PhenFlex challenge) were inconsistent, i.e. some biomarkers were positively influenced, and others negatively affected [88,96]. In addition, Weseler et al. (2011) integrated multiple biomarkers into a vascular health index, which unveiled an improvement in overall vascular health from flavanols, which was less clear from the analysis of individual outcomes [19,97]. These results highlight the importance of implementing integrative outcome measures to unravel the subtle, pleiotropic effects of nutrition.

Besides the integration of well-known biomarkers into a health index, novel biomarkers that are sensitive and easy-to-use are essential to further elucidate the health effects of nutrition in the general population. In **Chapter 5,** it was shown that urinary 11-dehydro-thromboxane B_2 (TXB_2) is such an innovative, non-invasive, and suitable biomarker to investigate the effects of phytonutrients on platelet responsiveness in healthy participants within hours. The study showed that a single administration of broccoli sprouts reduced 11-dehydro-TXB_2 levels by clinically relevant amounts in healthy participants challenged with a standardized caloric load. Increased production of thromboxanes contributes to vasculopathy by adversely affecting endothelial function and promoting vascular inflammation [98]. For that reason, targeting thromboxane production via antiplatelet agents has become a cornerstone of cardiovascular disease treatment [99,100].

Consistent with our results, Medina et al. (2015) showed that higher doses (30 g and 60 g) of broccoli sprouts significantly reduced urinary 11-dehydro-TXB_2 levels by 91% and 94%, respectively, within 12 hours in healthy subjects [101]. 11-dehydro-TXB_2 is produced from the breakdown of thromboxane A and can be used to monitor the response to aspirin (ASA) therapy [102,103], but it has hardly been used in nutritional intervention studies. Davì et el. (1999) demonstrated that vitamin E supplementation (600 mg/d) significantly reduced 11-dehydro-TXB_2 levels in diabetic patients after two weeks [104]. Moreover, O'Kennedy et al. (2017) showed that water-soluble tomato concentrate (WSTC) significantly reduced 11-dehydro-TXB_2 concentrations after a single dose [105,106]. Unlike most pharmaceuticals, the antiplatelet effect of these phytochemicals is reversible, making them potentially safe for use in a variety of clinical scenarios, including primary prevention of cardiovascular diseases in the general population [99,105,107].

One key point for discussion is our finding that sulforaphane induced a mild pro-inflammatory effect seen in blood biomarkers which was accompanied by vagal withdrawal and sympathetic dominance while reducing urinary 11-dehydro-TXB_2 levels by clinically relevant amounts in healthy participants (**Chapters 4-6**). Prostaglandin H_2 (PGH_2) is produced by both COX isoforms and is the common substrate for a series of specific synthase enzymes that produce prostaglandins and thromboxanes [108,109]. The five principal bioactive prostaglandins and thromboxanes generated *in vivo* are: prostaglandin E_2 (PGE_2), prostacyclin (PGI_2), prostaglandin D_2 (PGD_2), prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) and thromboxane A_2 (TXA_2) [108,109]. It is the differential expression of these specific synthase enzymes within cells present at sites of inflammation that will determine the profile of prostanoids production [108]. To illustrate, macrophages mainly produce PGE_2 and TXA_2, while mast cells predominantly generate PGD_2 [108]. Interestingly, while resting macrophages produce TXA_2 in excess of PGE_2, this ratio changes to favor PGE_2 production after LPS activation [108]. These *in vitro* results could explain the reduction of 11-dehydro-TXB_2 by the mild pro-inflammatory effect of sulforaphane and could even be confirmed as tetranor-prostaglandin E metabolite (tetranor-PGEM) levels, the main urinary metabolite of PGE_2, were increased. Nevertheless, this seems unlikely given that, in addition to a decrease in 11-dehydro-TXB_2, Medina et al. (2015) also observed a decrease in tetranor-PGEM and 11b-PGF_{2a} after intake of broccoli sprouts, while ten other eicosanoids remained unchanged [101]. In addition, Vazzana et al. (2013) showed that both exercise and fenofibrate, a lipid-lowering drug with antithrombotic properties, significantly reduced urinary 11-dehydro-TXB_2 and 8-iso-PGF_{2a} in healthy subjects, in parallel with an HDL increase [110]. These apparently contradictory results observed between bodily fluids demonstrate the complex pleiotropic effects that substances can exert in our bodies and emphasize the importance of implementing integrated outcome measures.

7

Sulforaphane has generated considerable interest due to its ability to target multiple pathways, e.g., Nrf2 and NF-κB, among others [101,111–113]. NF-κB regulates multiple aspects of innate and adaptive immune functions and serves as a crucial mediator of inflammatory responses [114]. NF-κB activity is induced by a range of stimuli including intercellular inflammatory cytokines, enzymes, and pathogen-derived substances [115–119]. Nrf2 appears to participate in a complex regulatory network and plays a pleiotropic role in the regulation of metabolism, inflammation, autophagy, mitochondrial physiology, and immune responses [120]. We hypothesize that sulforaphane's impact on multiple pathways during caloric overload might not result in simultaneous and equal effects on all pathways affected and may therefore explain the decrease in 11-dehydro-TXB_2 observed in healthy participants. As a result, 11-dehydro-TXB_2 may have been reduced through multiple pathways, while the circulating inflammatory biomarkers were still in a mild pro-inflammatory state following initial Nrf2 activation (Figure 1 and 2). Moreover, Navarro et al. (2014) observed variable effects on inflammatory biomarkers following cruciferous vegetable interventions in healthy young adults and speculated that overlap with other (anti-inflammatory) pathways that may be differentially regulated and the inflammatory state of their participants may have obscured an effect of their interventions [121]. However, further research is needed to determine whether these multi-pathway dissimilarities could be explained by the pleotropic effects of sulforaphane and the inflammatory status of participants.

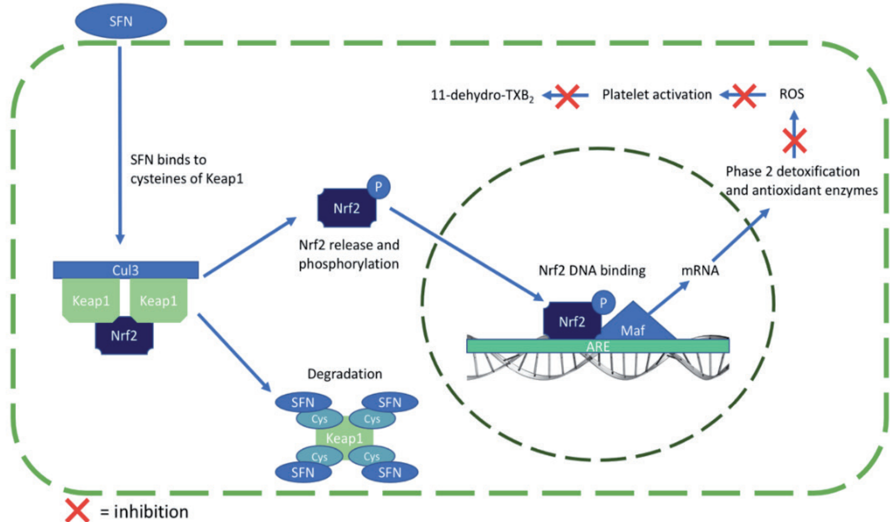

Figure 1: *Mechanism of action of sulforaphane on the Nrf2-pathway.*

Sulforaphane binds to Keap1, resulting in the degradation of Keap1 and the release of Nrf2. Nrf2 gets phosphorylated and translocates to the nucleus for transcription of phase 2 detoxification and antioxidant enzymes. These enzymes decrease ROS and in turn potentially platelet activation and 11-dehydro-TXB_2. SFN: sulforaphane, Cul3: cullin 3-based ubiquitin E3 ligase complex, Keap1: Kelch-like ECH-associated protein 1, Nrf2: nuclear factor erythroid 2, Cys: cysteine residues, P: phosphate group, ARE: antioxidant response element, Maf: Maf protein, ROS: reactive oxygen species.

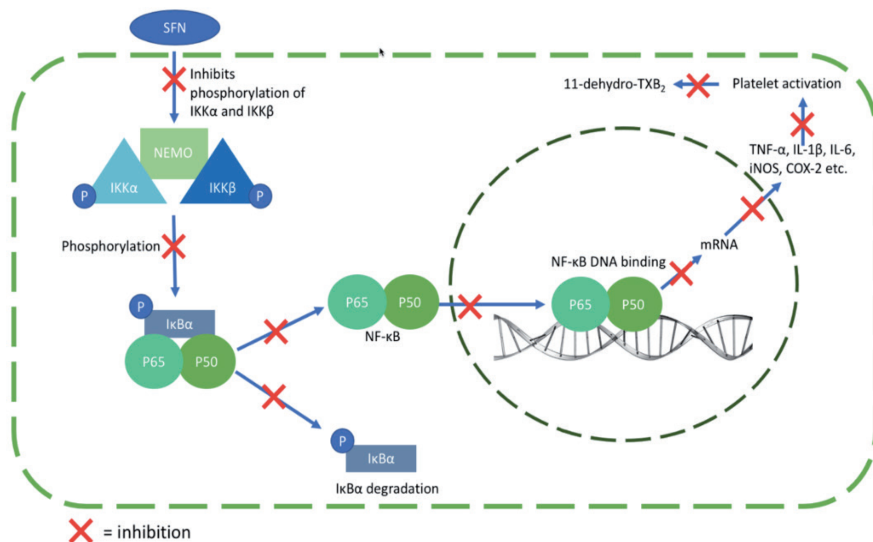

***Figure 2**: Mechanism of action of sulforaphane on the NF-κB-pathway.*

Sulforaphane inhibits phosphorylation of the IKK complex, subsequently reducing activation and translocation to the nucleus of NF- κB. This results in less transcription of pro-inflammatory mediators and enzymes and potentially less platelet activation and 11-dehydro-TXB_2. SFN: sulforaphane, NEMO: NF-κB essential modulator, IKK: I-κB kinase, P: phosphate group, NF-κB: nuclear factor kappa-B.

In addition, the inflammatory status has been associated with autonomic activity, e.g. the influence of the cholinergic anti-inflammatory pathway (CAP) in response to injury, pathogens, and tissue ischemia [122]. However, heart rate variability (HRV) monitoring for the assessment of inflammation in humans has rarely been used. In **Chapter 6**, correlations between changes in HRV parameters and circulatory biomarker levels of hs-CRP demonstrated that HRV is a sensitive apparatus for investigating the inflammatory response during challenge testing. In addition, administration of broccoli sprouts containing sulforaphane prior to the caloric challenge caused a pro-inflammatory effect which was accompanied by vagal withdrawal and sympathetic dominance. The pea sprouts without sulforaphane in combination with the standardized caloric load induced other changes in autonomic nervous system regulation in healthy young individuals. The caloric overload mainly affected sympathetic nervous system activity during the first 30 minutes of the postprandial phase, followed by the reciprocation of this activity in the subsequent 90 minutes. The dissimilarities in HRV dynamics show that HRV is promising to non-invasively investigate the physiological effects of macronutrients and phytonutrients and their role in the prevention of inflammation over a short period of time. Insight into the relationship between nutrients (carbohydrates, fats, proteins, phytonutrients) and interactive physiological parameters (HRV) can lead to more personalized nutrition through

innovative biotechnological methods in the future. Modern biotechnological methods (e.g. wearables) further promote gathering insight on HRV as an important indicator of health [123–126]. Wearable healthcare technology refers to non-invasive health monitoring devices worn on the body. These devices use biosensors to collect different data from the wearer, such as heart rate, blood pressure, sleep patterns, and activity [127]. While wearable and mobile chemical sensors have experienced tremendous growth over the past decade, their potential for tracking and guiding nutrition has emerged only over the past five years [128]. In addition, the use of wearables has yet to be validated against ECG-derived HRV, the gold standard assessment [125,129]. During non-stationary conditions, the comparison between ECG-derived HRV and wearables showed weak correlations, which may also prevent the use of wearables from providing an accurate picture of the effect on autonomic activity during challenge testing [125]. Currently, guidelines from physicians and dietitians represent the most common approach to maintaining optimal nutritional status. However, such recommendations are based on population averages and do not account for individual variability in responding to nutrients. Precision nutrition has recently emerged to address the wide heterogeneity in individuals' responses to nutrition, tailoring nutrition to each person's specific needs. It aims to prevent and manage disease by formulating personalized dietary interventions for individuals based on their metabolic profile, background, and environmental exposure. Non-invasive wearable and mobile electrochemical sensors, capable of monitoring temporal chemical variations upon the intake of food and supplements, are prime candidates to bridge the gap between digital and biochemical analyses for a successful personalized nutrition approach [128]. As a result, physiological effects (such as HRV) and nutrient status can be related (with possible causal relationships) in a non-invasive way in the future.

In conclusion, these studies demonstrate that integrative and novel outcome measures can be a more efficient approach to study the effects of phytonutrients on a personal level compared to classical methodologies adopted from pharmaceutical research. For future nutritional research, these innovative measurements could be widely used in the general population without a clinical setting and/or using modern biotechnological methods. Subsequently, the evidence provided can be reviewed by regulatory authorities for potential substantiation of health claims.

7.2.3 As nutritional science is evolving, so should scientific assessment strategies

In Europe, the use of voluntary information stating, suggesting, or implying a certain nutritional content (nutrition claim) or health benefit (health claim) of a food is regulated under the Nutrition and Health Claim Regulation (NHCR), Regulation (EC) No 1924/2006. Before health claims can be used on foods, they need to be authorized by the European Commission, based on the scientific substantiation of this health benefit of a food [130].

Food business operators interested in making claims therefore need to submit a scientific dossier along with their request for authorizing a newly proposed claim. This scientific dossier needs to contain all relevant information about the product upon which a claim is proposed, and the evidence showing a health benefit of consuming such a product. The European Commission subsequently asks the European Food Safety Authority (EFSA) to evaluate the evidence on the proposed claims [131]. This evaluation involves a critical review of three main criteria: (1) the bioactive substance is sufficiently characterized, (2) the proposed claim is well characterized and should comprise a beneficial physiological effect, and (3) the cause-and-effect relationship between the bioactive substance and the beneficial physiological effect should be established [130,132]. So far, no health claims have been authorized for fruits or vegetables, whilst sufficient consumption of such products has been associated with improved health outcomes and prevention of disease.

In **Chapter 5**, broccoli sprouts containing sulforaphane were shown to be effective in targeting platelet responsiveness after a single intake. In addition, the correlations between sulforaphane and 11-dehydro-TXB_2 in urine suggest a cause-and-effect relationship. Other health products have also been shown to positively affect platelet functionality, with WSTC seen as the first functional food that contributes to healthy blood flow by maintaining normal platelet aggregation, a claim considered scientifically substantiated by EFSA's NDA Panel [133]. According to the Panel, a reduction in platelet aggregation in subjects with platelet activation during sustained exposure to the food/constituent (at least 4 weeks) is a beneficial physiological effect. Other outcome variables, such as thromboxane A_2 (TXA_2) or plasma soluble P-selectin, are not considered established markers of platelet aggregation but can be used as supporting evidence for the scientific substantiation of these claims [134,135]. When follow-up studies on the effects of sulforaphane using light transmission aggregometry yield positive results, fresh broccoli sprouts or other products containing sufficient sulforaphane per serving, could apply for a claim similar to the authorized claim for WSTC.

In **Chapter 3**, applications for health claims related to support of the immune system, stimulation of the immune system, and defense against pathogenic microorganisms were further elucidated. All applications for purported health claims related to immune system stimulation and defense against pathogenic microorganisms have so far been rejected by the NDA panel. As put forward in EFSA's guidance documents, the infectious nature of the disease should be established, e.g., by clinical differential diagnosis in itself or combining this with microbiological data and/or the use of validated questionnaires, depending on the study context and type of infection [136]. Determining the infectious nature of the disease has been the major limitation for fungal beta-glucan applications in defense against common colds and upper respiratory tract infections (URTI). In practice, however, it is unusual to perform routine laboratory tests for the diagnosis of these diseases, as

they can be caused by many different agents (adenovirus, coronavirus, influenza virus, rhinovirus, etc.) [137,138]. As EFSA's NDA panelists tend to follow the scientific consensus, removing this criterion from the assessment could potentially stimulate innovation in immune stimulation in the EU food sector.

In addition to potential simplifications that can be made in the assessment of claims related to the immune system, the assessment of claims regarding the reduction of inflammation by consuming food ingredients can also be further improved. To date, no claims referring to the reduction of inflammation have been approved for use in the EU. Only one claim related to a healthy inflammatory response has been approved based on 'generally accepted scientific evidence', falling into the category of Article 13.1 health claims. As described in EFSA's scientific opinion on vitamin D, a cause-and-effect relationship has been established between the dietary intake of vitamin D and contribution of normal function of the immune system and healthy inflammatory response. The scientific evidence that has led to the approval of this claim consists of three review studies [139–142]. However, close examination of the individual studies shows that these effects have only been demonstrated *in vitro*, animal models or conflicting observational studies at most [139–142]. Furthermore, the studies are not unanimous about the effects that vitamin D may have in humans [139–142]. It seems that in this evaluation, the NDA Panel has been rather lenient regarding the assessment of the three main criteria [130,132]. The opposite is however true for the scientific dossier supporting non-authorized claims on the anti-inflammatory effects of whole grains [143]. Hoevenaars et al. (2020) described that, although the effects of whole grains on inflammation have been studied in RCTs [144–146], no health claims have been granted for the anti-inflammatory effects of whole grains [143]. In general, the main reasons for the non-authorization of claims on the reduction of inflammation occurred in the second and third criteria of the assessment. In other words, either the beneficial physiological effect was missing or insufficiently defined, or a causal relationship between the bioactive substance and a beneficial physiological effect was not demonstrated. In order to provide stakeholders with greater clarity on which health effects related to inflammation could be studied to support health claims, in 2016, a guidance was published by EFSA's NDA Panel that provides more detailed guidelines for the evaluation of health claims regarding inflammation [136]. This guidance states the following (p. 8):

> "*In this context, outcome variable(s) which can be measured in vivo in humans by generally accepted methods but do not refer to a benefit on specific functions of the body cannot constitute the only basis for the scientific substantiation of a health claim. These include changes in immune markers, changes in markers of inflammation. Changes in these outcome variable(s) should be accompanied by evidence of a beneficial physiological effect or clinical outcome in the application*".

This clarification, however, does not mention any example of a physiological effect that can be beneficially influenced. In addition, the Panel states that measuring markers of inflammation does not suffice for the constitution of a health claim [136]. In conclusion, novel methods that provide quantitative interpretation for effects on these markers are needed [17,143]. In **Chapter 6**, it was shown that HRV is a promising tool to investigate the physiological effects of phytonutrients and their role in the prevention of inflammation over a short period of time. Evidence from clinical studies showed that reduction of HRV associated with an increase of the LF component is linked to increased inflammatory activity [147]. At the same time, vagal stimulation was proposed to be used in treatment of CLGI-associated disease due to its ability to trigger an anti-inflammatory response [148]. These findings demonstrate the functionality of the cholinergic anti-inflammatory pathway [122,149] and also provide grounds for assessing inflammation through HRV, as it may provide insights in the physiological effects associated with inflammation.

Finally, in general it is challenging to demonstrate a beneficial health effect in nutritional intervention studies, since nutritional effects are often subtle and long term [90,150]. Health is no longer seen as simply a fixed entity of complete physical, mental, and social well-being, but redefined as our body's ability to cope with everyday challenges [14,16,17,19,90,150]. The concept of this phenotypic flexibility or resilience implies that health can be measured by the ability to maintain homeostasis through a highly energy-dependent, rapid, and orchestrated adaptation to continuous environmental changes and challenges [2,81,90,151,152]. Although this progressive shift in thinking about health is increasingly recognized, this shift has not yet been reflected in the methods of scientific substantiation of health effects, e.g. in the EU under the Nutrition and Health Claim Regulation (NHCR), Regulation (EC) No 1924/2006. Stroeve et al. (2015) identified a composition for a nutrition stress test that assesses phenotypic flexibility by eliciting responses in all processes considered relevant, based on detailed comparison of different challenge tests [150]. A key factor is determining the physiological relevance of specific changes in phenotypic flexibility in a population, in response to a nutritional intervention. Ideally, this is pursued through validation against established health characteristics, but these have not been established [150]. In addition, differences in phenotypic flexibility between individuals (influenced by gender, age, nutritional status, food intake, stress, physical activity, and genetic background, among others) will cause people to respond differently to acute and chronic stressors and develop a personalized trajectory of metabolic-inflammatory health and disease [90]. For comparison of nutritional interventions on health, a crossover design is therefore perhaps for challenge tests most suitable. By combining this into an integrated readout (as measured by a composite scoring system consisting of a range of biomarkers), a phenotypic flexibility marker can be obtained that has a broader value, both for substantiating the effects of food on health, but also for application in personalized nutrition [90,150].

In summary, in some areas the guidelines for health claims are clearly described and 'gold standard' methodologies can be used to demonstrate the effects of nutrition, e.g. the percentage of inhibition of platelet aggregation using light transmission aggregometry. In other areas, considering and adopting new methodologies that can be used to support claims submission would stimulate research into innovative foods and personalized nutrition. The current methods advised and used in scientific dossiers to support such claims are still quite pharmaceutical, in other words: they focus on one disease - one target - one substance, while nutrition exerts subtle and pleiotropic effects on phenotypic flexibility.

7.3 METHODOLOGICAL CONSIDERATIONS

Different methods have been used in the research described in this thesis. First, the systematic review described in **Chapter 2** aimed to elucidate the relationship between circulating lycopene and inflammation. The strength of this systematic approach is that it allows to provide a summary of all available evidence in an attempt to answer research questions fully and reproducibly. Another strength of this study is that in this review, a clear distinction is made between effects reported in observational studies versus intervention studies. However, it is important to acknowledge that there is a risk for publication bias in the intervention studies. Secondly, **Chapter 3** describes a narrative review on the relationship between beta-glucans from fungi and immune-related health effects. This approach was chosen because the ability to consider different types of research and topics allows generating a broader and more inclusive picture of the available research. Nevertheless, this approach did not allow direct comparison between findings reported in the studies.

Results from the PRO SANI study, a cross-over, double-blind, randomized controlled intervention study with healthy subjects, were described in **Chapters 4-6**. As described above, in the PRO SANI study we aimed to investigate the efficacy of broccoli sprouts – as a source of sulforaphane – on biomarkers of platelet activation and inflammation, and other markers of phenotypic flexibility in healthy participants who were subjected to a standardized caloric load. In this approach, it was important to analyze the effects of the product using a composite scoring system, as this may be a more sensitive way of assessing the effects of fruits and vegetables on the health status of the low-risk healthy population. In **Chapters 4 and 6**, sulforaphane was shown to enable the development of a mild pro-inflammatory state during the caloric challenge. We hypothesized that the pro-inflammatory effect may be due to the initial pro-oxidative action of sulforaphane (to activate Nrf2). In the PRO SANI study, however, we were unable to demonstrate this because blood was only drawn at two time points. In the design of this clinical study, a

careful choice was made to draw blood from participants a maximum of two times, so that venipuncture could be used instead of cannulation. Chabot et al. (2018) showed that when blood was collected by venous punctures, IL-6 concentrations remained unchanged, whereas the concentrations increased progressively over time when collected through a cannula in healthy participants [153]. In follow-up studies, a longer observation time (6, 8, 12, 24 hours) could help to demonstrate the hormetic response of sulforaphane, but it should be considered that cannulation might affect the effect of interest. Nonetheless, we do speculate that during longer observation the anti-inflammatory effects of sulforaphane will become evident since such sustained anti-inflammatory effects of sulforaphane have been shown in previous research [154,155]. Moreover, the organization of follow-up studies of longer duration including the testing of biomarkers of multiple pathways could shed more light on the mechanism of action of sulforaphane in humans and would help to further characterize its longer-term effects in people with, or those at risk of developing, CLGI [156].

Lastly, it is challenging to find an impeccable placebo in nutritional intervention studies, especially for fresh produce in a double-blinded setting [157]. In the PRO SANI study, the placebo had to match the broccoli sprouts in terms of nutrient value, except for glucoraphanin content. In **Chapter 4**, it was shown that after administration of the pea sprouts (placebo), the metabolic challenge did not significantly affect plasma adhesion marker levels in healthy participants. In contrast, previous research has shown that a single administration of the same challenge increased the levels of sVCAM-1 and sICAM-1 after two hours in healthy volunteers [89]. A possible explanation for our findings is the fact that the subjects in the current study were given whole food products (sprouts of broccoli or pea) before undergoing the challenge and thus were not fasted when consuming the PhenFlex. Both products contain retinol, vitamin E and ascorbic acid, which may have counteracted the expected transient disruption of post-exposure endothelial homeostasis that was observed in other studies. This is supported by the findings of Nappo et al. (2002), who showed that supplementation with vitamin C and E prevented an increase in sICAM-1 and sVCAM-1 in healthy middle-aged subjects after a high-fat meal [92]. In addition, Rubin et al. (2008) found no changes in plasma adhesion markers in young participants (25 years old on average), after a standardized lipid-rich meal which contained retinol [158]. Thus, in our study, the effect of sulforaphane on endothelial homeostasis may have been influenced by the other nutrients present in the whole food product. Furthermore, in **Chapter 5** the expected increase in urinary 11-dehydro-TXB_2 after administration of placebo (pea sprouts) also failed to materialize. Despite our efforts, the amount of β-carotene and some vitamins in the pea sprouts may have counteracted the expected post-challenge increase in 11-dehydro-TXB_2 [159,160]. So far, whole foods and fresh produce have not shown a clear protective effect against the PhenFlex challenge [88,96]. In fact, dietary interventions high in hormetins facilitated the development of a pro-inflammatory state

during caloric overload, e.g. broccoli sprouts and garlic extract [88]. In future studies it is essential to determine the nutritional composition of whole food interventions and to measure multiple nutrients in bodily fluids. This will help better substantiate the expected response to a metabolic challenge.

7.4 CONCLUSION AND FUTURE PERSPECTIVES

The goal of the experiments performed in this thesis was to further elucidate the role of fruit and vegetables in the prevention of CLGI and subsequently, the development of NCDs. Since CLGI seems to affect apparently healthy people as a result of poor lifestyle choices, healthy lifestyle modifications may be more effective than medication in preventing this 'silent killer' [4,10–12]. A wealth of observational data indicates that diets rich in fruits and vegetables have a particularly positive effect on inflammatory status and on the development of various NCDs [26,32–37]. In addition to these well-known observational data, future methodologies using multifaceted approaches will shed new light on the health effects of fruits and vegetables and even demonstrate cause-and-effect relationships. At the same time, it is the task of scientific risk assessors to keep pace with these developments. For this, it is essential to continuously review guidelines, implement new measurement methods and acknowledge differences between pharmaceuticals and whole foods. For the latter, for example, an exception to criterion 1 (characterization of the bioactive substance) for fresh produce could be adopted by EFSA when assessing health claims dossiers. This may also lead to more approved health claims due to the wide variety of bioactive substances in the food matrices and the synergy or interactions between the different mechanisms of action of these compounds in the body. Finally, this can provide an opportunity for fruit and vegetables to be distinguished from other products and could reduce the temptation to add already approved substances (e.g. vitamins and minerals) to unhealthy foods. If this new approach is implemented and scientific evidence is subsequently provided, policymakers will be strong in defending legislative changes, such as global targets for increasing fruit and vegetable consumption, a VAT exemption for fruit and vegetables, and placing free fruits and vegetables in schools and workplaces.

This thesis presents just one piece of the puzzle to elucidating health effects of nutrition. New discoveries are being made at a rapid pace that may provide solutions to remaining bottlenecks, such as interpersonal differences. Relevant scientific developments to tackle such issues include precision nutrition using -omics approaches [161,162] and nutritional epigenetics [163–165]. Incorporating these advanced scientific methodologies into multifaceted approaches would allow more accurate quantification of the effects of fruits and vegetables in relation to inflammation and metabolic processes and could reveal the protective effects of nutrition on development of NCDs.

In conclusion, the research described in this thesis showed that inflammation related effects of phytonutrients from vegetables can be studied within hours in healthy participants. The use of integrative outcomes measures, as well as non-invasive biomarkers, such as 11-dehydro-TXB_2 and HRV monitoring may offer improved ways of measuring and reporting the subtle and pleiotropic effects of nutrition on inflammation. Considering, validating, and adopting innovative integrative research approaches (e.g. composite scores, wearables, n-of-1 designs) would enhance our understanding of the hormetic principles of bioactive compounds and provide the much-needed evidence to develop research portfolios that will inform new product development and associated health claims.

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8

Impact paragraph

8.1 THE SOCIETAL IMPACT OF PREVENTING NON-COMMUNICABLE DISEASES

Non-communicable diseases (NCDs) are the leading cause of death worldwide, accounting for 71% of total deaths each year [1]. The four major NCDs with the highest number of deaths are cardiovascular diseases (CVD) (17.9 million deaths annually), cancers (9.0 million), respiratory diseases (3.9 million), and type II diabetes (T2DM) (1.6 million) [1]. NCDs threaten progress towards the 2030 Agenda for Sustainable Development, which includes a target of reducing the probability of death from any of the four main NCDs between ages 30 and 70 years by one-third. NCDs place a significant and growing burden on the health care system and the overall global economy [2]. Therefore, primary prevention of NCDs is essential to reduce morbidity, mortality, and disability related to those prevalent illnesses. Therapeutic targeting of chronic inflammatory processes, which were shown to be a significant risk factor for NCDs, has been attracting much interest as a promising preventive strategy [3–13].

Many advances have been made in the prevention of chronic low-grade inflammation (CLGI), including the implementation of dietary interventions. Adopting a healthy diet may support preventing the onset and progress of such conditions [3,6,14–20]. As previously highlighted in this thesis, whilst observational data indicate that diets rich in fruits and vegetables have a particularly positive effect on the inflammatory status and prevent the development of various NCDs [21–31], causal relationships have so far not yet been established [32–34]. This is partially attributed to the use of classical methodologies in nutrition research [17,20,21,32–41]. Nutrition can be expected to exert subtle effects on many pathways involved in the development of chronic diseases, whilst pharmacology is still dominated by the "one disease - one target - one drug" paradigm [15–20,42]. Methodologies in nutrition research however often follow this pharmacological paradigm. Thus, there is an urgent need for more novel approaches to investigate the health-promoting effects of nutrition, in particular for fresh produce.

In the PRO SANI study described in this thesis, we provide evidence that the effects of fresh produce on inflammation can be measured within hours with the highest degree of evidence (a double-blind RCT). We found that the use of integrative outcomes measures, non-invasive biomarkers, and heart rate monitoring can be seen as an efficient new approach to studying the subtle and pleiotropic effects of nutrition on inflammation. Challenging our resilience, e.g. the standardized caloric load used in our RCT, sheds new light on the health effects of fruits and vegetables and even allow for establishing cause-and-effect relationships. These results emphasize the need to study fruits and vegetables as a whole, rather than individual components often found in supplements. This can

promote consumer acceptance of fruit and vegetables and may break the notion that unhealthy diets supplemented with food supplements are healthy.

In addition, these effects were demonstrated in healthy participants, which is essential for substantiating potential health claims on food products. To support understanding of health benefits of foods and potentially functional food innovation, scientific risk assessment has to make use of these new developments. For this, it is fundamental to continuously review guidelines and implement new measurement methods in assessment procedures. Once challenge testing is validated and implemented, and scientific evidence is subsequently provided, future claims on fruit and vegetables are within reach. Subsequently, consumers can be exposed to these claims in the supermarket. This may lead individuals to make healthier choices, which is in line with most global discussions that address the risk factors of self–management and focus on the role of individual responsibility to manage the risk factors of NCDs. At the same time, policymakers can use this empirical substantiation of the health benefits of these products in defending legislative changes, such as VAT exemption on fruit and vegetables. Taken together, the new knowledge generated in this thesis can support information provision to consumers of health benefits and the development of evidence-based health policies. Both can contribute to increasing the consumption of fruits and vegetables, in turn reducing the global rise in NCDs and its health-related and economic consequences.

8.2 SCIENTIFIC AND COMMERCIAL IMPACT – NEXT STEPS TOWARDS INNOVATION

The research field for the prevention and treatment of NCDs shows a paradigm shift from studying each disease separately, towards a more holistic approach to understanding NCDs are multifactorial and caused by complex gene-environment interactions [43]. This proposed holistic strategy encompasses comprehensive patient-centered integrated care and multi-scale, multi-modal and multi-level systems approaches to tackle NCDs as a common group of diseases. In other words, intertwined gene-environment, socioeconomic interactions, and comorbidities leading to individual-specific complex phenotypes will be taken into account [43].

In the PRO SANI trial, the clear associations between heart rate parameters and circulatory inflammation biomarkers demonstrate the involvement of multiple organs and systems in the inflammatory response. The mild pro-inflammatory effect observed in the circulatory system and the beneficial effect on urinary platelet response caused by sulforaphane further demonstrate the complex interaction between integrated networks and mechanisms. For future nutritional research, these innovative measurements could

be widely used in the general population moving testing outside of the clinical setting. The scientific insights of the current work, together with future studies using integrative research approaches, may ultimately provide the much-needed evidence to develop research portfolios that will support the development of new healthy food products and associated health claims.

Already whilst undertaking this research project, different researchers and companies have shown interest in the methodology and findings of the PRO SANI study. In addition, this research project helped to strengthen collaborations between different partners, including academic, private, and public institutions. This is not only beneficial for future research projects but also allows for supporting the swift translation of research findings into real-world innovations. The research in this thesis is partly funded by industrial partners (food companies) that could use the presented findings as leads for future product development and health claim portfolios. Furthermore, the experiments performed in this thesis are relatively fast, accessible, and inexpensive compared to other, more pharmacological approaches. Since food business operators – often SMEs – usually don't have the resources for research and development that pharmaceutical companies do, this accessibility can be a decisive factor in stimulating research into their products.

The approach taken in the PRO SANI study and the results from our work have been and will be actively shared within the academic community and outside of that, with students, organizations active in the fruit and vegetable sector, as well as the general public. So far, this has resulted in the development of new PBL cases and lectures in University and University of Applied Sciences educational programs, coverage on social media and articles, columns and interviews in trade journals and other pressed media. The findings were shared within the scientific community via (inter)national conferences and by publishing all work open access. These activities will further promote the dissemination of information on the health effects of fruits and vegetables to scientists, health care professionals, food business operators and the general population.

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Appendices

NEDERLANDSE SAMENVATTING

Volgens de Wereldgezondheidsorganisatie (WHO) zijn niet-overdraagbare aandoeningen (NCD's) wereldwijd de belangrijkste doodsoorzaak, goed voor 71% van alle sterfgevallen per jaar. Voorbeelden van deze aandoeningen, ook wel welvaartsziekten genoemd, zijn diabetes type 2, hart- en vaatziekten, bepaalde vormen van kanker en chronische luchtwegaandoeningen. Welvaartsziekten, zoals de naam al aangeeft, worden vaak in verband gebracht met een hogere levensstandaard en worden geassocieerd met een modern en sedentair leefstijlpatroon. Aangezien er nog steeds geen ideale behandelingen beschikbaar zijn voor deze aandoeningen, is het van cruciaal belang om meer onderzoek te doen naar manieren om ze te voorkomen. NCD’s ontstaan vaak door langdurige blootstelling aan risicofactoren zoals ongezonde voeding, gebrek aan lichaamsbeweging, roken, overmatig alcoholgebruik en psychosociale stress. De betrokkenheid van aanhoudende chronische ontstekingsreacties heeft recent veel aandacht getrokken, omdat deze verstoring van een gezond evenwicht lijkt plaats te vinden vóór de manifestatie van de meeste ziekten. Dit maakt het een veelbelovend doelwit voor preventiestrategieën. Ongezonde voeding kan een rol spelen bij de ontwikkeling van NCD's en er zijn aanwijzingen dat gezonde voedingsveranderingen effectief kunnen zijn bij het voorkomen van dergelijke aandoeningen, met name bij NCD's waarin ontstekingen een rol spelen.

Een overvloed aan observationele studies wijst erop dat diëten die rijk zijn aan groenten en fruit een bijzonder positief effect hebben op de ontstekingsstatus en zo de ontwikkeling van verschillende NCD's kunnen voorkomen. Niettemin blijft het onduidelijk in hoeverre specifieke versproducten en voedingsstoffen in groenten en fruit (fytonutriënten) verantwoordelijk zijn voor bepaalde gezondheidseffecten. Bovendien blijft het een uitdaging om causale verbanden tussen voeding en een vermindering van NCD's in de algemene bevolking aan te tonen met behulp van traditionele methoden die ontleend zijn aan de farmaceutische sector. Dit komt doordat de farmacologie nog steeds gedomineerd wordt door het paradigma van "één ziekte - één doelwit - één geneesmiddel", terwijl voeding vaak subtiele effecten uitoefent op vele netwerken en mechanismen die betrokken zijn bij de ontwikkeling van chronische ziekten. Dit complexe karakter van voeding maakt het uitdagend om de oorzakelijke verbanden aan te tonen die nodig zijn voor de wetenschappelijke onderbouwing van gezondheidsclaims. Daarom zijn nieuwe benaderingen nodig om de gunstige effecten van voeding op de gezondheid te onderzoeken en indien mogelijk gezondheidsclaims te ondersteunen voor communicatie naar consumenten. Het onderzoek in dit proefschrift is gericht op een diepere verkenning van de rol van groenten en fruit in de ontwikkeling en preventie van NCD's en het onderzoeken van methoden om de gezondheidsbevorderende effecten van fytonutriënten met betrekking tot ontstekingen te meten.

Hoofdstuk 1 geeft een algemene inleiding van het onderwerp en een voorbeschouwing van de onderzoeksvragen. In eerste instantie werd onderzocht of bepaalde gezondheidseffecten kunnen worden toegeschreven aan specifieke fytonutriënten. **Hoofdstuk 2** behandeld de invloed van lycopeen, een veel bestudeerde antioxidant, op ontstekingen, en onderzoekt het effect van het consumeren van tomatenproducten en/of lycopeen supplementen op markers van ontstekingen. Er werden lagere lycopeen concentraties en hogere niveaus van ontstekingsbiomarkers gevonden in het bloed van patiënten met NCD's, bijvoorbeeld stabiele angina pectoris en ischemische beroerte, in vergelijking met gezonde controles. Hoewel interventiestudies aantoonden dat suppletie met lycopeen of een verhoogde inname van tomatenproducten leidde tot een verhoging van circulerend lycopeen, werd weinig bewijs gevonden dat de toename van lycopeen ook leidde tot verlichting van ontstekingen. Niettemin kan uitputting van lycopeen een van de eerste tekenen zijn van chronische ontstekingen. De beschikbare gegevens suggereren daarom dat het gunstig is om af en toe lycopeenrijke voedingsmiddelen te consumeren om het circulerende lycopeen op een basaal niveau te houden.

In **Hoofdstuk 3** werden de effecten van beta-glucanen uit schimmels op ontstekingsreacties en het immuunsysteem geëvalueerd. Bij inname beïnvloeden beta-glucanen op een vergelijkbare manier als antigenen het mucosale immuunsysteem in het maagdarmkanaal, en initiëren zo een ontstekingsreactie. Traditionele Chinese geneeskunde waardeert beta-glucanen echter al eeuwenlang vanwege hun gunstige effecten op het immuunsysteem. De review wees uit dat schimmel beta-glucanen een rol kunnen spelen in het ondersteunen en behouden van een gezond immuunsysteem. Zo verminderde beta-glucaansuppletie het aantal symptomatische verkoudheden bij gezonde deelnemers, evenals het aantal luchtweginfecties bij kinderen en atleten, door het activeren van het aangeboren immuunsysteem. Hoewel de beoordeelde studies een duidelijk beschermend effect van beta-glucanen tegen toekomstige ziekteverwekkers aantoonden, zijn de mogelijke moleculaire mechanismen die ten grondslag liggen aan de inductie van het aangeboren immuungeheugen door beta-glucanen een complexe interactie tussen immunologische, metabolische en epigenetische veranderingen via vele tot nu toe onbekende routes. Door deze onzekerheden zijn er beperkingen bij de onderbouwing van gezondheidsclaims met betrekking tot de werking van het immuunsysteem. Echter, met behulp van moderne immunologische en biotechnologische methoden worden steeds meer inzichten verkregen in immunomodulerende beta-glucanen, met potentiële toepassingen zowel in voedingsmiddelen als farmaceutische producten.

Hoofdstukken 4, 5 en 6 behandelen de resultaten van de PROtective effects of SulforAphaNe on chronic low-grade Inflammation (PRO SANI) studie. De PRO SANI studie is een cross-over, dubbelblinde gerandomiseerde gecontroleerde studie die de effectiviteit van broccolikiemen als bron van sulforafaan op biomarkers van ontstekingen onderzocht

bij gezonde deelnemers die werden blootgesteld aan een gestandaardiseerde calorierijke belasting.

In **Hoofdstuk 4** werden de effecten van broccolikiemen en calorierijke overbelasting op circulerende ontstekingsbiomarkers en metabolische parameters beoordeeld, evenals de relaties tussen deze factoren. Daarnaast werd onderzocht of integratieve uitkomstmaten een betere benadering bieden om de subtiele en veelzijdige effecten van fytonutriënten te bestuderen. Het werd aangetoond dat het gebruik van integratieve uitkomstmaten, zoals een systemische chronische ontstekingsscore, beschouwd kan worden als een efficiënte benadering om de subtiele en veelzijdige effecten van stoffen die ontsteking beïnvloeden te bestuderen. In dit onderzoek zorgde sulforafaan voor de ontwikkeling van een milde pro-inflammatoire toestand na de calorierijke uitdaging. Interessant genoeg weerspiegelde de score nauwkeuriger het pro-inflammatoire effect van broccolikiemen dan de individuele biomarkers. Bovendien werden de verbanden tussen risicofactoren voor de ontwikkeling van NCD's, zoals een hoog visceraal vetgehalte en roken, duidelijker door het gebruik van de score. Deze resultaten lijken te bevestigen dat geïntegreerde uitkomstmaten geschikter zijn als markers van ontstekingen. We vermoeden dat de pro-inflammatoire toestand tijdelijk is en kan worden veroorzaakt door de initiële pro-oxidatieve werking van sulforafaan (om Nrf2 te activeren). We denken dat voedingsmiddelen die fytonutriënten bevatten die later ook onze eigen antioxidantverdediging versterken via Nrf2-activatie, mogelijk meer gezondheidseffecten teweeg kunnen brengen.

Naast het integreren van bekende biomarkers in een gezondheidsindex, zijn nieuwe biomarkers die gevoelig en gemakkelijk te gebruiken zijn essentieel om de gezondheidseffecten van voeding in de algemene bevolking verder te verduidelijken. In **Hoofdstuk 5** werd aangetoond dat 11-dehydro-thromboxane B_2 (TXB_2) een innovatieve, niet-invasieve en geschikte urinaire biomarker is om de effecten van fytonutriënten op de bloedplaatjesfunctionaliteit bij gezonde deelnemers binnen enkele uren te onderzoeken. Het onderzoek toonde aan dat een enkele toediening van broccolikiemen 11-dehydro-TXB_2 niveaus met klinisch relevante hoeveelheden verminderde bij gezonde deelnemers die werden blootgesteld aan een gestandaardiseerde calorierijke belasting. Deze bevindingen zijn interessant omdat verhoogde productie van thromboxanen bijdraagt aan vasculopathie door een negatieve invloed op de endotheel functie en het bevorderen van vaatontsteking. Om deze reden is het verminderen van thromboxanen met medicatie een belangrijk aspect geworden in de behandeling van hart- en vaatziekten. In tegenstelling tot medicamenteuze therapie is het effect van de meeste fytonutriënten omkeerbaar, waardoor ze potentieel veilig zijn voor toekomstig gebruik in een verscheidenheid aan situaties, waaronder primaire preventie van hart- en vaatziekten in de algemene bevolking.

De ontstekingsstatus is geassocieerd met autonome activiteit. Toch is hartslagvariabiliteit (HRV) monitoring voor de beoordeling van ontstekingen bij mensen zelden gebruikt. In **Hoofdstuk 6** werden correlaties tussen veranderingen in HRV-parameters en circulerende ontstekingsbiomarker-niveaus aangetoond, waarbij HRV een gevoelig instrument bleek te zijn om de ontstekingsreactie tijdens uitdagingstests te onderzoeken. Bovendien veroorzaakte toediening van broccolikiemen met sulforafaan vóór de calorierijke uitdaging een pro-inflammatoir effect, dat gepaard ging met vagale terugtrekking en sympathische dominantie. De erwtenkiemen zonder sulforafaan (placebo) in combinatie met de gestandaardiseerde calorierijke belasting veroorzaakten andere veranderingen in de regulatie van het autonome zenuwstelsel bij de gezonde jonge individuen. De verschillen in dynamiek tonen aan dat HRV veelbelovend is om niet-invasief de fysiologische effecten van fytonutriënten te onderzoeken bij de preventie van ontstekingen. Inzicht in de relatie tussen voedingsstoffen en interactieve fysiologische parameters kan leiden tot meer gepersonaliseerde voeding door innovatieve biotechnologische methoden in de toekomst.

Het onderzoek in dit proefschrift had als doel de subtiele en veelzijdige gezondheidseffecten van groenten en fruit bij gezonde mensen in kaart te brengen, om zo de rol van groenten en fruit in de preventie van NCD's gerelateerd aan ontstekingen beter te begrijpen.

Concluderend toonde het onderzoek beschreven in dit proefschrift aan dat de ontstekingsgerelateerde effecten van fytonutriënten uit groenten en fruit binnen enkele uren bij gezonde deelnemers kunnen worden bestudeerd. Het gebruik van integratieve uitkomstmaten, evenals niet-invasieve biomarkers zoals 11-dehydro-TXB_2 en HRV-monitoring, kan verbeterde manieren bieden om de subtiele en veelzijdige effecten van voeding op ontstekingen te meten en te rapporteren. Het overwegen, valideren en toepassen van innovatieve onderzoeksbenaderingen (bijvoorbeeld met samengestelde scores of draagbare technologieën) zou ons begrip van de complexe effecten van voeding vergroten en het broodnodige bewijs leveren om onderzoeksportfolio's te ontwikkelen die nieuwe informatie zullen verschaffen voor productontwikkeling en bijbehorende gezondheidsclaims.

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CURRICULUM VITAE

Hidde van Steenwijk was born on the 1st of December in 1993, in Helmond. He attended secondary school in Helmond at the Dr-Knippenberg College where he received his Atheneum degree in 2012. In that same year, he started his Bachelor of Science in Pharmacy at Rijksuniversiteit Groningen. After obtaining his diploma in 2016, he continued his studies at Rijksuniversiteit Groningen with the Master of Science Pharmacy. During his master thesis at the Department of Pharmacokinetics, Toxicology and Targeting he investigated mechanisms of hepatotoxicity of organophosphorus compounds *ex vivo* using precision-cut tissue slices, which was published in 2022.

After graduating from his Master of Science in 2019, he started his PhD project at the Food Claims Centre Venlo of Maastricht University Campus Venlo, under supervision of Prof. dr. Aalt Bast, dr. Alie de Boer, and dr. Khrystyna Semen. Besides the interdisciplinary research into the subject he was also teaching in various courses in the bachelor's programme University College Venlo and master's programme Health Food Innovation Management. He obtained his University Teaching Qualification during his time as a PhD student. Hidde presented his work at several national and international conferences. His research focused on the role of fruit and vegetables in the prevention of non-communicable diseases, where he specifically focused on chronic low-grade inflammation as potential target for preventive strategies, and is presented in this thesis.

LIST OF PUBLICATIONS

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