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Immune health

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Immune health

Effects of dietary approaches and determinants throughout life

The research described in this dissertation was performed within the Physiology of Human Nutrition (PHuN) research group at the Department of Nutrition and Movement Sciences, which is embedded within NUTRIM Research Institute of Nutrition and Translational Research in Metabolism. Part of the research was funded within the Health Holland framework and part of the research was funded by Raisio Nutrition Ltd.

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Immune Health

Effects of dietary approaches and determinants throughout life

DISSERTATION

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Prof. dr. Pamela Habibović
in accordance with the decision of the Board of Deans,
to be defended in public
on Thursday the 1st of February 2024, at 10:00 hours

by

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Chapter 1

General introduction

The human immune system

The immune system is complex and consists of multiple organs, tissues, and cells that produce various substances (e.g., cytokines and chemokines) to protect the human body from foreign invaders, such as bacteria and viruses [1]. It can be divided into two parts: the innate and the adaptive immune system. These two parts have different functions, but there is also overlap and interaction between these two domains in various immunological processes [1, 2]. Surface markers present on foreign substances causing immune responses are called antigens, and the innate immune system provides the first responses when antigens are recognized. The innate immune system mainly consists of immune cells such as natural killer (NK) and mast cells that can produce aspecific compounds aiming to eliminate foreign invaders as quickly as possible (Figure 1.1: innate immunity) [3]. However, aspecific reactions can also damage healthy tissues [1]. The innate immune system also contains antigen presenting cells, which are able to "consume" and degrade foreign invaders, and subsequently present parts of the foreign invaders' antigens on their plasma membrane (Figure 1.1: phagocytes and dendritic cells). This antigen presentation occurs on so-called major histocompatibility complex molecules to alert cells of the adaptive immune system, such as cluster of differentiation (CD)4+ and CD8+ T cells [4]. Thus, the innate immune system responds fast and aspecific, when foreign substances enter the body, and activates the adaptive immune system [3, 4]. The adaptive immune system has more specific modes of action to eliminate foreign substances (e.g., neutralization by antibodies produced by activated B cells [i.e., plasma cells] or actions of cytotoxic T cells; Figure 1.1), which may cause less harm to healthy tissues compared to actions of the innate immune system [1]. Memory cells are also part of the adaptive immune system and remember the antibody that should be produced to attack a specific antigen [3, 5]. This is how the adaptive immune system trains itself to react faster upon exposure to an antigen that it has already encountered before [5]. Vaccinations are therefore an effective strategy to create immunological memory against antigens that may be encountered in the future [6].

Development of the immune system

The development of the innate immune system starts as early as in utero. The first innate immune responses already occur prenatally, indicating that innate immune cells are already active before birth. The innate immune system continues to develop throughout childhood and reaches full development in teenagers [8]. The cells of the adaptive immune system also develop in utero, but require (repeated) antigen exposure to fully develop and to optimize well-balanced and specific adaptive immune responses, and memory [3, 5, 8]. At birth, immune responses are not yet balanced, though balanced immune responses are essential for immune health. For example, T cell subset helper (Th)2 is overactive in newborns [9], but this disbalance or Th2 skewing of immune responses will change over time as the immune system matures. Repeated exposure to antigens causes the Th2 skewing to disappear and allows infants to further develop Th1 responses [8]. Thus, innate and adaptive immune responses are both immature after birth, but mature gradually throughout childhood and adolescence. The youngest

children rely on their immature immune responses and passive immunity (e.g., maternal immunoglobulin [Ig]G antibodies in breast milk). Repeated exposure to foreign substances, e.g., through infections and vaccinations, cause gradual development of stronger innate and adaptive immune responses [10].

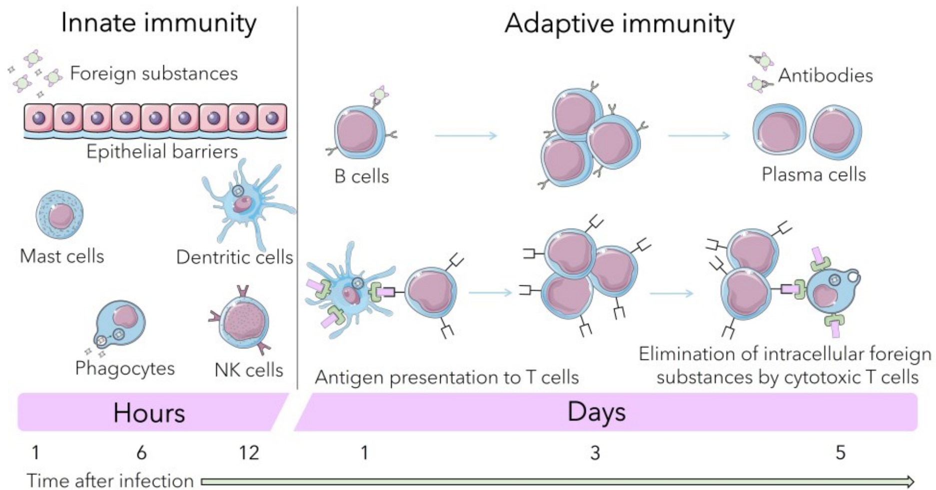

Figure 1.1. Timeline of the principal mechanisms of the innate and adaptive immune systems after infection with a foreign substance. Adapted from Abbas et al., 2015, Figure 1-3, page 4 [7].

How can we measure immune health?

Dysfunctions in the immune system can be genetic from origin [11] or triggered by other factors, such as aging or obesity [12]. Immune health is pivotal for human health, as dysfunctions in the immune system can lead to serious health consequences varying from disease development [13, 14] to an increased risk of hospitalization and severe complaints after infection with COVID-19 [15, 16]. There are several methods used to estimate immune health. A specific task force discussed this topic in 2005, and suggested to use specific antibody responses to vaccinations as the gold standard to measure immune health *in vivo* in humans [17]. Vaccinations are suitable to measure immune health *in vivo* since they activate a variety of immune cells that all respond and interact with each other, including B cells, T cells, NK cells, but also memory cells [17]. In addition, the specific task force advised to also include supporting measures for immune health (e.g., *ex vivo* cytokine production by PBMCs) to obtain indications of underlying mechanisms [17].

Dysfunctional T cell responses in allergic asthma patients

As already indicated in a previous section, T cells can be subdivided into various subsets. Some important subsets are Th1, Th2, Th17, and regulatory T cells (Tregs). In a healthy situation, a balance exists between the activity of the various subsets, and unbalanced

situations (i.e., skewing towards activity of one subtype) can lead to diseases. Tregs and their main cytokine interleukin (IL)-10 play an essential role in this balance, since they regulate Th cells responses. Healthy Th1, Th2, and Th17 responses are critical in the elimination of foreign invaders [18]. However, disbalances in activity of T cell subsets can cause a broad range of diseases, which are characterized by overproduction of Th cytokines, such as interferon (IFN)-γ (Th1), IL-4 (Th2), or IL-17 (Th17; Table 1.1). Overactivity of Th1 and Th17 cells may result in autoimmune diseases [13], whereas overactivity of Th2 cells may result in allergic diseases [14], such as allergic asthma [11]. Allergic asthma is characterized by overproduction of Th2 associated cytokines IL-4, IL-5, and IL-13, which ultimately contribute to a cascade of reactions leading to asthma exacerbations, including immune cell recruitment to the airways, IgE synthesis by plasma cells, and histamine release by mast cells [19]. Treatment with inhaled corticosteroids (ICS) is the gold-standard therapy for asthma in clinical practice [20]. However, when used on the longer term, this therapy has systemic side effects including decreased bone mineral density and cataracts [21]. Treatment with biologicals is recommended for severe asthma, e.g., anti-IgE, anti-IL5, anti-IL13 [20, 22]. These therapies are often effective, though costs are very high [23]. Therefore, there is a need for other and safer complementary strategies for patients with allergic diseases such as allergic asthma to alleviate symptoms and reduce drug use.

Table 1.1. Overview of cytokines, functions, and diseases associated with subsets of Th cells and Tregs [18].

Subset	Main cytokines	Function	Diseases associated with overactivity
Th1	IFN-γ, IL-2	Elimination of intracellular bacteria and viruses	Autoimmune diseases
Th2	IL-4, IL-5, IL-13	Elimination of extracellular parasites and venoms	Asthma and allergies
Th17	IL-17	Elimination of extracellular bacteria and fungi	Autoimmune diseases
Treg	IL-10	Immune regulation and tolerance	-

IFN: interferon; IL: interleukin; Th1: T helper 1; Th2: T helper 2; Th17: T helper 17; Treg: regulatory T cell.

Immunomodulation by dietary approaches to restore immune function in asthma patients

Lifestyle interventions, such as dietary approaches, result in general in less side effects and lower costs in comparison to drug prescription and have been shown to be promising in prevention or treatment of several allergic diseases [24-26]. For example, the immunomodulatory properties of plant sterols and stanols have gained much attention over the past years. Plant sterols and stanols are plant-based nutrients with structural similarities to cholesterol, which lower intestinal cholesterol absorption by e.g., competing with cholesterol for incorporation into mixed micelles [27]. Moreover, besides lowering cholesterol absorption, sitostanol also increased concentrations of Th1 cytokines IL-2 and IFN-γ, and increased IL-10 production by Tregs when added to peripheral blood mononuclear cells (PBMCs) of allergic asthma patients [28]. Furthermore, sitosterol suppressed the production of inflammatory mediator tumor necrosis factor (TNF)-α, and of Th2 cytokines IL-4 and IL-5 in serum and bronchoalveolar

lavage fluid in a guinea pig asthma model. In the same model, airway inflammation and immune cell infiltration was inhibited, while tidal volume increased and respiration rate decreased [29]. A study using *ex vivo* stimulated human PBMCs reported that Toll-like receptor (TLR)2 activation was essential in these Th1 and Treg stimulating effects of plant sterols and stanols [30]. TRLs play an important role in recognizing antigens and mediating immune responses [31]. Finally, a randomized-controlled trial in allergic asthma patients showed that consumption of 4 grams plant stanol esters per day improved specific antibody responses to a hepatitis A vaccination and decreased plasma total IgE concentrations, as well as concentrations inflammatory mediators TNF-α and IL-1β . In addition, increases in serum plant stanol concentrations correlated with decreases in Th2 cytokine IL-13 and a switch toward Th1 responses in the Th1/Th2 balance [32]. Altogether these studies suggest that plant sterols and stanols have the potential to improve immune cell behavior in asthma patients by shifting immune responses away from the Th2 dominant allergic asthma responses via a TLR-2 dependent mechanism (Figure 1.2). Therefore, plant sterols and stanols are a promising dietary approach for patients with allergic diseases characterized by Th2 dominant immune responses.

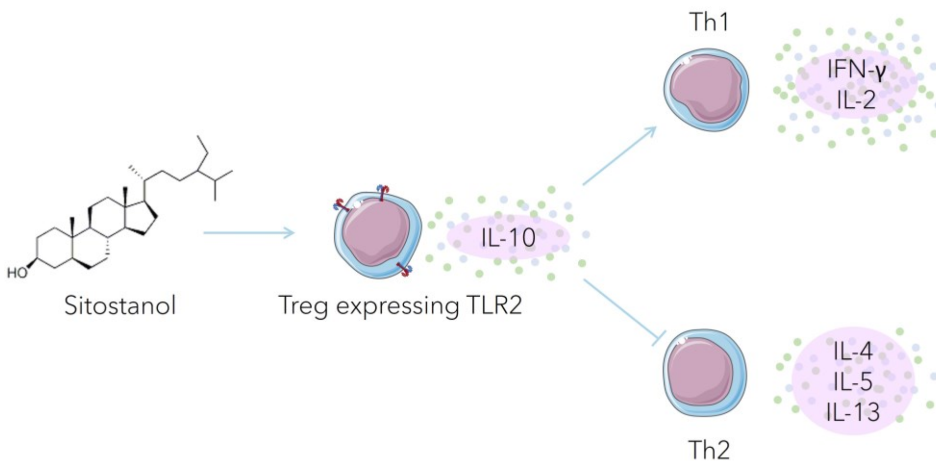

Figure 1.2 Suggested TLR2 dependent mechanism by which plant sterols and stanols alter immune responses in allergic asthma patients.

Thesis outline

The aim of this thesis was to study the effects of dietary approaches and determinants on immune health throughout life, with special attention given to the mediating effects of non-cholesterol sterols. In Chapter 2, a systematic review provides an overview of various dietary approaches that simultaneously improve asthma-related outcomes and immunological parameters in pediatric and adult asthma patients. This combination of these two outcomes lead to speculations that the effects of these dietary approaches on

asthma-related outcomes have an immunomodulating origin. In Chapter 3, non-cholesterol sterols were measured in breast milk sampled one-month postpartum in order to explore associations between non-cholesterol sterol concentrations in breast milk and the development of allergic outcomes in breastfed children in the first two years of life. In Chapter 4, non-cholesterol sterols (standardized for total cholesterol) were measured in serum samples of two studies with pediatric populations (mean ages 3 and 12). These data were used to study associations between cholesterol metabolism with asthma-related parameters and airway inflammation during childhood. The influence of body mass index standard deviation scores (BMI-SDS) on these associations was also explored. Chapter 5 describes a randomized controlled trial, where overweight or obese adults were provided with either plant stanols (4 g/day) or control in order to study the effects of plant stanol ester consumption on the vaccination response to a COVID-19 vaccine. Other study outcomes included immunological parameters (e.g., hs-CRP, stimulated and unstimulated cytokine production by PBMCs), leukocyte counts (total and differential counts), and metabolic parameters (e.g., serum lipid and lipoprotein profiles, glucose and insulin). Chapter 6 describes secondary analyses in samples obtained from two earlier intervention trials. Here, the effects of different doses of plant stanols (recommended versus high dose) were studied on immunological parameters in healthy adults, to determine whether plant stanol esters could potentially disturb the immune cell response and affect the Th1/Th2 cytokine balance in healthy subjects without a priori skewed Th1/Th2 immune responses. Finally, Chapter 7 summarizes the main findings of this thesis. The discussion and interpretation of the results of this thesis are used to draw overall conclusions and propose future directions.

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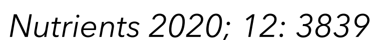
Chapter 2

Nutritional interventions to improve asthma-related outcomes through immunomodulation: a systematic review

Lieve van Brakel, Ronald P. Mensink, Geertjan Wesseling, and Jogchum Plat



Nutrients 2020; 12: 3839

Abstract

Asthma is a chronic inflammatory disease of the airways, characterized by T-helper (Th) 2 inflammation. Current lifestyle recommendations for asthma patients are to consume a diet high in fruits and vegetables and to maintain a healthy weight. This raises the question of whether other nutritional interventions may also improve asthma-related outcomes and whether these changes occur via immunomodulation. Therefore, we systematically reviewed studies that reported both asthma-related outcomes as well as immunological parameters and searched for relations between these two domains. A systematic search identified 808 studies, of which 28 studies met the inclusion criteria. These studies were divided over six nutritional clusters: herbs, herbal mixtures and extracts (N=6); supplements (N=4); weight loss (N=3); vitamin D3 (N=5); omega-3 long-chain polyunsaturated fatty acids (LCPUFAs) (N=5); and whole-food approaches (N=5). Fifteen studies reported improvements in either asthma-related outcomes or immunological parameters, of which eight studies reported simultaneous improvements in both domains. Two studies reported worsening in either asthma-related outcomes or immunological parameters, of which one study reported a worsening in both domains. Promising interventions used herbs, herbal mixtures or extracts, and omega-3 LCPUFAs, although limited interventions resulted in clinically relevant results. Future studies should focus on further optimizing the beneficial effects of nutritional interventions in asthma patients, e.g., by considering the phenotypes and endotypes of asthma.

Introduction

Asthma is a chronic inflammatory disease of the respiratory system, which affects over 300 million people worldwide [1]. The inflammation is characterized by infiltration of immune cells into the airways, among others the T-helper (Th) cells. In asthma patients, these cells predominantly secrete the Th2 cytokines interleukin (IL) 4, IL-5 and IL-13 [2]. The release of these cytokines activates a cascade of reactions, including mast cell activation and immunoglobulin E (IgE) production. Ultimately, airway inflammation leads to symptoms such as wheezing, cough and shortness of breath [2]. The Global Initiative for Asthma (GINA) provides treatment steps to determine the type of treatment [3]. Frequently, asthma patients have prescribed a combination of short-acting β2-agonists for short-term relief and inhaled corticosteroids in order to suppress the airway inflammation, thereby preventing exacerbations. Compliance to inhaled corticosteroids is generally low and was previously estimated to be between 22% and 63%, whereas short-acting β2-agonists are often used too frequently [4,5]. Noncompliance to inhaled corticosteroids could lead to a gradual worsening of the airway and even systemic inflammation in asthma patients over time [4,6]. This does not only worsen asthma severity, but long-term continuous low-grade systemic inflammation may also contribute to the simultaneous development of disorders related to low-grade inflammation, such as cardiovascular diseases [7]. Therefore, asthma patients could benefit from acceptable and easily applicable strategies in conjunction with pharmacological treatment to decrease airway inflammation and asthma symptoms.

In recent years, there has been a growing interest in the effects of lifestyle and, more particularly, nutrition in the prevention of noncommunicable diseases [8–10]. For example, the importance of nutrition in the prevention of common lifestyle-related diseases such as diabetes and cardiovascular diseases is well known and has been described in various reviews and meta-analyses [10–13]. The role of nutrition in relation to asthma has also been studied extensively. Over the past decades, numerous nutrients, foods, diets or even dietary patterns have been suggested to lower exacerbation rates, improve lung function and asthma control, or even decrease inflammatory markers [14,15]. According to several reviews and meta-analyses, vitamin D [16], omega-3 long-chain polyunsaturated fatty acids (LCPUFAs) [17], and increased fruit and vegetable intake [18] are promising interventions for asthma patients. In line with these observations, the current GINA guidelines state that the use of non-pharmacological strategies on top of asthma medication could contribute to the improvement of asthma control [3]. These guidelines advise clinicians to recommend their asthma patients to follow a healthy lifestyle. More specifically, they recommend to quit smoking [19,20], lose weight in case of obesity (with [21,22] or without [23] bariatric surgery), and consume a diet high in fruit and vegetables. Furthermore, regular physical activity is recommended to reduce cardiovascular disease risk [24] and improve quality of life [25]. However, as mentioned above, asthma severity may be reduced by many more dietary approaches than just by increasing the intake of fruit and vegetables. Moreover, it is relevant to emphasize that most of these interventions potentially affect

the immune system, which could explain the decrease in inflammation of the respiratory system and the consequent reduction in asthma complaints. An example is a suggestion made in a review that vitamin D could have a direct effect on the immune system, since a variety of immune cells involved in asthma pathology express the vitamin D receptor [26]. However, despite promising results of vitamin D interventions [16], data are inconsistent. This may at least partly be related to the different doses of vitamin D supplemented in the various studies [26,27]. Therefore, a better understanding of mechanisms underlying the immunomodulatory effects of nutritional components is needed to support dietary approaches to improve asthma control. However, a systematic evaluation of randomized controlled trials studying these effects is missing. Therefore, the aim of this review is to provide an overview of nutritional interventions in asthma patients and reported asthma-related outcomes as well as immunological parameters and to search for possible relations.

Methods

Search strategy

The preferred reporting items for systematic review and meta-analyses (PRISMA) checklist was used to structure this systematic review [28]. The aim of the search was to find controlled intervention studies in which the effects of a dietary intervention on asthma-related outcomes as well as on immunological parameters in asthma patients were reported. Three databases (Medline, Embase and the Cochrane Controlled Register of Trials) were used to conduct the search strategy. The abstracts of papers present in these databases were searched in August 2019 without any restriction on publication date. The following search string was used: asthma and immune system or immune function or immunology or inflammation or inflammatory or immunity and trial or clinical study or intervention or RCT and nutrition* or food or diet* or supplement* or micronutrient* or antioxidant*.

Selection of studies

After the removal of duplicates, two researchers (LvB and JP) screened the articles that were retrieved from the databases. The screening consisted of two rounds. First, titles and abstracts were screened to determine potential eligible papers. Second, all these papers were read and included in the systematic review if they met all inclusion criteria. Relevant papers present in the reference lists of selected articles were screened as well. The following inclusion criteria were used: (1) randomized-controlled trials, (2) written in English, (3) scientific papers, (4) publication in a peer-reviewed journal, (5) used a nutritional intervention, (6) the subjects used medication for allergic asthma, (7) at least one immunological parameter was reported, (8) at least one asthma-related outcome was reported. Studies were excluded if they did not have a control group or were not randomized. Additionally, conference abstracts or posters were excluded. Differences in selection by the two researchers were solved by discussion. If two articles referred to the same study, both were included in the review, provided that both articles presented

either different asthma-related outcomes or immunological parameters. Data of articles describing the same study were merged and presented as one study in tables. In this systematic review, this occurred twice and has been mentioned as a footnote of the corresponding table.

Data extraction

After the second screening, data were extracted from the eligible papers and transferred to Excel. The following characteristics were extracted: (1) study information (first author, year of publication, study design, duration, subgroups, intervention, type of asthma, participants' health status, medication use, dietary requirements during the study) (2) baseline characteristics (number of subjects, gender, age, body mass index (BMI)), (3) immunological parameters measured, and (4) asthma-related outcomes measured. For each paper, data were extracted for the experimental and control groups separately. If outcome parameters were only presented in a figure, corresponding means or medians were estimated using a pixel ruler [29]. If not presented in the paper, within-group changes were calculated by subtracting the mean or median outcome of the baseline measurement from the mean or median of the final measurement in that group for both parallel and crossover studies [30–32].

For asthma-related outcomes, a wide variety of parameters was reported. Therefore, we decided that the three most reported questionnaires (asthma control test (ACT), asthma control questionnaire (ACQ) and asthma quality of life questionnaire (AQLQ)) and the three most reported lung function parameters (forced expiratory volume in one second (FEV_1), forced vital capacity (FVC) and peak expiratory flow (PEF)) were the focus of this review. Moreover, a study was classified to result in improved asthma-related outcomes if at least one of these parameters was significantly improved as compared to control treatment. For immunological outcomes, there was an even wider variation in outcome parameters. Therefore, we decided to focus on markers that were reported by at least five different studies. A study was classified to result in improved immunological outcomes if at least one of these five parameters was significantly improved as compared to control treatment. If a study did not report one of the three most used asthma questionnaires or lung function parameters, results were still included in the systematic review and listed under the heading "other" in the corresponding tables. The same applies to studies that only used immune markers that were not measured in five or more studies. Interventions that observed statistically significant changes in both asthma-related outcomes and immunological parameters were used to search for possible relations between these two outcome domains.

FEV_1 and FVC were reported as the percentage of the predicted value unless the original paper only reported these outcomes in liters. If FEV_1 or FVC were reported in milliliters, the unit was transformed into liters. PEF was also reported as the percentage of the predicted value unless the original paper reported PEF in L/min. The total score of the AQLQ was calculated from domain scores if the total score was not presented in the original paper. Units for the immunological parameters IgE, IL-10 and C-reactive

protein (CRP) were transformed into IU/mL, pg/mL and mg/L, respectively. Units for eosinophils in blood were transformed into 10^9 cells/L unless the original paper reported eosinophils as a percentage of total leukocytes.

Methodological quality assessment

The methodological quality of the trials was assessed by calculating the Jadad score [33]. Studies received a total score ranging from 0 to 5 based on methodological aspects, including randomization, blinding and description of withdrawals. Outcomes are described in Supplemental Table S2.1.

Results

The search resulted in 808 records, and six other papers were identified through other sources. In the end, 30 articles were included in this review after removal of duplicates, the title and abstract screening (first screening round) and the full-text screening (second screening round) (Figure 2.1). These articles described 28 individual studies.

These 28 studies were clustered based on the nutritional interventions used. The following six clusters (with their respective number of articles) were formed: herbs, herbal mixtures and extracts (N=6); supplements (N=4); weight loss (N=3); vitamin D3 (N=5); omega-3 LCPUFAs (N=5); and whole-food approaches (N=5). An overview of the clusters and selected studies with their characteristics is presented in Table 2.1.

Herbs, herbal mixtures and extracts

Asthma-related outcomes: The results for the cluster "Herbs, Herbal Mixtures and Extracts" are shown in Table 2.2. Lung function parameters were reported in five studies in the "herbs, herbal mixtures or extracts" cluster. Saffron, Nigella sativa or an extract of B. serrata gum resin and A. marmelos fruit caused an increase in these parameters as compared to the control group [36,40,41]. In contrast, no effect of Nigella sativa was found on airway function in another study [39]. Only within-group changes were reported in a study using an extract of propolis. Therefore, no statement could be made if the change of the intervention group differed significantly from the change in the control group [38]. Improvements in asthma control as measured via the ACT were reported in three studies using Nigella sativa [35,39,40]. Furthermore, an extract of B. serrata gum resin and A. marmelos fruit improved asthma-related quality of life [41].

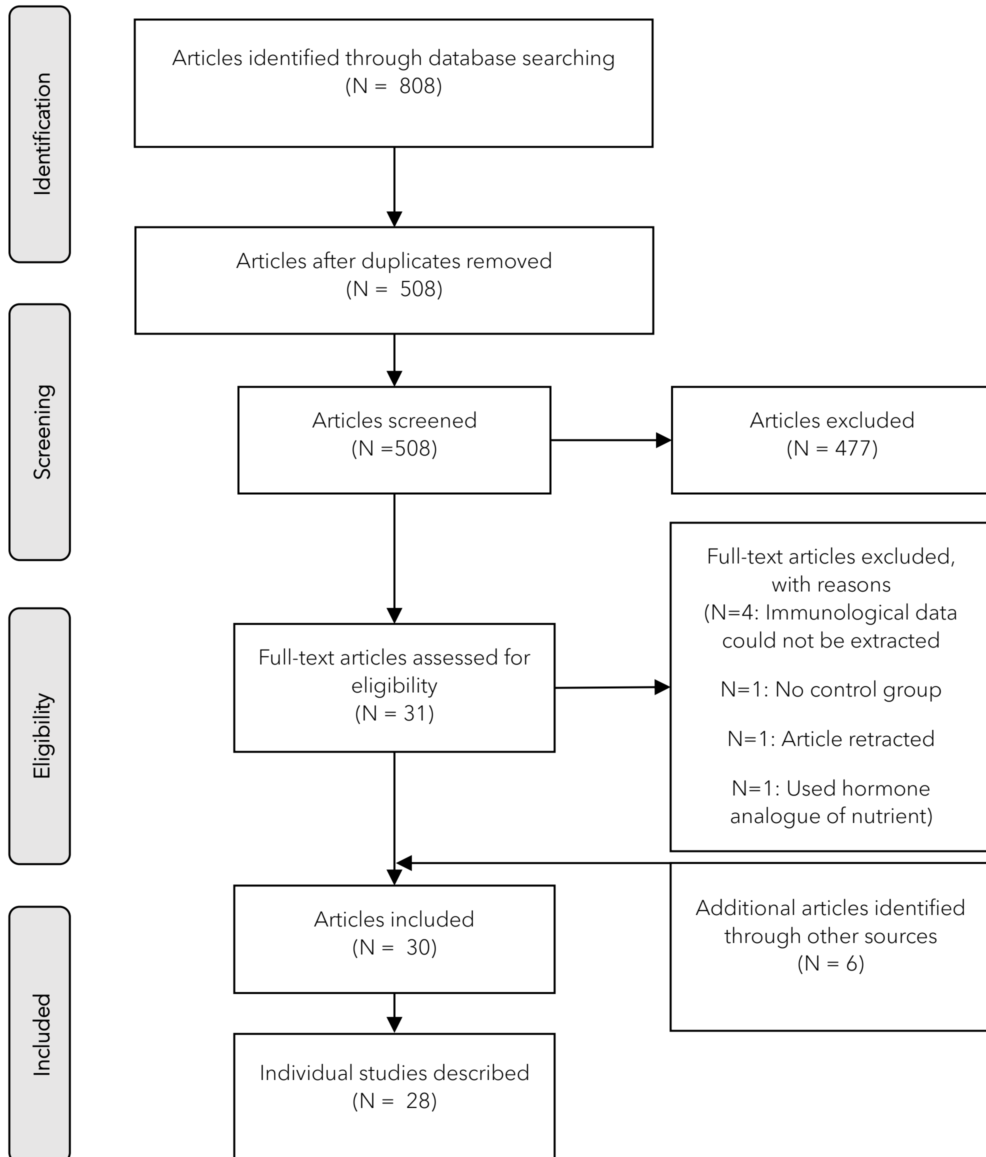


Figure 2.1. PRISMA flow diagram of study selection. In total, 30 articles describing 28 studies were included in the review.

Table 2.1. Study characteristics.

Cluster	First author (year)	Study design	Population	Asthma diagnosis	Intervention and dose##	Study duration	n	Age (years)	Male (%)
Herbs, herbal mixtures and extracts	Barlianto (2017)* Barlianto (2018)* [34, 35]	Parallel	Children with asthma	GINA guidelines	*Nigella Sativa* oil 15-30 mg/kg/day	8 weeks	28	9#	39#
	Hosseini (2018)** Zilaee (2019)** [37, 38]	Parallel	Adults with mild-to-moderate asthma	GINA guidelines	Saffron 100 mg/day	8 weeks	76	41#	63#
	Khayyal (2003) [39]	Parallel	Adults with mild-to-moderate asthma	National Institutes of Health and GINA guidelines	Aqueous extract of propolis 13% solution, equivalent to active constituents in 2 mL of aqueous extract of propolis per day	2 months	46	Range: 19 - 52	78#
	Koshak (2017) [41]	Parallel	Adults with asthma	GINA guidelines and ACT score	*Nigella Sativa* oil 1 g/day	4 weeks	80	41#	41#
	Salem (2017) [42]	Parallel, 3 arms	Adults with asthma	Previous physician's diagnosis and National Institutes of Health criteria	*Nigella Sativa* (low dose) 1 g/day *Nigella Sativa* (high dose) 2 g/day	12 weeks	76	38#	34#
	Yugandhar (2017) [43]	Parallel	Adults with bronchial asthma	Previous physician's diagnosis	Extract of *B. serrata gum* resin and *A. marmelos* fruit 200 mg/day	56 days	29	39#	41#
Supplements	Ghaffari (2014) [44]	Parallel	Children with moderate asthma	Previous physician's diagnosis	Vitamin E 50 mg/day	8 weeks	240	9#	54#
	Pearson (2004) [45]	Parallel	Adults with asthma	Previous physician's diagnosis and medication use	Vitamin E 500 mg/day	6 weeks	72	48	46
	Smith (2015) [46]	Parallel	Children and adults with asthma	Previous physician's diagnosis, symptoms and medication use	Soy isoflavone 100 mg/day	6 months	386	36	34
	Wood (2008) [47]	Cross-over, 3 arms	Adults with stable asthma	Previous physician's diagnosis, symptoms and airway hyper-responsiveness	Tomato extract 45 mg lycopene/day Tomato juice 45 mg lycopene/day	7 days	22	52	36
Weight loss	Dias-Junior (2014) [48]	Parallel	Obese adults with severe asthma	Previous physician's diagnosis and treatment according to GINA guidelines	Low calorie intake, use of sibutramine (10 mg/day) and use of orlistat (max. 120 mg/day)	6 months	33	43#	6#

Abbreviations: ACT = Asthma Control Test; ACQ = Asthma Control Questionnaire; DHA = docosahexaenoic acid; EPA = eicosapentaenoic acid; GINA = Global Initiative for Asthma; IU = international unit; (LC)PUFA = long-chain polyunsaturated fatty acid; PEFR = Peak Expiratory Flow Rate; 1 = exercise arm not included in this review; 2 = not all data of this article could be extracted; */** = articles were based on the same study; # = calculated; ## = Habitual intake and reference data for nutritional interventions is presented in Supplemental Table S2.2

Table 2.1. Continued

Cluster	First author (year)	Study design	Population	Asthma diagnosis	Intervention and dose##	Study duration	n	Age (years)	Male (%)
	Jensen (2013) [49]	Parallel	Obese children with asthma	Previous physician's diagnosis	Energy reduction (-500 kcal/day) and counseling sessions	10 weeks	28	12#	61#
	Toennesen (2018)[1] [50]	Parallel, 4 arms	Adults with asthma	ACQ score and positive diagnostic test	High protein and low glycemic index diet Combination of diet and exercise	8 weeks	125	40#	31#
Vitamin D3	Bar Yoseph (2015) [51]	Parallel	Children with mild asthma	Previous physician's diagnosis, positive methacholine challenge test	Vitamin D3 14.000 IU/week	6 weeks	39	13#	64#
	Castro (2014) [52]	Parallel	Adults with symptomatic asthma	Previous physician's diagnosis, evidence of bronchodilator reversibility or airway hyper-responsiveness	Vitamin D3 100.000 IU once, followed by 4000 IU/day	28 weeks	408	40#	32#
	de Groot (2015) [53]	Parallel	Adults with nonatopic asthma	Evidence of bronchodilator reversibility or airway hyper-responsiveness	Vitamin D3 (Cholecalciferol) 400.000 IU single dose	9 weeks	44	56#	59#
	Kerley (2016) [54]	Parallel	Children with uncontrolled asthma	Previous physician's diagnosis and medication use according to GINA guidelines	Vitamin D3 2000 IU/day	15 weeks	39	8#	62#
	Martineau (2015) [55]	Parallel	Adults with asthma	Previous physician's diagnosis, evidence of bronchodilator reversibility	Vitamin D3 (Vigantol oil) 120.000 IU/ 2 months	1 year	250	48#	44#
Omega-3 LCPUFA	Emelyanov (2002) [56]	Parallel	Adults with mild-to-moderate atopic asthma	American Thoracic Society asthma definition	Lipid extract of the New Zealand green-lipped mussel 200 mg/day EPA+DHA	8 weeks	46	39#	26#
	Hodge (1998) [57]	Parallel	Children with asthma and a history of episodic wheeze in the last 12 months and airway hyperresponsiveness to histamine	Symptoms and airway hyper-responsiveness	Omega-3 fatty acid rich diet and omega-3 fatty acid supplementation 1200 mg/day EPA+DHA	6 months	39	10#	41#
	Mickleborough (2013) [58]	Cross-over	Adults with mild-to-moderate persistent asthma	Previous physician's diagnosis	Marine lipid fraction PCSO-524™ 400 mg/day omega-3 LCPUFA, of which 120 mg/day EPA+DHA	3 weeks	20	23	60

Abbreviations: ACT = Asthma Control Test; ACQ = Asthma Control Questionnaire; DHA = docosahexaenoic acid; EPA = eicosapentaenoic acid; GINA = Global Initiative for Asthma; IU = international unit; (LC)PUFA = long-chain polyunsaturated fatty acid; PEFR = Peak Expiratory Flow Rate; 1 = exercise arm not included in this review; 2 = not all data of this article could be extracted; */** = articles were based on the same study; # = calculated; ## = Habitual intake and reference data for nutritional interventions is presented in Supplemental Table S2.2

Table 2.1. Continued

Cluster	First author (year)	Study design	Population	Asthma diagnosis	Intervention and dose##	Study duration	n	Age (years)	Male (%)
	Moreira (2007) [59]	Parallel	Adults with stable persistent asthma	Previous physician's diagnosis and use of inhaled corticosteroids	N-3 PUFA 780 mg/day EPA+DHA 10 mg/day vitamin E	2 weeks	20	38#	0
	Schubert (2009)[2] [60]	Parallel	Adults with asthma and house dust mite allergy	Unknown	N-3 PUFA-enriched fat blend 750 mg/day (of which 630 mg/day EPA+DHA)	3 weeks	23	24#	43#
Whole food approaches	Bseikri (2018) [61]	Parallel	Obese adolescents with asthma	Previous physician's diagnosis and ACQ score	Nutrient dense bar (CHORI-bar) 2 bars/day	2 months	56	15#	55#
	Papamichael (2019) [62]	Parallel	Children with mild asthma	Previous physician's diagnosis and GINA guidelines	Two meals with fatty fish per week as part of the Greek Mediterranean diet	6 months	64	8#	52#
	Sexton (2013) [63]	Parallel, 3 arms	Adults with symptomatic asthma	Previous physician's diagnosis, bronchodilator reversibility or PEFR variability during run-in	High intervention: encouraged to adopt a Mediterranean diet and received intensive initial advice and 41 hours of consultation sessions with a dietitian Low intervention: received less intensive advice and spent 2 hours with a dietitian	12 weeks	35	38#	29#
	Sudini (2016) [64]	Parallel	Adults with asthma and a positive skin test to an indoor allergen	Previous physician's diagnosis	Broccoli sprouts 100 g/day	3 days	40	34#	40#
	Wood (2012)[2] [65]	Parallel	Adults with stable asthma	Previous physician's diagnosis, symptoms and airway hyper-responsiveness	High antioxidant diet	14 days	137	57#	42#

Abbreviations: ACT = Asthma Control Test; ACQ = Asthma Control Questionnaire; DHA = docosahexaenoic acid; EPA = eicosapentaenoic acid; GINA = Global Initiative for Asthma; IU = international unit; (LC)PUFA = long-chain polyunsaturated fatty acid; PEFR = Peak Expiratory Flow Rate; 1 = exercise arm not included in this review; 2 = not all data of this article could be extracted; */** = articles were based on the same study; # = calculated; ## = Habitual intake and reference data for nutritional interventions is presented in Supplemental Table S2.2

Immunological parameters: Nigella sativa oil did not change fractional exhaled nitric oxide (FeNO) [40] but caused a decrease in the number of eosinophils in blood [39].Th1 cytokines were reported in four studies. Nigella sativa and an extract of B. serrata gum resin and A. marmelos fruit increased interferon-γ (IFN-γ) [34,40,41]. Th1 cytokines were reported in a study using an extract of propolis, but between-group changes were not presented. Therefore, no statement could be made if the change of the intervention group differed significantly from the change in the control group [38]. Th2 cytokines or IgE were reported in four studies. Nigella sativa and an extract of B. serrata gum resin and A. marmelos fruit caused decreases in these parameters [34,41]. In contrast to the Nigella sativa intervention by Barlianto et al. (2017) [34], two other Nigella sativa interventions did cause a significant effect on Th2 cytokines or IgE [39,40]. Treg cytokines were analyzed in two studies. Nigella sativa had no effect on these parameters [40]. Only within-group changes were reported in a study using an extract of propolis [38]. Proinflammatory markers were reported in three studies and only decreased after saffron supplementation [36]. Nigella sativa did not change these markers [40]. Only within-group changes were reported in a study using an extract of propolis [38].

Overlap between both domains: Five out of six studies from this herb, herbal mixture, or extract cluster showed significant improvements in both asthma-related as well as immunological parameters, compared to the control group [34–37,39–41]. Nigella sativa (N=3) increased ACT score in children with 1.9 points [34,35], and in adults with 1.3, 1.4 [40] or 2.1 points [39]. It also increased PEF variability with 8.2 and 5.8 L/min in adults, depending on the dose used [40]. Nigella sativa also increased IFN-γ with 7.8 pg/mL in children [34,35] and with 0.5 or 0.9 pg/mL in adults [40]. It decreased IL-4 with 0.3 pg/mL in children [34,35] and eosinophils in blood with 65 cells/μL in adults. Saffron (N=1) increased FEV1 with 5% and FVC with 1% and decreased CRP with 37.5 ng/mL in adults [36,37]. A mixture of B. serrata gum resin and A. marmelos fruit (N=1) improved PEF with 46.2 L/min, the AQLQ score with 0.5 points, increased IFN-γ with 7.6 pg/mL and decreased IL-4 with 0.4 pg/mL in adults [41].

Supplements

Asthma-related outcomes: The results for the cluster "Supplements" are shown in Table 2.3. All six studies that used supplements as intervention reported lung function parameters. Soy isoflavone supplementation decreased FVC [44]. Using vitamin E, tomato juice, or a tomato extract did not cause changes in lung function [43,45]. Another study with vitamin E supplements only reported within-group changes. Therefore, no statement could be made if the change of the intervention group differed significantly from the change in the control group [42]. No effects were found of soy isoflavones on ACT score [44] or a tomato extract or juice on ACQ score [45].

Immunological parameters: Two studies reported the effect of their intervention on FeNO, but no effects were reported for a tomato extract or tomato juice [45]. Soy isoflavone supplementation even worsened FeNO concentrations [44]. The number of immune cells in sputum or blood was reported by two studies. Tomato extract and tomato juice decreased the number of immune cells in sputum [45]. Soy isoflavones had no effect [44]. Two studies reported Th2 cytokines or IgE, and one study reported proinflammatory markers. Vitamin E did not change IgE levels [43]. Another study using vitamin E supplements only reported within-group changes. It is unknown if the change of the intervention group differed significantly from the change in the control group [42]. Soy isoflavones did not change proinflammatory markers [44].

Overlap between both domains: None of the studies reported improvements in both asthma-related outcomes and immunological parameters. In adults, soy isoflavones worsened both asthma-related outcomes and immunological outcomes, as FVC was decreased by 0.1 L and FeNO increased with 4.9 ppb compared to the control group [44].

Weight loss

Asthma-related outcomes: The results for the cluster "Weight Loss" are shown in Table 2.4. Lung function parameters were reported in all three studies of the weight loss cluster. Low caloric intake combined with weight loss medication (sibutramine and orlistat) caused a reduction in BMI of 5.3 kg/m^2 in the intervention group and improved FVC compared to the control group [46]. A high protein and low glycemic index diet, high-intensity interval training, or a combination of this diet and high-intensity interval training caused weight losses of 2.3 kg, 1.0 kg and 3.1 kg, respectively [48]. Combining energy restriction and counseling sessions resulted in a weight loss of 3.4 kg and a reduction in BMI z-score of 0.2 [47]. No changes were found in lung function parameters in these two studies [47,48]. Additionally, asthma control was evaluated in all studies. Low caloric intake combined with weight loss medication and energy restriction together with counseling sessions improved asthma control [46,47]. The study evaluating diet and high-intensity interval training reported an improvement in asthma control in the group that combined the diet with high-intensity interval training [48]. Asthma-related quality of life was increased after this same diet combined with high-intensity interval training [48], but not after energy reduction combined with counseling sessions [47].

Immunological parameters: FeNO was reported as an immunological outcome in all studies, but no significant improvements were found. The number of immune cells was also evaluated by all three studies. Energy reduction, combined with counseling sessions, decreased sputum lymphocyte numbers [47]. The other interventions did not change immune cell numbers [46,48]. Low caloric intake, combined with weight loss medication, did not change IgE levels [46]. Proinflammatory markers were reported in all studies. Only energy reduction in combination with counseling sessions decreased CRP [47].

Overlap between both domains: Energy reduction in combination with counseling sessions in children significantly decreased ACQ score by 0.6 points, decreased sputum lymphocyte numbers with 0.1×10^6 cells/mL, and decreased CRP with 1.1 mg/L compared to the control group [47].

Vitamin D3

Asthma-related outcomes: The results for the cluster "Vitamin D3" are shown in Table 2.5. Lung function parameters were evaluated in four out of five studies from the vitamin D3 cluster, but no significant improvements were found [50–53]. The effect of vitamin D3 on asthma control was also evaluated in four studies, but none found significant improvements [50–53]. The effect of vitamin D3 on asthma-related quality of life was reported in two studies, although no changes were reported [51,52]. Finally, Bar Yoseph et al. (2015) did not find an effect of vitamin D3 on the provocative dose causing a 20% fall in FEV_1.

Immunological parameters: FeNO was reported in three studies, but no changes were found [49,51,53]. Only a single dose of 400.000 IU vitamin D3 decreased sputum eosinophilia after adjustment for baseline values [51]. Other interventions did not change the number of eosinophils in sputum [50] or blood [49]. Th2 cytokines and IgE were reported twice [49,51], as well as Treg cytokines [49,52]. No changes in these parameters were reported. Two studies reported proinflammatory markers. One study using a daily dose of 2.000 IU vitamin D3 reported an increase in CRP [52], whereas no effect on CRP was found in another study using the same dose of vitamin D3 [49].

Overlap between both domains: None of the vitamin D3 interventions resulted in improvements in both asthma-related outcomes and immunological parameters.

Omega-3 LCPUFAs

Asthma-related outcomes: The results for the cluster "Omega-3 LCPUFAs" are shown in Table 2.6. Lung function parameters were evaluated in all five studies of the omega-3 LCPUFA cluster [54–58]. PEF increased after using a lipid extract of the New Zealand green-lipped mussel [54,56]. Omega-3 LCPUFA supplementation, an omega-3 LCPUFA rich diet or an omega-3 LCPUFA enriched fat blend did not improve lung function parameters [55,57,58]. ACQ score did not change after omega-3 LCPUFA supplementation [57].

Table 2.2. Changes in asthma-related outcomes and immunological parameters as compared to the control group for the herbs, herbal mixtures and extracts cluster.

First author (year)	Jadad score ***	Intervention and dose	Asthma-related outcomes					Immunological parameters					
			Lung function			Asthma control	QoL						
			FEV_1	FVC	PEF	ACT	(P)AQLQ	FeNO	Cells (sputum, blood)	Th1	Th2, IgE	Treg	Pro-inflammatory markers
Barlianto (2017)* Barlianto (2018)* [34, 35]	2	*Nigella Sativa* oil 15-30 mg/kg/day				↑[1]				↑	↓		
Hosseini (2018)** Zilaee (2019)** [37, 38]	5	Saffron 100 mg/day	↑	↑					=				↓
Khayyal (2003) [39]	2	Aqueous extract of propolis 13% solution, equivalent to active constituents in 2 mL of aqueous extract of propolis per day	N/A	N/A	N/A					N/A		N/A	N/A
Koshak (2017) [41]	5	*Nigella Sativa* oil 1 g/day	=		=	↑			↓		=		
Salem (2017) [42]	3	*Nigella Sativa* (low dose) 1 g/day	=	=	↑	↑		=		↑	=	=	=
		Nigella Sativa (high dose) 2 g/day	=	=	↑	↑		=		↑	=	=	=
Yugandhar (2017) [43]	4	Extract of *B. serrata gum* resin and *A. marmelos* fruit 200 mg/day	=		↑		↑			↑	↓		

Abbreviations: FEV_1 = forced expiratory flow in one second; FVC = forced vital capacity; PEF = peak expiratory flow; ACT = asthma control test; (P)AQLQ = (Pediatric) Asthma Quality of Life Questionnaire; FeNO = fractional exhaled nitric oxide; Th = T helper cell; IgE = immunoglobulin E; Treg = regulatory T cell; QoL = Quality of Life; N/A = between group changes not reported; * = based on the same study; ** = based on the same study; *** = Jadad score was calculated to assess the methodological quality of the RCTs. The calculation of the Jadad score can be found in Supplemental Table S2.1; 1 = both articles presented ACT data, only between-group changes are shown here since comparing changes results in less bias than comparing scores at the final timepoint. Quantitative data showing the within-group changes is presented in Supplemental Tables S2.3 and S2.4.

Table 2.3. Changes in asthma-related outcomes and immunological parameters as compared to the control group for the supplements cluster.

First author (year)	Jadad score*	Intervention and dose	Asthma-related outcomes					Immunological parameters			
			Lung function			Asthma control					
			FEV_1	FVC	PEF	ACT	ACQ	FeNO	Cells (sputum, blood)	Th2, IgE	Pro-inflammatory markers
Ghaffari (2014) [44]	3	Vitamin E 50 mg/day	N/A	N/A						N/A	
Pearson (2004) [45]	5	Vitamin E 500 mg/day	=	=	=					=	
Smith (2015) [46]	5	Soy isoflavone 100 mg/day	=	↓	=	=		↑	=		=
Wood (2008) [47]	3	Tomato extract 45 mg lycopene/day	=	=			=	=	↓; =		
		Tomato juice 45 mg lycopene/day	=	=			=	=	↓; =		

Abbreviations: FEV_1 = forced expiratory flow in one second; FVC = forced vital capacity; PEF = peak expiratory flow; ACT = asthma control test; ACQ = asthma control questionnaire; FeNO = fractional exhaled nitric oxide; Th = T helper cell; IgE = immunoglobulin E; N/A = between group changes not reported; * = Jadad score was calculated to assess the methodological quality of the RCTs. The calculation of the Jadad score can be found in Supplemental Table S2.1. Quantitative data showing the within-group changes is presented in Supplemental Tables S2.3 and S2.4.

Table 2.4. Changes in asthma-related outcomes and immunological parameters as compared to the control group for the weight loss cluster.

First author (year)	Jadad score*	Intervention and dose	Asthma-related outcomes					Immunological parameters			
			Lung function		Asthma control		QoL				
			FEV_1	FVC	ACT	ACQ	(P)AQLQ	FeNO	Cells (sputum, blood)	Th2, IgE	Pro-inflammatory markers
Dias-Junior (2014) [48]	3	Low calorie intake, use of sibutramine (10 mg/day) and use of orlistat (max. 120 mg/day)	=	↑	↑	↓		=	=	=	=
Jensen (2013) [49]	3	Energy reduction (-500 kcal/day) and counseling sessions	=	=		↓	=	=	↓; =		↓; =
Toennesen (2018) [50]	3	High protein and low glycemic index diet	=	=		=	=	=	=		=
		Combination of diet and exercise	=	=		↓	↑	=	=		=

*Abbreviations: FEV_1 = forced expiratory flow in one second; FVC = forced vital capacity; ACT = asthma control test; ACQ = asthma control questionnaire; (P)AQLQ = (Pediatric) Asthma Quality of Life Questionnaire; FeNO = fractional exhaled nitric oxide; Th = T helper cell; IgE = immunoglobulin E; QoL = Quality of Life; * = Jadad score was calculated to assess the methodological quality of the RCTs. The calculation of the Jadad score can be found in Supplemental Table S2.1. Quantitative data showing the within-group changes is presented in Supplemental Tables S2.3 and S2.4.*

Table 2.5. Changes in asthma-related outcomes and immunological parameters as compared to the control group for the vitamin D3 cluster.

First author (year)	Jadad score*	Intervention and dose	Asthma-related outcomes							Immunological parameters				
			Lung function			Asthma control		QoL						
			FEV_1	FVC	PEF	ACT	ACQ	(P)AQLQ	Other	FeNO	Cells (sputum, blood)	Th2, IgE	Treg	Pro-inflammatory markers
Bar Yoseph (2015) [51]	4	Vitamin D3 14.000 IU/week							$=^{1}$	=	=	=		=
Castro (2014) [52]	5	Vitamin D3 100.000 IU once, followed by 4000 IU/day	=			=					=			
de Groot (2015) [53]	4	Vitamin D3 (Cholecalciferol) 400.000 IU single dose	=				=	=		=	$\downarrow^{2}$	=		
Kerley (2016) [54]	3	Vitamin D3 2000 IU/day	=	=		=		=					=	↑
Martineau (2015) [55]	5	Vitamin D3 (Vigantol oil) 120.000 IU/ 2 months	=		=	=				=				

*Abbreviations: FEV_1 = forced expiratory flow in one second; FVC = forced vital capacity; PEF = peak expiratory flow; ACT = asthma control test; ACQ = asthma control questionnaire; (P)AQLQ = (Pediatric) Asthma Quality of Life Questionnaire; FeNO = fractional exhaled nitric oxide; Th = T helper cell; IgE = immunoglobulin E; Treg = regulatory T cell; QoL = Quality of Life; * = Jadad score was calculated to assess the methodological quality of the RCTs. The calculation of the Jadad score can be found in Supplemental Table S2.1; 1 = provocative dose causing a 20% fall in FEV_1; 2 = significant difference after correction for baseline. Quantitative data showing the within-group changes is presented in Supplemental Tables S2.3 and S2.4.*

Immunological parameters: FeNO was reported by three studies and decreased after using an extract of the New Zealand green-lipped mussel and a fat blend enriched with omega-3 fatty acids [56,58]. Another study did not find any changes [57]. Furthermore, the number of sputum eosinophils was not changed after increasing omega-3 LCPUFA intake [55,58]. Finally, Emelyanov et al. showed that an extract of the New Zealand green-lipped mussel decreased exhaled H2O2 [54].

Overlap between both domains: Two studies that used lipid extracts of the New Zealand green-lipped mussel as intervention found significant improvements in asthma-related outcomes as well as immunological parameters in adults, compared to the control group [54,56]. Combined morning and evening PEF increased with 21.8 L/min and FeNO decreased with 9.9 ppb in one study [56], whereas morning PEF increased with 80.4 L/min and exhaled H2O2 decreased with 0.1 uM in the other study [54].

Whole food approaches

Asthma-related outcomes: The results for the cluster "Whole Food Approaches" are shown in Table 2.7. Lung function parameters were reported in all five studies using a whole-food approach intervention [59–63]. Consuming a high antioxidant diet increased FEV1 and FVC [63]. The other interventions did not change lung function parameters. Additionally, asthma control was evaluated in all studies. However, no changes in ACT or ACQ score were found. The Mediterranean diet did not improve asthma-related quality of life [60,61].

Immunological parameters: FeNO was reported in four studies [59,60,62,63]. Consuming two meals with fatty fish per week as part of the Mediterranean diet decreased FeNO [60]. A nutrient-dense bar [59], broccoli sprouts [62] and a high antioxidant diet [63] did not have an effect on FeNO concentrations. The Mediterranean diet and a high antioxidant diet did not change Th1 cytokines [61,63]. In the same study on the Mediterranean diet, no changes in Treg cytokines and immune cell count were found [61]. Using a nutrient-dense bar or broccoli sprouts did not change Th2 cytokines or IgE [59,62]. Proinflammatory markers were not changed after using a nutrient-dense bar, the Mediterranean diet, a high antioxidant diet or broccoli sprouts as an intervention [59,61–63].

Overlap between both domains: None of the studies from this whole-food approach cluster found improvements in asthma-related outcomes and immunological outcomes simultaneously.

Effect sizes in the context of minimal clinically important difference

Eight studies found changes in asthma-related outcomes as well as in immunological parameters, which may indicate a link between immunological parameters with asthma-related outcomes. The magnitude of changes in asthma-related outcomes in comparison to the minimal clinically important difference is shown in Figure 2.2. In three of these eight studies, the changes as compared to those of the control group had an effect size that exceeded the minimal clinically important difference. These three interventions were part of the following clusters: herbs, herbal mixtures and extracts (*N*=1), weight loss (*N*=1) and omega-3 LCPUFAs (*N*=1) and showed effects on PEF (extract of *B. serrata* gum resin and *A. marmelos* fruit; lipid extract of the New Zealand green-lipped mussel), ACQ (energy reduction combined with counseling) and AQLQ (extract of *B. serrata* gum resin and *A. marmelos* fruit). A minimal clinically important difference of FVC could not be found in the literature.

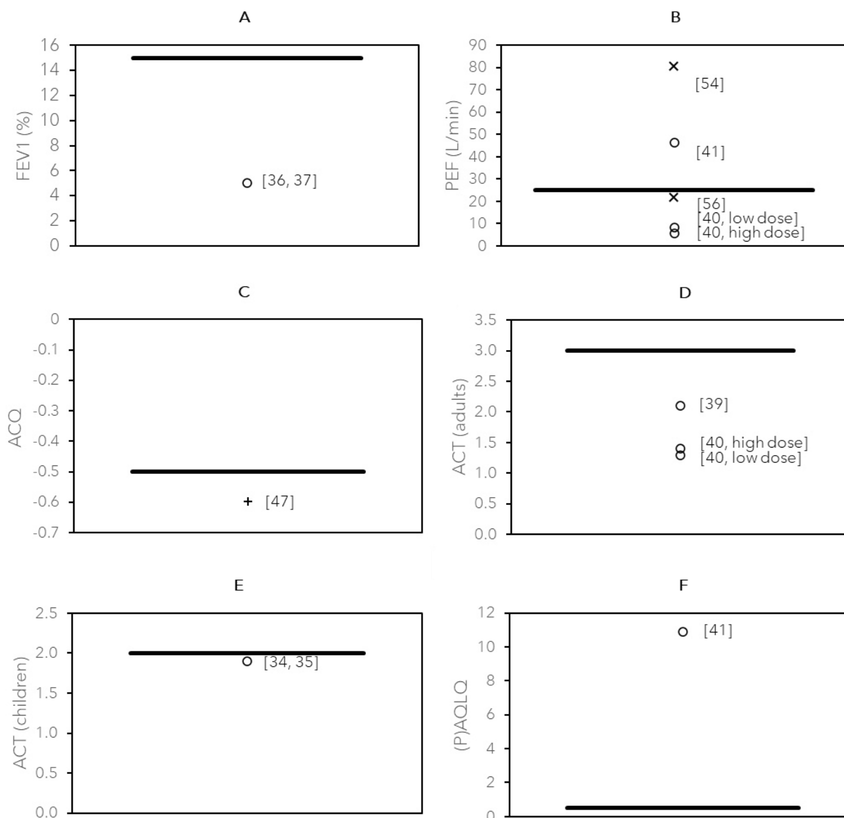


Figure 2.2. The magnitude of changes in asthma-related outcomes compared to the minimal clinically important difference for these outcomes. The symbols (◦ = herbs, herbal mixtures and extracts; x = omega-3 LCPUFA; + = weight loss) represent individual studies and the line (-) indicates the minimal clinically important difference. Reference numbers for individual studies are noted next to the corresponding symbol.

Table 2.6. Changes in asthma-related outcomes and immunological parameters as compared to the control group for the omega-3 LCPUFA cluster.

First author (year)	Jadad score*	Intervention and dose	Asthma-related outcomes			Immunological parameters		
			Lung function		Asthma control			
			FEV_1	PEF	ACQ	FeNO	Cells (sputum, blood)	Other
Emelyanov (2002) [56]	5	Lipid extract of the New Zealand green-lipped mussel 200 mg/day EPA+DHA	=	↑; =				↓[1]
Hodge (1998) [57]	4	Omega-3 fatty acid rich diet and omega-3 fatty acid supplementation 1200 mg/day EPA+DHA	=				=	
Mickleborough (2013) [58]	5	Marine lipid fraction PCSO-524™ 400 mg/day omega-3 LCPUFA, of which 120 mg/day EPA+DHA		↑		↓		
Moreira (2007) [59]	5	N-3 PUFA 780 mg/day EPA+DHA 10 mg/day vitamin E	=		=	=		
Schubert (2009) [60]	4	N-3 PUFA-enriched fat blend 750 mg/day (of which 630 mg/day EPA+DHA)	=			↓	=	

*Abbreviations: FEV_1 = forced expiratory flow in one second; PEF = peak expiratory flow; ACQ = asthma control questionnaire; FeNO = fractional exhaled nitric oxide; * = Jadad score was calculated to assess the methodological quality of the RCTs. The calculation of the Jadad score can be found in Supplemental Table S2.1; 1 = exhaled H_2O_2. Quantitative data showing the within-group changes is presented in Supplemental Tables S2.3 and S2.4.*

Table 2.7. Changes in asthma-related outcomes and immunological parameters as compared to the control group for the whole food approach cluster.

First author (year)	Jadad score*	Intervention and dose	Asthma-related outcomes						Immunological parameters					
			Lung function			Asthma control		QoL						
			FEV_1	FVC	PEF	ACT	ACQ	(P)AQLQ	FeNO	Cells (sputum, blood)	Th1	Th2, IgE	Treg	Pro-inflammatory markers
Bseikri (2018) [61]	2	Nutrient dense bar (CHORI-bar) 2 bars/day	=	=		=			=			=		=
Papamichael (2019) [62]	3	Mediterranean diet	=	=	=		=	=	↓[1]					
Sexton (2013) [63]	2	Mediterranean diet (high intervention)[2]	=	=			=	=		=	=		=	=
		Mediterranean diet (low intervention)[2]	=	=			=	=		=	=		=	=
Sudini (2016) [64]	4	Broccoli sprouts 100 g/day	=	=		=			=			=		=
Wood (2012) [65]	3	High antioxidant diet	↑	↑			=		=	=	=			=

*Abbreviations: FEV_1 = forced expiratory flow in one second; FVC = forced vital capacity; PEF = peak expiratory flow; ACT = asthma control test; ACQ = asthma control questionnaire; (P)AQLQ = (Pediatric) Asthma Quality of Life Questionnaire; FeNO = fractional exhaled nitric oxide; Th = T helper cell; IgE = immunoglobulin E; Treg = regulatory T cell; QoL = Quality of Life; * = Jadad score was calculated to assess the methodological quality of the RCTs. The calculation of the Jadad score can be found in Supplemental Table S2.1; 1 = significant difference after correction for baseline; 2 = High intervention: encouraged to adopt a Mediterranean diet and received intensive initial advice and 41 hours of consultation sessions with a dietitian. Low intervention: received less intensive advice and spent 2 hours with a dietitian. Quantitative data showing the within-group changes is presented in Supplemental Tables S2.3 and S2.4.*

Discussion

The aim of this systematic review was to provide an overview of studies that examined the effects of nutritional interventions in asthma patients on both changes in asthma-related outcomes as well as immunological parameters and search for possible relations. The reason for this approach was that we hypothesized that nutritional interventions might affect asthma severity via modulation of the immune system. Therefore, interventions that improved asthma-related outcomes, as well as immunological parameters, were considered as indications of a link between these two outcome domains, without claiming causality. Current guidelines for asthma patients do contain lifestyle recommendations, but only to a limited extent. It is only advised to increase fruit and vegetable intake and to maintain a healthy weight in order to improve asthma-related outcomes. Based on the results of our systematic review, it may be interesting to consider whether these lifestyle recommendations could be extended in the future. As shown in Tables 2.2–2.7, fifteen out of 28 controlled dietary intervention studies reported an improvement in at least one asthma-related outcome or immunological parameter. However, also two studies reported a worsening in one of the domains, of which one study using soy isoflavones observed a worsening in both domains. With respect to our hypothesis that nutritional interventions likely affect asthma severity via modulation of the immune system, we identified eight studies that showed a simultaneous improvement in asthma-related outcomes and immunological parameters. These studies used Nigella sativa (N=3), saffron (N=1), an extract of B. serrata gum resin and A. marmelos fruit (N=1), energy reduction in combination with counseling sessions (N=1) or a lipid extract of the New Zealand green-lipped mussel (N=2) as an intervention. Regarding the clinical relevance of these results, we showed in Figure 2.2 that three out of the eight studies were able to find clinically relevant changes in asthma-related outcomes. Clinically relevant changes were found in lung function parameters, asthma control and quality of life.

In the context of identifying nutritional interventions that improve asthma-related outcomes via immunomodulation, the "herbs, herbal mixtures and extracts" cluster showed the most consistent and promising results. When interpreting the effects of this cluster, however, it should be noted that exact concentrations of the active compounds of herbal extracts are not always known, and concentrations of extracts could be variable. The use of Nigella sativa resulted in an improvement in asthma control and PEF, which was accompanied by a reduction in eosinophils numbers in blood, an increase of IFN-γ and an increase in IL-4 [34,35,39,40]. An intervention with saffron increased FEV1 and FVC and simultaneously decreased CRP levels [36,37]. An increase in PEF and asthma-related quality of life was observed after using an extract of B. serrata gum resin and A. marmelos fruit. These changes in asthma-related outcomes occurred simultaneously with an increase in the Th1 cytokine IFN-γ and a decrease in the Th2 cytokine IL-4 [41]. These results suggest that the improvement in asthma-related outcomes after using these herbal interventions is mediated by affecting the Th1/Th2 balance, thereby dampening the Th2 driven pathological process. Since the Th1/Th2

balance in asthma is disturbed as asthma is characterized by Th2 mediated inflammation, asthma patients could benefit from interventions that increase Th1 activity, thereby contributing to restoring the Th1/Th2 balance [64]. Based on mouse models, it has been suggested that increased production of Th1 cytokines, such as IFN-γ, could contribute to a decrease in immune cell infiltration in the lungs and eventually to a decrease in local inflammation [64–66]. This suggested mechanism is supported by the findings in this review and is in accordance with previous research. For example, the main constituent of Nigella sativa oil, thymoquinone, stimulates IFN-γ production and decreases IL-4 production in animal models of asthma [64,67,68]. Moreover, lowering Th2 cytokine concentrations is part of the suggested mechanism underlying the effects of crocin, which is the main active component of saffron [69]. However, there are additional pathways that can be modulated by thymoquinone [70] and crocin [71], which indicates that herbal interventions could reduce asthma-related complaints via several mechanisms.

The second cluster that showed promising results was the omega-3 LCPUFA cluster. Two studies using a lipid extract of the New Zealand green-lipped mussel both showed an increase in PEF, which occurred simultaneously with a decrease in FeNO concentrations in one study [56] and a decrease in exhaled H2O2 in the other study [54]. FeNO and exhaled H2O2 are both markers for airway inflammation and can be analyzed in exhaled breath, and correlate positively with eosinophils in induced sputum [72,73]. Furthermore, FeNO can provide information on the asthmatic state that is consistent with other biomarkers for inflammation in asthma [74] and also decreases after treatment with inhaled corticosteroids [75,76]. The results found in this cluster suggest that the effects of interventions with omega-3 LCPUFAs on asthma-related outcomes are mediated by a decrease in airway immune cell infiltration and local inflammation. These anti-inflammatory effects of omega-3 LCPUFAs and their mediators, such as resolvins and protectins, are generally acknowledged and are in line with earlier findings [77]. Several findings in mouse models confirm the results of the studies described in this review. For example, a study in mice suggested that administration of resolvin E1 to an experimental asthma model resulted in an increased IFN-γ production and a decrease in the proinflammatory lipid mediator leukotriene B4 [78]. Leukotriene B4, as well as other leukotrienes, may be involved in immune cell recruitment in lung tissue of asthma patients [79,80]. Also, leukotriene B4 production in neutrophils was previously found to be reduced after human subjects were supplemented with omega-3 LCPUFA and omega-6 short-chain fatty acids [81]. Another study in Fat-1 transgenic mice, which can endogenously produce omega-3 from omega-6 fatty acids, showed that Fat-1 mice had decreased concentrations of Th2 cytokines IL-5 and IL-13 in their lung tissue as compared to wildtype mice [82]. In short, these results suggest that mediators of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) stimulate IFN-γ production by Th1 cells and inhibit the production of Th2 cytokines and other proinflammatory markers such as leukotrienes, at least in asthma conditions. This could ultimately lead to an inhibition of immune cell

recruitment into the airways and therefore contribute to an improvement in asthma-related outcomes. However, not all omega-3 LCPUFA intervention studies in this review showed improvements in asthma-related outcomes that were mediated by the immune system. Strikingly, the three studies with the lowest daily doses of EPA and DHA were the studies that showed beneficial effects on asthma-related outcomes as well as immunological parameters. These studies used intakes between 120 and 300 mg/day. Additionally, one study using a daily dose of EPA and DHA of 630 mg only reported a decrease in FeNO concentrations, but not in asthma-related outcomes [58]. Studies using higher daily intakes of 780 mg or even 1200 mg did not report changes in asthma-related outcomes or in immunological parameters [55,57]. The Food and Agriculture Organisation/World Health Organization set an acceptable macronutrient distribution range for omega-3 LCPUFA between 250 and 2.000 mg/day [83]. Based on the results of this systematic review, asthma patients may benefit from the immunomodulatory effects of omega-3 LCPUFAs when doses on the lower side of this range are consumed. Regarding the effects of higher doses, Yin et al. (2009) showed that supplementing the diet of mice with high doses of fish oil (2% w/w and 4% w/w) increased the levels of Th2 cytokines IL-5 and IL-13 in lung tissue [84]. It is possible that this mechanism explains why the studies using higher doses of EPA and DHA described in this review did not find the improvements in asthma-related outcomes as observed with the lower doses. The underlying mechanism of the dose-response effect of omega-3 LCPUFAs in asthma patients should be explored further.

The least promising clusters were the supplement cluster, the vitamin D3 cluster and whole-food approaches. Since many different interventions were part of the supplement and whole-food approach clusters, we cannot conclude that these types of interventions will never affect asthma-related outcomes. For vitamin D3, the evidence remains contradictory. We found no indications that vitamin D3 is beneficial for asthma patients. However, future studies should take into account the vitamin D status at the start of the study, dose, timing of the dose (single dose versus multiple doses) and parameters that could be affected by vitamin D3, e.g., immune cells that express the vitamin D receptor.

The current GINA guidelines for asthma state that a diet high in fruit and vegetable intake, as well as weight loss, could improve asthma-related outcomes. Indeed, all interventions of the weight loss cluster had a positive effect on asthma-related outcomes and, more specifically, asthma control. This agrees with the systematic review of Okoniewski and colleagues, who reported that weight loss improved a variety of asthma-related outcomes [85]. However, these beneficial effects hardly coincided with improvements in the response of the immune system in our current review. An explanation for this could be that obesity-related asthma has lower eosinophilic inflammation compared to other phenotypes, and therefore the asthma-related changes after weight-loss were mediated by other mechanisms [86]. It has been suggested that adipokines such as leptin and adiponectin could be involved, which can

directly affect airway reactivity [87]. Furthermore, two of the whole-food approaches targeted fruit and vegetable intake of asthma patients [62,63]. Only one of these studies, which used a high antioxidant diet, reported changes in asthma-related outcomes. None of these studies, unfortunately, reported changes in immunological parameters. The effect of fruit and vegetable intake on asthma has been described previously and indeed improved asthma-related outcomes, which has been attributed to their high antioxidant and fiber contents [18]. The high antioxidant capacity of fruit and vegetables, as well as the short-chain fatty acids formed from fiber by the microbiota have been suggested to reduce airway inflammation [18]. These suggestions are, unfortunately, not in accordance with the results of this review. However, the number of studies included in this review evaluating fruit and vegetable intake was limited, and these studies mainly addressed the short-term intake of fruits and vegetables. It remains unclear if fruit and vegetables may influence asthma-related outcomes via other routes than the immune system.

Finally, as shown in this review, studies using the same nutritional intervention do not always find similar effects on asthma-related outcomes and immunological parameters. This could be explained by several factors that influence the success of nutritional interventions in asthma patients in general. These factors that were unintendedly part of the study populations may certainly have influenced the interpretation of the results described. An example of such a factor is asthma severity at baseline. Scott and co-workers found that a 10-week weight loss intervention was more successful in participants with more severe asthma at baseline. They suggested that asthma severity could have been a motivator for this group of patients since the burden of the severity of asthma was a motivation for weight loss [88]. Moreover, nutritional status at the start of the intervention may influence the success of the nutritional intervention in asthma patients. Poor diet quality, which may lead to deficiencies of several micronutrients, has been associated with severe asthma [89]. Therefore, dietary interventions might be more successful in asthma patients that have deficiencies at baseline [16]. In addition, study duration could have influenced study outcomes. The studies in this review had durations varying from three days to one year but were, in general, relatively short (3–6 months). Therefore, we cannot exclude that longer-term nutritional interventions could result in more beneficial effects for asthma patients. Furthermore, pathophysiologic mechanisms differ between asthma endotypes [90], suggesting that characteristics of the study population (e.g., age, obesity) influence the success of nutritional interventions. We here show the data for the studies presented in this review did not suggest that the success of nutritional interventions was depended on age. However, other characteristics of the study population could have been of influence. Other examples are indications that vitamin C may be relevant in the prevention of viral-induced exacerbations [91], whereas weight loss interventions may be most relevant for the obesity-induced asthma phenotype [86]. Information on asthma phenotypes or endotypes was missing in many of the trials included in this review. A recommendation

for future research is to provide this information since it is crucial in order to interpret the effect of nutritional interventions in asthma patients.

In summary, this review provides an overview of studies that examined nutritional interventions in asthma patients and reported changes in asthma-related outcomes as well as immunological parameters. Certain components from the herbs, herbal mixtures, and extract cluster, as well as the omega-3 LCPUFAs, are promising interventions in the context of improving asthma-related outcomes via immunomodulation. Only three interventions showed clinically relevant improvements. Future studies should now focus on how to optimize the beneficial effects of nutritional interventions in asthma patients, e.g., by considering the phenotypes and endotypes of asthma in the study population. The potential of these interventions and underlying pathways should be explored further before any of these interventions could be added to lifestyle guidelines for asthma patients.

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Supplementary Materials

Table S2.1. Calculation of the Jadad score.

Cluster	First author (year)	Intervention	Q1	Q2	Q3	Q4	Q5	Total score
Herbs, herbal mixtures and extracts	Barlianto (2017)* Barlianto (2018)*	*Nigella Sativa* oil	1	0	0	0	1	2
	Hosseini (2018)** Zilaee (2019)**	Saffron	1	1	1	1	1	5
	Khayyal (2003)	Aqueous extract of propolis	1	0	0	0	1	2
	Koshak (2017)	*Nigella Sativa* oil	1	1	1	1	1	5
	Salem (2017)	*Nigella Sativa*	1	1	0	0	1	3
	Yugandhar (2017)	Extract of *B. serrata* gum resin and *A. marmelos* fruit	1	0	1	1	1	4
Supplements	Ghaffari (2014)	Vitamin E	1	0	1	0	1	3
	Pearson (2004)	Vitamin E	1	1	1	1	1	5
	Smith (2015)	Soy isoflavone	1	1	1	1	1	5
	Wood (2008)	Tomato extract and tomato juice	1	1	0	0	1	3
Weight loss	Dias-Junior (2014)	Low calorie intake, use of sibutramine and use of orlistat	1	1	0	0	1	3
	Jensen (2013)	Energy reduction and counselling sessions	1	1	0	0	1	3
	Toennesen (2018)	High protein + low glycemic index diet and combination of diet and exercise	1	1	0	0	1	3
Vitamin D3	Bar Yoseph (2015)	Vitamin D3	1	0	1	1	1	4
	Castro (2014)	Vitamin D3	1	1	1	1	1	5
	de Groot (2015)	Vitamin D3 (Cholecalciferol)	1	0	1	1	1	4
	Kerley (2016)	Vitamin D3	1	0	1	0	1	3
	Martineau (2015)	Vitamin D3 (Vigantol oil)	1	1	1	1	1	5

Q1 = Was the study described as randomized?; Q2 = Was the method used to generate the sequence of randomization described and appropriate?; Q3 = Was the study described as double blind?; Q4 = Was the method of double blinding described and appropriate?; Q5 = Was there a description of withdrawals and dropouts?; Points were deducted in the following cases: if the method used to generate the sequence of randomization was described and it was inappropriate or if the study was described as double blind but the method of blinding was inappropriate. Abbreviations: (LC)PUFA = (long-chain) polyunsaturated fatty acid.

Table S2.1. Continued.

Cluster	First author (year)	Intervention	Q1	Q2	Q3	Q4	Q5	Total score
Omega-3 LCPUFA	Emelyanov (2002)	Lipid extract of the New Zealand green-lipped mussel	1	1	1	1	1	5
	Hodge (1998)	Omega-3 fatty acid rich diet and omega-3 fatty acid supplementation	1	0	1	1	1	4
	Mickleborough (2013)	Lipid extract of the New Zealand green-lipped mussel (marine lipid fraction PCSO-524™)	1	1	1	1	1	5
	Moreira (2007)	N-3 PUFA	1	1	1	1	1	5
	Schubert (2009)	N-3 PUFA-enriched fat blend	1	1	1	1	0	4
Whole food approaches	Bseikri (2018)	Nutrient dense bar (CHORI-bar)	1	0	0	0	1	2
	Papamichael (2019)	Two meals with fatty fish per week as part of the Greek Mediterranean diet	1	1	0	0	1	3
	Sexton (2013)	Mediterrenean diet	1	0	0	0	1	2
	Sudini (2016)	Broccoli sprouts	1	0	1	1	1	4
	Wood (2012)	High antioxidant diet	1	1	0	0	1	3

Q1 = Was the study described as randomized?; Q2 = Was the method used to generate the sequence of randomization described and appropriate?; Q3 = Was the study described as double blind?; Q4 = Was the method of double blinding described and appropriate?; Q5 = Was there a description of withdrawals and dropouts?; Points were deducted in the following cases: if the method used to generate the sequence of randomization was described and it was inappropriate or if the study was described as double blind but the method of blinding was inappropriate. Abbreviations: (LC)PUFA = (long-chain) polyunsaturated fatty acid.

Table S2.2. Habitual intakes of adults living in Europe and reference intakes for the interventions described in this review.

Cluster	First author (year)	Intervention	Intake habitual diet (adults)	Reference value
Herbs, herbal mixtures and extracts	Barlianto (2017) Barlianto (2018)	*Nigella Sativa*	10.8 mg/day ¶,#	N/A
	Salem (2017)			
	Koshak (2017)			
	Khayyal (2003)	Aqueous extract of propolis	N/A	N/A
	Hosseini (2018) Zilaee (2019)	Saffron	2.3 mg/day ¶,##	N/A
	Yugandhar (2017)	Extract of *B. serrata* gum resin and *A. marmelos* fruit	N/A	N/A
Supplements	Ghaffari (2014)	Vitamin E	Males: 8.2 – 16.0 mg/day Females: 7.8 – 12.5 mg/day*	AI adult men and boys 10-18 y/o: 13 mg/day AI adult women and girls 10-18 y/o: 11 mg/day AI children 1-3 y/o: 6 mg/day AI children 3-10 y/o: 9 mg/day *
	Pearson (2004)			
	Smith (2015)	Soy isoflavone	N/A	N/A
	Wood (2008)	Tomato extract and tomato juice	Tomato extract: N/A Tomato juice: 963.4 mg/day ¶,###	N/A
Weight loss	Dias-Junior (2014)	Low calorie intake, use of sibutramine and use of orlistat	N/A	N/A
	Jensen (2013)	Energy reduction and counselling sessions		
	Toennesen (2018)	High protein + low glycemic index diet and combination of diet and exercise		
Vitamin D3	Bar Yoseph (2015)	Vitamin D3	From diet: 1.1 – 8.2 µg/day From diet and supplementation: 3.1 – 23.5 µg/day**	AI adults: 15 µg/day AI children (1-17 y/o): 15 µg/day **
	Castro (2014)	Vitamin D3		
	de Groot (2015)	Vitamin D3 (Cholecalciferol)		
	Kerley (2016)	Vitamin D3		
	Martineau (2015)	Vitamin D3 (Vigantol oil)		
Omega-3 LCPUFA	Emelyanov (2002)	Lipid extract of the New Zealand green-lipped mussel	EPA and DHA from diet: 127 - 295 mg/day	AMDR: 250 – 2000 mg/day****

Abbreviations: AI = Adequate intake; AMDR = acceptable macronutrient distribution range; N/A = data not available; (LC)PUFA = (long-chain) polyunsaturated fatty acid; *Derived from EFSA (2015): Scientific Opinion on Dietary Reference Values for vitamin E as α-tocopherol; **Derived from EFSA (2016): Dietary reference values for vitamin D; ***Derived from EFSA (2012): Scientific opinion on the Tolerable Upper Intake Level of eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA) and docosapentaenoic acid (DPA); ****Derived from FAO/WHO (2010): Expert Consultation on Fats and Fatty Acids in Human Nutrition: Fats and Fatty Acids in Human Nutrition: Report of an expert consultation; ¶ Data obtained from the EFSA Comprehensive European Food Consumption Database; #Weighed average was calculated based on available data for Austria, Belgium, Croatia, France, Ireland, Portugal, Slovenia, United Kingdom; ##Weighed average was calculated based on available data for Austria, France, Italy, Portugal, Slovenia, Spain, United Kingdom; ###Weighed average was calculated based on available data for Austria, Belgium, Croatia, Cyprus, Denmark, Estonia, Finland, France, Germany, Hungary, Ireland, Italy, Latvia, the Netherlands, Portugal, Romania, Slovenia, Spain, Sweden, United Kingdom.

Table S2.2. Continued.

Cluster	First author (year)	Intervention	Intake habitual diet (adults)	Reference value
	Hodge (1998)	Omega-3 fatty acid rich diet and omega-3 fatty acid supplementation		
	Mickleborough (2013)	Lipid extract of the New Zealand green-lipped mussel (marine lipid fraction PCSO-524™)		
	Moreira (2007)	N-3 PUFA		
	Schubert (2009)	N-3 PUFA-enriched fat blend		
Whole food approaches	Bseikri (2018)	Nutrient dense bar (CHORI-bar)	N/A	N/A
	Papamichael (2019)	Two meals with fatty fish per week as part of the Greek Mediterranean diet		
	Sexton (2013)	Mediterrenean diet		
	Sudini (2016)	Broccoli sprouts		
	Wood (2012)	High antioxidant diet		

Abbreviations: AI = Adequate intake; AMDR = acceptable macronutrient distribution range; N/A = data not available; (LC)PUFA = (long-chain) polyunsaturated fatty acid; *Derived from EFSA (2015): Scientific Opinion on Dietary Reference Values for vitamin E as α-tocopherol; **Derived from EFSA (2016): Dietary reference values for vitamin D; ***Derived from EFSA (2012): Scientific opinion on the Tolerable Upper Intake Level of eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA) and docosapentaenoic acid (DPA); ****Derived from FAO/WHO (2010): Expert Consultation on Fats and Fatty Acids in Human Nutrition: Fats and Fatty Acids in Human Nutrition: Report of an expert consultation; ¶ Data obtained from the EFSA Comprehensive European Food Consumption Database; #Weighed average was calculated based on available data for Austria, Belgium, Croatia, France, Ireland, Portugal, Slovenia, United Kingdom; ##Weighed average was calculated based on available data for Austria, France, Italy, Portugal, Slovenia, Spain, United Kingdom; ###Weighed average was calculated based on available data for Austria, Belgium, Croatia, Cyprus, Denmark, Estonia, Finland, France, Germany, Hungary, Ireland, Italy, Latvia, the Netherlands, Portugal, Romania, Slovenia, Spain, Sweden, United Kingdom.

Table S2.3a. Within-group changes in lung function parameters.

First author (year)	Study group	FEV$_1$ (%predicted)			FVC (%predicted)			PEF (%predicted)		
		BL	FU	Change	BL	FU	Change	BL	FU	Change
Herbs, herbal mixtures and extracts										
Hosseini (2018) Zilaee (2019)	Saffron	76.0 ± 10.9	80.2 ± 12.6	3.0 [0.0 - 5.3] ↑	82.0 ± 12.9	84.3 ± 13.0	0.0 [-1.0 - 2.0] =			
	Placebo	76.4 ± 10.5	73.2 ± 12.2	-2.0 [-4.3 - 2.0] ↓	81.1 ± 11.7	79.7 ± 12.7	-1.0 [-4.0 - 0.3] ↓			
Khayyal (2003)	Aqueous extract of propolis	55.6 ± 2.3 [2,3]	71.6 ± 2.9 [2,3]	16.0 ↑ [2,3]	67.5 ± 1.7 [2,3]	80.5 ± 1,7 [2,3]	13.0 ↑ [2,3]	53.9 ± 2.3 [2,3]	70.4 ± 2.3 [2,3]	16.6 ↑ [2,3]
	Placebo	55.2 ± 2.4 [2,3]	55.8 ± 3.6 [2,3]	0.6 = [2,3]	73.6 ± 2.4 [2,3]	75.3 ± 2.4 [2,3]	1.7 = [2,3]	54.0 ± 1.8 [2,3]	57.5 ± 2.4 [2,3]	3.5 = [2,3]
Koshak (2017)	Nigella Sativa oil	N/A	N/A	4.0 [-1.3 - 8.8]				N/A	N/A	6.5 [0.3 - 22.8]
	Placebo	N/A	N/A	1.0 [-2.0 - 5.0]				N/A	N/A	2.0 [0.0 - 14.5]
Salem (2017)	Nigella Sativa (low dose)	85.5 ± 17.3	87.7 ± 15.8	2.2 = [1]	92.8 ± 17.3	94.8 ± 14.8	2.0 = [1]	73.5 ± 10.7 [4]	83.6 ± 8.7 [4]	10.1 ↑ [1,4]
	Nigella Sativa (high dose)	78.1 ± 21.4	85.5 ± 22.9	7.4 ↑ [1]	88.7 ± 21.9	93.0 ± 22.9	4.3 = [1]	73.7 ± 11.2 [4]	81.4 ± 8.7 [4]	7.7 ↑ [1,4]
	Placebo	81.1 ± 19.1	80.8 ± 20.6	-0.3 = [1]	90.1 ± 13.7	89.1 ± 13.7	-1.0 = [1]	76.6 ± 7.3 [4]	78.5 ± 8.8 [4]	1.9 = [1,4]
Yugandhar (2017)	Extract of *B. serrata* gum resin and *A. marmelos* fruit	1.7 ± 0.1 [3,5]	N/A	0.1 ± 0.1 [3,5]				179.4 ± 16.6 [3,5]	N/A	90.0 ± 13.0 [3,5]
	Placebo	1.6 ± 0.2 [3,5]	N/A	-0.0 ± 0.1 [3,5]				158.1 ± 16.0 [3,5]	N/A	43.9 ± 12.7 [3,5]
Supplements										
Ghaffari (2014)	Vitamin E	71.6 ± 4.1	83.1 ± 5.4	11.5 ↑ [1]	95.3 ± 2.4	100.2 ± 1.1	4.9 = [1]			
	Placebo	72.4 ± 3.4	74.8 ± 2.7	2.4 = [1]	96.1 ± 1.7	95.8 ± 1.6	-0.3 = [1]			

Values are presented as mean ± SD, mean (lower bound 95% CI; upper bound 95% CI), median [Q1 – Q3] or median [minimum; maximum]. = indicates that within-group changes were not significantly different from baseline; ↑ indicates a significant increase compared to baseline; ↓ indicates a significant decrease compared to baseline; blank indicates that within-group changes were not reported in the article; N/A indicates data could not be extracted. Abbreviations: BL = baseline; FU = follow-up; FEV$_1$ = forced expiratory flow in one second; FVC = forced vital capacity; PEF = peak expiratory flow; (LC)PUFA = (long-chain) polyunsaturated fatty acid; MD = Mediterranean diet; 1 = calculated; 2 = estimated using pixel ruler; 3 = ± SEM; 4 = PEF variability; 5 = unit is liters; 6 = unit transformed; 7 = n at follow-up is smaller than n at baseline; 8 = morning PEF; 9 = evening PEF

Table S2.3a. Continued.

First author (year)	Study group	FEV$_1$ (%predicted)			FVC (%predicted)			PEF (%predicted)		
		BL	FU	Change	BL	FU	Change	BL	FU	Change
Pearson (2004)	Vitamin E	N/A	N/A	0.0 ± 0.2 = [5,6]	N/A	N/A	0.0 ± 0.2 = [5,6]	N/A	N/A	1.0 ± 24.0 = [5]
	Placebo	N/A	N/A	0.0 ± 0.2 = [5,6]	N/A	N/A	0.0 ± 0.3 = [5,6]	N/A	N/A	-4.0 ± 21.0 = [5]
Smith (2015)	Soy isoflavone	N/A	N/A	0.0 (-0.1; 0.1) = [5]	N/A	N/A	0.0 (-0.1; 0.0) = [5]	N/A	N/A	9.6 (-0.4; 19.6) = [5]
	Placebo	N/A	N/A	0.0 (0.0; 0.1) = [5]	N/A	N/A	0.0 (0.0; -0.1) ↑ [5]	N/A	N/A	15.8 (4.4; 27.2) = [5]
Wood (2008)	Tomato extract	76.5 (68.9; 84.1)	79.7 (72.0; 87.5) [7]	N/A	90.4 (84.3; 96.5)	91.3 (83.9; 98.7) [7]	N/A			
	Tomato juice	76.5 (68.9; 84.1)	80.0 (71.1; 88.9) [7]	N/A	90.4 (84.3; 96.5)	91.7 (83.6; 99.8) [7]	N/A			
	Placebo	76.5 (68.9; 84.1)	80.9 (72.7; 89.2) [7]	N/A	90.4 (84.3; 96.5)	92.3 (85.2; 99.4) [7]	N/A			
Weight loss										
Dias-Junior (2014)	Low calorie intake, use of sibutramine and use of orlistat	64.1 ± 3.4 [3]	70.0 ± 4.9 [3]	5.9 = [1]	82.4 ± 3.2[3]	87.8 ± 3.0 [3]	5.3 = [1]			
	Placebo	59.2 ± 4.2 [3]	61.5 ± 3.2 [3]	2.3 = [1]	74.5 ± 1.5[3]	74.4 ± 2.0 [3]	-0.1 = [1]			
Jensen (2013)	Energy reduction and counselling sessions	2.4 [2.0 -2.9] [5]	N/A	0.0 [-0.2 - 0.1] = [5]	3.4 [2.7 - 3.5] [5]	N/A	0.1 ± 0.2 = [5]			
	Placebo	2.6 [2.2 - 2.9] [5]	N/A	0.0 [-0.2 - 0.1] = [5]	3.3 [2.9 -3.5] [5]	N/A	0.0 ± 0.2 = [5]			

Values are presented as mean ± SD, mean (lower bound 95% CI; upper bound 95% CI), median [Q1 – Q3] or median [minimum; maximum]. = indicates that within-group changes were not significantly different from baseline; ↑ indicates a significant increase compared to baseline; ↓ indicates a significant decrease compared to baseline; blank indicates that within-group changes were not reported in the article; N/A indicates data could not be extracted. Abbreviations: BL = baseline; FU = follow-up; FEV$_1$ = forced expiratory flow in one second; FVC = forced vital capacity; PEF = peak expiratory flow; (LC)PUFA = (long-chain) polyunsaturated fatty acid; MD = Mediterranean diet; 1 = calculated; 2 = estimated using pixel ruler; 3 = ± SEM; 4 = PEF variability; 5 = unit is liters; 6 = unit transformed; 7 = n at follow-up is smaller than n at baseline; 8 = morning PEF; 9 = evening PEF

Table S2.3a. Continued.

First author (year)	Study group	FEV$_1$ (%predicted)			FVC (%predicted)			PEF (%predicted)		
		BL	FU	Change	BL	FU	Change	BL	FU	Change
Toennesen (2018)	High protein and low glycemic index diet	87.6 ± 14.5	89.4 ± 13.4	1.8 = [1]	95.8 ± 11.5	99.2 ± 11.6	3.4 = [1]			
	Combination of diet and exercise	82.6 ± 15.2	84.5 ± 16.2	1.9 = [1]	94.3 ± 15.3	96.8 ± 14.0	2.5 ↑ [1]			
	Placebo	81.9 ± 12.3	81.6 ± 12.8	-0.3 = [1]	96.0 ± 12.5	95.0 ± 13.5	-1.0 = [1]			
Vitamin D3										
Castro (2014)	Vitamin D3	80.7 [2]	79.7 [2]	-1.0 [1]						
	Placebo	80.4 [2]	80.1 [2]	-0.3 [1]						
de Groot (2015)	Vitamin D3 (Cholecalciferol)	99.1 ± 15.7	97.4 ± 15.7	-1.7 = [1]						
	Placebo	97.6 ± 18.1	94.0 ± 17.1	-3.6 ↓ [1]						
Kerley (2016)	Vitamin D3	105.0 [92.0 - 114.0]	N/A	-4.0 [-6.3 - (-1.0)]	94.5 [87.0 - 191.0]	N/A	-2.5 [-8.3 - 3.0]			
	Placebo	96.0 [90.0 - 104.0]	N/A	2.5 [-4.3 - 6.5]	93.0 [85.0 - 98.0]	N/A	0.0 [-5.0 - 4.5]			
Martineau (2015)	Vitamin D3 (Vigantol oil)	82.0 ± 18.7	81.6 ± 18.5	-0.4 [1]				383.0 ± 106.0 [5]	388.1 ± 116.8 [5]	5.1 [1,5]
	Placebo	81.0 ± 20.4	80.1 ± 22.8	-0.9 [1]				379.0 ± 123.0 [5]	387.7 ± 122.9 [5]	8.7 [1,5]

Values are presented as mean ± SD, mean (lower bound 95% CI; upper bound 95% CI), median [Q1 – Q3] or median [minimum; maximum]. = indicates that within-group changes were not significantly different from baseline; ↑ indicates a significant increase compared to baseline; ↓ indicates a significant decrease compared to baseline; blank indicates that within-group changes were not reported in the article; N/A indicates data could not be extracted. Abbreviations: BL = baseline; FU = follow-up; FEV$_1$ = forced expiratory flow in one second; FVC = forced vital capacity; PEF = peak expiratory flow; (LC)PUFA = (long-chain) polyunsaturated fatty acid; MD = Mediterranean diet; 1 = calculated; 2 = estimated using pixel ruler; 3 = ± SEM; 4 = PEF variability; 5 = unit is liters; 6 = unit transformed; 7 = n at follow-up is smaller than n at baseline; 8 = morning PEF; 9 = evening PEF

Table S2.3a. Continued.

First author (year)	Study group	FEV$_1$ (%predicted)			FVC (%predicted)			PEF (%predicted)		
		BL	FU	Change	BL	FU	Change	BL	FU	Change
Omega-3 LCPUFA										
Emelyanov (2002)[8]	Lipid extract of the New Zealand green-lipped mussel	82.9 ± 4.2 [3]	82.9 ± 3.6 [3]	-0.0 ± 2.9 [3]				361.3 ± 17.4 [3,8]	408.3 ± 18.7 [3,8]	47.0 ± 11.7 [3,8]
	Placebo	92.3 ± 2.9 [3]	90.5 ± 3.2 [3]	-1.8 ± 4.4 [3]				384.3 ± 21.5 [3,8]	350.9 ± 21.3 [3,8]	-33.4 ± 6.2 [3,8]
Emelyanov (2002)[9]	Lipid extract of the New Zealand green-lipped mussel	82.9 ± 4.2 [3]	82.9 ± 3.6 [3]	-0.0 ± 2.9 [3]				375.4 ± 18.2 [3,9]	406.5 ± 19.7 [3,9]	31.1 ± 14.6 [3,9]
	Placebo	92.3 ± 2.9 [3]	90.5 ± 3.2 [3]	-1.8 ± 4.4 [3]				399.6 ± 16.7 [3,9]	403.9 ± 18.3 [3,9]	4.3 ± 10.3 [3,9]
Hodge (1998)	Omega-3 fatty acid rich diet and omega-3 fatty acid supplementation	81.1 (75.3; 86.9)	83.7 (78.4; 89.0)	2.6 [1]						
	Placebo	86.1 (79.1; 93.1)	83.5 (78.3; 88.7)	-2.6 [1]						
Mickleborough (2013)	Marine lipid fraction PCSO-524™							N/A	386.3 ± 22.8 [5]	N/A
	Placebo							N/A	364.5 ± 17.2 [5]	N/A
Moreira (2007)	N-3 PUFA	96.7 (85.4; 108.0)	100.7 (87.9; 113.6)	4.0 (-3.7; 11.7) =						
	Placebo	90.9 (75.9; 105.8)	94.5 (75.9; 113.0)	3.7 (-4.6; 12.9) =						
Schubert (2009)	N-3 PUFA-enriched fat blend	4.3 ± 0.3 [2,3,5]	4.3 ± 0.3 [2,3,5]	0.0 [1,5]						
	Placebo	4.1 ± 0.2 [2,3,5]	4.2 ± 0.2 [2,3,5]	0.1 [1,5]						

Values are presented as mean ± SD, mean (lower bound 95% CI; upper bound 95% CI), median [Q1 – Q3] or median [minimum; maximum]. = indicates that within-group changes were not significantly different from baseline; ↑ indicates a significant increase compared to baseline; ↓ indicates a significant decrease compared to baseline; blank indicates that within-group changes were not reported in the article; N/A indicates data could not be extracted. Abbreviations: BL = baseline; FU = follow-up; FEV$_1$ = forced expiratory flow in one second; FVC = forced vital capacity; PEF = peak expiratory flow; (LC)PUFA = (long-chain) polyunsaturated fatty acid; MD = Mediterranean diet; 1 = calculated; 2 = estimated using pixel ruler; 3 = ± SEM; 4 = PEF variability; 5 = unit is liters; 6 = unit transformed; 7 = n at follow-up is smaller than n at baseline; 8 = morning PEF; 9 = evening PEF

Table S2.3a. Continued.

First author (year)	Study group	FEV$_1$ (%predicted)			FVC (%predicted)			PEF (%predicted)		
		BL	FU	Change	BL	FU	Change	BL	FU	Change
Whole food approaches										
Bseikri (2018)	Nutrient dense bar (CHORI-bar)	92.3 ± 15.6	97.5 ± 13.2	5.2 = [1]	110.5 ± 14.0	115.9 ± 12.1	5.4 = [1]			
	Placebo	97.4 ± 16.1	96.9 ± 16.3	-0.6 = [1]	113.0 ± 17.4	114.5 ± 18.7	1.5 = [1]			
Papamichael (2019)	Two meals with fatty fish per week as part of the Greek MD	97.2 ± 8.8	100.2 ± 9.4	2.8	94.6 ± 8.7	96.9 ± 9.2	2.5	94.3 ± 19.3	100.6 ± 21.0	6.1
	Placebo	99.1 ± 10.6	100.1 ± 8.8	0.6	96.3 ± 11.1	96.8 ± 9.1	-0.1	93.5 ± 18.8	101.2 ± 21.7	7.1
Sexton (2013)	High-intervention MD	N/A	N/A	0.1 ± 0.1 [3,5]	N/A	N/A	0.1 ± 0.1 [3,5]			
	Low intervention MD	N/A	N/A	0.0 ± 0.1 [3,5]	N/A	N/A	0.0 ± 0.1 [3,5]			
	Placebo	N/A	N/A	0.0 ± 0.1 [3,5]	N/A	N/A	0.0 ± 0.1 [3,5]			
Sudini (2016)	Broccoli sprouts	3.0 ± 0.8	3.0 ± 0.8	0.0 ± 0.1	4.0 ± 0.9	3.90 ± 0.86	-0.05 ± 0.10			
	Placebo	2.9 ± 0.9	2.9 ± 0.9	-0.0 ± 0.2	3.8 ± 1.0	3.8 ± 1.0	0.0 ± 0.2			

Values are presented as mean ± SD, mean (lower bound 95% CI; upper bound 95% CI), median [Q1 – Q3] or median [minimum; maximum]. = indicates that within-group changes were not significantly different from baseline; ↑ indicates a significant increase compared to baseline; ↓ indicates a significant decrease compared to baseline; blank indicates that within-group changes were not reported in the article; N/A indicates data could not be extracted. Abbreviations: BL = baseline; FU = follow-up; FEV$_1$ = forced expiratory flow in one second; FVC = forced vital capacity; PEF = peak expiratory flow; (LC)PUFA = (long-chain) polyunsaturated fatty acid; MD = Mediterranean diet; 1 = calculated; 2 = estimated using pixel ruler; 3 = ± SEM; 4 = PEF variability; 5 = unit is liters; 6 = unit transformed; 7 = n at follow-up is smaller than n at baseline; 8 = morning PEF; 9 = evening PEF

Table S2.3b. Within-group changes in asthma control and quality of life.

First author (year)	Study group	Asthma control						Quality of life		
		ACT			ACQ			(P)AQLQ		
		BL	FU	Change	BL	FU	Change	BL	FU	Change
Herbs, herbal mixtures and extracts										
Barlianto (2017)*,	Nigella Sativa oil	16.6 ± 2.53	20.3 ± 1.82	3.7 ↑ [1]						
Barlianto (2018)*	Placebo	17.6 ± 1.22	19.4 ± 1.15	1.8 ↑ [1]						
Koshak (2017)	Nigella Sativa oil	16.0 ± 3.9	21.1 ± 2.6	5.1 [1]						
	Placebo	16.6 ± 3.6	19.6 ± 3.7	3.0 [1]						
Salem (2017)	Nigella Sativa (low dose)	17.5 ± 1.3 [2]	21.1 ± 2.1 [2]	3.6 ↑ [1]						
	Nigella Sativa (high dose)	17.4 ± 1.4 [2]	21.1 ± 1.6 [2]	3.6 ↑ [1]						
	Placebo	17.2 ± 1.3 [2]	19.4 ± 2.2 [2]	2.2 ↑ [1]						
Yugandhar (2017)	Extract of *B. serrata* gum resin and *A. marmelos* fruit							25.4 [4]	N/A	16.2 [4]
	Placebo							25.1 [4]	N/A	5.4 [4]
Supplements										
Smith (2015)	Soy isoflavone	N/A	N/A	2.2 (1.5; 2.9) =						
	Placebo	N/A	N/A	2.0 (1.4; 2.5) =						
Wood (2008)	Tomato extract				1.4 (1.0; 1.8)	1.1 (0.8; 1.5) [5]	N/A			
	Tomato juice				1.4 (1.0; 1.8)	1.0 (0.6; 1.31) [5]	N/A			
	Placebo				1.4 (1.0; 1.8)	1.1 (0.8; 1.4) [5]	N/A			
Weight loss										
Dias-Junior (2014)	Low calorie intake, use of sibutramine and use of orlistat	12.3 ± 1.1 [3]	17.4 ± 1.1 [3]	5.2 ↑ [1]	3.0 ± 0.3 [3]	1.6 ± 0.2 [3]	-1.4 ↓ [1]			
	Placebo	11.2 ± 1.2 [3]	12.1 ± 0.7 [3]	0.9 = [1]	2.9 ± 0.3 [3]	2.9 ± 0.2 [3]	-0.0 = [1]			
Jensen (2013)	Energy reduction and counselling sessions				N/A	N/A	-0.4 ± 0.5 ↓	5.5 [4.7 - 6.3]	N/A	0.7 ± 1.2 =
	Placebo				N/A	N/A	0.1 ± 0.5 ↑	6.0 [5.7 - 6.5]	N/A	0.1 ± 0.7 =

Values are presented as mean ± SD, mean (lower bound 95% CI; upper bound 95% CI), median [Q1 – Q3]. = indicates that within-group changes were not significantly different from baseline; ↑ indicates a significant increase compared to baseline; ↓ indicates a significant decrease compared to baseline; blank indicates that within-group changes were not reported in the article; N/A indicates data could not be extracted. Abbreviations: BL = baseline; FU = follow-up; ACT = asthma control test; ACQ = asthma control questionnaire; (P)AQLQ = (pediatric) asthma quality of life questionnaire; (LC)PUFA = (long-chain) polyunsaturated fatty acid; MD = Mediterranean diet; 1 = calculated; 2 = estimated using pixel ruler; 3 = ± SEM; 4 = total score calculated from domain scores; 5 = n at follow-up is smaller than n at baseline.

Table S2.3b. Continued.

First author (year)	Study group	Asthma control						Quality of life		
		ACT			ACQ			(P)AQLQ		
		BL	FU	Change	BL	FU	Change	BL	FU	Change
Toennesen (2018)	High protein and low glycemic index diet				2.0 ± 0.6	1.3 ± 0.8	-0.7 ↓ [1]	5.3 ± 0.8	5.9 ± 0.9	0.6 ↑ [1]
	Combination of diet and exercise				1.9 ± 0.7	1.0 ± 0.7	-0.9 ↓ [1]	5.2 ± 0.8	6.2 ± 0.7	1.0 ↑ [1]
	Placebo				1.8 ± 0.8	1.5 ± 0.8	-0.3 ↓ [1]	5.2 ± 0.8	5.7 ± 0.7	0.5 ↑ [1]
Vitamin D3										
Castro (2014)	Vitamin D3	N/A	N/A	0.5 (-0.1; 1.2)						
	Placebo	N/A	N/A	-0.1 (-0.1; 0.0)						
de Groot (2015)	Vitamin D3 (Cholecalciferol)				0.9 [0.4 - 1.9]	0.8 [0.4 - 1.3]	-0.1 = [1]	6.0 [5.1 - 6.4]	6.3 [6.0 - 6.6]	0.3 ↑ [1]
	Placebo				1.2 [0.7 - 1.6]	1.1 [0.8 - 1.6]	-0.1 = [1]	5.7 [5.2 - 6.3]	6.0 [5.6 - 6.2]	0.3 = [1]
Kerley (2016)	Vitamin D3	19.0 [17.0 - 21.0]	N/A	2.0 [-2.0 - 4.0]				5.6 [5.0 - 6.2]	N/A	0.5 [-0.2 - 0.8]
	Placebo	17.0 [14.3 -19.0]	N/A	3.5 [0.0 - 5.0]				5.4 [3.8 - 6.0]	N/A	0.9 [-0.3 - 1.5]
Martineau (2015)	Vitamin D3 (Vigantol oil)	19.2 ± 3.9	20.4 ± 4.0	1.2 [1]						
	Placebo	18.9 ± 3.9	20.4 ± 4.2	1.5 [1]						
Omega-3 LCPUFA										
Moreira (2007)	N-3 PUFA				1.4 (0.8; 2.1)	1.0 (0.4; 1.5)	-0.5 (-0.9; -0.1) ↓			
	Placebo				1.7 (1.0; 2.5)	1.1 (0.4; 1.8)	-0.6 (-1.2; -0.1) ↓			
Whole food approaches										
Bseikri (2018)	Nutrient dense bar (CHORI-bar)	15.0 ± 3.0	20.3 ± 3.1	5.3 ↑ [1]						
	Placebo	13.4 ± 3.4	19.7 ± 3.2	6.3 ↑ [1]						
Papamichael (2019)	Two meals with fatty fish per week as part of the Greek MD				0.4 ± 0.3	0.2 ± 0.5	-0.1	6.8 ± 0.3	6.8 ± 0.6	0.1
	Placebo				0.4 ± 0.4	0.2 ± 0.3	-0.2	6.7 ± 0.4	6.9 ± 0.2	0.2

Values are presented as mean ± SD, mean (lower bound 95% CI; upper bound 95% CI), median [Q1 – Q3]. = indicates that within-group changes were not significantly different from baseline; ↑ indicates a significant increase compared to baseline; ↓ indicates a significant decrease compared to baseline; blank indicates that within-group changes were not reported in the article; N/A indicates data could not be extracted. Abbreviations: BL = baseline; FU = follow-up; ACT = asthma control test; ACQ = asthma control questionnaire; (P)AQLQ = (pediatric) asthma quality of life questionnaire; (LC)PUFA = (long-chain) polyunsaturated fatty acid; MD = Mediterranean diet; 1 = calculated; 2 = estimated using pixel ruler; 3 = ± SEM; 4 = total score calculated from domain scores; 5 = n at follow-up is smaller than n at baseline.

Table S2.3b. Continued.

First author (year)	Study group	Asthma control						Quality of life		
		ACT			ACQ			(P)AQLQ		
		BL	FU	Change	BL	FU	Change	BL	FU	Change
Sexton (2013)	High-intervention MD	N/A	N/A	-0.2 ± 0.2 [3]				N/A	N/A	0.5 ± 0.2 [3]
	Low intervention: MD	N/A	N/A	-0.1 ± 0.2 [3]				N/A	N/A	0.2 ± 0.2 [3]
	Placebo	N/A	N/A	-0.1 ± 0.2 [3]				N/A	N/A	0.2 ± 0.2 [3]
Sudini (2016)	Broccoli sprouts	21.0 [20.0 - 22.0]	21.0 [19.0 -22.0]	0.0 [-1.0 - 1.3]						
	Placebo	20.0 [18.0 - 23.0]	22.0 [20.0 -23.0]	0.0 [0.0 - 1.5]						
Wood (2012)	High antioxidant diet				0.7 [0.4 - 1.4]	0.9 [0.4 - 1.4]	0.2 = [1]			
	Placebo				0.9 [0.4 - 1.4]	0.9 [0.4 - 1.6]	0.0 = [1]			

Values are presented as mean ± SD, mean (lower bound 95% CI; upper bound 95% CI), median [Q1 – Q3]. = indicates that within-group changes were not significantly different from baseline; ↑ indicates a significant increase compared to baseline; ↓ indicates a significant decrease compared to baseline; blank indicates that within-group changes were not reported in the article; N/A indicates data could not be extracted. Abbreviations: BL = baseline; FU = follow-up; ACT = asthma control test; ACQ = asthma control questionnaire; (P)AQLQ = (pediatric) asthma quality of life questionnaire; (LC)PUFA = (long-chain) polyunsaturated fatty acid; MD = Mediterranean diet; 1 = calculated; 2 = estimated using pixel ruler; 3 = ± SEM; 4 = total score calculated from domain scores; 5 = n at follow-up is smaller than n at baseline.

Table S2.4a. Within-group changes in immunological parameters in breath and immune cells.

First author (year)	Study group	Breath			Cells								
		FeNO (ppb)			sEOS (%)			sNEU (%)			bEOS (10^9 cells/L)		
		BL	FU	Change	BL	FU	Change	BL	FU	Change	BL	FU	Change
Herbs, herbal mixtures and extracts													
Koshak (2017)	Nigella Sativa oil										N/A	N/A	-0.1 [-0.2 – 0.0] [1]
	Placebo										N/A	N/A	0.0 [-0.1 - 0.1] [1]
Salem (2017)	Nigella Sativa (low dose)	23.0 ± 13.3	18.1 ± 8.2	-4.9 ↓ [2]									
	Nigella Sativa (high dose)	27.6 ± 30.60	26.9 ± 29.1	-0.7 = [2]									
	Placebo	34.9 ± 32.8	34.8 ± 26.9	-0.1 = [2]									
Hosseini (2018) Zilaee (2019)	Saffron										5.6 [3.0 - 7.5] [3]	4.55 [2.8 - 6.0] [3]	-0.85 [-1.62 - 0.15] ↓ [3]
	Placebo										4.9 [3.9 - 6.5] [3]	4.9 [3.0 - 6.0] [3]	0.1 [-1.6 - 0.6] = [3]
Supplements													
Smith (2015)	Soy isoflavone	N/A	N/A	1.4 (-1.7; 4.5) =							N/A	N/A	0.0 (0.0; 0.0) = [1]
	Placebo	N/A	N/A	-3.5 (-6.0; -1.0) ↓							N/A	N/A	0.0 (0.0; 0.0) = [1]
Wood (2008)	Tomato extract	19.9 (16.4; 27.5)	19.6 (13.1; 31.6) [4]	N/A	1.0 (0.0; 3.1)	0.9 (0.1; 1.8) [4]	N/A	41.0 (24.2; 56.6)	39.8 (18.4; 77.5) [4]	N/A			
	Tomato juice	19.9 (16.4; 27.5)	19.7 (11.0; 25.9) [4]	N/A	1.0 (0.0; 3.1)	0.9 (0.0; 17.8) [4]	N/A	41.0 (24.2; 56.6)	42.0 (21.0; 67.8) [4]	N/A			
	Placebo	19.9 (16.4; 27.5)	19.1 (12.9; 31.4) [4]	N/A	1.0 (0.0; 3.1)	0.4 (0.0; 1.5) [4]	N/A	41.0 (24.2; 56.6)	55.1 (35.0; 91.1) [4]	N/A			

Values are presented as mean ± SD, mean (lower bound 95% CI; upper bound 95% CI), median [Q1 – Q3] or median (IQR). = indicates that within-group changes were not significantly different from baseline; ↑ indicates a significant increase compared to baseline; ↓ indicates a significant decrease compared to baseline; blank indicates that within-group changes were not reported in the article; N/A indicates data could not be extracted. Abbreviations: BL = baseline; FU = follow-up; FeNO = fractional exhaled nitric oxide; sEOS = sputum eosinophils; sNEU = sputum neutrophils; bEOS = blood eosinophils; PUFA = (long-chain) polyunsaturated fatty acid; MD = Mediterranean diet; 1 = unit transformed; 2 = calculated; 3 = unit is percentage; 4 = n at follow-up is smaller than n at baseline; 5 = estimated using pixel ruler; 6 = ± SEM.

Table S2.4a. Continued.

First author (year)	Study group	Breath			Cells								
		FeNO (ppb)			sEOS (%)			sNEU (%)			bEOS (10^9 cells/L)		
		BL	FU	Change	BL	FU	Change	BL	FU	Change	BL	FU	Change
Weight loss													
Dias-Junior (2014)	Low calorie intake, use of sibutramine and use of orlistat	19.6 ± 3.7	26.8 ± 5.2	7.2 = [2]	15.0 ± 4.9	14.4 ± 4.7	-0.6 = [2]	40.2 ± 5.8	41.9 ± 7.3	1.7 = [2]			
	Placebo	20.1 ± 4.9	19.3 ± 3.2	-0.8 = [2]	11.9 ± 3.9	12.5 ± 3.2	0.6 = [2]	41.7 ± 4.0	53.6 ± 4.1	11.9 = [2]			
Jensen (2013)	Energy reduction and counselling sessions	13.1 [8.4 - 41.8]	N/A	-2.6 [-11.3 - 0.4] =	0.8 [0.5 - 5.3]	N/A	-0.1 [-0.5 - 5.1] =	10.5 [8.0 - 18.8]	N/A	-4.8 [-7.5 – (-0.6)] =			
	Placebo	27.2 [10.5 - 46.7]	N/A	-1.9 [-4.0 - 0.3] =	0.8 [0.3 - 8.5]	N/A	0.0 [-0.8 - 2.3] =	10.3 [2.8 - 27.5]	N/A	1.0 [-4.5 - 14.0] =			
Toennesen (2018)	High protein and low glycemic index diet	20.5 (13.0)	18.0 (19.5)	-2.5 = [2]	0.5 (5.8)	5.5 (2.5)	0.0 = [2]	54.3 (26.0)	61.0 (45.5)	6.7 = [2]	0.2 (0.2)	0.1 (0.2)	0.0 = [2]
	Combination of diet and exercise	32.5 (29.0)	27.0 (32.3)	-5.5 = [2]	7.8 (14.9)	4.80 (13.1)	-3.0 = [2]	43.0 (52.4)	46.3 (33.6)	3.3 = [2]	0.21(0.1)	0.2 (0.1)	0.0 = [2]
	Placebo	20.8 (35.6)	20.3 (22.3)	-0.5 = [2]	1.5 (6.7)	0.8 (5.2)	-0.7 = [2]	60.3 (41.0)	55.4 (33.5)	-4.9 = [2]	0.2 (0.2)	0.2 (0.3)	0.0 = [2]
Vitamin D3													
Bar Yoseph (2015)	Vitamin D	36.6 ± 39.1	34.2 ± 26.8	-2.4 = [2]							0.6 ± 0.9	0.3 ± 0.2	-0.2 = [1,2]
	Placebo	58.6 ± 54.7	51.0 ± 40.2	-7.6 = [2]							0.4 ± 0.3	0.3 ± 0.3	-0.1 = [1,2]
Castro (2014)	Vitamin D3				N/A	N/A	-0.3 (-1.4; 0.8)						
	Placebo				N/A	N/A	0.2 (-0.9; 1.3)						
de Groot (2015)	Vitamin D3 (Cholecalciferol)	24.0 [19.0 - 36.0]	22.0 [17.0 - 29.0]	-2.0 = [2]	3.1 [0.3 - 13.3]	0.7 [0.2 - 11.4]	-2.4 = [2]	64.8 [44.6 - 76.4]	65.5 [45.1 - 86.2]	0.7 = [2]	0.2 [0.1 - 0.3]	0.2 [0.1 - 0.3]	0.0 = [2]
	Placebo	33.0 [15.0 - 67.0]	26.0 [11.0 - 60.0]	-7.0 = [2]	6.7 [0.2 - 39.7]	3.9 [0.2 - 50.9]	-2.8 = [2]	67.8 [33.8 - 75.6]	50.5 [22.5 - 72.1]	-17.3 = [2]	0.2 [0.1 - 0.4]	0.2 [0.1 - 0.4]	0.0 = [2]

Values are presented as mean ± SD, mean (lower bound 95% CI; upper bound 95% CI), median [Q1 – Q3] or median (IQR). = indicates that within-group changes were not significantly different from baseline; ↑ indicates a significant increase compared to baseline; ↓ indicates a significant decrease compared to baseline; blank indicates that within-group changes were not reported in the article; N/A indicates data could not be extracted. Abbreviations: BL = baseline; FU = follow-up; FeNO = fractional exhaled nitric oxide; sEOS = sputum eosinophils; sNEU = sputum neutrophils; bEOS = blood eosinophils; PUFA = (long-chain) polyunsaturated fatty acid; MD = Mediterranean diet; 1 = unit transformed; 2 = calculated; 3 = unit is percentage; 4 = n at follow-up is smaller than n at baseline; 5 = estimated using pixel ruler; 6 = ± SEM.

Table S2.4a. Continued.

First author (year)	Study group	Breath			Cells								
		FeNO (ppb)			sEOS (%)			sNEU (%)			bEOS (10⁹ cells/L)		
		BL	FU	Change	BL	FU	Change	BL	FU	Change	BL	FU	Change
Martineau (2015)	Vitamin D3 (Vigantol oil)	38.1 ± 29.1	37.5 ± 26.9	-0.6 [2]									
	Placebo	37.0 ± 26.0	38.5 ± 36.9	1.5 [2]									
Omega-3 LCPUFA													
Hodge (1998)	Omega-3 fatty acid rich diet and omega-3 fatty acid supplementation										0.9 [0.5 - 1.2]	0.7	-0.3 [-0.3 – 0.0] [2,5]
	Placebo										0.6 [0.6 - 0.9]	0.8	0.2 [-0.1 - 0.3] [2,5]
Mickleborough (2013)	Marine lipid fraction PCSO-524™	N/A	15.3 ± 10.7	N/A									
	Placebo	N/A	25.2 ± 19.1	N/A									
Moreira (2007)	N-3 PUFA	27.6 (16.6; 38.6)	30.0 (15.8; 44.2)	2.4 (-3.5; -8.3) =									
	Placebo	20.4 (10.0; 30.1)	25.0 (12.3; 37.7)	4.6 (0.2; 8.9)↑									
Schubert (2009)	N-3 PUFA-enriched fat blend	N/A	N/A	-2.1 ± 3.6	1.5 [5]	0.3 [5]	-1.2 [2]				4.4 ± 0.6 [3,5]	3.8 ± 0.5 [3,5]	-0.6 [2,3]
	Placebo	N/A	N/A	10.8 ± 3.1	1.5 [5]	0.7 [5]	-0.8 [2]				6.0 ± 0.7 [3,5]	5.6 ± 0.9 [3,5]	-0.5 [2,3]
Whole food approaches													
Bseikri (2018)	Nutrient dense bar (CHORI-bar)	35.8 ± 23.5	30.5 ± 20.2	-5.3 = [2]									
	Placebo	24.0 ± 11.5	22.0 ± 13.9	-2.0 = [2]									
Papamichael (2019)	Two meals with fatty fish per week as part of the Greek MD	17.9 ± 17.6	14.6 ± 15.1	-3.8									
	Placebo	10.2 ± 7.2	18.1 ± 29.4	8.1									

Values are presented as mean ± SD, mean (lower bound 95% CI; upper bound 95% CI), median [Q1 – Q3] or median (IQR). = indicates that within-group changes were not significantly different from baseline; ↑ indicates a significant increase compared to baseline; ↓ indicates a significant decrease compared to baseline; blank indicates that within-group changes were not reported in the article; N/A indicates data could not be extracted. Abbreviations: BL = baseline; FU = follow-up; FeNO = fractional exhaled nitric oxide; sEOS = sputum eosinophils; sNEU = sputum neutrophils; bEOS = blood eosinophils; PUFA = (long-chain) polyunsaturated fatty acid; MD = Mediterranean diet; 1 = unit transformed; 2 = calculated; 3 = unit is percentage; 4 = n at follow-up is smaller than n at baseline; 5 = estimated using pixel ruler; 6 = ± SEM.

Table S2.4a. Continued.

First author (year)	Study group	Breath			Cells								
		FeNO (ppb)			sEOS (%)			sNEU (%)			bEOS (10^9 cells/L)		
		BL	FU	Change	BL	FU	Change	BL	FU	Change	BL	FU	Change
Sexton (2013)	High-intervention MD										N/A	N/A	-0.0 ± 0.0 [6]
	Low intervention MD										N/A	N/A	0.0 ± 0.0 [6]
	Placebo										N/A	N/A	-0.1 ± 0.1 [6]
Sudini (2016)	Broccoli sprouts	21.0 [15.0 - 42.0]	22.0 [15.9 - 34.5] [2]	1.0 [2]									
	Placebo	25.5 [15.0 - 42.0]	19.50 [17.0 - 45.3] [2]	-6.0 [2]									
Wood (2012)	High antioxidant diet	17.0 [12.0 - 30.0]	19.0 [15.0 - 31]	2.0 = [2]	1.0 [0.3 - 2.8]	0.8 [0.3 - 2.3]	-0.2 = [2]	46.1 ± 26.2	42.0 ± 26.8	-4.1 = [2]			
	Placebo	23.0 [15.0 - 38.0]	24.0 [16.0 - 35.0]	1.0 = [2]	1.8 [0.5 - 6.4]	1.3 [0.3 - 5.5]	-0.5 ↓ [2]	42.0 ± 20.9	45.7 ± 19.9	3.7 = [2]			

Values are presented as mean ± SD, mean (lower bound 95% CI; upper bound 95% CI), median [Q1 – Q3] or median (IQR). = indicates that within-group changes were not significantly different from baseline; ↑ indicates a significant increase compared to baseline; ↓ indicates a significant decrease compared to baseline; blank indicates that within-group changes were not reported in the article; N/A indicates data could not be extracted. Abbreviations: BL = baseline; FU = follow-up; FeNO = fractional exhaled nitric oxide; sEOS = sputum eosinophils; sNEU = sputum neutrophils; bEOS = blood eosinophils; PUFA = (long-chain) polyunsaturated fatty acid; MD = Mediterranean diet; 1 = unit transformed; 2 = calculated; 3 = unit is percentage; 4 = n at follow-up is smaller than n at baseline; 5 = estimated using pixel ruler; 6 = ± SEM.

Table S2.4b. Within-group changes in Th1 and Th2 cytokines and IgE.

First author (year)	Study group	Th1 cytokines			Th2 cytokines and IgE					
		IFN-γ (pg/ml)			IL-4 (pg/ml)			IgE (IU/ml)		
		BL	FU	Change	BL	FU	Change	BL	FU	Change
Herbs, herbal mixtures and extracts										
Barlianto (2017) Barlianto (2018)	Nigella Sativa oil	12.5 ± 4.4	20.0 ± 6.4	7.5	1.4 ± 0.3	1.1 ± 0.2	-0.3 [1]			
	Placebo	10.1 ± 2.2	9.8 ± 3.3	-0.3	1.3 ± 0.5	1.4 ± 0.5	0.1 [1]			
Koshak (2017)	Nigella Sativa oil							N/A	N/A	-0.7 [-25.2 - 6.3] [3]
	Placebo							N/A	N/A	-10.0 [-50.6 - 5.7] [3]
Salem (2017)	Nigella Sativa (low dose)	3.8 ± 5.8	4.7 ± 6.0	0.9 ↑ [1]	2.4 ± 6.7	2.3 ± 6.8	-0.1 = [1]	4.5x10^5 ± 7.1x10^5 [5]	3.9x10^5 ± 6.2x10^5 [5]	-6.0 x10^4 = [1,5]
	Nigella Sativa (high dose)	2.8 ± 5.8	3.3 ± 6.0	0.5 ↑ [1]	2.2 ± 6.5	2.1 ± 6.4	-0.1 = [1]	3.9x10^5 ± 4.7x10^5 [5]	3.2x10^5 ± 3.7x10^5 [5]	-7.2 x10^4 ↓ [1,5]
	Placebo	3.0 ± 5.5	2.6 ± 5.4	-0.4 = [1]	1.6 ± 5.7	1.6 ± 5.7	-0.0 = [1]	6.2 x10^5 ± 8.0x10^5 [5]	6.0x10^5 ± 7.5x10^5 [5]	-2.8 x10^4 = [1,5]
Yugandhar (2017)	Extract of *B. serrata* gum resin and *A. marmelos* fruit	12.7 ± 0.6 [6]	22.0 ± 1.4 [6]	1.8 [1,6]	1.6 ± 0.2 [6]	1.1 ± 0.2 [6]	-0.5 [1,6]			
	Placebo	13.6 ± 0.5 [6]	15.4 ± 0.4 [6]	9.4 [1,6]	1.5 ± 0.2 [6]	1.4 ± 0.2 [6]	-0.1 [1,6]			
Supplements										
Ghaffari (2014)	Vitamin E							154.5 ± 33.8	118.3 ± 14.4	-36.2 = [1]
	Placebo							147.2 ± 27.6	127.0 ± 22.3	-20.2 = [1]
Pearson (2004)	Vitamin E							N/A	N/A	1.0 ± 1.2 = [6]
	Placebo							N/A	N/A	1.0 ± 1.5 = [6]

Values are presented as mean ± SD, median [Q1 – Q3] or median [minimum; maximum]. = indicates that within-group changes were not significantly different from baseline; ↑ indicates a significant increase compared to baseline; ↓ indicates a significant decrease compared to baseline; blank indicates that within-group changes were not reported in the article; N/A indicates data could not be extracted. Abbreviations: BL = baseline; FU = follow-up; Th = T helper cell; Ig = immunoglobulin; IFN=interferon; IL = interleukin; MD = Mediterranean diet; 1 = calculated; 2 = ELISA units; 3 = estimated using pixel ruler; 4 = unit is ng/ml; 5 = unit transformed; 6 = ± SEM; 7 = unit was missing in original article and was estimated based on other articles of the same research group.

Table S2.4b. Continued.

First author (year)	Study group	Th1 cytokines			Th2 cytokines and IgE					
		IFN-γ (pg/ml)			IL-4 (pg/ml)			IgE (IU/ml)		
		BL	FU	Change	BL	FU	Change	BL	FU	Change
Weight loss										
Dias-Junior (2014)	Low calorie intake, use of sibutramine and use of orlistat							24.4 ± 6.5	23.80 ± 6.3	-0.6 = [1]
	Placebo							22.4 ± 5.9	17.7 ± 5.3	-4.7 = [1]
Vitamin D3										
Bar Yoseph (2015)	Vitamin D							432.8 ± 465.7	398.1 ± 412.6	-34.7 = [1]
	Placebo							433.8 ± 455.0	539.5 ± 651.8	105.7 = [1]
de Groot (2015)	Vitamin D3 (Cholecalciferol)							29.0 [13.0 - 117.0] [5]	29.0 [13.0 - 88.0] [5]	0.0 = [1,5]
	Placebo							69.0 [1.0 - 2110.0] [5]	47.0 [4.0 - 264.0] [5]	-22.0 = [1,5]
Whole food approaches										
Bseikri (2018)	Nutrient dense bar (CHORI-bar)							518.8 ± 712.2	560.0 ± 734.2	41.5 = [1]
	Placebo							294.3 ± 516.6	247.3 ± 497.2	-47.0 ↓ [1]
Sexton (2013)	High-intervention MD	N/A	N/A	-0.8 ± 0.3 [6]						
	Low intervention MD	N/A	N/A	-0.6 ± 0.4 [6]						
	Placebo	N/A	N/A	-0.4 ± 0.4 [6]						
Sudini (2016)	Broccoli sprouts				1.4 [0.8 - 2.3]	2.2 [1.1 - 3.1]	0.6 [0.1 - 1.0]			
	Placebo				1.8 [0.7 - 2.2]	2.0 [1.3 - 2.6]	0.5 [0.3 - 1.0]			

Values are presented as mean ± SD, median [Q1 – Q3] or median [minimum; maximum]. = indicates that within-group changes were not significantly different from baseline; ↑ indicates a significant increase compared to baseline; ↓ indicates a significant decrease compared to baseline; blank indicates that within-group changes were not reported in the article; N/A indicates data could not be extracted. Abbreviations: BL = baseline; FU = follow-up; Th = T helper cell; Ig = immunoglobulin; IFN=interferon; IL = interleukin; MD = Mediterranean diet; 1 = calculated; 2 = ELISA units; 3 = estimated using pixel ruler; 4 = unit is ng/ml; 5 = unit transformed; 6 = ± SEM; 7 = unit was missing in original article and was estimated based on other articles of the same research group.

Table S2.4c. Within-group changes in Treg cytokines and pro-inflammatory markers.

First author (year)	Study group	Treg cytokines			Pro-inflammatory markers					
		IL-10 (pg/ml)			IL-6 (pg/ml)			CRP (mg/L)		
		BL	FU	Change	BL	FU	Change	BL	FU	Change
Herbs, herbal mixtures and extracts										
Hosseini (2018) Zilaee (2019)	Saffron							0.1 [0.0 - 0.1] [1]	0.0 [0.0 - 0.1] [1]	-0.0 [-0.0 – 0.0] ↓[1]
	Placebo							0.1 [0.0 - 0.1] [1]	0.1 [0.0 - 0.1] [1]	-0.0 [-0.0 - 0.0] = [1]
Khayyal (2003)	Aqueous extract of propolis	88.1 ± 14.3 [2,3]	273.8 ± 35.7 [2,3]	185.7 ↑ [2,3,4]	59.1 ± 7.1 [2,3]	33.5 ± 3.5 [2,3]	-25.6 ↓ [2,3,4]			
	Placebo	100.0 ± 19.1 [2,3]	142.9 ± 28.6 [2,3]	42.9 ↑ [2,3,4]	88.2 ± 17.7 [2,3]	109.4 ± 29.1 [2,3]	-3.0 = [2,3,4]			
Salem (2017)	Nigella Sativa (low dose)	2.4 ± 6.1	2.8 ± 6.3	0.4 = [4]						
	Nigella Sativa (high dose)	1.7 ± 6.6	1.5 ± 6.6	-0.2 = [4]						
	Placebo	2.2 ± 5.8	1.6 ± 6.3	-0.6 = [4]						
Supplements										
Smith (2015)	Soy isoflavone				N/A	N/A	1.0 (0.9; 1.1) =	N/A	N/A	1.0 (0.9; 1.1) =
	Placebo				N/A	N/A	1.0 (0.9; 1.1) =	N/A	N/A	1.0 (0.9; 1.2) =
Weight loss										
Dias-Junior (2014)	Low calorie intake, use of sibutramine and use of orlistat							286.5 ± 62.0	292.8 ± 80.7	24.3 = [4]
	Placebo							409.1 ± 107.0	507.5 ± 124.3	98.4 = [4]
Jensen (2013)	Energy reduction and counselling sessions				1.2 [0.7 - 2.7]	N/A	0.3 [-0.3 - 0.4] =	2.1 [1.5 - 3.3]	N/A	-0.4 [-0.5 - 0.4] =
	Placebo				1.4 [0.7 - 2.0]	N/A	-0.1 [-0.5 - 0.4] =	2.1 [0.7 - 4.0]	N/A	0.7 [-0.1 - 1.9] ↑

Values are presented as mean ± SD, mean (lower bound 95% CI; upper bound 95% CI), median [Q1 – Q3], or median (IQR). = indicates that within-group changes were not significantly different from baseline; ↑ indicates a significant increase compared to baseline; ↓ indicates a significant decrease compared to baseline; blank indicates that within-group changes were not reported in the article; N/A indicates data could not be extracted. Abbreviations: BL = baseline; FU = follow-up; Treg = regulatory T-cell; IL = interleukin; CRP = C-reactive protein; MD = Mediterranean diet; 1 = unit transformed; 2 = estimated using pixel ruler; 3 = ± SEM; 4= calculated.

Table S2.4c. Continued.

First author (year)	Study group	Treg cytokines			Pro-inflammatory markers					
		IL-10 (pg/ml)			IL-6 (pg/ml)			CRP (mg/L)		
		BL	FU	Change	BL	FU	Change	BL	FU	Change
Toennesen (2018)	High protein and low glycemic index diet				1.5 (1.0)	1.3 (0.7)	-0.1 = [4]	1.1 (1.6)	0.9 (1.8)	-0.2 = [4]
	Combination of diet and exercise				1.5 (0.8)	1.6 (0.9)	0.1 = [4]	0.9 (2.1)	1.2 (1.4)	0.3 = [4]
	Placebo				1.70 (1.6)	1.5 (0.8)	-0.2 = [4]	1.1 (2.0)	1.1 (1.3)	-0.1 = [4]
Vitamin D3										
Bar Yoseph (2015)	Vitamin D							2.0 ± 1.0	2.3 ± 1.7	0.3 = [4]
	Placebo							2.1 ± 1.3	2.1 ± 0.9	0.1 = [4]
Kerley (2016)	Vitamin D3	$1.1x10^5$ [$8.9x10^4$ - $1.3x10^5$]	N/A	$-1.3x10^4$ [$-2.5x10^4$ - $3.0x10^3$]				0.3 [0.2 - 0.6]	N/A	0.1 [-0.1 - 0.9]
	Placebo	$1.1x10^5$ [$8.6x10^4$ - $1.5x10^5$]	N/A	$-1.7x10^4$ [$-2.6x10^4$ – ($-2.7x10^4$)]				0.8 [0.5 - 1.5]	N/A	-0.4 [-0.9 - 0.2]
Whole food approaches										
Bseikri (2018)	Nutrient dense bar (CHORI-bar)							3.9 ± 4.3	4.4 ± 6.1	0.5 = [4]
	Placebo							3.8 ± 5.7	3.5 ± 4.9	-0.4 = [4]
Sexton (2013)	High-intervention MD	N/A	N/A	-0.1 ± 0.6 [3]	N/A	N/A	-1.2 ± 0.7 [3]	N/A	N/A	2.1 ± 1.9 [3]
	Low intervention MD	N/A	N/A	-0.4 ± 0.6 [3]	N/A	N/A	-0.5 ± 0.7 [3]	N/A	N/A	2.9 ± 1.7 [3]
	Placebo	N/A	N/A	-0.2 ± 0.6 [3]	N/A	N/A	-0.3 ± 0.7 [3]	N/A	N/A	-0.6 ± 1.7 [3]
Sudini (2016)	Broccoli sprouts				0.6 [0.1 - 1.6]	0.7 [0.0 - 1.4]	-0.1 [-0.6 - 0.0]			
	Placebo				1.6 [0.5 - 2.6]	1.3 [0.4 - 2.8]	0.0 [-1.5 - 0.5]			
Wood (2012)	High antioxidant diet				1.9 [1.1 - 2.2]	1.9 [1.3 - 2.5]	0.0 = [4]	4.2 [0.9 - 9.1]	3.0 [1.3 - 9.5]	-1.2 = [4]
	Placebo				1.9 [1.3 - 3.0]	2.0 [1.3 - 2.9]	0.1 = [4]	2.5 [1.1 - 6.0]	3.3 [1.5 - 6.4]	0.8 = [4]

Values are presented as mean ± SD, mean (lower bound 95% CI; upper bound 95% CI), median [Q1 – Q3], or median (IQR). = indicates that within-group changes were not significantly different from baseline; ↑ indicates a significant increase compared to baseline; ↓ indicates a significant decrease compared to baseline; blank indicates that within-group changes were not reported in the article; N/A indicates data could not be extracted. Abbreviations: BL = baseline; FU = follow-up; Treg = regulatory T-cell; IL = interleukin; CRP = C-reactive protein; MD = Mediterranean diet; 1 = unit transformed; 2 = estimated using pixel ruler; 3 = ± SEM; 4= calculated.

Chapter 3

Non-cholesterol sterols in breast milk and risk of allergic outcomes in the first two years of life

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Abstract

This study aimed to explore associations between non-cholesterol sterol concentrations in breast milk and allergic outcomes in children aged two. Data from the KOALA Birth Cohort Study, the Netherlands, were used. Non-cholesterol sterols were analyzed by gas-liquid chromatography–mass spectrometry in breast milk sampled one-month postpartum (N=311). Sterols were selected for each allergic outcome, i.e., eczema, wheeze, and allergic sensitization, prior to analyses. Associations between the selected sterols with allergic outcomes were analyzed using multiple logistic regression to calculate odds ratios (ORs). The odds of eczema in the first two years of life were lower with higher concentrations of cholestanol (OR (95%CI): 0.98 (0.95; 1.00), p=0.04), lanosterol (0.97 (0.95; 1.00), p=0.02), lathosterol (0.93 (0.87; 0.99), p=0.02), and stigmasterol (0.51 (0.29; 0.91), p=0.02) in breast milk sampled one-month postpartum. None of the sterols were associated with wheeze in the first two years of life. The odds of allergic sensitization at age two were lower with higher concentrations of campesterol in breast milk (OR (95%CI): 0.81 (0.70; 0.95), p=0.01). In conclusion, our data suggests that exposure to higher non-cholesterol sterol concentrations in breast milk may indeed be associated with the prevention of allergic outcomes in the first two years of life.

Introduction

Breastfeeding is the preferred nutrition for newborns and infants [1]. The World Health Organization therefore recommends exclusive breastfeeding for the first six months of life and to combine breastfeeding with complementary foods for children aged from six months to two years and beyond [2]. Breastfeeding has several health benefits for infants. For example, breastfeeding has been associated with a decreased risk of child mortality in the first two years of life [3]. In addition, probiotic bacteria in breast milk play an essential role in developing the gut microbiota in early life by seeding the infant gut [4]. Breastfeeding even has health benefits tracking into adulthood. Breastfeeding has been associated with a lower risk of several non-communicable diseases in adults, such as cardiovascular diseases [5, 6], obesity [7, 8], and type 2 diabetes [7, 9]. Moreover, in recent years there has been increasing interest in the potential role of breastfeeding for the prevention of allergic outcomes in newborns and infants [10-15].

Although results are inconclusive [10], breastfeeding has been associated with a reduced risk of developing asthma [11-13], eczema [13, 14], and allergic diseases [15]. It is therefore important to identify compounds within breast milk that could be responsible for the supposed reduced risk of developing allergic diseases. However, identification of these compounds is difficult, since breast milk composition is highly variable, especially during the first month of breastfeeding [16]. Variability in composition is highest in the milk produced during the first three weeks postpartum: colostrum (produced in first 4-7 days) and transitional milk (produced approximately from day 7-21 postpartum) [17]. This variability may reflect the infant's needs, e.g., for infant growth [18]. The composition of mature milk (produced from approximately day 21 postpartum onwards) is less variable and contains approximately 3-5% (w/w) fat, 6.9-7.2% carbohydrates, 0.8-0.9% protein, and 0.2% mineral constituents [17, 19]. Lipids in breast milk are the most important energy source for infants [16]. The lipid fraction of breast milk mainly consists of triacylglycerol and for approximately 0.5% of cholesterol [20]. It also contains plant sterols, which surprisingly do not reflect the circulating plasma plant sterol concentrations of the mother [21]. In addition, mRNA expression for sterol transporters ABCG5/G8 was previously observed in bovine mammary glands [22]. When this is also the case in human mammary glands, it could explain the presence of the specific plant sterol concentrations in breast milk [21]. Altogether, these findings suggest a regulated transport process of plant sterols into breast milk.

Today, plant sterols are mainly recognized for their LDL-cholesterol lowering effects [23]. However, in a paper published by our group, Plat and colleagues have suggested that plant sterols in breast milk may have a perinatal role, e.g., in growth and development of the child [21]. This rationale was, among others, based on that plant sterols have been shown to interact with immune cells [24]. In more detail, plant sterols and stanols (saturated derivatives of plant sterols) may affect T-helper cell behavior, potentially by interacting with regulatory T-cells (Tregs) [25-27]. This effect of plant sterols and stanols could be relevant in conditions characterized by a disbalance between T-helper cell subsets Th1 and Th2. For instance, a disbalance in T-helper cell activity towards the Th2

profile has been related to increased immunoglobulin E (IgE) concentrations and allergic diseases, such as allergic asthma [28]. Brüll and colleagues studied the effects of plant stanols on immune cells of allergic asthma patients. Based on their *in vitro* and *in vivo* observations, they suggested that plant stanols stimulated Treg and Th1 cell activity, while inhibiting Th2 cell activity [25, 26]. Furthermore, plant sterols share a structural similarity with cholesterol precursors, which are intermediates in the endogenous cholesterol synthesis pathways [21]. Previous research has shown that some of these compounds can also interact with immune cells, thereby affecting immune responses. For example, desmosterol was found to inhibit inflammatory cascades within macrophages [29]. Moreover, mevalonate, which is another intermediate in the cholesterol synthesis pathway, was found to be crucial to induce trained immunity [30]. As with plant sterols, cholesterol precursors are also present in breast milk [21]. Together, these sterol compounds can be grouped as non-cholesterol sterols. However, it is important to consider these non-cholesterol sterols in breast milk as nutrients and not as markers for intestinal cholesterol absorption and endogenous synthesis for which their serum concentrations have been validated [31].

It is unknown whether the suggested effects of non-cholesterol sterols on immune cell behavior translate into a benefit for children when exposed to these compounds in early life. This raises the question whether the amount of non-cholesterol sterols in breast milk could influence immune maturation, alter T-helper cell behavior and immune responses in early life, and thereby prevent allergic diseases. Therefore, this study aimed to determine the association between non-cholesterol sterol concentrations in breast milk and allergic outcomes in breastfed children in the first two years of life.

Methods

Study population

The cohort used in this study is part of the "Kind, Ouders en gezondheid: Aandacht voor Leefstijl en Aanleg" (KOALA) Birth Cohort Study, the Netherlands, which has been described in detail elsewhere [32]. Briefly, recruitment of pregnant women started in October 2000. Participants with a conventional (N=2343) or an 'alternative' lifestyle (N=491) with regard to e.g., child rearing practices or diet (organic or vegetarian) were recruited and enrolled between the 14th and 18th week of gestation. Participants were followed during gestation and up to several years postpartum and completed relevant questionnaires during follow-up. From January 2002, we started collecting biosamples, such as maternal blood at 36 weeks of pregnancy. In this subcohort (KOALA-SUB), other samples were also obtained, including a breast milk sample from the mother one-month postpartum and a venous blood sample from the child at age two. For this study, we used these two samples and the data collected from questionnaires until the age of two. The KOALA study was approved by the Medical Ethical Committee of Maastricht University Medical Center, Maastricht, the Netherlands (MEC 01-139 and 00-182) and the Central Committee on Research Involving Human Subjects, The Hague, the Netherlands (CCMO P01.1265L). Inclusion criteria for the present study were

participation in KOALA-SUB and an available one-month postpartum breast milk sample. Exclusion criteria were prematurity (<37 weeks gestation) and diseases or disorders such as cystic fibrosis, Down's syndrome, and arthritis. The current study included N=311 children (N=141 mothers with conventional lifestyle, N=166 mothers with alternative lifestyle, N=4 mothers have missing data on lifestyle).

Study outcomes

Allergic outcomes of interest were eczema and wheeze during the first two years of life, and allergic sensitization at age two. At that age, the immune system has had the opportunity to mature while being exposed to different concentrations of non-cholesterol sterols in breast milk [33]. The International Study of Asthma and Allergies in Childhood Questionnaire (ISAAC) was used to determine the presence of eczema and wheeze at 3, 7, 12 and 24 months postpartum, as described previously [34]. In short, if parents ever reported symptoms of eczema (itchy rash that was coming and going) or wheeze (wheezing or whistling in the chest) in this questionnaire, the child was defined as a case of eczema or wheeze, respectively. Children who only had diaper rash, rash around the eyes, or scalp scaling were not considered to have eczema. Allergic sensitization against hen's egg, cow's milk, peanut, birch, grass pollen, cat, dog, or house dust mite was determined in a venous blood sample at age two. Allergic sensitization was defined as having specific serum IgE levels >0.30 IU/mL against one of the allergens tested. As described earlier, IgE was measured with a detection limit of 0.10 IU/mL [35, 36].

Breast milk sampling and analysis of non-cholesterol sterols

Methods for breast milk sampling and storage have been described elsewhere [37]. Briefly, breast milk was collected in the morning in sterile tubes (Greiner Bio-One, Kremsmuenster, Austria). A sample was collected from the contra-lateral breast since the last feeding, before breastfeeding the child. The milk samples were kept in the refrigerator (4°C) and picked up by a researcher on the same day. During transport, the milk samples were stored in a cooler (Coleman Company, Inc., Breda, the Netherlands) on packed ice (4°C) until processing on the same day at the Biobank Maastricht. After measuring the volume of the sample, it was mixed (gently shaking by hand) and five Eppendorf tubes (2 ml) were filled with whole milk for storage. Two Eppendorf tubes were filled for creamatocrit measurement. The remaining sample was centrifuged (400g, 12 minutes, no brake, 4°C) to separate the lipid and aqueous fraction. The lipid layer was trimmed off with a pipette and released in plastic storage vials (Sarstedt, Nümbrecht, Germany) and stored at -80°C in the Biobank Maastricht until further processing. Creamatocrit was determined as described previously [38, 39]. In short, milk samples were centrifuged for 15 minutes at 12000 g. The length of the total milk column and of the cream layer were measured directly after centrifuging. Creamatocrit was determined by calculating which percentage of the total length of the milk sample consisted of cream.

For non-cholesterol sterol analysis, the frozen breast milk samples were transported on dry ice and delivered on the same day at the Institute of Clinical Chemistry and Clinical Pharmacology of the University Hospital Bonn, Germany. Here, plant sterol (sitosterol, campesterol, stigmasterol, brassicasterol), cholestanol and cholesterol precursor (lanosterol, lathosterol and desmosterol) concentrations in breast milk were analyzed by gas-liquid chromatography–mass spectrometry (GC-MS), as described elsewhere [40]. Sterol concentrations were corrected for the lipid levels (creamatocrit) of the breast milk sample by dividing the sterol concentrations by creamatocrit expressed as fraction.

Statistical analysis

Two types of exploratory analyses were first conducted to determine which sterols could be associated with allergic outcomes. First, factor analysis was performed to determine correlations between sterol concentrations in breast milk. A varimax rotation was used to maximize between-subject variance and the minimal eigenvalue was set to 1. The obtained factors were then used in multiple logistic regression analysis to determine factors associated with the allergic outcomes, and to calculate odds ratios (ORs) and their corresponding 95% confidence intervals (95%CIs). Second, independent sample t-tests were performed to determine differences in sterol concentrations between cases and controls for each allergic outcome. Based on these two exploratory analyses, sterols were selected for further analysis with $p<0.10$ as selection threshold. Sterols were selected when they: 1) were present in factors that were associated with an allergic outcome in the multiple logistic regression analyses (trend [$p<0.10$] or significant association [$p<0.05$]), or 2) differed in concentration between cases and controls in the independent sample t-tests (trend [$p<0.10$] or significant association [$p<0.05$]). Next, multiple logistic regression models were made for each selected sterol to determine which sterols were significantly associated with allergic outcomes ($p<0.05$). ORs and corresponding 95%CIs were calculated. A priori, confounders to be used in these regression models were determined by drawing and analyzing causal diagrams (DAGs). Confounders that were tested in the models included: study group, smoking, season of milk sampling, gestational age, prepregnancy BMI, maternal age, atopy of parents, maternal education, gender of child, gravidity, duration of breastfeeding, and birthweight. If confounders changed the regression coefficient $\beta 1$ by at least 10%, they were added to regression models. Finally, Spearman correlations were used to explore relations between non-cholesterol sterol and cholesterol concentrations in breast milk.

A result was considered significantly different when $p<0.05$. All analyses were conducted using IBM SPSS Statistics for Windows, Version 25.0 (Armonk, New York, USA).

Results

Baseline characteristics and flow chart

The selection of participants from the KOALA study is shown in Figure 3.1. Of the total cohort (N=2834), the women with an available breast milk sample were selected (N=315). The 311 women who fulfilled the criteria for the current study were selected.

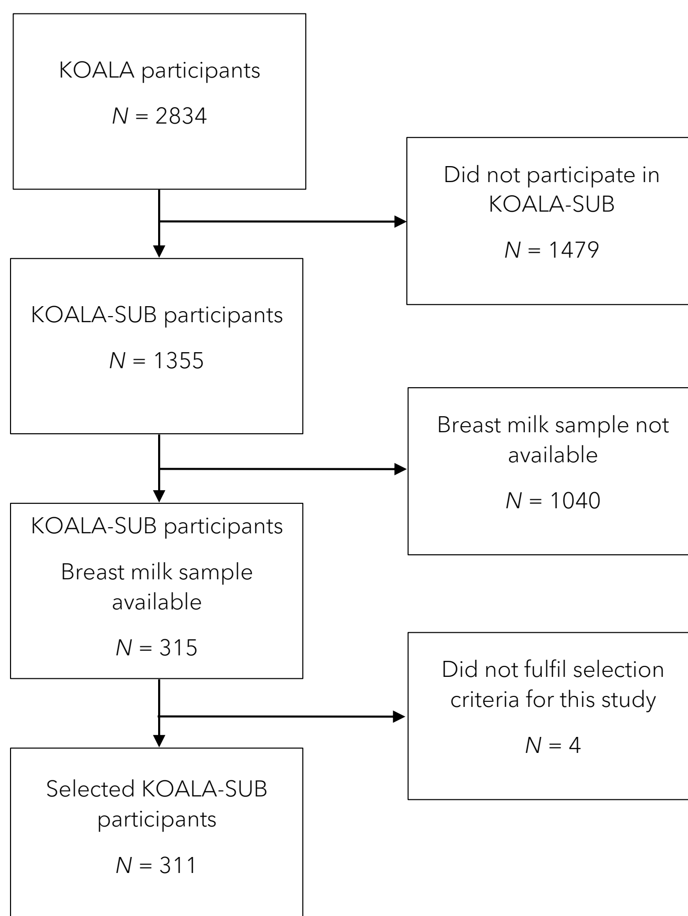

Figure 3.1. Flow chart of participants included in this study.

Characteristics of the study population are shown in Table 3.1. The mean (SD) maternal age and median BMI (IQR) of the 311 mothers at the start of pregnancy were 32.4 (3.9) years and 22.4 (20.6-24.5) kg/m2, respectively. In total, 91 children developed eczema and 79 children developed wheeze in the first two years of life, and 49 children were allergically sensitized against common allergens at age two. The baseline characteristics were comparable between the women with either a conventional or alternative lifestyle. Only sitosterol was higher in the alternative lifestyle group (Table 3.1).

Desmosterol was the non-cholesterol sterol with the highest concentration in breast milk (median (IQR): 52.2 (37.4-70.3) μmol/L), which was 25- to 1000-fold higher as compared to the other sterols. Stigmasterol was the sterol with the lowest concentration (0.05 (0.04-0.06) μmol/L). Overall, non-cholesterol concentrations in breast milk were similar in the women with a conventional lifestyle and an alternative lifestyle.

Selection process of sterols

Exploratory factor analysis

To explore which of the eight non-cholesterol sterols that were analyzed in breast milk were intercorrelated, exploratory factor analysis was performed. Two factors were found based on the sterol concentration in breast milk corrected for creamatocrit (Table 3.2). The two factors separated brassicasterol, stigmasterol, campesterol, and lathosterol (factor 1); and lanosterol and desmosterol (factor 2). Cholestanol and sitosterol loaded on both factors, although to a higher extent on factor 1 than on factor 2.

Multiple logistic regression using the factors

Multiple logistic regression was performed to explore relations between factors 1 and 2 with the allergic outcomes of interest (i.e., eczema and wheeze in the first two years of life, and allergic sensitization at age two) (Table 3.3). None of the factors were significantly associated with eczema, wheeze, or allergic sensitization. However, trends were observed for associations between factor 1 and eczema (OR (95%CI): 0.69 (0.46; 1.03), p=0.07), factor 2 and eczema (0.69 (0.46; 1.04), p=0.08), and factor 1 and allergic sensitization (0.52 (0.26; 1.07), p=0.07).

Independent sample t-tests

Independent sample t-tests were performed to explore which of the individual sterols differed between cases and controls for each allergic outcome (Table 3.4). Lathosterol (p=0.06) and stigmasterol (p=0.08) concentrations in breast milk tended to be lower in eczema cases compared to controls. For wheeze, all sterol concentrations were similar in cases and controls. For allergic sensitization, campesterol concentrations in breast milk were significantly lower in cases compared to controls (p=0.03).

Table 3.2. Factor loadings after varimax rotation. Non-cholesterol sterol concentrations were corrected for creamatocrit.

Non-cholesterol sterol (μmol/Lf)	Factor 1	Factor 2
Cholestanol	0.88	0.34
Brassicasterol	0.83	
Stigmasterol	0.78	
Sitosterol	0.65	0.31
Campesterol	0.64	
Lathosterol	0.37	
Lanosterol		0.93
Desmosterol		0.85

μmol/Lf = μmol per liter milk fat; only factor loadings ≥0.30 are shown; there were no factor loadings ≤-0.30.

Table 3.1. Baseline characteristics of the KOALA-SUB cohort selected for this study. Data are shown as mean (SD) or median (Q1-Q3), unless otherwise indicated.

	Total (N=311)	Conventional lifestyle[1] (N=141)	Alternative lifestyle[1] (N=166)
Maternal age, years (SD)	32.4 (3.9)	31.5 (3.4)	33.1 (4.2)
Prepregnancy BMI, kg/m² (IQR)	22.4 (20.6-24.5)	23.0 (21.5-25.2)	21.7 (20.1-24.0)
Smoking during pregnancy[2], N (%)	5 (2%)	4 (3%)	1 (1%)
Atopic history parents[1], N (%)			
None	113 (37%)	50 (36%)	62 (38%)
Only father	78 (25%)	37 (26%)	40 (24%)
Only mother	64 (21%)	31 (22%)	32 (20%)
Both	52 (17%)	22 (16%)	30 (18%)
Gender child female, N (%)	161 (52%)	74 (53%)	83 (50%)
Duration breastfeeding, N (%)			
1-3 months	64 (21%)	46 (33%)	17 (10%)
4-6 months	70 (23%)	40 (28%)	29 (18%)
7-9 months	70 (23%)	28 (20%)	41 (25%)
10-12 months	53 (17%)	17 (12%)	36 (22%)
≥13 months	53 (17%)	10 (7%)	43 (26%)
Maternal education, N (%)			
Lower	12 (4%)	7 (5%)	5 (3%)
Middle	96 (31%)	53 (38%)	41 (25%)
Higher vocational	131 (42%)	60 (43%)	69 (42%)
Academic	66 (21%)	19 (14%)	47 (28%)
Other	6 (2%)	2 (1%)	4 (2%)
Season breast milk sampling[1], N (%)			
December 2002 – February 2003	112 (37%)	64 (45%)	48 (29%)
March – May 2003	120 (39%)	59 (42%)	61 (37%)
June – September 2003	75 (24%)	18 (13%)	57 (34%)
Gravidity, N (%)			
1	123 (40%)	65 (46%)	58 (34.9)
2	110 (35%)	51 (36%)	57 (34.3)
≥3	78 (25%)	25 (18%)	51 (30.7)
Eczema in first two years[3], N (%)	91 (30%)	41 (30%)	50 (31.1)
Wheeze in first two years[4], N (%)	79 (26%)	37 (27%)	42 (26%)
Allergic sensitization at age 2[5], N (%)	49 (24%)	25 (28%)	24 (22%)
Creamatocrit value[6], % (IQR)	7 (5-9%)	7 (5-9%)	7 (5-9%)
Cholesterol concentration breast milk, mmol/L (IQR)	0.35 (0.28-0.42)	0.35 (0.27-0.43)	0.36 (0.29-0.41)
Cholesterol concentration breast milk corrected for creamatocrit[7], mmol/Lf (IQR)	4.81 (4.14-5.90)	4.81 (4.17-5.93)	4.83 (5.84-6.87)
Non-cholesterol sterol concentrations breast milk, µmol/L (IQR)			
Brassicasterol	0.23 (0.18-0.27)	0.24 (0.19-0.29)	0.22 (0.17-0.25)
Campesterol[7]	0.32 (0.20-0.52)	0.37 (0.21-0.60)	0.28 (0.19-0.46)
Cholestanol	1.51 (1.28-1.73)	1.50 (1.26-1.75)	1.52 (1.31-1.73)
Desmosterol	52.2 (37.4-70.3)	51.7 (36.3-68.3)	54.3 (38.1 -71.8)
Lanosterol	2.03 (1.43-2.89)	2.01 (1.31-2.85)	2.04 (1.50-2.93)
Lathosterol	0.62 (0.40-0.84)	0.62 (0.38-0.87)	0.62 (0.40-0.84)
Sitosterol	0.70 (0.49-1.41)	0.59 (0.45-0.87)	0.82 (0.55-1.48)
Stigmasterol	0.05 (0.04-0.06)	0.05 (0.04-0.06)	0.05 (0.04-0.06)
Non-cholesterol sterol concentrations breast milk corrected for creamatocrit, µmol/Lf (IQR)			
Brassicasterol[7]	3.18 (2.44-4.16)	3.32 (2.53-4.25)	2.95 (2.31-4.10)
Campesterol[8]	4.90 (3.01-7.07)	5.34 (3.50-7.87)	4.27 (2.61-6.13)
Cholestanol[7]	21.0 (17.2-27.7)	22.5 (17.3-27.9)	20.5 (17.0-27.9)
Desmosterol[7]	776.5 (592.7-997.9)	716.1(561.6-957.0)	807.2 (633.6-1026.5)
Lanosterol[7]	29.1 (22.7-37.5)	28.4 (22.8-37.3)	29.7 (22.7-37.5)
Lathosterol[7]	8.69 (6.25-11.9)	8.80 (6.47-12.2)	8.31 (5.99-11.6)
Sitosterol[7]	10.3 (7.17-18.7)	8.26 (6.60-12.6)	13.7 (7.88-23.8)
Stigmasterol[7]	0.68 (0.51-0.93)	0.67 (0.51-0.93)	0.69 (0.51-0.94)

BMI = body mass index; IQR = interquartile range; SD = standard deviation; mmol/Lf = mmol per liter milk fat; 1. Missing data N=4; 2. Missing data N=1; 3. Missing data N=12; 4. Missing data N=9; 5. Missing data N=110; 6. Missing data N=37; 7. Missing data N=8; 8. Missing data N=45.

Table 3.3. Results of the multiple logistic regression analyses to determine relations between the factors and allergic outcomes.

	Factor 1			Factor 2		
Outcome variable	OR	95% CI	*p*-value	OR	95% CI	*p*-value
Eczema (N=256)[1]	0.69	0.46; 1.03	0.07*	0.69	0.46; 1.04	0.08*
Wheeze (N=259)[2]	1.04	0.77; 1.40	0.82	0.99	0.69; 1.42	0.95
Allergic sensitization (N=171)[3]	0.52	0.26; 1.07	0.07*	1.17	0.79; 1.73	0.43

OR: odds ratio; 95% CI: 95% confidence interval; 1: adjusted for season, atopy of parents, maternal education, and duration of breastfeeding; 2: adjusted for smoking, season, gestational age, prepregnancy BMI, atopy of parents, maternal education, gender of child, gravidity, and duration of breastfeeding; 3: adjusted for study group, smoking, season, prepregnancy BMI, atopy of parents, maternal education, gender of child, gravidity, duration of breastfeeding, and birthweight. *p<0.10.

Table 3.4. Results of the independent sample t-tests to explore differences between non-cholesterol sterol concentrations in breast milk between cases and controls for eczema (N=80 cases, N=179 controls), wheeze (N=67 cases, N=195 controls), and allergic sensitization (N=41 cases, N=130 controls). Non-cholesterol sterol concentrations were adjusted for creamatocrit.

	Eczema			Wheeze			Allergic sensitization		
Sterol (µmol/Lf)	Mean difference	95% CI	*p*-value	Mean difference	95% CI	*p*-value	Mean difference	95% CI	*p*-value
Brassicasterol	-0.11	-0.76; 0.55	0.75	0.16	-0.52; 0.84	0.64	-0.25	-1.06; 0.55	0.54
Campesterol	-0.76	-1.85; 0.32	0.17	0.11	-1.03; 1.26	0.84	-1.78	-3.40; -0.16	0.03**
Cholestanol	-3.19	-7.33; 0.94	0.13	0.30	-4.08; 4.67	0.89	-2.68	-8.04; 2.68	0.33
Desmosterol[1]	-0.04	-0.14; 0.06	0.44	0.01	-0.11; 0.13	0.83	-0.05	-0.22; 0.12	0.57
Lanosterol	-2.03	-5.55; 1.48	0.26	-0.36	-4.82; 4.10	0.88	0.94	-4.77; 6.65	0.75
Lathosterol	-1.18	-2.39; 0.03	0.06*	-0.93	-2.23; 0.36	0.16	-0.53	-2.22; 1.16	0.54
Sitosterol	-0.13	-0.28; 0.03	0.11	-0.10	-0.26; 0.07	0.25	-0.13	-0.31; 0.04	0.14
Stigmasterol	-0.01	-0.02; 0.00	0.08*	0.00	-0.01; 0.01	0.89	-0.01	-0.02; 0.01	0.36

95% CI: 95% confidence interval; µmol/Lf = micromoles per liter milk fat; 1. concentration in mmol/Lf; *p<0.10; **p<0.05

Multiple logistic regression using selected sterols

Eczema

Based on the multiple logistic regression analysis using the obtained factors (Table 3.3), all sterols included in factors 1 and 2 were selected for eczema. In addition, based on the independent sample t-tests (Table 3.4), lathosterol and stigmasterol were selected for eczema. Thus, all eight sterols were included in the final multiple logistic regression analyses. Separate models were made for each individual sterol (Table 3.5). The odds of eczema in the first two years of life were significantly lower with higher concentrations of cholestanol (OR (95%CI): 0.98 (0.95; 1.00), *p*=0.04), lanosterol (0.97 (0.95; 1.00), *p*=0.02), lathosterol (0.93 (0.87; 0.99), *p*=0.02), and stigmasterol (0.51 (0.29; 0.91), *p*=0.02) in breast milk one-month postpartum. The other sterols did not affect the odds of eczema during the first two years of life.

Table 3.5. Results of the multiple logistic regression analyses using the selected sterols for eczema. Separate models were made for each individual sterol. Non-cholesterol sterol concentrations were adjusted for creamatocrit.

Sterol (μmol/Lf)	N	OR	95% CI	*p*-value
Brassicasterol[1]	264	0.95	0.87; 1.03	0.22
Campesterol[2]	259	0.95	0.88; 1.03	0.25
Cholestanol[3]	264	0.98	0.95; 1.00	0.04*
Desmosterol[a,1]	264	0.52	0.22; 1.22	0.13
Lanosterol[4]	267	0.97	0.95; 1.00	0.02*
Lathosterol[5]	267	0.93	0.87; 0.99	0.02*
Sitosterol[6]	264	0.98	0.95; 1.00	0.09
Stigmasterol[6]	264	0.51	0.29; 0.91	0.02*

OR: odds ratio; 95% CI: 95% confidence interval; μmol/Lf = micromoles per liter milk fat; a: unit is mmol/Lf; 1: adjusted for season, atopy of parents, maternal education, duration of breastfeeding, and gravidity; 2: adjusted for season and maternal education; 3: adjusted for atopy of parents and maternal education; 4: adjusted for season, maternal education, and duration breastfeeding; 5: adjusted for maternal education; 6: adjusted for season, atopy of parents, and maternal education; *$p<0.05$.

Wheeze

None of the factors from exploratory factor analysis were associated with wheeze, nor were there differences in sterol concentrations in breast milk between cases and controls. Therefore, none of the sterols were evaluated in further analysis for wheeze.

Allergic sensitization

Based on the multiple logistic regression analysis using the obtained factors (Table 3.3), the sterols included in factor 1 were selected for allergic sensitization. In addition, based on the independent sample t-tests (Table 3.4), campesterol was selected for allergic sensitization. Thus, brassicasterol, campesterol, cholestanol, lathosterol, sitosterol, and stigmasterol were included in the final multiple logistic regression analyses. Separate models were made for each individual sterol (Table 3.6). The odds of allergic sensitization at age 2 were significantly lower with a higher concentration of campesterol in breast milk one-month postpartum (OR (95%CI): 0.81 (0.70; 0.95), *p*=0.01). The other sterols did not affect the odds of allergic sensitization at age 2.

Table 3.6. Results of the multiple logistic regression analysis using the selected sterols for allergic sensitization. Non-cholesterol sterol concentrations were adjusted for creamatocrit.

Sterol (µmol/Lf)	N	OR	95% CI	*p*-value
Brassicasterol[1]	176	0.93	0.78; 1.12	0.47
Campesterol[2]	171	0.81	0.70; 0.95	0.01*
Cholestanol[3]	176	0.98	0.95; 1.01	0.26
Lathosterol[4]	176	0.99	0.91; 1.07	0.77
Sitosterol[5]	176	0.97	0.93; 1.01	0.13
Stigmasterol[5]	176	0.77	0.42; 1.40	0.38

OR: odds ratio; 95% CI: 95% confidence interval; µmol/Lf = micromoles per liter milk fat; 1: adjusted for study group, season, and duration breastfeeding; 2: adjusted for season and duration breastfeeding; 3: adjusted for smoking; 4: adjusted for smoking, season, and duration breastfeeding; 5: adjusted for smoking and season $^*p<0.05$.

Cholesterol and allergic outcomes

Non-cholesterol sterol concentrations were significantly correlated to cholesterol concentrations (corrected for creamatocrit) in breast milk, except for lanosterol and lathosterol concentrations (Table 3.7). Therefore, relationships between cholesterol concentrations (corrected for creamatocrit) in breast milk and allergic outcomes were also considered. However, the odds of having eczema or wheeze in the first two years of life were not lower with higher cholesterol concentrations, nor were the odds for allergic sensitization at age two (data not shown). Hence, non-cholesterol sterols did not act as a marker for cholesterol. The reported associations can instead be attributed specifically to the non-cholesterol sterols.

Table 3.7. Spearman correlations between cholesterol and non-cholesterol sterols.

Sterol (µmol/Lf)	Spearman's ρ Cholesterol (mmol/Lf)
Brassicasterol	-0.42**
Campesterol[1]	0.40**
Cholestanol	-0.45**
Desmosterol	-0.13*
Lanosterol	-0.07
Lathosterol	-0.04
Sitosterol	-0.37**
Stigmasterol	-0.51**

N=311. µmol/Lf = micromoles per liter milk fat; 1. N=303; $^*p<0.05$; $^{**}p<0.001$.

Discussion

The aim of this study was to determine the association between non-cholesterol sterols in breast milk and allergic outcomes in breastfed children in the first two years of life. We found that the odds of eczema during the first two years of life were significantly lower with higher concentrations of cholestanol, lanosterol, lathosterol, and stigmasterol in breast milk one-month postpartum. We also showed that the odds of allergic sensitization at age 2 were significantly lower with a higher concentration of campesterol in breast milk. None of the sterols were associated with wheeze during the first two years of life. Study groups (women with a conventional or 'alternative' lifestyle

with regards to e.g., child rearing practices) were not further compared, since study group did not seem to influence the reported associations. *A priori*, we hypothesized that exposure of the immune system to non-cholesterol sterols through breastfeeding early in life influences the maturation of the immune system and thereby prevents allergic outcomes later in life. Our results presented here support this hypothesis and are in line with previous suggestions that non-cholesterol sterols may play a role in infant health [21].

Non-cholesterol sterols in serum are known for their relationship with cholesterol metabolism [21, 23, 24]. This group of sterols can be divided into two subgroups: i.e., some are diet derived and considered as markers for intestinal cholesterol absorption (brassicasterol, campesterol, cholestanol, sitosterol, stigmasterol), while others are endogenously synthesized and markers for cholesterol synthesis (desmosterol, lanosterol, lathosterol). However, these two subgroups were not identified when exploratory factor analysis was performed using concentrations of these non-cholesterol sterols in breast milk (with or without correction for creamatocrit). This finding is in line with our hypothesis that sterols provided by breast milk should be considered as nutrients (and not as markers for intestinal cholesterol absorption and endogenous synthesis), which may have specific effects in the body in early life, e.g., involvement in the maturation of the immune system. In addition, studies in adults have also reported effects of non-cholesterol sterols on the immune system. Brüll and colleagues [25] used antibody production to a hepatitis A vaccine as a measure for immune function in adult asthma patients that received either plant stanols or placebo. They reported that daily intake of 4 grams of plant stanols increased antibody production by 22% compared to placebo [25]. In addition, changes in serum plant stanol concentrations were positively correlated to the Th1/Th2 cytokine balance towards more Th1 activity [25]. These results, together with our current findings, indicate that consuming plant sterols and stanols may not only affect cholesterol metabolism, but may also be related to developing and sustaining immune function throughout life.

Results of studies evaluating the effect of breastfeeding on allergic outcomes in children are inconclusive, and information on the maternal diet during breastfeeding is often missing [41]. Therefore, a clear recommendation for future studies is to include data about maternal diet composition during pregnancy and breastfeeding, and/or breast milk composition. Unfortunately, no studies have related non-cholesterol sterols in breast milk and infant feeding with allergic outcomes in children, which makes it difficult to compare our study results. However, results can be compared with studies evaluating the effect of children's intake of diets or foods rich in these non-cholesterol sterols on allergic outcomes. Our results are in accordance with a review, which stated that plant-based diets and diets similar to the Mediterranean diet, which are generally rich in plant sterols, could reduce inflammation and asthma symptoms in children [42]. Another study reported a negative association between fruit and vegetable intake and allergic symptoms in children [43]. Moreover, the results of our study could be compared to

studies evaluating the effects of non-cholesterol sterols on immune-related outcomes in other parts of the body, such as the gut. Van Gorp and colleagues found that intra amniotic administration of β-sitosterol and campesterol prevented gut inflammation in fetal lambs that were intra-amniotically infected with *Ureaplasma parvum* [44]. Plasma IL-6, influx of mucosal myeloperoxidase-positive cells, and intestinal damage were all lowered by the intra amniotic administration of plant sterols [44]. In addition, de Smet and colleagues showed that an acute intake of plant stanols down-regulated genes regulating T-cell functioning in the jejunum of healthy volunteers [45]. These two studies also indicate that non-cholesterol sterols are able to influence immune cell behavior, although the exact mechanisms remain unclear.

Considering our results and the studies described above, it is tempting to suggest that higher intakes of non-cholesterol sterols via breast milk would result in better health outcomes. Infant formulas sometimes also contain high concentrations of plant sterols, especially when produced with vegetable oil as fat source [46], while formula feeding has not been associated to better health outcomes compared to breastfeeding [47-49]. Claumarchirant and colleagues reported that total plant sterol concentrations (sum of brassicasterol, campesterol, β-sitosterol, stigmasterol, and sitostanol) in various infant formulas ranged between 3.1-5.0 mg/100mL (78.1-132.3 μmol/L). These concentrations are higher than those reported in our study, where the median total plant sterol concentration in breast milk (sum of brassicasterol, campesterol, sitosterol, and stigmasterol) was 1.3 μmol/L. However, desmosterol (0.2-0.4 mg/100mL (6.2-11.1 μmol/L)) and cholesterol (1.6-5.1 mg/100mL (0.04-0.1 mmol/L)) concentrations in infant formulas were lower as compared to the concentrations we found in breast milk (desmosterol: 52.2 μmol/L; cholesterol 0.35 mmol/L). Hence, not only absolute concentrations, but also ratios between individual sterols differ between breast milk and infant formula. In more detail, the ratio between cholesterol and sitosterol concentrations in infant formula ranges from approximately 0.6 to 1.8 [46], whereas in our study the median cholesterol concentration was 500 times higher than the sitosterol concentration (350 μmol/L and 0.70 μmol/L, respectively). The lower concentration of cholesterol in infant formulas as compared to breast milk induces higher endogenous cholesterol synthesis in formula fed infants, whereas breastfed infants have a higher intestinal cholesterol absorption [50, 51]. To the best of our knowledge, the bioavailability of non-cholesterol in breast milk and infant formula has not been studied. Therefore, it is currently not completely understood how serum non-cholesterol concentrations in children are affected by either breastfeeding or formula feeding.

Additionally, it is currently unknown whether the differences in sterol concentrations between breast milk and infant formula are associated with the difference in the immune responses of breastfed versus formula fed children. However, it should be kept in mind that breast milk and infant formula differ in many more aspects that could affect immune system development than solely non-cholesterol sterol and cholesterol concentrations. For example, breast milk contains human immune factors, which help forming the

neonatal immune system [52]. Ultimately, the relation between early infant feeding and allergic outcomes is not fully understood. Future studies should further evaluate: 1) whether the reported effects of non-cholesterol sterols on allergic outcomes in our study can be attributed to their concentration in breast milk, 2) whether the ratio between the different sterols could also play a role, and 3) whether other components in breast milk are potentially involved in this association.

Although the associations between sterols in breast milk, eczema, and allergic sensitization were statistically significant, none of the sterols were significantly associated with wheeze in the first two years of life. There are several wheezing phenotypes, based on the age at which wheezing first occurs [53]. For example, phenotypes such as transient early wheeze and prolonged early wheeze are characterized by wheezing only in the first years of life, while wheezing disappears as the child gets older. Other phenotypes such as intermediate onset wheeze or late onset wheeze are characterized by wheezing occurring at a later age (18-42 months old). The phenotypes characterized by later onset wheeze are strongest associated with allergic outcomes later in life [53]. For this study, it means some of the children could have suffered from the phenotypes characterized by early onset wheeze, which could also be caused by viral infections [54]. It would be interesting to evaluate the association between non-cholesterol sterols in breast milk and wheezing or even asthma at a later age, and to take wheezing phenotypes into account. Unfortunately, the number of late onset wheeze and asthma cases in the subgroup with breast milk samples was insufficient to allow proper statistics. The associations between non-cholesterol sterols, wheeze, and asthma should therefore be evaluated in a larger study.

Another limitation of this study was the extensive selection of sterols and that multiple allergic outcomes were tested. The exploratory nature of this study may have increased the chance of type I errors. Therefore, data should be interpreted with care and additional studies are needed to confirm or refute our findings. In addition, future studies should consider if there are optimal sterol concentrations in breast milk, if ratios between different sterols in breast milk play a role in the prevention of allergic outcomes, and how breast milk composition fits into this association.

In conclusion, our data suggests that exposure to higher non-cholesterol sterol concentrations (corrected for creamatocrit) in breast milk may indeed contribute to the prevention of allergic outcomes, such as eczema and allergic sensitization at the age of two. Evidence regarding the elaborate role of sterols in human health rapidly grows and should be explored in further detail. Future studies should consider a role for breast milk composition and maternal diet during pregnancy and lactation in the association between breastfeeding and allergic outcomes in children. The effects of sterol intake via breastfeeding versus bottle feeding on allergic disease prevention should also be studied in more detail.

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Chapter 4

Associations between cholesterol metabolism and lung function differ between asthmatic and non-asthmatic children

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Submitted

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Chapter 5

Plant stanol consumption increases anti-COVID-19 antibody responses, independent of changes in serum cholesterol concentrations: a randomized controlled trial

Lieve van Brakel, Ronald P. Mensink, Dieter Lütjohann, and Jogchum Plat

Abstract

Background: People with overweight/obesity generally have impaired immune responses, resulting amongst others in an increased risk of severe COVID-19 complaints and hospitalization, as well as decreased antibody production after vaccinations. Plant stanol ester previously increased the combined immunoglobulin M and G (IgM/IgG) antibody titers towards a hepatitis A vaccination in allergic asthma patients, but the underlying mechanism is unknown.

Objective: We evaluated whether plant stanol ester consumption improves the immune response in subjects with overweight/obesity after a COVID-19 vaccination.

Methods: A double-blind, randomized, placebo-controlled trial was performed (registered at clinicaltrials.gov [NCT04844346]). Thirty-two subjects with overweight/obesity consumed products with added plant stanols (4g/day; provided as plant stanol ester) or control at least two weeks before receiving their COVID-19 vaccination until 4 weeks after vaccination. Antibody titers were analyzed weekly and statistically analyzed using mixed models. Serum metabolic markers and cytokine profiles were also analyzed.

Results: IgM concentrations against the COVID-19 spike protein differed between groups, with the largest difference observed 2 weeks after vaccination (31.2 [0.43; 62.1] BAU/mL, or +139%; Group*Time: p=0.031). Subjects that produced very low IgM antibodies produced as expected hardly any IgG antibodies. In those with IgG sero-conversion, IgG spike concentrations also differed between groups (71.3 [2.51; 140.1] BAU/mL; Group p=0.043). Stimulated cytokine concentrations decreased in the plant stanol ester group compared to control in all three cytokine domains (i.e., pro-inflammatory, Th1/Th17, Th2/Treg). Between-group differences in serum LDL-C or other metabolic markers were not observed.

Conclusion: Consuming plant stanols (4g/day) affects immune responses to COVID-19 vaccinations, translating into increased serum anti-COVID-19 IgM concentrations in subjects with overweight/obesity. Only in IgG seroconverted subjects, serum anti-COVID-19 IgG concentrations also increased. These effects were independent of reductions in LDL-C. These results suggest that this high-risk group for COVID-19 complications could benefit from plant stanol consumption.

Introduction

Globally, 39% and 13% of adults are overweight or obese, and its prevalence is expected to increase even further [1-3]. During the COVID-19 pandemic it became evident that the impaired immune system of people with obesity translated into more severe illness and hospitalization after a COVID-19 infection [4, 5]. The decreased immune responses of people with overweight or obesity can be measured best by analyzing changes in specific antibody titers in response to a vaccination [6]. Immunoglobulin M (IgM) is the first antibody produced upon vaccination [7] and after class-switching, a more pronounced IgG response will occur [8-10]. The production of specific antibodies in response vaccination is called seroconversion. Individuals with obesity in general have suboptimal vaccination responses, potentially because of impaired T cell activation or functioning [11, 12]. These suboptimal responses have also been observed in other population groups with impaired immune responses, such as frail older adults [13]. Seroconversion to a vaccine has become increasingly important during the COVID-19 pandemic, especially since an early COVID-19 vaccination trial showed that the initial antibody production as response to the BioNTech/Pfizer COVID-19 vaccine was impaired for people with a higher body mass index (BMI) [14]. This finding raised the question how antibody titers to COVID-19 vaccinations can be increased.

Lifestyle interventions may improve immunological outcomes in people with a compromised immune function [15-19]. Our group previously showed that consumption of a plant stanol enriched yoghurt by asthma patients increased antibody production following a hepatitis A vaccination [20]. This effect was most pronounced in a subgroup of participants with overweight or obesity [20, 21], indicating that especially those with a compromised response benefit from immunomodulation by plant stanol ester. Toll-like receptor 2 (TLR-2) activation was essential in the underlying mechanism to activate regulatory T cells (Tregs) and ultimately the Th1/Th2 balance [20, 22]. In contrast, high dietary cholesterol intakes decreased immune responses in mice, leading to an increased morbidity after infection with influenza A [23]. Therefore, it is also possible that the observed effects of plant stanol ester on hepatitis A antibody titers could be attributed to the plant stanol induced reduction in intestinal cholesterol absorption and corresponding lowering of serum low-density lipoprotein cholesterol (LDL-C) concentrations [20, 22]. Irrespective of the underlying mechanism, these findings suggest that consuming products enriched with plant stanol ester can be beneficial for people with altered immune responses that are at risk for severe COVID-19, such as people with overweight or obesity [4, 5, 24, 25].

Therefore, the primary aim of this study was to demonstrate clinical benefits of plant stanol ester consumption on the vaccination response to a COVID-19 vaccine in adults with overweight or obesity aged 18 years or older. The secondary aims of this study were to evaluate the effect of plant stanol ester on: 1) immunological parameters and leukocyte count (e.g., hs-CRP, stimulated and unstimulated cytokine production, leukocyte total and differential count), and 2) metabolic parameters after vaccination (e.g., serum lipid and lipoprotein profiles, glucose and insulin).

Methods

Study population

Forty-eight men and women were recruited via advertisements or approached by us if they had previously participated in other studies from our department and consented to be approached for future intervention studies. Participants were eligible for participation if they: 1) were 18 years of age or older, 2) had a BMI between 27 and 35 kg/m2, 3) did not use any products enriched with added plant sterols or stanols at the moment of inclusion, 4) were willing to abstain from commercially available products enriched with added plant sterols or stanols during the study, and 5) were willing not to change the intake of fish oil and other supplements during the study. Participants were excluded when they: 1) had already received a COVID-19 vaccination, 2) had previously been tested positive for COVID-19, irrespective of the type of test used, 3) were allergic to the study product, 4) had donated blood in the month prior to the study or were planning to donate blood during the study, 5) were pregnant or breastfeeding, 6) consumed more than 20 alcohol drinks per week, or 7) regularly used soft and/or hard drugs. Participants were first screened via telephone questionnaires and invited to participate if they fulfilled all criteria. Height and weight were measured at the first study visit to calculate their BMI. Written informed consent was provided by all participants at the start of the first study visit. This study was approved by the medical ethical committee of Maastricht University and Medical Centre (NL76906.068.21) and registered at clinicaltrials.gov (NCT04844346).

Study design and measurements

This randomized, placebo-controlled, double-blind, parallel design had an intervention period of at least 6 weeks (Figure 5.1) and was carried out between April 2021 and January 2022. The study consisted of six visits to measure anthropometrics (height, weight, waist and hip circumference) and to collect a venous blood sample. Participants were fasted for at least 12 hours and abstained from alcohol the day before each visit. Participants kept a diary throughout the study, in which they noted the number of products consumed, medical complaints if any (also side effects of the COVID-19 vaccine), medication use, and general practitioner's or hospital visits. A validated food frequency questionnaire was completed at the start and end of the study to estimate dietary intake. Participants were instructed not to change their dietary habits during the study.

Visit 1 (T=-1) was at least ten days prior to receiving the COVID-19 vaccination, visit 2 (T=0) was scheduled approximately one day before (but never after) receiving the COVID-19 vaccination, and visits 3 to 6 (T=1 to T=4) in the four weeks after the COVID-19 vaccination, with one visit per week. The scheduling of the COVID-19 vaccination was imposed by the Dutch health authorities and not known when participants were included. Therefore, the interval between visits 1 and 2 could differ between

participants, but was at least 10 days. Participants started consuming the experimental products on the day of visit 1 (T=-1).

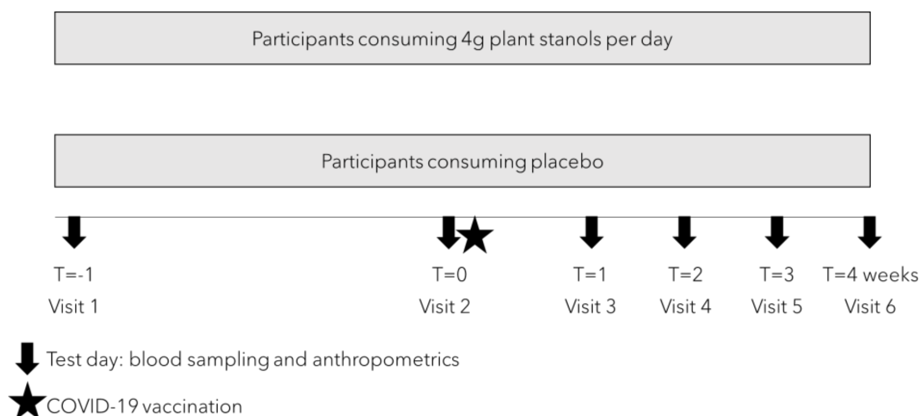

Figure 5.1. Study design of the randomized, placebo-controlled, double-blind, parallel study. Study duration depended on the timing of the COVID-19 vaccination (regulated by the Dutch health authorities).

Intervention products

Participants were randomly assigned to the plant stanol ester or control groups (1:1 allocation), stratified for age and sex. The test products were oat-based proprietary drinks produced by Raisio Nutrition Ltd, Raisio, Finland. The plant stanol oat-based drinks (100 mL) contained 2 grams of plant stanols. Plant stanols were provided as plant stanol ester, esterified to rapeseed oil fatty acids. Control oat-based drinks (100 mL) contained rapeseed oil without added plant stanols. Participants consumed two drinks per day: one at breakfast and one at dinner, with or directly after the meal to optimize the bioavailability of the plant stanols. Drinks had to be stored in the refrigerator. Any unused study products had to be returned at the end of the study and counted to estimate compliance.

Biochemical analyses

During each visit, blood was collected in serum separator tubes and ethylenediaminetetraacetic acid (EDTA) tubes (Becton, Dickson and Company, Franklin Lanes, NY, United States). Serum separator tubes (8.5 mL) clotted at room temperature for at least 30 minutes prior to centrifugation at 1300xg for 10 minutes at 21°C. At each visit, one of the EDTA tubes (4 mL) was directly placed on ice after sampling and centrifuged within 15 minutes at 1300xg for 10 minutes at 4°C. Serum and EDTA plasma aliquots were snap frozen in liquid nitrogen and stored at -80°C until further analysis. Directly after withdrawal, another EDTA tube (4 mL) was used for leukocyte total and differential cell count. In addition, an anti-CD3/CD28 TruCulture tube (Rules-Based

Medicine, Austin, TX, United States) was collected during visits 1, 2, and 6. After sampling, these tubes were placed in an incubator for 48 hours at 37°C, after which a TruCulture filter (Rules-Based Medicine, Austin, TX, United States) was placed in the tube 1-3 mm above the sediment. TruCulture tubes with an inserted filter were stored at -80°C until further analysis, according to instructions by the manufacturer.

Serum non-cholesterol sterols

Non-cholesterol sterols were measured in all serum samples at the Institute of Clinical Chemistry and Clinical Pharmacology of the University Hospital Bonn, Germany. Serum plant stanols (sitostanol, campestanol), plant sterols (sitosterol, campesterol, stigmasterol, brassicasterol), cholestanol, intermediates in endogenous cholesterol synthesis pathways (desmosterol, lanosterol, and lathosterol), cholesterol, and bile acid precursors (7α-hydroxycholesterol [7α-OH-CH] and 27-hydroxycholesterol [27OH-CH]) were analyzed by gas-liquid chromatography–mass spectrometry (GC-MS), as described elsewhere [26, 27]. Serum non-cholesterol sterol concentrations were standardized for serum total cholesterol (TC) concentrations. Participants in the intervention group without increases in TC-standardized plant stanol levels were considered to be non-compliant.

Antibody responses

Serum anti-COVID-19 IgM and IgG (SARS-CoV-2 Nucleocapsid [N], SARS-CoV-2 S1 receptor binding domain [RBD], and SARS-CoV-2 Spike) were measured in samples from the day before vaccination (visit 2, T=0) until the final study visit (visit 6, T=4 weeks after vaccination). All antibodies were measured using V-PLEX Kits (IgM: V-PLEX SARS-CoV-2 Panel 2 [IgM] Kit; IgG: V-PLEX SARS-CoV-2 Panel 2 [IgG] Kit; Meso Scale Discovery, Rockville, MD, United States).

Cytokines, hs-CRP, and leukocyte count

Unstimulated cytokines (IL-6, IL-8, TNF-α) were analyzed in serum samples before vaccination (start of study [T=-1], the day before vaccination [T=0]), and at the final visit (T=4) according to the protocol of the manufacturer (V-PLEX Human Inflammatory Panel II [4-plex]; Meso Scale Discovery, Rockville, MD, United States).

Stimulated cytokines were analyzed in the supernatant of anti-CD3/CD28 stimulated TruCulture tubes at the Rules-Based Medicine Lab, Austin, TX, United States sampled at T=-1 or 0 versus T=4. Cytokines were analyzed using two Multi-Analyte-Profiling (MAP) assays (CytokineMAP A and CytokineMAP B; Rules-Based Medicine, Austin, TX, United States). A standardized Th1/Th2 ratio was calculated with interferon-γ (IFN-γ) and IL-2 as Th1 cytokines and IL-4 and IL-10 as Th2 cytokines, as described elsewhere [28].

hs-CRP concentrations (CRP CP, Horiba ABX, Montpellier, France) were analyzed in all serum samples. Leukocyte total and differential count were also analyzed in all samples shortly after blood drawing using the hematology analyzer Sysmex XN900 (Sysmex

Europe GmbH, Norderstedt, Germany) at the Laboratory of Central Diagnostics at the MUMC+, Maastricht, the Netherlands.

Metabolic markers

Serum lipids and lipoproteins were analyzed in samples at all time points. Serum triacylglycerol (TAG) concentrations were analyzed (GPO-PAP, Roche Diagnostic Systems, Mannheim, Germany) at the Laboratory of Central Diagnostics at the MUMC+, Maastricht, the Netherlands. Serum TC and high-density lipoprotein cholesterol (HDL-C) were analyzed enzymatically using the CHOD-PAP method (Roche Diagnostic Systems, Mannheim, Germany) and - for HDL-C - after precipitation of apoB-containing lipoproteins (phosphotungstate precipitant; Roche Diagnostic Systems, Mannheim, Germany). Serum LDL-C concentrations were calculated using the Friedewald formula [29]. Serum non-HDL-C concentrations were calculated by subtracting HDL-C from TC concentrations. Plasma glucose (Glucose HK CP, Horiba ABX, Montpellier, France) and serum insulin (human-specific ELISA, Crystal Chem, Zaandam, the Netherlands) were also analyzed in samples at all time points. HOMA-IR was calculated using the formula: (glucose [mmol/L]*insulin [mU/L])/22.5 [30].

Statistical analysis

Power calculations were based on effect sizes and standard deviations observed in a study related to the immunogenicity of the AstraZeneca COVID-19 vaccine and in an earlier study of our group related to effect sizes after plant stanol ester intake on hepatitis A vaccination responses [20, 31]. To detect a true difference of 40% (standard deviation [SD]: 63.2%) in IgG responses between the plant stanol ester and control groups, with a power of 80% and a two-sided significance level of 5%, a total sample size of N=80 participants was estimated (i.e., N=40 per group).

All data are presented as means ± SDs or median (interquartile range [IQR]) unless indicated otherwise. Normality was checked using the Shapiro-Wilk test. Differences in baseline characteristics between study groups were analyzed using independent sample t-tests, Mann-Whitney U tests, or chi-square tests as appropriate. Linear mixed model analyses were performed to determine a potential significant difference in changes over time and between groups. Covariance structures were chosen for each model using a top-down approach. In all models, age, sex, time, and study group were included as fixed factors and a group*time interaction term was included. The interaction term was removed from the model when it did not reach statistical significance. The group effect was reported, unless the interaction term reached statistical significance. A Bonferroni correction was applied when the interaction term was statistically significant to determine at which time points responses between groups differed. Time between the start of the study and vaccination date were added to all models to correct for variation in study duration. This variable was removed when not statistically significant. All models were corrected for corresponding baseline concentrations, except for the anti-COVID-19 IgM and IgG models, since baseline IgM

and IgG concentrations were nearly zero. In addition to the main analyses, a sensitivity analysis was performed for anti-COVID-19 IgG titers. Only participants that showed IgG seroconversion (i.e., IgG titers >50 binding antibody units [BAU]/mL) were used in these models. Differences in characteristics between participants that did or did not show IgG seroconversion (i.e., responders and non-responders) were analyzed using independent sample t-tests, Mann-Whitney U tests, or chi-square tests as appropriate. Differences in cytokine concentrations and dietary intakes between groups were compared using two-sided independent sample t-tests, or Mann-Whitney U tests as appropriate. P-values <0.05 were considered to be statistically significant. All analyses were performed using IBM SPSS Statistics version 26.0 for Windows (IBM Corp., Armonk, NY, United States).

Results

Participants

Forty-eight participants were screened before the first round of COVID-19 vaccinations for all age groups in the Netherlands had ended, of which 38 started the study. These participants were randomized to receive either the plant stanol ester oat-based drink or the control oat-based drink. In total, 4 participants dropped out due to various reasons and 34 participants completed the trial. Data from 32 participants were included in the analyses (Figure 5.2). In addition, LDL-C concentrations for one participant could not be calculated, because serum TAG concentrations were too high for reliable use of the Friedewald formula [29]. Also, two participants were excluded from analysis of all antibody data (but not of the other study outcomes), since increases in antibody concentrations throughout the study were too high to be considered as primary immune response to COVID-19 (i.e., >2000 BAU/mL) [20].

The mean age of the total study population was 47±14 years and the median BMI was 30.2 (28.7-32.0) kg/m^2. Most participants received the Pfizer/BioNTech COVID-19 vaccine (75%). Baseline characteristics were comparable between study groups (Table 5.1), except for fasting plasma glucose concentrations (plant stanol ester group: 5.26 [5.13-5.45] mmol/L; control group: 5.61 [5.34-6.24] mmol/L; p=0.006).

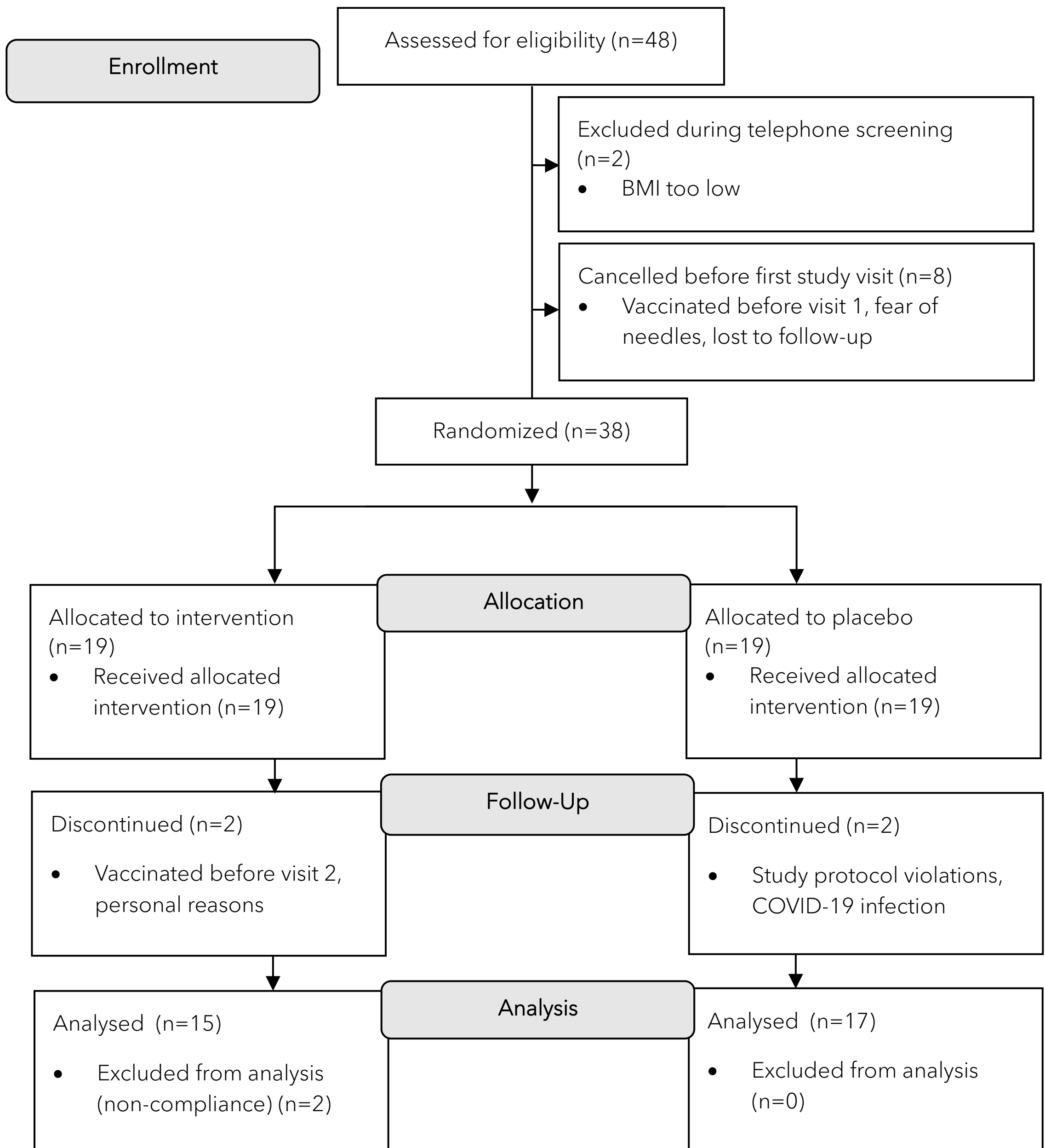

Figure 5.2. CONSORT flow diagram. In total, 34 participants completed the study and 32 participants were included in the analysis.

Table 5.1. Baseline characteristics of the total study population and per group.

	Total group (N=32)	Plant stanol ester (N=15)	Control (N=17)	Between group *p*-value
Age, years (SD)[a]	47 (14)	48 (17)	47 (11)	0.858
Sex (male), N (%)[b]	17 (53.1)	7 (46.7)	10 (58.8)	0.492
Type of COVID-19[b] vaccination, N (%)				0.383
Pfizer/BioNTech	24 (75.0)	13 (86.7)	11 (64.7)	
Moderna	2 (6.3)	0 (0.0)	2 (11.8)	
AstraZeneca	2 (6.3)	1 (6.7)	1 (5.9)	
Janssen	4 (12.5)	1 (6.7)	3 (17.6)	
BMI, kg/m^2 (IQR)[c]	30.2 (28.7-32.0)	29.6 (28.8-31.6)	30.6 (28.4-32.9)	0.710
TAG, mmol/L (IQR)[c,d]	1.34 (1.05-2.20)	1.20 (0.93-2.22)	1.44 (1.09-2.31)	0.545
TC, mmol/L (SD)[a]	5.59 (0.89)	5.56 (0.64)	5.61 (1.09)	0.872
HDL-C, mmol/L (SD)[a]	1.28 (0.23)	1.30 (0.23)	1.26 (0.24)	0.614
Non-HDL-C, mmol/L (SD)[a]	4.31 (0.85)	4.26 (0.57)	4.36 (1.06)	0.752
LDL-C, mmol/L (SD)[a,d]	3.55 (0.68)	3.58 (0.52)	3.52 (0.82)	0.814
Glucose, mmol/L (IQR)[c]	5.44 (5.20-5.75)	5.26 (5.13-5.45)	5.61 (5.34-6.24)	**0.006***
Insulin, mU/L (IQR)[c]	11.8 (6.20-16.8)	12.1 (10.1-14.7)	8.81 (5.72-18.6)	0.433
HOMA-IR (IQR)[c]	2.75 (1.59-4.22)	3.04 (2.32-3.54)	2.25 (1.39-4.61)	0.602
CRP, mg/mL (IQR)[c]	1.25 (0.83-2.74)	1.20 (0.92-2.79)	1.30 (0.80-3.38)	0.941
Smoking, N (%)[b]				0.271
Never smoked	17 (53.1)	10 (66.7)	7 (41.2)	
Quitted smoking	10 (31.3)	4 (26.7)	6 (35.3)	
Current smoker	5 (15.6)	1 (6.7)	4 (23.5)	

[a] Independent sample *t*-test; [b] Chi-square test; [c] Mann-Whitney U test; [d] Total group N=31, plant stanol group N=15, control group N=16. Abbreviations: SD=standard deviation, IQR=interquartile range presented as Q1-Q3, BMI=body mass index, TAG=triacylglycerol, TC=total cholesterol, HDL-C=high-density lipoprotein cholesterol, LDL-C=low-density lipoprotein cholesterol, HOMA-IR=homeostatic model assessment of insulin resistance, CR*P*=C-reactive protein.

Compliance, non-cholesterol sterols, and precursors of bile acids

High compliance was confirmed by changes in serum plant stanol and other non-cholesterol sterol levels in the plant stanol ester group versus the control group (Table 5.2). Serum TC-standardized sitostanol levels increased in the plant stanol ester group and changes were significantly different from those of the control group from visit 2 onwards (Group*Time p=0.007). Also, serum TC-standardized campestanol levels were higher in the plant stanol ester group (estimated marginal difference compared to control [95%CI]: 13.4 [9.76-17.0] μmol/mmol*100; Group p<0.001). Serum TC-standardized plant sterols (sitosterol, campesterol, brassicasterol) and cholestanol were consistently lower in the plant stanol ester group compared to control (Table 5.2), except for serum stigmasterol. Serum TC-standardized lathosterol and lanosterol were increased in the plant stanol ester group compared to control (lathosterol: 104 [28.8-179] μmol/mmol*100, Group p=0.008; lanosterol: 1.92 [0.12; 3.73] μmol/mmol*100, Group p=0.037). Changes in serum TC-standardized desmosterol levels did not differ between groups (Table 5.2). TC-standardized bile acid precursor 7α-OH-CH levels differed between groups, depending on time point (Group*Time p=0.002). However, TC-standardized 7α-OH-CH levels did not differ between groups at any timepoint after Bonferroni correction. Finally, serum TC-standardized 27OH-CH levels were higher in the plant stanol ester group compared to control (16.7 [7.72-25.8] nmol/mmol; Group p=0.001).

Table 5.2. Non-cholesterol sterols and bile acid precursors.

	Estimated marginal difference compared to control (95%CI)	Group
*Compliance markers (TC-standardized plant stanol levels; μmol/mmol*100)*		
Sitostanol[a]	N/A	N/A
Campestanol	13.4 (9.76; 17.0)	**<0.001***
*TC-standardized plant sterol and cholestanol levels (μmol/mmol*100)*		
Sitosterol	-82.0 (-128; -36.0)	**0.001***
Campesterol	-95.1 (-144; -46.1)	**<0.001***
Stigmasterol	-1.12 (-5.29; 3.06)	0.587
Brassicasterol	-11.6 (-16.9; -6.40)	**<0.001***
Cholestanol	-28.8 (-53.8; -3.79)	**0.025***
*TC-standardized intermediates in endogenous cholesterol synthesis pathways (μmol/mmol*100)*		
Lathosterol	104 (28.8; 179)	**0.008***
Desmosterol[b]	-2.58 (-32.2; 27.0)	0.859
Lanosterol	1.92 (0.12; 3.73)	**0.037***
TC-standardized bile acid precursors (nmol/mmol)		
7α-OH-CH[c]	N/A	N/A
27OH-CH	16.7 (7.72; 25.8)	**0.001***

All models were corrected for group, time, baseline levels, age, sex. [a] Significant differences between groups at visit 2, 3, 4, 5, and 6 after Bonferroni correction; [b] Model corrected for time between the start of the study and the COVID-19 vaccination date; [c] No statistical differences between groups at all timepoints after Bonferroni correction. Abbreviations: 95%CI= 95% confidence interval, 7α-OH-CH=7α-hydroxycholesterol, 27OH-CH=27-hydroxycholesterol, N/A=not applicable in this model since interaction term reached statistical significance. TC-standardized levels at each visit are shown in Supplemental Table S5.1.

Antibodies

The difference in anti-COVID-19 IgM titers against the Spike protein (IgM Spike) reached statistical significance two and three weeks after receiving the COVID-19 vaccination (Group*Time p=0.031; Figure 5.3). The largest difference between groups (31.2 [0.43 –

62.1] BAU/mL, or +139%) was observed after two weeks. IgM RBD and IgM N did not differ between groups. The difference in anti-COVID-19 IgG titers against the Spike protein (IgG Spike) was borderline significantly higher in the plant stanols ester group compared to control (estimated marginal difference compared to control [95%CI]: 52.1 [-1.48 – 106] BAU/mL; Group p=0.056). IgG RBD and IgG N did not differ between groups (Table 5.3). In a follow-up sensitivity analysis, where non-responders with an IgG response below 50 BAU/mL were removed, IgG Spike significantly increased in the plant stanol ester group compared to control (IgG Spike: 71.3 [2.51-140] BAU/mL; Group p=0.043; Figure 5.3). Based on the sensitivity analysis, baseline characteristics between responders and non-responders were analyzed. Non-responders had a statistically significant higher age, waist-to-hip ratio (WHR), TC, non-HDL-C, LDL-C, and smoking history compared to responders. No differences were observed in other baseline characteristics, such as serum non-cholesterol sterols concentrations and unstimulated serum cytokine concentrations (Supplementary Table S5.3).

Table 5.3. Anti-COVID-19 IgM and IgG.

	Estimated marginal difference compared to control in BAU/mL (95%CI)	Group p-value
IgM Spike[a]	N/A	N/A
IgM RBD	-5.94 (-14.3; 2.42)	0.156
IgM N	-8.68 (-45.8; 28.5)	0.635
IgG Spike	52.1 (-1.48; 106)	0.056
IgG RBD	66.4 (-10.5; 143.3)	0.088
IgG N	-0.08 (-1.57; 1.41)	0.911
Sensitivity analysis		
IgG Spike[b]	71.3 (2.51; 140.1)	0.043*
IgG RBD[c]	73.8 (-16.2; 163.7)	0.102

All models were corrected for group, time, age, sex. Only visit 4, 5, and 6 were included, since antibody titers at visits 2 and 3 were nearly zero. In the sensitivity analysis, only responders to the COVID-19 vaccination (subjects that produced at least 50 BAU/mL IgG) were used. [a] Significant differences between groups at visit 4 and 5 after Bonferroni correction [b] Plant stanol group N=11, control group N=10; [c] plant stanol group N=12, control group N=11. Abbreviations: Ig=immunoglobulin, RBD=receptor binding domain, N=nucleocapsid, 95%CI=95% confidence interval, N/A=not applicable in this model since interaction term reached statistical significance. Concentrations per visit are shown in Supplemental Table S5.2.

Cytokines, hs-CRP, and leukocyte count

Changes in unstimulated serum cytokines (IL-6, IL-8, TNF-α) did not differ between groups throughout the study (Table 5.4). Cytokines after anti-CD3/CD28 stimulation using the TruCulture system were clustered into Th1/Th17 cytokines (IFN-γ, IL-1β, IL-1 receptor antagonist [IL-1ra], IL-2, IL-17), Th2/Treg cytokines (IL-4, IL-5, IL-10), and pro-inflammatory cytokines (IL-6, IL-8, TNF-α). Of the Th1/Th17 cytokines, IL-1β decreased compared to control (median change [IQR]: plant stanol ester group: -6.00 [-112; 2.00] pg/mL; control group: 14.6 [-0.75; 62.3] pg/mL; p=0.032). IL-1ra also decreased compared to control (mean change [SD]: plant stanol ester group: -291 [692] pg/mL; control group: 357 [622] pg/mL; p=0.028). The decrease in IFN-γ in the plant stanol ester group compared to control was borderline significant (plant stanol ester group: -4.50 [-154; 24.0] pg/mL; control group: 36.0 [-10.4; 457] pg/mL; p=0.059). IL-2 and IL-17 did not differ between groups (Table 5.5). Of the Th2/Treg cytokines, IL-4 decreased in the plant stanol ester group compared to control (plant stanol ester group: -60.0 [-230; 241] pg/mL; control group: 380 [0.00; 769] pg/mL; p=0.016). IL-10 also decreased

in the plant stanol ester group compared to control (plant stanol ester group: -4.00 [-50.0; 11.0] pg/mL; control group: 18.5 [0.00; 126] pg/mL; p=0.009). Changes in IL-5 were borderline significant (plant stanol ester group: 0.00 [-26.0; 23.0] pg/mL; control group: 46.5 [0.00; 124] pg/mL; p=0.051) (Table 5.5). All pro-inflammatory cytokines decreased significantly in the plant stanol ester group compared to control. IL-6 decreased with -29.0 (-738; -9.00) pg/mL in the plant stanol ester group, compared to -1.58 (-283; 16.3) in the control group (p=0.027). IL-8 decreased with -23044 (59329) pg/mL in the plant stanol ester group, whereas it increased with 64909 (91797) pg/mL in the control group (p=0.013). TNF-α decreased with -488 (-11922; -353) pg/mL in the plant stanol ester group, whereas it increased with 13.8 (-1621; 1335) in the control group (p=0.013) (Table 5.5). Finally, the standardized Th1/Th2 ratio did not differ between groups (Table 5.5). No differences between groups were observed for hs-CRP, leukocyte total and differential count (Table 5.6).

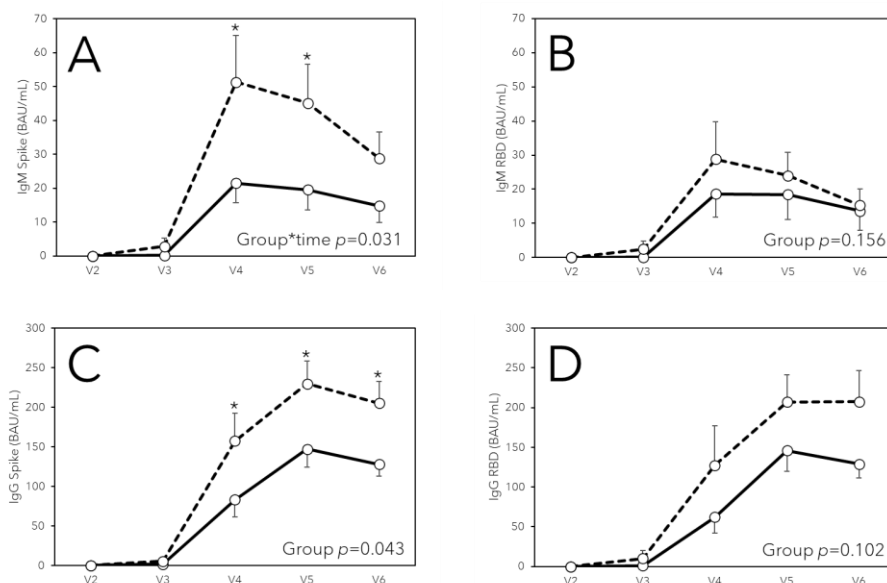

*Figure 5.3. Changes in antibody titers over time. Analysis in total population: A: IgM Spike, B: IgM RBD (plant stanol group: N=15, control group: N=15), and sensitivity analysis C: IgG Spike (plant stanol group: N=11, control group N=10), and D: IgG RBD (plant stanol group: N=12, control group N=11), *p<0.05, - - - plant stanol group, ——— control group.*

Table 5.4. Unstimulated serum cytokine concentrations.

	Plant stanol ester group (N=15)			Control group (N=17)			Between groups	
pg/mL (SD) or (IQR)	Start study (V1)	Day before vaccination (V2)	End of study (V6)	Start study (V1)	Day before vaccination (V2)	End of study (V6)	*p*-value V2-V1	*p*-value V6-V1
IL-6[a]	0.64 (0.56-0.94)	0.64 (0.51-0.91)	0.70 (0.51-0.81)	0.62 (0.44-0.88)	0.54 (0.38-0.72)	0.61 (0.43-0.78)	0.655	0.370
IL-8[a]	5.09 (4.17-5.61)	4.83 (4.16-5.89)	4.03 (3.62-4.97)	4.68 (3.61-5.81)	4.10 (3.18-6.21)	4.50 (3.59-5.10)	0.551	0.576
TNF-**α**[b]	0.90 (0.18)	0.85 (0.14)	0.83 (0.16)	0.87 (0.19)	0.85 (0.22)	0.90 (0.23)	0.597	0.113

[a] Mann-Whitney U test; [b] Independent sample *t*-test. Abbreviations: SD=standard deviation, IQR=interquartile range presented as Q1-Q3, IL=interleukin, TNF=tumor necrosis factor.

Table 5.5. Stimulated cytokine concentrations (TruCulture anti-CD3/CD28).

	Plant stanol ester group (N=11)		Control group (N=12)		Between groups
	Before vaccination	End of study	Before vaccination	End of study	*p*-value
Th1/Th17 cytokines, pg/mL (SD) or (IQR)					
IFN-γ[a]	111 (50-348)	74 (29-312)	123 (34.5-239)	166.5 (58.8-709)	0.059
IL-1β[a]	92 (46-482)	90 (45-499)	148 (5.90-372)	120.5 (6.68-994)	0.032*
IL-1ra[b]	2386 (1261)	2095 (1271)	2197 (1659)	2554 (1815)	0.028*
IL-2[a]	753 (299-1110)	729 (232-954)	811 (236-2308)	1078 (209-2588)	0.190
IL-17[a]	139 (55-295)	93 (21-199)	121 (20.1-376)	177 (21.0-579)	0.118
Th2/Treg cytokines, pg/mL (IQR)					
IL-4[a]	961 (255-2170)	1150 (233-1940)	702.5 (88.5-897)	1113.5 (79.8-1698)	0.016*
IL-5[a]	90 (38-196)	121 (19-164)	45 (8.00-67.8)	91.5 (8.00-211)	0.051
IL-10[a]	66 (11-145)	52 (7.0-77)	59.5 (10.1-162)	92.5 (7.20-309)	0.009*
Pro-inflammatory cytokines, pg/mL (SD) or (IQR)					
IL-6[a]	34 (16-77)	18 (6.50-99)	32.5 (7.03-88.8)	90.5 (10.1-277)	0.027*
IL-8[b]	94891 (78923)	71848 (67440)	116182 (121882)	181091 (190013)	0.013*
TNF-α[a]	875 (614-1900)	614 (220-3290)	1195 (135-2720)	2495 (236-3128)	0.013*
Th1/Th2 ratio[c] (IQR)					
Th1/Th2	0.84 (0.46-1.56)	0.70 (0.49-1.42)	0.92 (0.80-1.34)	0.90 (0.77-1.73)	0.347

[a] Mann-Whitney U test; [b] Independent sample *t*-test; [c]Th1/Th2 ratio was calculated as ratio between standardized concentrations of Th1 cytokines (IFN-γ and IL-2) and Th2 cytokines (IL-4 and IL-10). Standardization was done by setting the average baseline concentration of each cytokine at 100%. Abbreviations: SD=standard deviation, IQR=interquartile range presented as Q1-Q3, Th=T=helper cell, IFN=interferon, IL=interleukin, ra=receptor antagonist, Treg= regulatory T cell, TNF=tumor necrosis factor.

Table 5.6. hs-CRP, leukocytes total and differential count.

	Estimated marginal difference compared to control (95%CI)	Group *p*-value
hs-CRP (mg/mL)	0.66 (-0.69; 2.00)	0.324
Leukocytes (10^9/L)	-0.02 (-0.49; 0.44)	0.917
Neutrophils (%)	-1.25 (0.46; -4.67)	0.463
Lymphocytes (%)	0.83 (-2.60; 4.26)	0.624
Monocytes (%)	0.24 (-0.38; 0.85)	0.435
Eosinophils (%)	-0.02 (-0.57; 0.54)	0.953
Basophils (%)	0.10 (-0.06; 0.25)	0.200

All models were corrected for group, time, baseline values, age, sex. Abbreviations: 95%CI=95% confidence interval, CR*P*=C-reactive protein. Values per visit are shown in Supplemental Table S5.4.

Metabolic markers

No statistically significant differences in serum lipid and lipoprotein (TC, LDL-C, HDL-C, non-HDL-C, and TAG) concentrations were observed between groups (Table 5.7). Plasma glucose and serum insulin concentrations, and the HOMA-IR also did not differ between groups (Table 5.7).

Table 5.7. Metabolic markers.

	Estimated marginal difference compared to control (95%CI)	Group
TC[a], mmol/L	-0.14 (-0.37; 0.09)	0.214
LDL-C[b], mmol/L	-0.01 (-0.21; 0.20)	0.956
HDL-C, mmol/L	-0.02 (-0.05; 0.09)	0.517
Non-HDL-C, mmol/L	-0.06 (-0.26; 0.15)	0.571
TAG[b], mmol/L	-0.04 (-0.26; 0.18)	0.725
Glucose, mmol/L	0.09 (-0.10; 0.27)	0.365
Insulin, mU/L	-1.34 (-4.26; 1.57)	0.352
HOMA-IR[a]	-0.42 (-1.20; 0.36)	0.281

All models were corrected for group, time, baseline values, age, sex. [a] Model corrected for time between the start of the study and the COVID-19 vaccination date [b] Total group N=31, plant stanol group N=15, control group N=16. Abbreviations: 95% CI=95% confidence interval, TC=total cholesterol, LDL-C=low-density lipoprotein cholesterol, HDL-C=high-density lipoprotein cholesterol, TAG=triacylglycerol, HOMA-IR=homeostatic model assessment for insulin resistance. Concentrations for each visit are shown in Supplemental Table S5.5.

Anthropometrics and dietary habits

Anthropometric measures (weight, BMI, waist circumference, hip circumference, and WHR) remained stable throughout the study and changes did not differ between groups (Table 5.8). Changes in dietary intakes slightly differed between groups (Table 5.9). Protein intake slightly increased in the plant stanol ester group (median [IQR]: 1.1 [-0.1; 2.2] EN%) compared to control (-0.7 [-1.7; 0.5] EN%; p=0.026). Trans fatty acid intake also slight increased in the plant stanol ester group (mean [SD]: 0.04 [0.1] EN%) compared to control (-0.02 [0.1] EN%; p=0.041). Finally, fiber intake slightly decreased in the plant stanol ester group (-3.3 [-8.3; -0.1] g) compared to control (0.0 [-1.5; 6.9] g; p=0.020).

Discussion

In this placebo-controlled double-blind intervention study we showed that consuming 4g plant stanols per day significantly increased serum anti-COVID-19 IgM Spike antibody titers (up to 139%) in adults with overweight or obesity. Only in people that showed IgG seroconversion, plant stanol consumption also increased serum anti-COVID-19 IgG Spike. Stimulated cytokine concentrations within all three domains (Th1/Th17, Th2/Treg, and pro-inflammatory cytokines) decreased compared to control, indicating that plant stanol intake decreased the overall inflammatory activity of the immune system, while simultaneously increasing antibody responses. Unexpectedly, the increase in antibody titers was independent of changes in serum LDL-C concentrations, since changes in serum lipid and lipoprotein concentrations were not different between the plant stanol ester and control groups. Altogether, these results suggest that this high-risk group for COVID-19 complications could benefit from plant stanol consumption to increase COVID-19 antibody titers.

In an earlier study we showed that consuming plant stanols (4g/day, provided as plant stanol ester) increased the combined IgM/IgG antibody titers (+73%) following a hepatitis A vaccination in a subgroup of participants with overweight or obesity [20, 21]. Future studies should consider if 4 g/day is the optimal daily dose of plant stanols or if lower doses achieve similar effects. Besides plant stanol ester, other lifestyle interventions may also be efficient strategies in optimizing immunological outcomes [15-19, 32]. Vitamin E (200 mg/day) increased antibody production after a hepatitis B (largest increase compared to control: ±300%) and tetanus vaccination (largest increase compared to control: ±160%) in older adults [33]. Additionally, trace elements alone or in combination with vitamins increased antibody production to an influenza vaccine in institutionalized older adults (largest increase compared to control: ±38%) [34]. The effect sizes of the current and previous studies are comparable and indicate a potential effect size of dietary interventions to increase antibody titers after vaccinations. These increases are highly relevant in the prevention and disease progression of COVID-19, as increased antibody titers after vaccination protect against severe illness and cause higher vaccine effectivity [13, 35]. It was striking to find that non-responders in our population were older, had higher serum TC, non-HDL-C, LDL-C, increased WHR, and a

Table 5.8. Anthropometric measures.

	Estimated marginal difference compared to control (95%CI)	Group
Weight, kg	0.56 (-0.15; 1.26)	0.118
BMI, kg/m^2	0.17 (-0.09; 0.43)	0.180
Waist circumference, cm	1.29 (-1.34; 3.91)	0.323
Hip circumference, cm	0.74 (-0.39; 1.86)	0.190
WHR	0.00 (-0.02; 0.02)	0.989

All models were corrected for group, time, baseline values, age, sex. Abbreviations: 95%CI=95% confidence interval, BMI=body mass index, WHR=waist-to-hip ratio. Values per visit are shown in Supplemental Table S5.6.

Table 5.9. Dietary intake of the total study population, and shown for each group.

	Total group		Plant stanol ester group		Control group		Between groups *p*-value
	V1	V6	V1	V6	V1	V6	
Energy, MJ (SD)[b]	9.5 (2.7)	9.2 (2.3)	9.9 (2.7)	9.3 (2.8)	9.2 (2.7)	9.1 (1.8)	0.430
Energy, kcal (SD)[b]	2260 (634)	2189 (545)	2352 (627)	2218 (669)	2179 (647)	2164 (427)	0.397
Protein, EN% (IQR)[b]	15.8 (13.8-18.7)	16.3 (15.2-17.5)	14 (13.7-16.5)	16.0 (13.8-16.8)	16.1 (15.0-19.5)	16.6 (15.2-18.5)	**0.026***
Carbohydrates, EN% (SD)[b]	43.3 (6.8)	42.1 (6.8)	44.9 (6.5)	42.7 (6.9)	41.8 (7.0)	41.6 (6.8)	0.263
Fat, EN% (SD)[b]	36.6 (5.8)	37.4 (5.9)	35.8 (6.5)	37.4 (5.5)	37.3 (5.3)	37.5 (6.4)	0.414
Saturated fatty acids, EN% (IQR)[a]	11.9 (10.5-13.1)	12.2 (10.9-12.9)	11.8 (10.5-13.0)	11.9 (10.9-13.0)	12.0 (10.3-13.4)	12.4 (11.1-12.9)	0.331
Trans fatty acids, EN% (SD)[b]	0.4 (0.1)	0.4 (0.1)	0.3 (0.1)	0.4 (0.1)	0.4 (0.1)	0.4 (0.1)	**0.041***
Monounsaturated fatty acids, EN% (SD)[a]	13.7 (3.0)	14.3 (3.9)	13.6 (3.5)	14.2 (3.5)	13.8 (2.6)	14.4 (4.3)	0.313
Oleic acid, EN% (IQR)[a]	12.0 (9.7-14.8)	12.4 (9.8-14.1)	11.8 (9.4-14.6)	12.3 (10.3-15.1)	12.0 (9.7-15.0)	12.7 (9.6-14.1)	0.370
Polyunsaturated fatty acids, EN% (SD)[b]	7.8 (1.6)	7.8 (1.4)	7.7 (1.9)	7.8 (1.5)	7.8 (1.3)	7.9 (1.4)	0.900
Linoleic acid, EN% (SD)[b]	6.4 (1.1)	6.5 (1.2)	6.5 (1.4)	6.5 (1.1)	6.4 (1.0)	6.4 (1.2)	0.823
Alpha-linoleic acid, EN% (IQR)[b]	0.7 (0.5-0.9)	0.7 (0.6-0.9)	0.7 (0.5-0.8)	0.6 (0.6-0.7)	0.7 (0.6-0.9)	0.8 (0.6-0.9)	0.612
EPA, g (IQR)[a]	0.04 (0.03-0.10)	0.06 (0.03-0.14)	0.04 (0.02-0.11)	0.03 (0.02-0.14)	0.05 (0.03-0.09)	0.06 (0.04-0.16)	0.682
DHA, g (IQR)[a]	0.05 (0.02-0.18)	0.08 (0.03-0.22)	0.05 (0.02-0.19)	0.03 (0.02-0.22)	0.06 (0.03-0.15)	0.10 (0.04-0.24)	0.766
Alcohol, EN% (IQR)[a]	1.0 (0.2-2.5)	1.0 (0.2-2.7)	1.1 (0.5-2.4)	1.1 (0.3-2.1)	0.6 (0.0-2.6)	0.8 (0.0-2.7)	0.278
Cholesterol, mg (IQR)[a]	213 (164-316)	229 (174-293)	185 (141-323)	187 (171-270)	247 (177-316)	247 (200-297)	0.295
Fiber, g (IQR)[a]	22.3 (17.5-31.2)	23.1 (16.4-27.5)	24.8 (17.6-36.8)	23.5 (15.7-30.3)	20.3 (16.0-29.2)	22.8 (16.7-27.5)	**0.020***
Fiber, EN% (SD)[b]	2.09 (0.50)	2.08 (0.53)	2.00 (0.42)	2.10 (0.61)	2.19 (0.56)	2.05 (0.44)	0.088

[a] Mann-Whitney U test was used since changes were not normally distributed; [b] Independent sample *t*-test. Abbreviations: MJ=mega joules, kcal=kilocalories, EN%=energy percentage, SD=standard deviation, IQR=interquartile range presented as Q1-Q3.

different smoking history compared to responders. These results suggest that abdominal obesity and an unhealthy metabolic profile at baseline could potentially predict alterations in immune responses. Furthermore, plant stanols may only improve immune responses (i.e., antibody production) if the immune system is able to produce antibodies after vaccination, as a group effect for serum IgG was only evident in those showing IgG seroconversion. In this light, evaluating additional measures for vaccine effectivity could be of interest, e.g., the decline in antibody titers [36, 37], T cell numbers and activity [38, 39], and memory responses to a booster vaccine. Adding these measures to future studies will create a better understanding of the full potential of dietary approaches to improve immune responses after vaccinations.

Besides antibody titers, lifestyle interventions may affect cytokine production as well. *in vitro*, Selenium increased IL-8 and decreased TNF-α production after cells were cultured with influenza antigens (32), and vitamin D3 decreased plasma TNF-α and IL-6 concentrations in vitamin D deficient older adults (18). Obesity is associated with elevated cytokines from all domains in either adipose tissue [25, 40-42] or serum [42-44]. We here reported reductions in all cytokine domains. Therefore, interaction of plant stanols with the immune system potentially lowers the overall higher inflammatory status (pro-inflammatory and Th1/Th17 cytokines) in people with overweight or obesity. Consequently, the normal physiological counteracting anti-inflammatory responses (Th2/Treg) are also decreased, which may allow cytokine concentrations to return to values consistent with a non-inflammatory state. The question is how the overall reduction in stimulated cytokine concentrations should be interpreted and what it implies for (immune) health. The immune system might handle (vaccination) challenges more efficiently once the chronic state of low-grade inflammation is reduced, explaining our results of simultaneous decreased cytokine production and increased antibody production. However, it should be noted that stimulated cytokines were produced at 37°C for 48 hours. This procedure allows cytokines production at maximum efficiency, but also leads to cytokine absorption or degradation by other immune cells before the end of incubation time [45]. Therefore, the stimulated cytokine results should be interpreted with care. Future studies could consider analyzing stimulated cytokines at earlier time points, i.e., after 12, 24, or 36 hours of incubation.

It is widely accepted that plant stanol ester lower cardiovascular disease risk by decreasing intestinal cholesterol absorption and consequently serum LDL-C concentrations [46-49]. However, our control oat-based drink lowered LDL-C as well, and changes in LDL-C throughout the study did not differ between groups (plant stanol ester group: -0.31 mmol/L or -8.7%; control group: -0.19 mmol/L or -5.4%). The effect size in the plant stanol ester group is slightly lower than the estimated LDL-C lowering effect of -0.48 mmol/L [50] or -13.6% (8.8-18.6%) [51] for 4 g/day (50), also compared to other intervention studies [20, 46, 52, 53]. The reduction in serum LDL-C concentrations can be attributed to reduced intestinal cholesterol absorption in the plant stanol group, but not in the control group. Therefore, we cannot exclude that changes in cholesterol absorption and synthesis play a role as underlying mechanism.

However, the fact that LDL-C was lowered by both study products strongly indicates that the observed effects on antibody titers and cytokine production in the plant stanol ester group were not related to reductions in serum LDL-C.

The question remains why LDL-C decreased in the control group. Both oat-based test products were proprietary products of Raisio Nutrition Ltd. and contained a small amount of soluble dietary fibers (daily intake 1.4 g/day from the test products), such as β-glucans (0.4 g/day), which are known to have serum LDL-C lowering effects. However, higher doses (3 g/day) would be needed to achieve the observed effect size of -0.19 mmol/L in the control group [54-56]. We also evaluated if the control affected bile acid synthesis and thereby reduced LDL-C. However, changes in bile acid precursors did not show a coherent pattern in the control group, making this mechanism unlikely. All in all, there were no changes in markers of cholesterol absorption and synthesis in the control group, and the other measurements conducted provided no information on the possible mechanism of action for the observed LDL-C lowering in the control group.

Since the observed effects of plant stanol ester on antibody titers and stimulated cytokines could not be attributed to the reduction in LDL-C, bile acid precursors might be involved in the underlying mechanism. The observed increased 27OH-CH levels are especially interesting in the context of the COVID-19 vaccination, since 27OH-CH (as well as other oxysterols [57]) showed antiviral activity against SARS-CoV-2 [58]. However, changes in 27OH-CH levels did not correlate with changes in IgM or IgG Spike antibody titers (data not shown). Furthermore, plant stanols might interact with immune cells in Peyer's patches (e.g., Tregs) during the process of being taken up from the intestinal tract and travelling (in very small amounts) to the blood stream [21]. We previously suggested that plant stanols interact with Tregs via TLR-2 and thereby stimulate Tregs to increase IL-10 production in asthma patients, which in turn affects the Th1/Th2 balance [20, 22]. However, in the current study IL-10 decreased compared to control and the Th1/Th2 ratio did not differ between groups. Clearly, the mechanism underlying the observed effects of plant stanol consumption on antibody titers and cytokine production in the current study is not fully understood and requires further attention.

In conclusion, consuming plant stanols (4g/day) effectively affects immune responses, which translates into increased anti-COVID-19 IgM and IgG Spike concentrations in subjects with overweight/obesity. These results imply that this high-risk group for COVID-19 complications could benefit from plant stanol consumption. We have shown here, for the first time, that the effects of plant stanols on immune responses occur independently of changes in serum LDL-C concentrations. Future studies should further unravel if changes in cholesterol metabolism and/or changes in immune cell behavior (e.g., within Peyer's patches) are involved in the underlying mechanism of action.

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Supplementary Materials

Table S5.1. Serum TC-standardized non-cholesterol sterol and bile acid precursor levels shown per visit for each group.

	Plant stanol ester group (N=15)						Control group (N=17)					
	V1	V2	V3	V4	V5	V6	V1	V2	V3	V4	V5	V6
*Compliance markers (TC-standardized plant stanol levels), μmol/mmol*100 (IQR)*												
Sitostanol	2.37	16.8	19.4	20.8	17.8	18.8	2.17	1.84	2.15	1.99	2.25	2.07
	(1.49-3.10)	(2.04-20.1)	(5.80-26.5)	(16.2-24.1)	(12.1-27.7)	(13.2-27.1)	(0.80-2.92)	(0.89-1.84)	(1.08-2.93)	(0.85-2.83)	(1.28-2.53)	(0.91-2.56)
Campestanol	4.42	14.5	13.6	19.2	16.8	18.4	5.41	4.61	3.94	4.42	4.91	4.54
	(2.16-5.94)	(3.85-24.5)	(9.92-21.9)	(15.2-24.3)	(12.1-19.9)	(12.0-19.7)	(2.76-6.65)	(3.23-6.35)	2.78-6.46)	(3.23-5.98)	(2.59-6.37)	(2.76-6.25)
*TC-standardized plant sterol and cholestanol levels, μmol/mmol*100 (IQR)*												
Sitosterol	231	142	121	134	131	145	149	151	156	170	165	140
	(123-390)	(111-207)	(100-146)	(98-196)	(98-178)	(95-154)	(96-216)	(80-229)	(119-205)	(95-239)	(122-240)	(88-210)
Campesterol	281	203	185	218	203	212	222	281	257	229	271	209
	(171-438)	(146-398)	(159-206)	(132-278)	(142-285)	(115-243)	(164-345)	(156-403)	(166-328)	(164-378)	(170-429)	(148-394)
Stigmasterol	6.61	9.07	5.66	5.98	6.94	7.02	3.28	3.43	3.85	3.52	3.67	3.13
	(3.26-31.8)	(3.07-10.7)	(2.66-10.6)	(2.22-7.93)	(2.94-9.02)	(2.25-8.76)	(2.16-6.74)	(2.43-8.89)	(2.52-7.36)	(1.98-16.0)	(2.14-12.9)	(1.75-8.14)
Brassicasterol	24.2	17.7	17.8	15.7	16.5	13.9	24.8	30.1	28.7	27.6	27.3	25.5
	(15.7-38.1)	(11.1-28.8)	(12.7-21.3)	(10.9-24.2)	(8.70-29.1)	(8.60-24.0)	(16.6-34.7)	(19.0-43.2)	(19.0-38.0)	(18.1-43.1)	(20.8-48.3)	(16.2-36.1)
Cholestanol	165	132	138	167	121	150	141	166	206	180	159	185
	(105-224)	(109-238)	(119-187)	(111-193)	(89-186)	(109-176)	(120-189)	(116-215)	(148-244)	(96-233)	(81-190)	(152-216)
*TC-standardized intermediates in endogenous cholesterol synthesis pathways, μmol/mmol*100 (IQR)*												
Lathosterol	259	470	386	467	531	444	340	332	321	303	326	301
	(156-412)	(332-527)	(304-485)	(335-680)	(353-632)	(353-589)	(235-489)	(182-517)	(225-425)	(223-428)	(223-505)	(206-444)
Desmosterol	145	184	169	194	178	181	194	198	198	162	198	188
	(96-161)	(158-222)	(144-194)	(151-238)	(166-273)	(149-221)	(146-221)	(148-239)	(111-242)	(128-233)	(124-250)	(122-232)
Lanosterol	14.8	16.9	17.2	17.8	18.0	17.2	16.3	16.6	16.6	18.1	17.5	17.2
	(14.2-16.0)	(16.1-20.9)	(16.5-19.3)	(16.1-21.4)	(15.9-19.6)	(15.4-21.6)	(13.8-20.3)	(14.0-21.8)	(15.3-18.9)	(14.5-20.9)	(15.2-20.0)	(13.6-19.6)
TC-standardized bile acid precursors, nmol/mmol (IQR)												
7α-OH-CH	19.6	20.5	24.2	26.1	23.9	22.0	27.9	30.1	30.7	26.0	30.9	26.5
	(14.7-24.5)	(16.9-27.6)	(19.0-29.2)	(18.3-33.9)	(18.5-26.8)	(17.9-27.3)	(19.0-37.2)	(21.7-37.2)	(21.0-37.2)	(18.5-34.2)	(19.7-41.8)	(21.7-41.8)
27OHCH	84.2	101	97.3	98.3	97.8	94.2	83.9	90.9	90.2	85.1	86.1	85.4
	(68.0-106)	(80.5-112)	(85.6-122)	(83.6-127)	(85.3-115)	(81.6-107)	(75.7-106)	(75.5-99.7)	(75.2-97.8)	(77.4-106)	(72.2-101)	(76.2-104)

Abbreviations: IQR=interquartile range presented as Q1-Q3, 7α-OH-CH=7-α-hydroxycholesterol, 27OHCH=27-hydroxycholesterol.

Table S5.2. Serum anti-COVID-19 IgM and IgG shown for each group per visit.

	Plant stanol ester group (N=15)					Control group (N=15)				
	V2	V3	V4	V5	V6	V2	V3	V4	V5	V6
IgM Spike	2.07	2.60	53.0	37.0	25.0	2.32	2.50	19.2	12.1	9.42
	(0.74-3.74)	(0.86-4.16)	(6.06-96.8)	(5.55-82.4)	(4.41-50.1)	(1.25-2.99)	(1.31-3.16)	(3.87-29.7)	(6.72-26.2)	(5.57-19.3)
IgM RBD	2.70	3.94	19.6	14.9	13.0	3.99	4.59	13.2	11.9	10.4
	(1.80-6.47)	(1.79-7.46)	(5.28-42.9)	(4.71-42.1)	(3.94-25.4)	(3.21-7.69)	(3.23-7.63)	(8.38-25.3)	(8.81-25.3)	(5.85-19.1)
IgM Nucleocapsid	44.7	58.7	60.2	55.4	59.2	57.5	67.1	61.7	73.4	62.1
	(27.7-94.8)	(32.1-100)	(32.9-106)	(38.7-104)	(40.5-95.8)	(32.3-90.2)	(33.9-129)	(40.4-122)	(37.8-145)	(39.5-115)
IgG Spike	0.33	0.69	85.7	180	147	0.25	0.36	27.0	87.8	104
	(0.15-0.61)	(0.32-1.46)	(7.57-153)	(39.3-257)	(42.2-220)	(0.16-0.47)	(0.18-0.95)	(10.6-92.2)	(28.7-172)	(39.0-160)
IgG RBD	0.34	0.55	37.5	142	143	0.48	0.48	20.1	72.3	98.4
	(0.29-0.52)	(0.25-1.01)	(7.93-88.3)	(51.4-244)	(58.8-248)	(0.26-1.00)	(0.31-1.32)	(3.93-78.1)	(27.3-218)	(37.9-163)
IgG Nucleocapsid	0.16	0.17	0.17	0.18	0.17	0.16	0.20	0.18	0.12	0.15
	(0.13-0.43)	(0.14-0.37)	(0.12-0.38)	(0.12-0.37)	(0.09-0.38)	(0.06-0.29)	(0.07-0.27)	(0.07-0.26)	(0.07-0.23)	(0.06-0.26)
Sensitivity analysis – antibodies BAU/mL (IQR)										
IgG Spike[a]	0.32	0.41	136	217	185	0.29	0.47	83.1	146	128
	0.15-0.49	(0.32-1.46)	(84.1-219)	(176-261)	(135-225)	(0.19-1.06)	(0.23-1.55)	(26.9-114)	(87.8-215)	(91.8-172)
IgG RBD[b]	0.34	0.53	41.8	196	173	0.48	0.48	47.6	158	137
	(0.29-0.52)	(0.25-0.66)	(25.0-226)	(106-265)	(111-257)	(0.26-1.00)	(0.24-2.50)	(14.0-92.7)	(40.8-226)	(58.7-183)

In the sensitivity analysis, only responders to the COVID-19 vaccination (subjects that produced at least 50 BAU/mL IgG) were added to the models. [a] Plant stanol group N=11, control group N=10; [b] plant stanol group N=12, control group N=11. Abbreviations: Ig=immunoglobulin, RBD=receptor binding domain, N=nucleocapsid, IQR=interquartile range presented as Q1-Q3.

Table S5.3. Differences in baseline characteristics between responders and non-responders to the COVID-19 vaccine.

	Responders (N=21)	Non-responders (N=9)	Between group *p*-value
Age, years (SD)[a]	42 (14)	59 (7.5)	**<0.001***
Sex (male), N (%)[b]	9 (43%)	7 (78%)	0.079
Type of COVID-19[b] vaccination, N (%)[b]			0.086
Pfizer/BioNTech	18 (86%)	5 (56%)	
Moderna	1 (5%)	0 (0%)	
AstraZeneca	0 (0%)	2 (22%)	
Janssen	2 (9%)	2 (22%)	
BMI, kg/m^2 (IQR)[c]	30.2 (28.5-32.8)	29.7 (28.8-31.8)	0.965
WHR (IQR)[c]	0.88 (0.84-0.95)	0.95 (0.91-1.93)	**0.025***
TAG, mmol/L (IQR)[c]	1.20 (0.91-1.93)	1.46 (1.25-2.56)	0.125
TC, mmol/L (SD)[a]	5.33 (0.81)	6.17 (0.72)	**0.013***
HDL-C, mmol/L (SD)[a]	1.26 (0.20)	1.31 (0.30)	0.601
Non-HDL-C, mmol/L (SD)[a]	4.07 (0.75)	4.86 (0.67)	**0.011***
LDL-C, mmol/L (SD)[a]	3.41 (0.66)	4.00 (0.48)	**0.024***
Glucose, mmol/L (IQR)[c]	5.45 (5.16-5.74)	5.43 (5.29-5.79)	0.594
Insulin, mU/L (IQR)[c]	12.1 (6.35-18.5)	10.1 (5.72-13.8)	0.397
HOMA-IR (IQR)[c]	3.07 (1.87-4.47)	2.34 (1.42-3.37)	0.476
CRP, mg/mL (IQR)[c]	1.17 (0.70-2.69)	2.20 (1.04-3.23)	0.244
Smoking, N (%)[b]			**0.028***
Never smoked	12 (57%)	3 (33%)	
Quitted smoking	4 (19%)	6 (67%)	
Current smoker	5 (24%)	0 (0%)	
Sitostanol, µmol/mmol*100 (IQR)[c]	2.53 (0.96-3.08)	2.07 (1.52-2.57)	0.790
Campestanol, µmol/mmol*100 (IQR)[c]	5.41 (2.56-6.05)	3.22 (2.18-8.93)	0.965
Sitosterol, µmol/mmol*100 (IQR)[c]	169 (93.7-357)	170 (119-234)	0.824
Campesterol, µmol/mmol*100 (IQR)[c]	268 (167-438)	211 (167-301)	0.372
Stigmasterol, µmol/mmol*100 (IQR)[c]	6.07 (2.68-20.1)	4.13 (2.13-5.93)	0.209
Brassicasterol, µmol/mmol*100 (IQR)[c]	27.8 (15.3-42.0)	24.2 (15.3-29.6)	0.449
Cholestanol, µmol/mmol*100 (IQR)[c]	165 (114-224)	121 (107-168)	0.244
Lathosterol, µmol/mmol*100 (IQR)[c]	338 (167-437)	312 (235-452)	0.594
Desmosterol, µmol/mmol*100 (IQR)[c]	147 (101-204)	172 (146-259)	0.104
Lanosterol, µmol/mmol*100 (IQR)[c]	14.7 (14.0-17.2)	16.0 (15.5-17.6)	0.263
7α-OH-CH, nmol/mmol (IQR)[c]	22.2 (15.1-29.3)	28.9 (17.7-36.2)	0.304
27OHCH, nmol/mmol (IQR)[c]	84.2 (70.4-109)	85.8 (79.6-104)	1.000
IL-6, pg/ml (IQR)[c]	0.61 (0.50-0.96)	0.62 (0.53-1.07)	0.594
IL-8, pg/ml (IQR)[c]	4.77 (3.66-5.42)	5.16 (4.19-7.74)	0.150
TNF-α, pg/ml (SD)[a]	0.85 (0.18)	0.95 (0.17)	0.148

[a] Independent sample *t*-test; [b] Chi-square test; [c] Mann-Whitney U test. Abbreviations: SD=standard deviation, IQR=interquartile range presented as Q1-Q3, BMI=body mass index, WHR=waist-to-hip ratio, TAG=triacylglycerol, TC=total cholesterol, HDL-C=high density lipoprotein cholesterol, LDL-C=low density lipoprotein cholesterol, HOMA-IR=homeostatic model assessment of insulin resistance, CR*P*=C-reactive protein, 7α-OH-CH=7-α-hydroxycholesterol, 27OHCH=27-hydroxycholesterol, IL=interleukin, TNF=tumor necrosis factor.

Table S5.4. Serum hs-CRP, whole blood leukocytes, and differential leukocyte counts shown per group for each visit.

	Plant stanol ester group (N=15)						Control group (N=17)					
	V1	V2	V3	V4	V5	V6	V1	V2	V3	V4	V5	V6
hs-CRP, mg/mL (IQR)	1.11 (0.60-2.79)	1.77 (0.44-4.75)	1.81 (0.94-0.33)	1.03 (0.63-2.72)	1.10 (0.73-2.31)	1.27 (0.34-2.04)	1.23 (0.62-3.88)	0.88 (0.67-2.16)	1.35 (0.99-5.99)	1.19 (0.61-1.77)	0.75 (0.44-4.26)	1.03 (0.58-4.41)
Leukocytes, 10^9/L (IQR)	5.1 (4.7-6.2)	5.4 (4.9-6.9)	5.4 (4.8-6.7)	5.8 (5.1-6.8)	6.0 (5.2-6.5)	5.4 (5.1-6.1)	5.5 (4.6-6.3)	5.4 (4.6-6.3)	5.6 (4.6-6.8)	5.8 (5.0-6.7)	5.9 (5.0-6.8)	5.7 (4.9-6.4)
Neutrophils, % (SD)	56.3 (9.0)	55.3 (6.9)	53.7 (6.8)	54.8 (6.1)	55.1 (8.1)	54.1 (6.9)	58.5 (5.1)	57.7 (5.8)	55.5 (6.5)	56.9 (5.3)	57.1 (7.2)	57.2 (5.9)
Lymphocytes, % (SD)	31.7 (7.9)	33.1 (6.6)	34.3 (5.5)	33.1 (6.3)	32.9 (6.7)	34.2 (5.9)	30.0 (6.2)	30.9 (6.3)	33.0 (7.6)	31.5 (6.1)	31.4 (8.2)	30.9 (6.6)
Monocytes, % (IQR)	8.0 (7.0-9.0)	8.0 (7.0-9.0)	7.0 (7.0-9.0)	8.0 (7.0-10)	8.0 (7.0-10)	8.0 (7.0-10)	8.0 (7.0-9.0)	8.0 (7.0-9.0)	8.0 (6.5-10)	8.0 (7.0-9.0)	8.0 (7.0-9.0)	8.0 (7.0-9.0)
Eosinophils, % (IQR)	3.0 (2.0-4.0)	3.0 (2.0-4.0)	3.0 (2.0-4.0)	3.0 (2.0-4.0)	3.0 (2.0-3.0)	3.0 (2.0-4.0)	3.0 (2.0-3.0)	3.0 (1.5-4.0)	3.0 (2.0-3.5)	3.0 (2.0-3.5)	3.0 (2.0-3.8)	3.0 (2.0-4.0)
Basophils, % (IQR)	1.0 (1.0-1.0)	1.0 (1.0-1.0)	1.0 (1.0-1.0)	1.0 (1.0-1.0)	1.0 (1.0-1.0)	1.0 (1.0-1.0)	1.0 (1.0-1.0)	1.0 (0.5-1.0)	1.0 (1.0-1.0)	1.0 (0.5-1.0)	1.0 (0.3-1.0)	1.0 (1.0-1.0)

Abbreviations: SD=standard deviation, IQR=interquartile range presented as Q1-Q3, CRP=C-reactive protein

Table S5.5. Metabolic marker concentrations shown per group for each visit.

	Plant stanol ester group (N=15)						Control group (N=17)					
	V1	V2	V3	V4	V5	V6	V1	V2	V3	V4	V5	V6
Lipids and lipoproteins, mmol/L (SD) or (IQR)												
TC	5.56 (0.64)	5.43 (0.8)	5.30 (0.64)	5.20 (0.71)	5.21 (0.55)	5.11 (0.55)	5.61 (1.09)	5.41 (1.09)	5.32 (0.98)	5.25 (1.05)	5.21 (1.10)	5.3 (1.02)
LDL-C[a]	3.58 (0.52)	3.48 (0.64)	3.37 (0.54)	3.29 (0.64)	3.30 (0.55)	3.27 (0.53)	3.52 (0.82)	3.42 (0.87)	3.32 (0.87)	3.28 (0.75)	3.18 (0.80)	3.33 (0.82)
HDL-C	1.30 (0.23)	1.29 (0.25)	1.25 (0.25)	1.28 (0.24)	1.26 (0.23)	1.25 (0.22)	1.26 (0.24)	1.24 (0.25)	1.19 (0.21)	1.19 (0.24)	1.19 (0.23)	1.18 (0.21)
Non-HDL-C	4.26 (0.57)	4.14 (0.68)	4.05 (0.59)	3.93 (0.69)	3.95 (0.54)	3.86 (0.57)	4.36 (1.06)	4.17 (1.09)	4.13 (1.02)	4.06 (1.05)	4.02 (1.10)	4.12 (1.04)
TAG[a]	1.20 (0.93-2.20)	1.42 (1.24-1.78)	1.38 (1.12-1.74)	1.16 (1.03-1.85)	1.25 (1.04-1.62)	1.21 (0.95-1.62)	1.44 (1.09-2.31)	1.57 (0.86-2.16)	1.40 (1.03-2.18)	1.38 (0.87-1.96)	1.49 (1.04-1.96)	1.25 (1.07-2.31)
Markers for glucose metabolism (IQR)												
Glucose, mmol/L	5.26 (5.13-5.45)	5.26 (5.13-5.41)	5.20 (5.07-5.39)	5.11 (5.01-5.32)	5.16 (5.09-5.57)	5.22 (4.97-5.69)	5.61 (5.34-6.24)	5.37 (5.10-6.13)	5.47 (5.20-5.72)	5.45 (4.96-5.72)	5.32 (5.11-5.77)	5.53 (4.97-5.79)
Insulin, mU/L	12.1 (10.1-14.7)	8.85 (6.97-13.2)	11.2 (7.15-16.5)	8.58 (5.73-11.6)	8.94 (5.84-14.9)	8.63 (6.75-12.6)	8.81 (5.72-18.6)	12.2 (6.89-18.3)	12.2 (5.80-18.6)	8.71 (4.36-16.6)	7.80 (6.07-15.1)	7.96 (4.42-17.4)
HOMA-IR	3.04 (2.32-3.54)	2.09 (1.59-3.08)	2.52 (1.59-3.93)	1.98 (1.30-2.61)	2.25 (1.34-3.37)	2.07 (1.60-2.90)	2.25 (1.39-4.61)	2.73 (1.56-4.77)	2.93 (1.41-4.67)	1.89 (1.09-4.05)	1.81 (1.44-3.86)	1.86 (0.99-4.16)

[a] Total group N=31, plant stanol group N=15, control group N=16. Abbreviations: 95% CI=95% confidence interval, TC=total cholesterol, LDL-C=low density lipoprotein cholesterol, HDL-C=high density lipoprotein cholesterol, TAG=triacylglycerol, HOMA-IR=homeostatic model assessment for insulin resistance, SD=standard deviation, IQR=interquartile range presented as Q1-Q3.

Table S5.6. Anthropometric measures shown for each group per visit.

	Plant stanol ester group (N=15)						Control group (N=17)					
	V1	V2	V3	V4	V5	V6	V1	V2	V3	V4	V5	V6
Weight, kg (SD)	88.8 (8.39)	88.9 (8.49)	89.0 (8.64)	89.0 (8.81)	88.7 (8.87)	88.8 (8.77)	92.2 (10.8)	92.0 (11.3)	91.8 (11.6)	91.6 (11.5)	91.0 (11.8)	91.4 (11.6)
BMI, kg/m² (IQR)	29.6 (28.8-31.6)	29.3 (28.7-31.6)	29.4 (28.7-32.0)	29.5 (28.7-31.6)	29.3 (28.6-31.5)	29.3 (28.6-31.3)	30.6 (28.4-32.9)	30.5 (28.4-33.2)	30.2 (28.1-33.0)	30.2 (28.1-33.0)	29.6 (27.9-33.3)	30.1 (28.2-32.9)
Waist circumference, cm (SD)	102 (6.29)	103 (4.84)	103 (5.96)	102 (5.75)	102 (5.38)	102 (5.43)	104 (8.87)	102 (8.54)	103 (3.39)	103 (8.37)	102 (8.83)	102 (9.17)
Hip circumference, cm (SD)	113 (4.29)	113 (3.75)	112 (4.41)	112 (3.98)	113 (4.78)	113 (4.12)	112 (7.01)	111 (7.71)	111 (6.93)	112 (6.90)	111 (8.00)	111 (8.21)
WHR (SD)	0.90 (0.07)	0.91 (0.05)	0.91 (0.06)	0.90 (0.06)	0.91 (0.06)	0.90 (0.05)	0.92 (0.07)	0.92 (0.08)	0.92 (0.08)	0.93 (0.07)	0.92 (0.07)	0.92 (0.07)

Abbreviations: BMI=body mass index, WHR=waist-to-hip ratio, SD=standard deviation, IQR=interquartile range presented as Q1-Q3.

Chapter 6

Recommended or high daily intakes of plant stanol esters do not affect ex vivo T cell derived cytokine production in immunologically healthy volunteers

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Submitted

EMBARGOED

Chapter 7

General discussion

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Appendices

Summary
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Summary

Optimal functioning of the immune system is a prerequisite for a healthy live. The immune system produces a variety of very different molecules that all play a different role in reacting to e.g., bacterial or viral infections that can cause illness. Normally these reactions are tightly regulated and well-balanced, but they can also be exaggerated, compromised or disbalanced, which can increase the risk at certain diseases. Previous studies have shown that certain plasma markers from dietary origin may reflect immune function, further referred to as dietary determinants of immune function. In addition, dietary approaches – such as the use of certain supplements or changing dietary patterns – are promising interventions to improve immune health.

Serum non-cholesterol sterols reflect cholesterol metabolism when standardized for serum total cholesterol concentrations. Non-cholesterol sterols that reflect intestinal cholesterol absorption can be retrieved from the diet (plant sterols, plant stanols) or are metabolites associated with cholesterol absorption (cholestanol). Contrary, non-cholesterol sterols that reflect endogenous cholesterol synthesis are intermediates of the human cholesterol synthesis pathways (lathosterol, lanosterol, desmosterol). Previous studies have shown that non-cholesterol sterols are able to affect immunological processes. For example, plant sterols and stanols have been shown to improve immune cell behavior in a subset of disbalanced immune cells typical for asthma patients. In addition, desmosterol was shown to reduce inflammatory responses of macrophages. Based on these previous findings, the aim of this thesis was to study the effects of dietary approaches and determinants on immune health throughout life, with special attention to the mediating effects of non-cholesterol sterols.

First, the potential of non-cholesterol sterols as dietary determinants that reflect or predict immune function throughout life was studied. Non-cholesterol sterols (standardized for creamatocrit) were analyzed in breast milk samples one month post partum and associations between non-cholesterol levels in breast milk and allergic outcomes in the first two years of life of breastfed children were analyzed (Chapter 3). The odds of developing eczema during the first two years of life were significantly lower with higher concentrations of cholestanol, lanosterol, lathosterol, and stigmasterol in breast milk one-month postpartum. In addition, the odds of being allergically sensitized (an early stage of allergy) to common allergens at age 2 were significantly lower with a higher concentration of campesterol in breast milk. None of the sterols in breast milk were associated with the development of wheeze during the first two years of life. In Chapter 4, serum non-cholesterol sterols (standardized for total cholesterol to reflect cholesterol metabolism) were measured in two studies that included children with or without asthma. The ADEM study included children aged 3 years and followed them until the age of 6 years. Then it was determined if these children had asthma, transient wheeze, or no complaints. Serum non-cholesterol sterols were measured at age 3 years. Characteristics of cholesterol metabolism were not associated with asthma-related

parameters in these children at the age of 3 or 6 years, nor with airway inflammation. The MIKADO study included children aged 6-16 years (mean age: 12 years). These children had obesity and either already had a confirmed asthma diagnosis or were at increased risk of developing asthma. Obesity was based on BMI-SDS scores, which is a measure of relative weight adjusted for age and sex, which can be determined using growth curves. Serum non-cholesterol sterols were measured before and after weight loss. At baseline, children with a lower BMI-SDS score had an higher intestinal cholesterol absorption (and vice versa). Only in children at risk of developing asthma, a higher cholesterol absorption associated with better lung function. After weight loss, the associations between changes in cholesterol metabolism and changes in lung function were opposite in children with a confirmed asthma diagnosis versus children at risk of developing asthma. Weight loss induced increases in cholesterol absorption related to improved lung function in children at risk of developing asthma. Contrary, weight loss induced decreases in endogenous cholesterol synthesis related to decreases in lung function in children with a confirmed asthma diagnosis. It is unknown why these associations differed in children with or without a confirmed asthma diagnosis, but these results indicate a possible relation between cholesterol metabolism with asthma development or disease progression.

Furthermore, the potential of dietary approaches to improve immune health was studied. In Chapter 2, dietary interventions that simultaneously evaluated effects on asthma-related outcomes and immunological parameters were systematically reviewed. Studies from the clusters "herbs, herbal mixtures, and extracts" and "omega-3 long-chain polyunsaturated fatty acids (LCPUFAs)" showed the highest potential to improve asthma-related outcomes via immunomodulation. However, it should be noted that only three studies were able to induce clinically relevant improvements in asthma-related outcomes. Contrary to these beneficial effects, it was suggested that soy isoflavones worsened asthma-related outcomes via immunomodulation. In Chapter 5, the potential of plant stanols as a dietary approach to improve immune function was studied in a randomized placebo-controlled double-blind intervention study in a population at risk of severe COVID-19 complications. Here, products enriched with plant stanols (4 g/day) or control were provided to people with overweight or obesity starting two weeks before receiving the COVID-19 vaccination until four weeks after the vaccination. Several immunological parameters were studied (e.g., anti-COVID-19 IgM and IgG titers, cytokine production), as well as metabolic parameters (e.g., serum lipid and lipoprotein concentrations, plasma glucose, serum insulin). After consuming 4 g of plant stanols per day, anti-COVID-19 IgM Spike antibody titers increased up to 139% in adults with overweight or obesity. Only in people that showed IgG seroconversion, plant stanol consumption also increased anti-COVID-19 IgG Spike titers. Furthermore, a decrease in stimulated cytokine production was observed, indicating that overall inflammatory responses where decreased, while simultaneously antibody production was increased. No changes in metabolic parameters were observed compared to control. These results imply that people with overweight or obesity at high risk of severe COVID-19 complications could benefit from plant stanols added to their diet to improve their

immune function. In Chapter 6, it was studied if plant stanols would affect immune cell behavior – and thereby potentially disturbed the balanced immune responses – in immunologically healthy volunteers. Samples from two studies were analyzed, either at recommended (2.5 g/day) or high (9.0 g/day) intakes of plant stanols and compared to placebo. Circulating levels of non-cholesterol sterols, lipids, and lipoproteins were also analyzed, as well as stimulated cytokine production. The results of this chapter showed that, as expected, plant stanol intakes lowered circulating serum total and LDL cholesterol concentrations. Cytokine production remained unaffected, indicating that plant stanols might only restore immune function when Th1/Th2 immune responses are imbalanced.

To summarize, the main conclusions of this thesis are:

1. Higher levels of non-cholesterol sterols (standardized for creamatocrit) in breast milk are associated with decreased risk of developing eczema and allergic sensitization in the first to years of life in breastfed children.
2. A high cholesterol absorption is associated with better lung function in children with obesity at risk of asthma. After weight loss, increases in cholesterol absorption are associated with improvements in lung function in children with obesity at risk of asthma. This association was opposite in children with a confirmed asthma, i.e., decreases in cholesterol synthesis were associated with decreases in lung function. Exact mechanisms and causality need to be determined.
3. Omega-3 LCPUFAs and certain herbs, herbal mixtures, and extracts are promising dietary interventions that can induce improvements in asthma-related outcomes via immunomodulation, whereas soy isoflavones induced a worsening in asthma-related outcomes via immunomodulation.
4. Plant stanols are promising dietary components to improve immune health in those with compromised immune responses. Although the exact mechanism by which plant stanols affect immune health remains unclear, LDL cholesterol lowering effects of plant stanols is not a likely explanation.
5. A higher dietary intake of plant stanols does not disturb the balanced immune response in immunologically healthy adults.

Samenvatting

Een optimaal werkend immuunsysteem is een voorwaarde om een gezond leven te leiden. Het immuunsysteem kan vele stoffen maken, die een verschillende rol vervullen wanneer het lichaam reageert op bijvoorbeeld bacteriële of virale infecties, die je ziek kunnen maken. Normaal gesproken zijn deze reacties strak gereguleerd en in balans, maar het kan ook zijn dat deze reacties te sterk, te zwak, of niet in balans zijn, hetgeen de kans op het krijgen van bepaalde ziektes verhoogt. Eerdere studies hebben aangetoond dat er bepaalde stoffen in onze voeding zijn die in het bloed zijn terug te vinden zijn en een indicatie kunnen geven of het immuunsysteem optimaal werkt. Deze stoffen zullen verder worden omschreven als voedingsdeterminanten. Daarnaast zijn voedingsinterventies –bijvoorbeeld het verstrekken van supplementen of het veranderen van voedingspatronen – veelbelovend om de immunologische gezondheid te verbeteren.

Non-cholesterol sterolen zijn markers voor bepaalde kenmerken van het cholesterolmetabolisme, indien hun serum concentraties gestandaardiseerd zijn voor het serum totaalcholesterolgehalte. Non-cholesterol sterolen die de hoogte van de cholesterolopname in de darmen weergeven zijn voornamelijk afkomstig uit onze voeding (plantensterolen, plantenstanolen) of zijn stofwisselingsproducten van cholesterol uit de darm (cholestanol). Er zijn echter ook non-cholesterol sterolen (lathosterol, lanosterol, desmosterol), die gemaakt worden tijdens de vorming van cholesterol door het menselijk lichaam en daarom een maat zijn voor de hoogte van de cholesterolsynthese. Eerdere studies hebben aangetoond dat non-cholesterol sterolen immunologische processen kunnen beïnvloeden. Zo is al aangetoond dat plantensterolen en plantenstanolen het gedrag van juist die immuuncellen van astmapatiënten die uit balans zijn, kunnen verbeteren. Daarnaast is aangetoond dat desmosterol ontstekingsreacties van macrofagen kan verminderen. Op basis van deze eerdere bevindingen was het doel van dit proefschrift om de effecten van voedingsdeterminanten en voedingsinterventies op immunologische gezondheid gedurende de levensloop te bestuderen. Hierbij werd aandacht gegeven aan non-cholesterol sterolen.

Allereerst is de potentie van non-cholesterol sterolen als voedingsdeterminanten die immuunfunctie reflecteren of voorspellen bestudeerd gedurende verschillende momenten van de levensloop. Non-cholesterol sterolen (gestandaardiseerd voor het vetgehalte van de melk) werden geanalyseerd in moedermelk samples die een maand na de bevalling werden verzameld (Hoofstuk 3). De kans op het ontwikkelen van eczeem in de eerste twee levensjaren was significant lager wanneer de concentraties van cholestanol, lanosterol, lathosterol en stigmasterol hoger waren in moedermelk. Daarnaast was de kans op het ontwikkelen van allergische sensitisatie (een voorstadium van allergie) tegen veelvoorkomende allergenen op leeftijd 2 jaar significant lager wanneer de concentraties van campesterol hoger waren in moedermelk. Er werd geen

relatie gevonden tussen non-cholesterol sterolen in moedermelk en de ontwikkeling van een piepende ademhaling gedurende de eerste twee levensjaren. In Hoofdstuk 4 zijn non-cholesterol sterolen gemeten in twee studies in het serum van kinderen met of zonder astma, waarbij de non-cholesterol sterolen waren gestandaardiseerd voor het serum totaalcholesterolgehalte, zodat zij een weerspiegeling waren van het cholesterolmetabolisme. Kinderen uit de ADEM studie zijn gevolgd van leeftijd 3 jaar tot leeftijd 6 jaar, waarbij er op leeftijd 6 jaar werd bekeken of de kinderen astma, een piepende ademhaling, of geen klachten hadden. Non-cholesterol sterolen werden gemeten in serum samples verzameld toen de kinderen 3 jaar oud waren. Karakteristieken van het cholesterolmetabolisme waren niet geassocieerd met astma-gerelateerde uitkomsten of luchtwegontsteking in deze kinderen zowel op de leeftijd van 3 als 6 jaar. Een andere groep kinderen van 6-16 jaar oud (gemiddelde leeftijd was 12 jaar) konden meedoen aan de MIKADO studie. Deze kinderen hadden allemaal obesitas in combinatie met ofwel reeds gediagnostiseerde astma ofwel een verhoogd risico op het ontwikkelen van astma. De mate van obesitas werd bepaald middels de BMI-SDS – een maat voor het gewicht van een kind in vergelijking met het gewicht van andere kinderen van dezelfde leeftijd en geslacht, bepaald aan de hand van groeicurves. Non-cholesterol sterolen zijn gemeten in serum samples die voor en na afvallen werden verzameld. Bij het begin van de studie hadden kinderen met een lagere BMI-SDS een hogere cholesterolabsorptie (en vice versa). Een hogere cholesterolabsorptie was geassocieerd met een betere longfunctie, maar alleen in kinderen met een verhoogd risico op het ontwikkelen van astma. Na afvallen waren de verbanden tussen de veranderingen in het cholesterolmetabolisme en de veranderingen in longfunctie omgekeerd in kinderen met astma versus kinderen met een verhoogd risico op het ontwikkelen van astma. Afvallen leidde tot een verhoging in de cholesterolabsorptie, hetgeen geassocieerd was met verbeteringen in longfunctie in kinderen met een verhoogd risico op het ontwikkelen van astma. Afvallen leidde ook tot een verlaging in de cholesterolsynthese, wat geassocieerd was met een verminderde longfunctie in kinderen met astma. Het is niet bekend waarom deze associaties tegenovergesteld waren in kinderen met en zonder astma, maar de resultaten impliceren dat er een verband lijkt te zijn tussen het cholesterolmetabolisme en de ontwikkeling en/of het ziekteverloop van astma.

Daarnaast is bekeken of verschillende voedingsinterventies de immunologische gezondheid konden verbeteren. In Hoofdstuk 2 is middels een systematische literatuurstudie bestudeerd of voedingsinterventies wellicht astma-gerelateerde uitkomsten en tevens immunologische parameters konden verbeteren. Voor studies uit de clusters "kruiden, kruidenmengsels en extracten" en "omega-3 langeketen meervoudig onverzadigde vetzuren" werd het meeste bewijs gevonden dat zij astma-gerelateerde uitkomsten verbeterden via immunomodulatie. Het moet echter vermeld worden dat er maar drie studies waren die tot klinisch relevante verbeteringen in astma-gerelateerde uitkomsten hebben geleid. Daarentegen verslechterde astma-gerelateerde uitkomsten via immunomodulatie na een interventie met soja isoflavonen. In Hoofdstuk 5 is middels een gerandomiseerde placebo-gecontroleerde dubbelblinde

interventiestudie bekeken of plantenstanolen gebruikt kunnen worden als voedingsinterventie om immuunfunctie te verbeteren in volwassenen met een verhoogd risico op ernstige COVID-19 klachten. In deze studie kregen volwassenen met overgewicht of obesitas 4 gram plantenstanolen of een placebo vanaf twee weken voordat ze de COVID-19 vaccinatie zouden krijgen, tot en met vier weken na het krijgen van de vaccinatie. Verschillende immunologische uitkomsten werden bestudeerd, waaronder antilichaam titers tegen COVID-19 en cytokine-productie. Ook werden metabole parameters bekeken, waaronder de concentraties circulerende lipiden en lipoproteïnen, glucose en insuline. Een dagelijkse inname van 4 gram plantenstanolen leidde tot een verhoging van IgM antilichaam titers tegen COVID-19 in volwassenen met overgewicht of obesitas, met een maximale verhoging van 139%. Alleen in mensen die IgG antilichaam titers tegen COVID-19 aanmaakten, leidde een dagelijkse inname van 4 gram plantenstanolen ook tot een verhoging van deze IgG titers. Ook werd een verlaging in gestimuleerde cytokineproductie gevonden, hetgeen suggereert dat de dagelijkse inname van plantenstanolen leidde tot een verlaging van ontstekingsreacties van het lichaam. Er werden geen verschillen in metabole parameters gevonden ten opzichte van de controlegroep. Deze resultaten impliceren dat mensen met een verhoogd risico op het ontwikkelen van ernstige COVID-19 klachten baat kunnen hebben bij het innemen van plantenstanolen als voedingsinterventie om immuunfunctie te verbeteren. In Hoofdstuk 6 is bekeken of plantenstanolen ook immuuncelgedrag beïnvloeden in mensen, die immunologisch gezien gezond zijn en dus al gebalanceerde reacties van het immuunsysteem hebben. Hierbij zijn twee studies bekeken: één waarbij een aanbevolen inname (2.5 gram/dag) en één waarbij een hoge inname (9.0 gram/dag) plantenstanolen werd vergeleken met een controle. Circulerende concentraties van non-cholesterol sterolen, lipiden, en lipoproteïnen werden geanalyseerd. Ook werd de gestimuleerde cytokineproductie bekeken. De resultaten beschreven in dit hoofdstuk toonden aan dat – zoals verwacht – circulerende concentraties totaal en LDL-cholesterol daalden na inname van plantenstanolen. Cytokineproductie veranderde niet, hetgeen suggereert dat plantenstanolen wellicht alleen de balans in reacties van het immuunsysteem herstellen wanneer deze verstoord is.

Samenvattend zijn dit de belangrijkste conclusies in het proefschrift:

1. Hogere concentraties van non-cholesterol sterolen (gestandaardiseerd voor het vetgehalte in de moedermelk) zijn geassocieerd met een verminderd risico op het ontwikkelen van eczeem en allergische sensitisatie (een voorstadium van allergie) in de eerste twee levensjaren in kinderen die borstvoeding hebben gekregen.
2. Een hogere cholesterolabsorptie is geassocieerd met een betere longfunctie in kinderen met obesitas en een verhoogd risico op het ontwikkelen van astma. De toename in cholesterolabsorptie na afvallen was geassocieerd met een verbetering in longfunctie in deze kinderen. Deze associatie had echter een tegenovergestelde richting in kinderen met een astmadiagnose. In deze

kinderen was juist de afname in cholesterolsynthese na afvallen geassocieerd met een vermindering in longfunctie. Exacte mechanismen en causaliteit moeten verder onderzocht worden.

3. Omega-3 langeketen meervoudig onverzadigde vetzuren en sommige kruiden, kruidenmengsels en extracten zijn veelbelovende voedingsinterventies die astma-gerelateerde uitkomsten kunnen verbeteren door middel van immunomodulatie. Daarentegen zijn soja isoflavonen geassocieerd met een verslechtering in astma-gerelateerde uitkomsten middels immunomodulatie.
4. Plantenstanolen zijn veelbelovend als voedingsinterventie om immunologische gezondheid te verbeteren in mensen waarbij de balans tussen reacties van het immuunsysteem verstoord is. Ondanks dat de exacte onderliggende mechanismen niet bekend zijn, kunnen we stellen dat het onwaarschijnlijk is dat de door plantenstanolen veroorzaakte verlaging in LDL-cholesterol hierbij betrokken is.
5. Een hogere inname van plantenstanolen verstoort gezonde en gebalanceerde reacties van het immuunsysteem in immunologisch gezonde mensen niet.

Impact

The aim of this thesis was to study the effects of dietary approaches and determinants on immune health throughout life, with special attention for non-cholesterol sterols. A systematic review, a randomized-controlled trial, and secondary analyses using data of two observational studies and three randomized-controlled trials have been described in this thesis. Briefly, higher non-cholesterol sterol levels in breast milk were associated with decreased risk of developing eczema (cholestanol, lanosterol, lathosterol, stigmasterol) and allergic sensitization (campesterol) in the first two years of life of breastfed children. Moreover, serum non-cholesterol sterols as markers for cholesterol metabolism were associated with lung function, but in opposite directions for children with or without asthma. In children with asthma, decreased cholesterol synthesis after weight loss was associated with decreased lung function. In children at risk of asthma with obesity, higher cholesterol synthesis (before weight loss) was associated with better lung function. Weight loss induced increases in cholesterol absorption related to improved lung function in children at risk of developing asthma, whereas weight loss induced decreases in cholesterol synthesis related to decreases in lung function in children with a confirmed asthma diagnosis. The associations in children at risk of developing asthma were as expected, since weight loss is associated with increased cholesterol absorption and improvements in asthma complaints. However, it remains unknown why the associations were opposite in children with a confirmed diagnosis in asthma. When used as dietary approach to improve immune function, consuming diets enriched with plant stanol esters improved immunological parameters in adults with overweight or obesity with a skewed immune system. An important question is what the effects of plant stanol consumption are in immunological healthy adults. We here showed that recommended or high intakes of plant stanols did not alter cytokine production in immunologically healthy adults. Based on our systematic review we concluded that other promising dietary interventions for those with compromised immune responses, such as asthma patients, included omega-3 long-chain polyunsaturated fatty acids (LCPUFAs), and certain herbs and extracts. The results described in this thesis will be discussed in this paragraph from a scientific and societal perspective.

Scientific relevance

The low-density lipoprotein (LDL) cholesterol lowering effects of plant sterols and stanols are well-known [1]. However, these non-cholesterol sterols that are derived from the diet may have other effects on human health, such as effects on the immune system [2-6]. This thesis showed that a relation between cholesterol metabolism and lung function, although causality was not determined. In addition, this thesis showed that plant stanols are a suitable dietary approach to increase specific antibody titers upon COVID-19 vaccinations and improve immune cell behavior in those with compromised immune responses. Exact underlying mechanisms remain unclear, although the findings

in Chapter 5 indicate that the LDL cholesterol lowering effects of plant stanols are not involved in immunomodulatory mechanisms. The results described in this thesis can be used to design new studies to further explore the involvement of non-cholesterol sterols in asthma. For example, it is unclear whether plant stanol supplementation in early life is safe on the long term and can prevent asthma development in children. In addition, underlying mechanisms of immunomodulatory effects of plant stanols should be further explored, more specifically the role of changes in cholesterol absorption and if plant stanols act on the immune system via changes in immune cell behavior or the gut microbiome. Finally, this thesis highlights the potential for plant stanol interventions to induce a multiple health effects in those with suboptimal immune and/or metabolic health. All studies presented in this thesis have been published or are in the process of being published in international peer-reviewed scientific journals. In addition, the results of the studies included in this thesis have been or will be presented at (inter)national conferences to scientific peers. These publications and presentations contribute to scientific knowledge utilization and is available for all scientists and other (health) professionals interested.

Societal relevance

A well-functioning immune system is a prerequisite for a healthy life, as dysfunctions in the immune system underlie various health problems. Dysfunctions in the immune system, and more specifically in T cell function, may translate into diseases such as asthma. Asthma is the most prevalent chronic respiratory disease in the youngest children [7, 8], severely affecting their quality of life [9]. From 1990 to 2017 the prevalence of chronic respiratory diseases has increased by 39.8% [10]. Moreover, in 2017, chronic respiratory diseases affected 544.9 million people globally and were ranked as third leading cause of death. Specifically for asthma, global prevalence was 3.6%, which surprisingly remained relatively stable since 1990 [10]. Although prevalence did not really increase, absolute numbers of cases have risen due to an increase in the global population. Therefore, it remains of importance to improve asthma-related complaints and quality of life of these patients.

There are various risk factors for asthma development, ranging from air pollution and antibiotics use during infancy to overweight and obesity [11]. Overweight and obesity are examples of modifiable risk factors, and several weight-loss interventions have already proven to be successful in improving the incidence and severity of asthma-related outcomes [12-14]. Overweight, obesity, and asthma have all been shown to contribute to an increased risk of cardiovascular diseases [15]. Especially for asthma, this increased risk relates to shared pathological mechanisms [15]. Since plant stanols also lower serum LDL cholesterol concentrations, which is a causal risk factor for CVD [16], the results of this thesis imply that plant stanols are promising dietary approaches to improve immune health and metabolic health simultaneously. As plant stanols interventions target multiple health outcomes simultaneously, they could potentially provide multiple health benefits.

A healthy immune system is also relevant for the economy. For example, the COVID-19 pandemic had an enormous impact on health care costs. A study in the USA showed that hospitalized COVID-19 patients had a median stay of 5 days in the hospital, with median hospital charges of $43 986, but in extreme cases reaching $198 394 [17]. Asthma also comes with a high economic burden, with estimated mean yearly costs per patient ranging from $1 900 in Europe to $3 100 in the USA [18]. These costs include direct costs of treatment, but also indirect costs, such as temporary or permanent disabilities or even early mortality [18]. This thesis highlights the relation between dietary determinants in early life (e.g., non-cholesterol sterol levels in breast milk) and asthma, although causality was not determined. It also highlights the relevance of dietary approaches to increase specific antibody titers to vaccinations and reduce asthma severity. Therefore, dietary determinants and approaches should be incorporated in health care to dampen the economic burden of diseases related to a compromised immune function.

Non-cholesterol sterols can either be produced by the human body (cholesterol synthesis markers, cholestanol) or are present in our diet (plant sterols and stanols). Food sources contributing most to plant sterol and stanol intake in the Netherlands are bread, vegetable oils, fruit, and vegetables [19]. Dietary intake of plant sterols is approximately 300 mg/day [19, 20]. People following (largely) plant-based diets are known to have the highest intakes of plant sterols and stanols, up to approximately 600 mg/day [21]. Diets containing more plant-based products than animal-based products – such as an ovolactovegetarian diet – or a completely plant-based vegan diet are more environmentally sustainable compared to (largely) animal-based diets. For example, greenhouse gas emissions are 35% lower for ovolactovegetarian diets, and even 50% lower for vegan diets, compared to omnivorous diets [22]. In general, these diets also require less land and water use compared to omnivorous diets [22]. Therefore, plant-based diets rich in plant sterols and stanols could contribute to increased environmental sustainability. Other dietary components that improved immune health described in this thesis include omega-3 LCPUFAs and herbs, herbal mixtures, and extracts. These approaches can also be incorporated into plant-based and environmentally sustainable diets, especially if omega-3 LCPUFAs are obtained from a plant-based source, such as (micro)algae [23].

Target groups

The effects of plant stanol interventions in diverse target groups were studied in this thesis: children with and without asthma, adults with overweight or obesity, and immunologically healthy adults. Based on the results of this thesis, those with compromised or skewed immune responses – e.g., people with obesity [24, 25], older adults [26], patients with HIV [27] or asthma [28] – may benefit most from dietary approaches to improve immune health. Immunologically healthy people might use these approaches to sustain immune health, as there were no undesired effects on cytokine production in immunologically healthy adults after plant stanol interventions, even at higher than recommended intakes (Chapter 6). Moreover, the LDL cholesterol

lowering effects of plant stanol interventions are well-known. Therefore, those with compromised immune health as well as metabolic health may benefit from a double health benefit of plant stanol interventions. These populations include e.g., patients with asthma and/or obesity, as both conditions are often characterized by elevated serum LDL cholesterol concentrations [29, 30], which is a risk factor for developing cardiovascular diseases [16].

Translation into practice

This thesis described two studies where non-cholesterol sterols were considered as dietary determinants to reflect or predict immune function, either as nutrients in breast milk or as characteristics of cholesterol metabolism. Higher concentrations of non-cholesterol sterols as nutrients in breast milk were associated with a decreased risk of developing eczema or allergic sensitization in the first two years of life. These results could be used in the future to update dietary recommendations for pregnant or lactating women. However, these results should first be confirmed using larger studies. Non-cholesterol sterol supplementation can be safe for pregnant or lactating women and their children [31], although the long-term safety should be confirmed before these results can be used in practice. The correlations observed between characteristics of cholesterol metabolism and lung function in children with asthma or at risk of developing asthma might suggest a link between cholesterol metabolism and asthma development / disease progression. Especially after weight loss, correlations between weight loss induced changes in cholesterol metabolism and lung function showed opposite patterns in children with a confirmed asthma diagnosis versus children at risk of developing asthma. In clinical practice, patterns in cholesterol metabolism might be used as indication to also check if the child suffers from asthma. It should also be explored if consumption of functional foods enriched with plant sterols or stanols could be beneficial for asthma patients. In addition, interventions to alter cholesterol metabolism might contribute to lowering asthma complaints, but well-designed randomized-controlled trials should be performed first to show causality between changes in cholesterol metabolism and asthma outcomes. This thesis also described three studies using dietary approaches to improve immune health. In summary, these studies showed that those with compromised immune responses benefit from dietary approaches, whereas those with healthy immune responses did not show adverse effects in immune function. Dietary approaches that may be used to improve immune health in populations with compromised immune health include plant stanols, omega-3 LCPUFAs, and herbal interventions. These results could be used to update dietary recommendations for asthma patients, as these guidelines are now limited to consuming a healthy diet rich in fruit and vegetables, and to lose weight for asthma patients with obesity. Clinicians and dietitians could use these recommendations in daily practice. However, as the underlying mechanisms of these dietary approaches remain unclear, these should be unraveled first before updating dietary recommendations.

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About the author

Lieve van Brakel was born on the 22nd of September 1994 in Veghel, the Netherlands. In 2012, she graduated from the Zwijsen College Veghel and started her BSc Nutrition and Health at Wageningen University. From September 2014 – February 2015 she did a minor Food Technology at Wageningen University. After obtaining her BSc degree in 2015, she continued at Wageningen University with the MSc Nutrition and Health and MSc Food Technology. As part of these MSc programs, she did internships at the Penn State Health Milton S. Hershey Medical Center (Hershey, PA, USA), the Laboratory of Food Chemistry (Wageningen, the Netherlands), and the HAN University of Applied Sciences (Nijmegen, the Netherlands). In 2018, she obtained the MSc Nutrition and Health with a specialization in Nutritional Physiology and Health Status, as well as the MSc Food Technology with a specialization in Food Chemistry. In September 2018 Lieve started her PhD at the Department of Nutrition and Movement Sciences at Maastricht University under the supervision of prof. dr. J. Plat and prof. dr. ir. R.P. Mensink. Her PhD research focused on the effects of dietary approaches and determinants on immune health throughout life, with special attention for the role of non-cholesterol sterols. In December 2023, Lieve started as a postdoctoral researcher at the Department of Nutrition and Movement Sciences of Maastricht University.

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