The Prophylactic Potential of a Mediterranean Diet Enriched with Fatty Fish in Childhood Asthma

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Glossary of Terms

Glossary term	*Definition*
Pulmonary	
FEV_1	Forced Expiratory Volume in 1 second
FVC	Forced Vital Capacity
FEV_1/FVC	Ratio of FEV_1/FVC
PEF	Peak Expiratory Flow rate
$FEF_{25-75\%}$	% Forced expiratory volume between 25% and 75% of vital capacity
$MEF_{50\%}$	Maximal expiratory flow at 50% of the forced vital capacity
FeNO	Fractional exhaled Nitric Oxide
$Sp0_2$	Erythrocyte oxygen saturation level
ICS	Inhaled Corticosteroids
SABA	Short-acting β_2 adrenergic agonists
LABA	Long-acting β_2 adrenergic agonists
Clinical	
BMI	Body Mass Index
FA	Fatty Acids
EPA	Eicosapentaenoic acid
DHA	Docosahexaenoic acid
AA	Arachidonic Acid
ALA	Alpha-linolenic acid
LA	Linoleic acid
MUFA	Mono-unsaturated fatty acids
PUFA	Poly-unsaturated fatty acids
25(OH)D	25-Hydroxy D
Ig	Immunoglobulin
Units	
g	grams
kg/m^2	kilograms per metres squared
ppb	parts per billion
L	litre
kcal	kilocalorie

Glossary term	*Definition*
Miscellaneous	
RCT	Randomized Controlled Trial
FFQ	Food Frequency Questionnaire
vs	versus
GINA	Global Initiative for Asthma
ISSAC	International Study of Asthma and Allergies in Childhood
NHANES	National Health and Nutrition Examination Survey
CDC	U.S Centre for Disease Control and Prevention
ERS	European Respiratory Society
ATS	American Thoracic Society
WHO	World Health Organization
RNI	Reference nutrient intake
RDI	Recommended dietary intake
EAR	Estimated average requirement
EI	Energy intake
GC-MS	Gas Chromatography- Mass Spectroscopy
RR	Relative Risk
OR	Odds Ratio
HR	Hazard Ratio

Abstract

Diet has contributed to the rise in paediatric asthma prevalence over the last three decades raising global public health concern.

The aim of this two-arm six-month randomized controlled trial was to investigate the effect of a Mediterranean diet enriched with fatty fish on pulmonary function and asthma symptoms in 72 Greek children (5-12 years old) suffering with mild asthma. The intervention group consumed two fatty fish meals weekly (≥ 150 g cooked fillet fish/meal) as part of the Greek Mediterranean diet; and the control group, their habitual diet. Pulmonary function was assessed using spirometry [ventilatory flow (FEV_1), capacity (FVC) and forced expiratory volume between 25% and 75% of vital capacity ($FEF_{25-75\%}$)]; pulmonary inflammation measured by Fractional exhaled Nitric Oxide (FeNO); and Mediterranean diet adherence with the KIDMED score (range 0-12). The final sample consisted of 64 children. At baseline, 54% were boys, 40% overweight/obese, only 17% of children had optimal Mediterranean diet adherence (score ≥ 8). Multiple linear regression analysis revealed, that in the intervention group consumption of 2 fatty fish meals/week (≥ 150 g fillet/meal) significantly reduced FeNO by 14 ppb ($p = 0.037$, 95% CI: -27.39, -0.91, $\beta = -14.15$) as compared to the control adjusting for age, sex, BMI and regular physical activity. Additionally, FEV_1/FVC improved by 4.89 units ($P = 0.013$; 95% CI_{adj}: 1.19-8.61; $\beta = 4.89$) and $FEF_{25-75\%}$ by 12.83 units ($p = 0.006$; 95% CI_{adj}: 4.27- 21.40; $\beta = 12.83$) in the intervention group with sufficient plasma 25(OH)D ≥ 25ng/mL baseline.

This study demonstrated that incorporating two fatty fish meals weekly in a Mediterranean-type diet might be an effective adjunct to conventional therapy targeting pulmonary inflammation in asthmatic children. Vitamin D status appears to be important in facilitating improvement in ventilatory function following a dietary intervention. Future longitudinal intervention studies in Mediterranean and non-Mediterranean regions are warranted to support our findings.

Acknowledgements

Firstly, I would like to express my sincere gratitude to my supervisor Professor Catherine Itsiopoulos for giving me the opportunity to undertake this PhD study and for having faith in me. Professor Itsiopoulos, your continual support, patience, motivation, immense knowledge, encouragement and words of wisdom will always be cherished. Your guidance helped me through my research and writing of this thesis. I consider it to be a great privilege having you as an advisor and mentor for my PhD study.

Besides my supervisor, I would like to thank my co-supervisor Professor Bircan Erbas, for her insightful comments, reinforcement, and statistical guidance as well as the opportunity to work with other international statisticians.

My sincere thanks go to Dr. Katsardis for his time and providing his clinic and patients. Without his precious support and knowledge it would not have been possible to conduct this research. And most importantly, to all the staff and asthmatic patients at the clinic for participating in this PhD project and to whom this thesis is dedicated. A special thanks goes to Dr.Tsoukalas who gave access to his clinic for biochemical testing that was provided free-of-charge to patients and introduced me to a new exciting field of Nutritional Medicine and Metabolomics. I also acknowledge financial assistance provided to disadvantaged families by Beropoulos and Sklavenitis Supermarkets (Hellas) for the provision of coupons and fresh fish for the purpose of this dietary intervention.

I would like to express my gratitude to my good friend Dr. E.M Theodoraki for her psychological support and for believing in me. And I would like to thank my parents, sister and family for supporting me spiritually throughout the PhD journey and my life in general.

Last but not the least, this work was supported by an Australian Government Research Training Program Scholarship. All research procedures reported in this thesis were approved by the relevant Ethics Committee, Safety Committee or authorised officer.

Statement of Original Authorship

This thesis includes work by the author that has been published or accepted for publication as described in the text. Except where reference is made in the text of the thesis, to the best of my knowledge and belief, this thesis contains no other material previously published elsewhere or extracted in whole or in part from a thesis accepted for the award of any other degree or diploma. No other person's work has been used without due acknowledgement in the main text of the thesis. This thesis has not been previously submitted for the award of any degree or diploma in any other tertiary institution.

Signature: _____

Date: 9 March 2020

External Investigators

Three external investigators located in Athens, Greece were approached and agreed to participate in this project. After a preliminary appointment which was scheduled to inform the investigators about project details, a declaration form was signed (Appendix 1).

External Investigator 1: Dr.Charis Katsardis MD (Assoc Professor of Pediatric Pneumology)

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Dr. Charis Katsardis, Paediatric Pneumologist kindly provided the patients and his clinic which is located in the city of Athens, Greece.

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Professor Catherine Itsiopoulos was the principal investigator and provided her nutrition and research skills.

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Professor Bircan Erbas provided her research skills and guided the statistical analysis.

Prologue

The link between food and health has been documented since Antiquity. The importance of food in medicine was recognized in the 5th Century BC by Hippocrates of Cos, the father of Western medicine [1]. In those times it was believed that the disease state was the result of natural causes including diet. In order to fight disease, Hippocratic physicians applied two interventions. Firstly, invasive interventions such as incisions, cauterization and medicines and secondly, dietetic interventions which included diet therapy and exercise [1; 2]. Thus, medicine in the Hippocratic era was indeed a dietetic intervention which gave rise to the renowned quote *"Let food be thy medicine and medicine be thy food"* and basis of modern dietetic practice. Medical research has demonstrated repeatedly the role of diet in the prevention and aetiology of common chronic diseases such as cardiovascular disease, obesity, cancer and diabetes.

There is universal consensus that the Mediterranean diet is a therapeutic sustainable dietary pattern based on the harmonious interaction among traditional foods, wine, cultures, people and environment of countries surrounding the Mediterranean Sea [3]. The Mediterranean dietary pattern has been promoted worldwide as one of the healthiest dietary patterns preventing chronic diseases and promising optimum health, well-being and longevity. Despite the different variants of the Mediterranean diet, there are certain common components and, in general, the Mediterranean diet is characterized by high consumption of seasonal fruits and fresh vegetables, legumes, whole grains, nuts and olive oil; moderate intake of fish and dairy products mostly in the form of cheese and yogurt and low intake of red meat, poultry, sugar and saturated fat accompanied by red wine mainly with meals [4]. Nevertheless, European studies

have reported that children and adolescents living in the Mediterranean countries are abandoning the Mediterranean diet and adopting a Western-type of diet [5].

According to the World Health Organization, asthma prevalence in children has escalated globally [6]. It is believed that asthma is caused by genetic and environmental factors including poor diet. The Mediterranean diet is increasingly being considered as an anti-inflammatory diet [7] but its potential in asthma is still in its infancy. Over the years there has been accumulating evidence from observational studies undertaken in Mediterranean regions supporting the beneficial effect of the Mediterranean diet on respiratory diseases including asthma [8]. However, intervention studies performed in asthmatic children are lacking. Fatty fish a rich source of omega-3 fatty acids possessing anti-inflammatory and immune-modulating effects, is a component of the Traditional Mediterranean dietary pattern [9]. Meta-analysis investigating the effect of omega-3 fatty acid supplementation on asthma in adults and children have produced inconclusive results due to scarcity of studies and heterogeneity among study designs [10; 11]. Therefore, the effect of increased fatty fish intake combined with the Mediterranean dietary pattern in childhood asthma is yet to be elucidated.

The purpose of this thesis was to explore the anti-inflammatory potential of a Mediterranean dietary intervention enriched with fatty fish (Ω3) in childhood asthma.

Thesis Format

This thesis is presented with publications as accepted that have been inserted as pdf in chapters and key points are summarized. In order to assist readability the main text of this thesis and appendices are presented in two separate files. References are numbered in order of appearance in text using the Vancouver referencing style. The format of the thesis incorporates eight chapters as follows:

- Chapter 1 introduces what is known about childhood asthma including prevalence, pathophysiology, aetiology, diagnosis and management.
- Chapter 2 presents characteristics of the Mediterraean diet and summarizes the current evidence on the impact of the Mediterranean diet regime in childhood asthma. Gaps in the literature are also critically reviewed.
- Chapter 3 assesses both qualitatively and quantitatively the scientific data on fish intake during childhood and asthma outcome. Fish consumption patterns in the Greek population are also explored.
- Chapter 4 states the aim of this study and hypotheses.
- Chapter 5 describes the study protocol and methods.
- Chapter 6 presents the results of data analysis together with published papers.
- Chapter 7 briefly summarizes and discusses the main findings of the dietary intervention.
- Chapter 8 proposes future directions, states conclusions and key messages.

List of Publications (As presented in this thesis)

CHAPTER 1 Introduction

1.1 Asthma definition, prevalence and burden of disease

According to the Global Initiative for Asthma (GINA), asthma is defined as a chronic inflammatory disorder of the lungs characterized by airway obstruction, increased bronchial hyperresponsiveness and underlying inflammation that is manifested with recurrent episodes of wheeze, cough, shortness of breath and tightness in the chest, especially at night and early morning [12].

Asthma affects over 300 million people worldwide [12]. It is estimated that an additional 100 million persons will suffer from asthma by the year 2025 [13]. Asthma accounts for about 250, 000 premature deaths per year worldwide (mainly over 45 years old), although rare in children ranging from 0 to 0.7 per 100, 000, and are preventable by good adherence to medication therapy [13; 14]. Adults are four times more likely to die from asthma than children [13].

Globally, over the last 30 years asthma has become the most frequent chronic lower respiratory disease in children [12]. Overall, 14 % of the world's children suffer from asthma [15]. Secular trends show that 1.1 million children in the UK and 6.2 million in US suffer from asthma [16; 17]. While here in Australia, it is estimated that 20.8 % of children aged 0 to 15 years have been 'ever diagnosed' with asthma (by symptoms of wheezing) and 11.3% of children within the same age group have a current diagnosis with prevalence being almost twice as high among indigenous Australians than in non-indigenous [18; 19]. In most children, asthma starts at about 5 years old, and in more than half, develops before age 3 years and can persist into adulthood [20]. It has a higher prevalence in boys younger than 14 years, after which asthma predominates in girls [21]. Diagnosing asthma in young

children is difficult because many of these children are transient wheezers, symptoms usually diminish by the time children reach preschool or early school years [22].

Current statistics show substantial morbidity among children with asthma, limiting daily activities and impacting negatively both the patient and family, socially and emotionally [23]. Asthma is one of the most common reasons for hospitalization, emergency department visits, absence from school and parent's days off work, that is attributed to increased severity, poor disease management and poverty [23]. Data from the Australian National Health Survey showed that in 2016-17 boys (0-14 years) were 1.7 times more likely than girls to be admitted to hospital for asthma, whereas girls 2.4 times more likely than boys during adolescence [24; 25]. An asthmatic child can lose from 3-5 school days due to asthma symptoms and parents the same time period for work absence to care for their child [26]. According to US data, 13.8 million school days were missed among children with asthma [27]. In England, it was reported that 69% of parents took time off work because of the child's asthma, and 13% had given up their jobs [23]. Asthma exacerbations are frightening for the child. Recurrent attacks cause sleeplessness, tiredness, lack of concentration and poor academic performance [6].

Asthma also causes considerable financial burden, due to increase in costs for hospital admissions, doctor's fees and medication [23]. It has been estimated that the mean direct expenditure for asthma (including emergency visits, hospitalization and medication) was approximately $5000 per patient annually in the US to $2,281 USD in Europe [26]. According to U.S statistics, it costed caretakers $726.1 million per year because of work absence due to asthma [28]. The estimated cost of asthma in Australia for 2015 was $11,740 AUD per person [29].

Currently, there is no cure for asthma. It can only be controlled by drug therapy, which on a long-term basis may cause side-effects [12]. Hence there is a need for an alternative

non-pharmacological prophylactic and therapeutic tool, as a preventive measure and to alleviate symptoms of asthma in children.

1.2 The ISAAC Study

The International Study of Asthma and Allergies in Childhood (ISAAC) is one of the largest epidemiological studies to date, investigating the worldwide prevalence and severity of asthma, rhino-conjunctivitis and eczema in children [30]. The programme was designed to allow population comparisons on the prevalence of allergic disease in different countries and trends over time, in an effort to uncover plausible hypotheses explaining the observed patterns and causes of asthma, rhinitis and eczema. The ISAAC study comprised of three phases. Phase One was undertaken between 1992-1997 and involved a total of 463,801 children aged 13-14 years in 155 centres in 56 countries and 256, 800 children aged 6-7 years in 91 centres in 38 countries [30]. It revealed striking geographic variations in the prevalence of asthma symptoms, even within genetically similar groups. Thus suggesting that environmental factors may be responsible for the observed variations [31]. According to the investigators, up to 20-fold variations in the prevalence of 'current wheeze' (in the last 12 months) were observed between centres worldwide [32]. Notably, the highest prevalence was from centres in English speaking countries (Australia, New Zealand, Ireland, United States, Great Britain), followed by most centres in North, Central and South America. In Europe, countries with the highest prevalence of asthma and allergy (>20%) were Finland, Germany, and recently Romania (Figure 1). In contrast, the lowest prevalences (< 5%) were reported in Mediterranean countries such as Greece, Albania, Italy, Malta, as well as in Estonia, Georgia, Lithuania, Spain , Sweden, China, Taiwan, Uzbekistan, Indonesia, India and Ethiopia [31]. Population level environmental factors found to be positively associated with symptoms for all three allergic conditions were gross national product per capita, trans fatty acids, paracetamol

and women smoking; and negatively associated with plant foods, pollen, immunization, air pollution and men smoking [33].

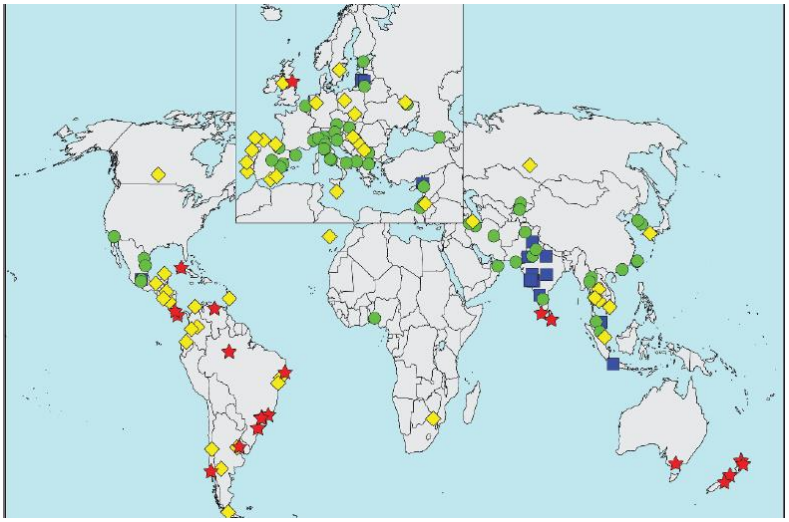

Figure 1 Prevalence of current wheeze in children aged 6-7 years

Key: Prevalence values of current wheeze: blue = 5 %; green = 5-10 %; yellow = 10-20 %; red= high prevalence >20 %.

Figure 1 illustrates the worldwide variation in prevalence of current wheeze for children aged 6-7 years. Current wheeze predominates in English-speaking countries, ≥ 20 % of children as indicated by the red star (Australia, UK, USA, New Zealand), is lowest in Mediterranean countries (Greece, Italy, Spain, Albania) and Asia-Pacific regions, < 5 % (blue star) .

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ISAAC study Phase two was undertaken in 30 centres in 22 countries that included 13 countries from the European Union (Albania, Estonia, France, Germany, Greece, Iceland, Italy, Latvia, Netherlands, Norway, Spain, Sweden and the United Kingdom) and 9 countries outside of Europe (Brazil, China, Ecuador, Georgia, Ghana, India, New Zealand, Turkey and Palestine) [34]. The purpose of Phase 2 was to investigate intensively possible aetiological factors in 10-12 year old children in an effort to explain the

variations in asthma prevalence and severity which emerged from Phase One. Comparisons between centres were undertaken using objective measures of disease and assessment of environment, lifestyle, genetic factors and clinical management. The 10-12 year old age group was selected as they were more likely to comprehend the procedures involved than younger children (6-7 year old) and to be more compliant than adolescents (13-14 years) [34]. An outstanding finding revealed from Phase 2 was that there was a strong relationship between BMI and wheeze in children from affluent countries as compared to non-affluent and that there was an adverse effect of overweight/obesity on airway obstruction biomarker (FEV_1/FVC) [35]. With respect to the diet and asthma, Nagel et al (2010) documented that fruit, fish, cooked green vegetables and the Mediterranean diet were associated with lower prevalence of wheeze and life-time asthma in children aged 8-12 years old [8]. Contrastingly, fast food, butter and burger intake were associated with increased asthma prevalence [8; 36]. These results are of utmost importance to this PhD study, since they provide promising evidence on the potential of the Mediterranean diet in protecting against wheeze and asthma in childhood, and merits further exploration in this PhD clinical trial.

Phase 3 was a repetition of phase 1 protocol over a period of 5-10 years with the addition of 128 new centres, specifically eight Pacific islands (Tonga, Fiji islands, Samoa, Cook Islands, Tokelau Islands, New French Polynesia and New Calendonia) [37; 38]. The scope of phase 3 was to investigate time trends in the prevalence of asthma, rhinoconjunctivitis and eczema in the countries that participated in phase 1, as well as describe the prevalence and severity of asthma in the new centres and explore the hypotheses at the individual level generated from the findings of Phase 1. The significance of this study was to explore the impact of economic development on the prevalence and severity of asthma symptoms in diverse socio-economic backgrounds. Phase 3 involved a total of 193,404 children, aged 6-7 years from 66 centres in 37 countries and 304, 679 adolescents aged 13-14 years, from 106 centres in 56 countries [31; 37]. Prevalence of asthma was based on symptoms of current

wheeze and severe wheeze during the last 12 months [31]. Results of Phase 3 also showed significant geographic variations in asthma prevalence. A slight increase in prevalence for current wheeze and severe wheeze was noted in both age groups (Figure 2). As compared to adolesecents, an increase in asthma prevalence was more common in young children (6-7 years) for centres in Asia-Pacific, India, North America, Eastern Mediterranean, Western Europe, whereas in Africa, Asia-Pacific, India, Latin America, Northern and Eastern Europe for the older-age group [37]. Nevertheless, for those countries participating in Phase 1, high prevalence remained in English-speaking countries, Latin America, North-West and South-East Europe [31]. Nevertheless, in adolescents with existing high prevalence, further increases were not observed [37], most likely due to increased awareness of the condition and improved diagnostic tools. Concerning new centres in Africa, Vietnam and Sri Lanka, the prevalence of severe asthma was among the highest in the world, whereas Asia-Pacific, India, Tibet and Mexico showed low prevalence [31]. A possible explanation for variation in asthma prevalence among countries could be related to the indoor and outdoor environment, economic status, microbial exposure and health literacy [37]. In low-income countries and among ethnic minorities in developing countries, there may be less awareness that wheezing is a symptom of asthma [38], along with poor living conditions such as exposure to dampness and mould in the home environment and use of open fires for cooking [21]. This hypothesis is supported by observations reporting that undiagnosed asthma among wheezers was common in children from lower income countries. Another reason, asthma care may not be available to all people in developing countries. Differences in exposure to air pollutants might also contribute to the greater severity observed in developing countries [38].

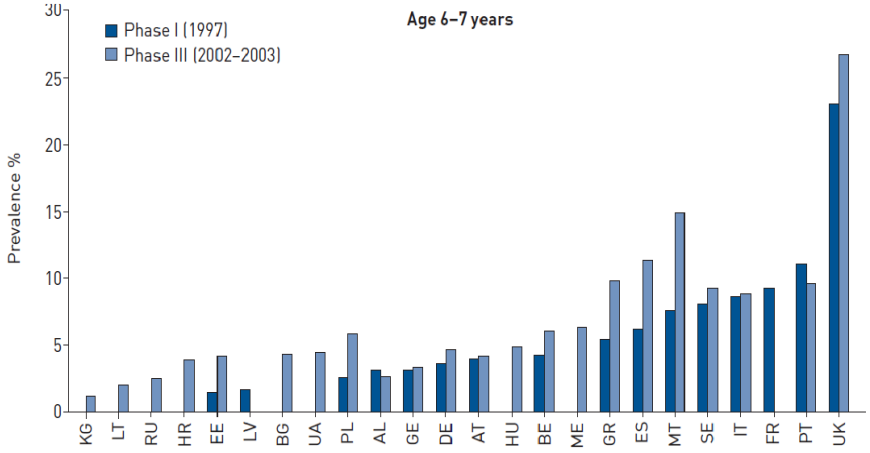

Figure 2 Prevalence of childhood asthma in 6-7 year old children in 1997 and 2002-2003

Key: Country Abbreviations: KG- India HR-North/East Europe, LT-Lithuania, RU-Russia; EE-Eastern Europe; LV-Latvia; BG-Bulgaria; UA-Asia/Pacific; PL-Poland; AL-Albania, GE-Germany; DE-Denmark; AT-Austria; HU-Hungary; BE-Belgium; ME- Mexico; GR-Greece; ES-Estonia; MT- Mediterranean East; SE- South East Asia ; IT-Italy; FR-France; PT-Portugal; UK-United Kingdom.

Source: Reproduced with permission of the © ERS 2020. In: The European Lung White Book Respiratory Health and Disease in Europe 2nd Edition, Edited by: G. John Gibson, Robert Loddenkemper, Yves Sibille, Bo Lundback, 2013 European Respiratory Society, Print ISBN: 978-1-84984-042-2, Online ISBN: 978-1-84984-043-9

In summary, the findings of the ISAAC study provide valuable insight to the global epidemiology of asthma and form a basis for further exploration in intervention studies. A strength of the ISAAC study was the standardized methodology that was replicated in surveys and achieved a high response rate, the large number of centres collaborating, high participation rate (89% for 6-7 year olds, 92% 13-14 years old) [33; 37], as well as homogeneity among participants (all school-children) which were randomly sampled.

Another strong point was the time frame (average of 7 years) which was adequate to detect changes in centres [37] .

Possible limitations of the ISAAC study were that most centres were urban which might not be representative of the country or region [37]. In Phases 1 and 3, details on environmental exposures, dietary habits and presence of asthma/wheeze were assessed using questionnaires which are prone to information and recall bias [39] as compared to objective measurements of lung function using the gold standard, spirometry [20]. Another drawback, the observational study design is unable to demonstrate potential causality of associations [33]. Nevertheless, interventions based on small associations could be beneficial in terms of public health or on an individual level and will be explored in this PhD study.

1.3 Pathophysiology

1.3.1 What is asthma?

The pathophysiology of asthma is complex involving mechanisms of adaptive and innate immunity that result in airway inflammation, airway obstruction, bronchial hyperresponsiveness as well as structural changes referred to as airway remodelling [20]. Airway inflammation is a central feature of asthma which varies according to phenotypic differences [40]. It is characterised by oedema, mucus production, infiltration by inflammatory cells, smooth muscle contraction and epithelial desquamation which initiate stenosis and resistance of airflow into and out of the lungs resulting in trapping of air and hyperinflation [20];[41] (Figure 3). The uneven distribution of air combined with hyperinflation cause dyspnea and subsequently lead to a state of alveolar hypoxia [42; 43].

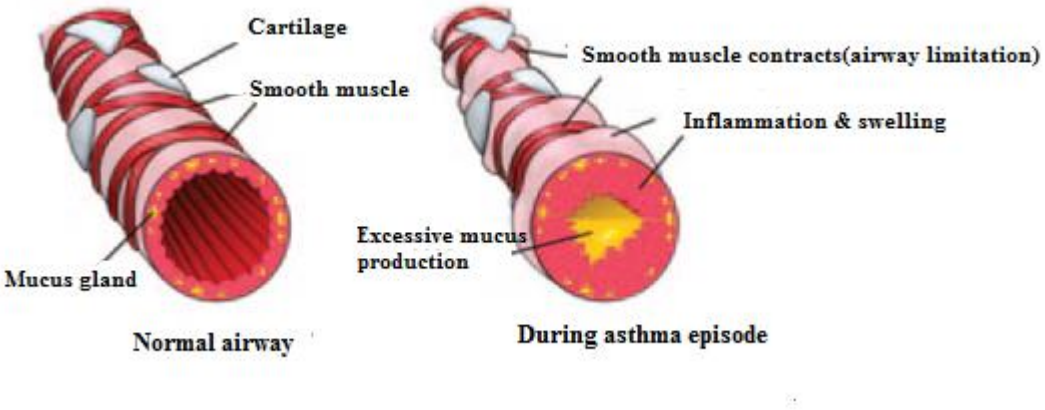

Figure 3 Asthma pathogenesis

Figure 3 depicts the hallmarks of asthma development. Asthma is recognized as a state of chronic inflammation.

Source:Modified from https://sites.google.com/a/abaoman.org/asthma-for-teenagers-a-guide/the-basics-of-asthma/what-happens-in-the-lungs-of-an-asthma-patient

1.3.2 The allergic response in asthma

The allergic response in airways is the result of a complex interaction of mast cells, eosinophils, T lymphocytes, macrophages, dendritic cells, and neutrophils [(44)] (Figure 4). On exposure to an allergen, an early allergic response in the airways occurs within minutes and peaks at 20 minutes following inhalation followed by asthma symptoms [(44)]. These effects are the result of mast cell–derived mediators. Four to 10 hours later, a late allergic response may occur, which is characterized by infiltration of inflammatory cells into the airway and is most likely caused by cytokine-mediated recruitment and activation of lymphocytes and eosinophils [(44)]. Antigen-presenting cells (such as macrophages, dendritic cells) in the airway capture, process, and present the antigen to helper T cells, which become activated and secrete cytokines. Helper T cells (Th) can be induced by cytokines (interferon-gamma, interleukin (IL-2)) to develop into Th1 or Th2 cells, however presence of an allergen activates $CD4^+$ and Th 2 cell responses. Th2 cells release pro-inflammatory cytokines interleukins (IL-4, IL-5, IL-6, IL-9 and IL13) which promote

synthesis of IgE from B-cells and eosinophil recruitment [44]. Subsequently, IgE binds to the high-affinity receptor for IgE, Fc-epsilon-RI, on the surface of mast cells and basophils [44]. This leads to degranulation of mast cells and basophils and finally the release of histamine and proteases that promote airway inflammation, narrowing of airways due to mucosal oedema, smooth-muscle constriction, mucus secretion and epithelial–cell shedding into the airways and structural changes that are responsible for the onset of characteristic symptoms of asthma including wheezing, cough, shortness of breath and tightness in the chest [44]. In addition, regulatory Tcells (Treg) participate in the Th2-cell allergic response and are important in suppression of airway inflammation and in prevention of airway remodelling [45]. Furthermore, proinflammatory cytokines (IL-3, IL-4, IL-5, tumor necrosis factor-alpha [TNF-α]) are released from mast cells and are generated de novo after mast-cell activation [44]. These cytokines contribute to the late allergic response by attracting neutrophils and eosinophils [44]. The eosinophils release major basic protein, eosinophil cationic protein, eosinophil derived neurotoxin, and eosinophil peroxidase into the airway, causing epithelial denudation and exposure of nerve endings [44]. The lymphocytes that are attracted to the airway continue to promote the inflammatory response by secreting cytokines and chemokines, which further drive cellular infiltration into the airway [44]. Recurrent inflammation eventually results in hypertrophy of smooth muscles, hyperplasia of mucous glands, thickening of basement membranes, and continuing cellular infiltration [44; 46]. These long-term changes in airways, also known as airway remodelling, lead to fibrosis and irreversible airway obstruction in patients and ultimately chronic, severe asthma and are responsible for resistant asthma which does not respond to conventional medication treatment [47].

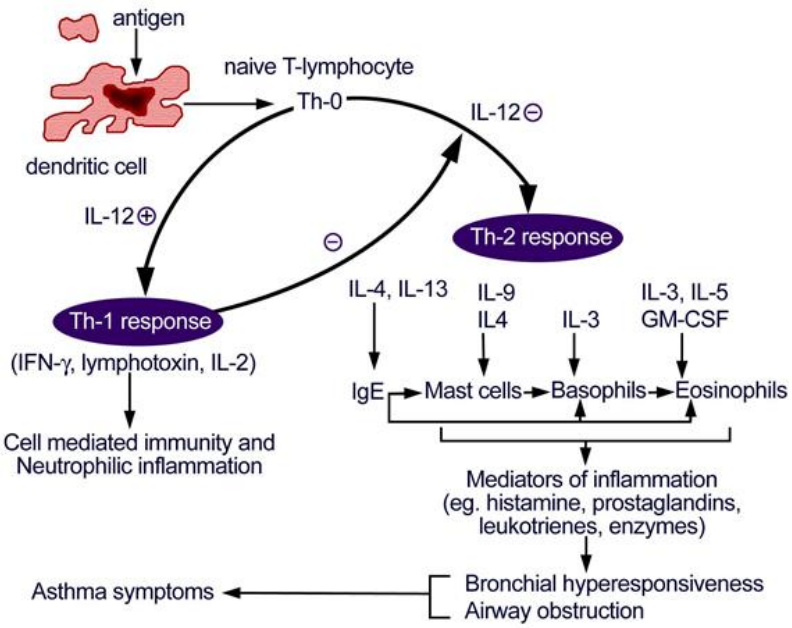

Figure 4 Pathogenesis of asthma- immune response to allergen exposure

Source: Image reproduced with permission from Medscape Drugs & Diseases (https://emedicine.medscape.com/), Asthma, 2019.

Available at: https://emedicine.medscape.com/article/296301-overview.

1.3.3 Oxidative stress and endoplasmic reticulum stress

Recent research has shown that oxidative stress induced by reactive oxygen species (ROS) and reactive nitrate species (RNS) play a central role in pulmonary disorders including bronchial asthma [48]. Firstly, an environmental exposure to an allergen (e.g air pollution, saturated fats) causes an imbalance between oxidant-antioxidant systems in the lungs, triggering excessive production of ROS and reactive nitrogen species RNS [49; 50]. ROS-mediate activation of signalling pathways such as NF-B and AP-1, producing pro-inflammatory mediators (TNF α, IL1, IL8), promoting tissue injury and inflammation in the airways [49]. The mechanism by which oxygen radicals cause asthma pathogenesis is by the oxidation or nitration of proteins, lipids, and DNA subsequently leading to

dysfunction of these molecules. In addition, the physiological antioxidant system, which under normal circumstances protects the organism from free radical damage, is impaired in asthma, possibly because of cellular inflammation [51]. Secondly, oxidative stress is closely linked to endoplasmic reticulum (ER) stress [50; 52]. In mice, saturated fats, stearic and palmitic acids are known inducers of ER stress in various cells [53]. The production of free radicals within the ER disturbs ER protein folding. The accumulation of mutated protein, disturbances in cellular redox regulation, build-up of ROS, hypoxia and changes in calcium regulation induce ER stress thereby activating the unfolded protein response (UPR) in an effort to restore ER homeostasis (Figure 5) [50; 52]. However, in allergen-induced bronchial asthma, prolonged ER stress elicits inflammatory responses via UPR pathways which ultimately trigger cell death [50; 52].

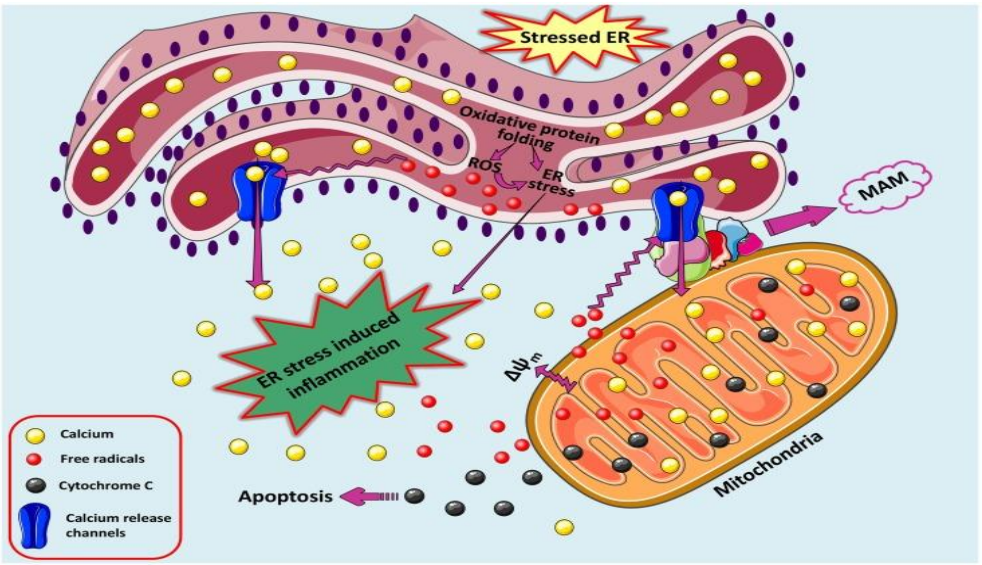

Figure 5 Oxidative stress, ER stress leading to inflammation in bronchial asthma

Figure 5 illustrates the mechanism leading to an inflammatory response: increased protein folding demand, calcium and ROS signalling integrated with UPR pathways.

Source: Reproduced from Chaudari, 2014. Front. Cell. Neurosci., https:// doi.org/10.3389/fncel.2014.00213 under Creative Commons Attribution License

Additive effect of immune response and oxidative stress

In contrast, allergen exposure triggers an inflammatory response that promotes recruitment of various inflammatory cells in the airway mucosa including epithelium, fibroblasts, airway smooth cells, immune regulatory cell types, dendritic cells(DCs), Th2 (T-helper cells 2), Th17, mast cells, granulocytes (eosinophils, neutrophils) and innate-type lymphoid cells (iLCs) are activated, which could all contribute to the generation of oxidative stress [(54)] (Figure 6). The high level of ROS and RNS, ER stress, mitochondrial dysfunction, might cause changes in signalling events and alterations in cellular metabolism, resulting in the generation of metabolites such as pro-inflammatory eicosanoids, lipid peroxidation and modification of proteins by oxidation/or nitration. These pro-inflammatory mediators sensitize cells causing them to become readily susceptible to stimulation by allergen exposure especially in genetically-susceptible individuals or those with epigenetic modifications [(54)].

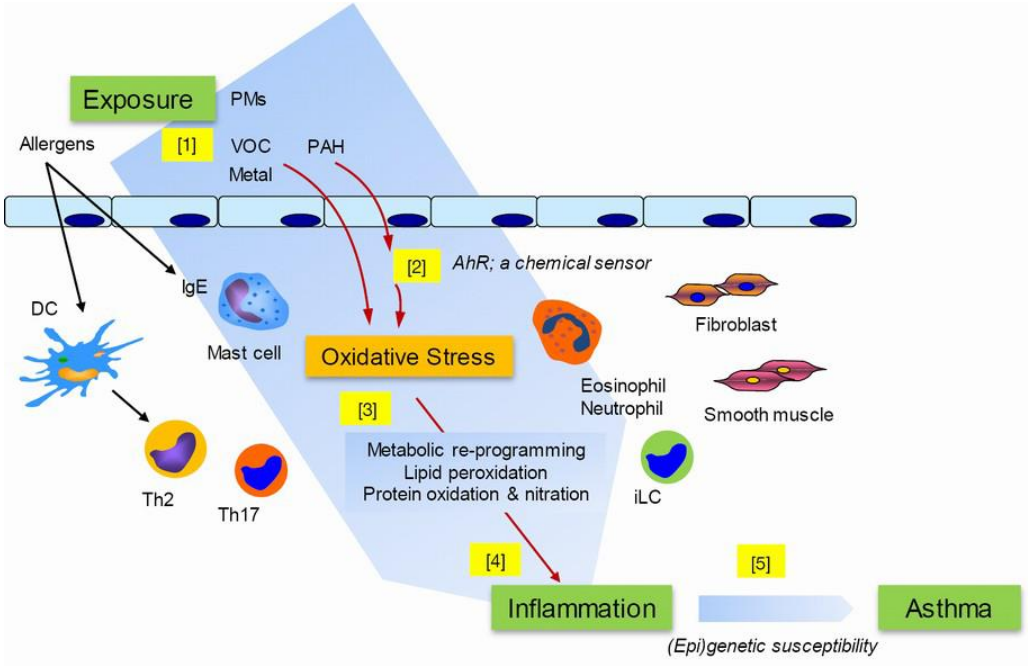

Figure 6 Asthma is the result of gene-environment interaction

Figure 6 describes the cascade of events following exposure to an allergen (e.g pollution Polycyclic Aromatic Hydrocarbons (PAH)) to the development of asthma which include: 1) Allergen exposure triggers oxidative stress 2) activation of immune response 3) metabolic reprogramming and alteration to cellular metabolism. 4) generation of pro-inflammatory metabolites, lipid peroxidation, modification of proteins, DNA damage 5) sensitization of cells promotes symptoms in genetically-susceptible individuals [(54)].

Source: Image reproduced with permission from AME Publishing Company for Huang et al, 2015 J Thorac Dis; 7(1): 23-33. doi: 10.3978/j.issn.2072-1439.2014.12.13.

Growing evidence suggests that ER stress, oxidative stress and inflammatory responses are cross-linked and that limiting one will affect the other [(48)]. Given that the latter mechanisms are associated with inflammatory disease, perhaps therapies that target both stresses may be effective in treating or relieving symptoms. Small molecules acting as chaperones have been identified that stabilize the misfolded proteins facilitating protein folding and alleviating ER stress. In mice, 4-phenylbutyrate an ER stress inhibitor reduced the lipopolysaccharide induced lung inflammation [(48)]. Similarly, post traumatic brain surgery administration of docosahexaenoic (DHA) reduced ER stress markers proteins in Sprague-Dawley rats [(52)]. Previous findings suggest that anti-oxidants can suppress disease-related oxidative and ER stress [(55)]. Recent data from the ISAAC II cohort studies undertaken in Greek children reported that high anti-oxidant intake as evaluated from Food Frequency Questionnaires (FFQ) was inversely associated with asthma [(56)]. The Mediterranean diet consists of a wide range of anti-oxidant and omega-3 fatty acid rich foods that deserves exploration in relation to oxidative stress and lung inflammation associated in respiratory diseases such as asthma.

1.4 Aetiology: Risk factors

Asthma has been called a "syndrome" resulting from a complex interplay between genetic, environmental and lifestyle factors with environmental triggers playing a major role more than genetics in the pathogenesis of this condition [21]. A number of mechanisms have been identified to explain asthma onset which will be briefly outlined. Determinants that are positively associated with asthma onset are presented in Table 1.

Table 1 Risk factors positively associated with asthma development

Demographics/lifestyle	**Risk factors**
Age	Pertussis
Sex	Less exposure to infection
Race/ethnicity	Medication early in life: antibiotics/paracetamol
Family history	**Diet**
Family size [57]	Fast food
Birth order [57]	Trans fats
Genetics	Salt
Urbanisation	**Inhaled exposure**
High-income lifestyle	Maternal smoking
Gut microbiota*	Paternal smoking
High BMI	Child smoking
Exercise/physical exertion	Air pollution
Sedentary lifestyle	House mites
Inhaled allergens	Cockroaches
Perinatal	Pollen
Low-birth weight (<2.5 kg)	Animal fur (cats/dogs)
Premature birth (<37 weeks)	Mould
Caesarean delivery	Dampness
Accelerated infant weight gain	Cold air
Pre-natal exposure to tobacco smoke	Household sprays/detergents/paint
Maternal age	Plants
Comorbidities	Wood dust
Rhinitis	Pet dander
Chronic sinusitis	**Psychological**
Gastroesophageal reflux disease	Stress
Atopic sensitization	Anger
Early respiratory infections	Fear
Respiratory syncytial virus	Excitement
Rhinovirus	Anxiety

Source: Adapted from Beasley et al, 2015. Risk factors for asthma: is prevention possible? Lancet 386, 1075-1085. doi: 10.1016/S0140-6736(15)00156-7

Strachan, 2000. Family size, infection and atopy. Thorax 55 S2-10

1.4.1 Genetic factors

Asthma is caused by multiple interacting genes, some having a protective effect and others contributing to the disease pathogenesis, with each gene having its own tendency to be influenced by the environment [58]. It has been estimated that 35-95% of heredity accounts for childhood asthma [59]. Many individuals are genetically predisposed to developing allergies on exposure to a particular allergen (for example pollen, antibiotics, perfumes) that do not generally elicit immune response [60]. We know that asthmatic mothers are more likely to have asthmatic offspring than non-atopic mothers [60] or asthmatic fathers [61; 62]. Also identical twins are more likely to be both asthmatic than non-identical twins [63]. Nevertheless, approximately 75% of identical twins with an asthmatic twin will themselves be asthmatic, indicating a contribution from both genetic and environmental factors [63]. Elevated levels of IgE have been detected in patients with allergic diseases which increases their propensity for hypersensitivity on exposure to an allergen [60]. Over the last two decades, genetic research has identified that multiple genes, gene-gene interactions, gene-environment interactions and epigenetic modifications are involved in asthma pathogenesis, severity and response to pharmacotherapy [64]. It has been proposed that the effect of one single polymorphism alone may be negligible, but the synergistic effect of many genes may enhance the development of disease [64]. As mentioned previously, asthma is not a single disease but has been described as an umbrella for multiple diseases with similar clinical features [59]. Genetic studies have identified many phenotypes to describe the various asthma subgroups. These phenotypes include bronchial hyperresponsiveness (BHR), lung function, serum IgE, allergen skin test response, drug response, frequency of exacerbations, age of onset, exercise-related asthma, degree of inflammation and blood eosinophil count [59].

Candidate-gene and linkage studies have isolated 53 genes and over 100 loci involved in atopy, bronchial hyperresponsiveness, elevated IgE, asthma onset as well as influencing spirometry parameters such as *ADAM33, VDR, DPP10, HLA-G* and *GPR154*.

These genetic markers are related to a variety of biological processes including cytokine signalling, T cell proliferation and differentiation (Th1/Th2), T reg cell function, HLA locus/immunity and Ig E response of B cells [64]. Genes important for Th1 versus Th2 T cell polarization, *GATA3, TBX21, IL4, IL4RA, STAT6*, and *IL12B*, have been implicated in asthma and allergy [64]. Another class of genes such as *CD14*, *toll-like receptor* (*TLR*) *2*, *TLR4*, *TLR6*, and *TLR10* [64] are involved in the detection of allergens and pathogens, while others contribute to airway remodelling by mediating the response to allergic inflammation, immunity and oxidant stress on the tissue level [64] . For example, A Disintegrin and Metalloprotease 33 (*ADAM 33)* which is expressed in lung fibroblasts and smooth muscle cells has been localized on chromosome 20p13 and encodes for asthma susceptibility [65]. This gene is associated with bronchial hyper-responsiveness, lung function decline and airway remodelling. ADAM33 is a multifunctional gene that is involved in activation, proteolysis, adhesion, fusion and intracellular signalling. Polymorphisms in ADAM33 are linked with an accelerated decline in forced expiratory volume in first second (FEV_1). It has been hypothesized that ADAM33 variants may influence lung function in early life and epithelial–mesenchymal dysfunction in airways thereby predisposing individuals to asthma [66].

With respect to the immune response, the gene for interleukin-4 (IL-4) is located on chromosome 5q31. IL-4 is a cytokine that is secreted by T helper cells type 2 (Th2) that stimulate the production of IgE and induce eosinophil-mediated attacks against allergens [67]. Polymorphisms in IL-4 are related to the severity of airway hyperresponsiveness [68]. Interleukin 21 (IL-21) that is found on chromosome 4q26-q27 affects growth and survival of immune cells as well as regulating IgE production [69; 70]. IL-21 is produced by activated DC4+T cells. Another gene associated with allergy, is β-chain of the high-affinity receptor for IgE (FcεRIβ) which is localized on chromosome 11q13 [71] . This gene is responsible for the immediate response during exposure to an allergen and is found on the surface of mast cells, basophils, eosinophils and Langerhan's cells. On exposure to an

allergen, the allergen binds to the receptor-bound IgE which leads to degranulation of mast cells and subsequent synthesis and release of cytokines (IL-4) and activated inflammatory cells [58].

Regarding inflammation, a number of genes have been isolated. A gene localized on chromosome 17q 11.2-q12, inducible nitric oxide synthase (iNOS) is expressed from T cells and macrophages. The resultant nitric oxide that is produced causes mucus hypersecretion, upregulation of Th2 and downregulation of Th1 responses [71; 72] resulting in smooth muscle constriction and asthma symptomology. Genes relating to pro-inflammatory cytokines, TNF-α and TNF-β have been identified on chromosome 6p21 [58]. Polymorphisms in these two genes are related with elevated Ig E levels in asthmatic patients [71; 73]). Another gene involved in inflammation and airway remodelling is mast cell chymase (CMA1) located on chromosome 14q11.2 which encodes for a serine protease expressed in mast cells [58] [71]. A relationship has also been observed between this gene and increased total Ig E in asthma [58] [74]. Similar functions have been found with N-acetyltransferase 2 (NAT2) gene which lies on chromosome 8p22 and is responsible for N-acetylation and influences susceptibility to atopic disorders. NAT2 has been linked with increased total IgE and eosinophilia associated with asthma [71; 75]. A different gene implicated in asthma is eotaxin (*SCYA11),* found on chromosome 17q21.1-q21.2 that encodes for the chemokine that is an attractant for eosinophils. Another chemo-attractant for inflammatory cells is the gene for acid mammalian chitinase (CHIA) that is localized on 1q13-21.3 and is important as an effector response for IL-13 and in shifting inflammation towards Th2 [76]. In contrast, the gene for the anti-inflammatory cytokine, Interleukin-10 (IL-10) produced by monocytes and macrophages is located on chromosome 1q31-q32 [77]. Another molecule with anti-inflammatory effects is the protein secreted from Clara cells in the respiratory system. The Clara cell secretory protein (CC16) gene is localized on chromosome 11q13 and encodes a protein which

limits the synthesis of leukotrienes and prostaglandins as well as inhibits chemotaxis of inflammatory cells involved in asthma pathogenesis [58; 78].

As for childhood asthma onset, an association was found with variation at the 17q21 asthma locus, encoding ORMDL3 and GSDML genes [59]. Regarding lung function as evaluated by spirometry, meta-analyses of GWAS studies have located nine loci, [THSD4, HHIP, GPR126, ADAM19, AGER-PPT2, FAM13A, PTCH1, PID1and HTR4] to be associated with FEV_1/FVC [79; 80], and two loci, HTR4 and INTS12-GSTCD-NPNT with FEV_1 [79; 80]. Table 2 presents chromosome regions involved in asthma pathogenesis identified by linkage analysis and single nucleotide polymorphism (SNPs) studies.

Table 2 Candidate genes in asthma with locations

Gene	Description	Location	Refs
ADAM33	Asthma susceptibility gene, airway remodelling	20p13	(65)
EGR-1	Early growth response protein 1	1p34	(71)
PTGER3	Prostaglandin E receptor 3	1p31	(71)
CLCA1	Chloride channel calcium activated family member	1p22-31	(71; 81; 82)
V-CAM 1	Vascular cell adhesion protein 1 precursor	1p21	(71)
GSTM1	Glutathione-S-transferase	1p13.3	(71)
A3AR	Adenosine A3 receptor	1p13	(71)
CHIA	An effector response for IL-13	1p13.1	(76)
LELP1	Late comified envelope-like proline-rich 1	1q21	(83)
FLG	Filaggrin	1q21.3	(84; 85)
IL-10	IL-10 gene	1q31	(77)
A1	Adenosine A1 receptor	1q32	(71)
CHI3L1	Chitinase 3-like 1	1q32	(86)
TGF-β2	Transforming growth factor beta- 2 precursor	1q41	(71)
TGF-β1	Transforming growth factor beta -1 precursor	6q11-q2	(87)
IL-1R-1	Interleukin -1 receptor	2ql1	(71)
INPP4A	Inositol polyphosphate 4 phosphatase type 1	2ql1.2	(88)
1L-1RN	Interleukin-1 receptor antagonist protein precursor	2q13	(71)
1L-1(α, β)	Interleukin-1 alpha & beta precursors	2q21	(71)
CTLA4	Cytotoxic T-lymphocyte antigen 4	2q 33	(85)
IL-8RA	High affinity interleukin-8 receptor A	2q35	(71)
DPP10	Dipeptidylpeptidase 10 isoform 1	2q14-2q32	(71; 82; 85)
CCR1	C-C chemokine receptor type 1	3p21	(71)
CCR2	Chemokine receptor 2	3p21.31	(89)
IL-8	Interleukin 8 precursor	4q13	(71)
APA	Aminopeptidase A	4q25	(71)
IL-21	IL-21 gene	4q26	(46)

Gene	Description	Location	Refs
IL-3,4,5,9,10,12,13	Interleukin-3, 4,5,9,10,12,13 precursors; Th mediated reponses	5q31	(84; 85; 90)
CD14	Monocyte differentiation antigen CD14 precursor	5q31	(71; 85)
SPINK5	Serine protease inhibitor Kazal-type 5 precursor	5q32	(71; 85)
ADRB2	Beta-2 adrenergic receptor	5q31-32	(91; 92)
UGRP1	Uteroglobin related protein 1	5q32	(93; 94)
GPX3	Plasma glutathione peroxidase precursor	5q33	(71)
CYFIP2	Cytoplasmic FMR 1 interacting protein 2	5q33	(86)
HAVCR1	Hepatitis A virus cellular receptor 1	5q33.2	(85)
SLP-2 LCP2	SH2 domain-containing leucocyte protein	5q35	(71)
SLP-76	Lymphocyte cytosolic protein 2	5q35	(71)
LTC4S	Leukotriene C4 synthase	5q35	(85)
TCRβV	T cell receptor Vβ	6p	(95)
IL-17	Interleukin -17 precursor	6p	(96)
HLA-DRB1	Major histocompatibility complex-class II-DR beta 1	6p 21	(97)
TNF-α, TNF-β	Tumor necrosis factor precursor	6p 21	(71; 73; 85; 95)
PIM1	Pim-1 oncogene	6p 21	(71)
PAF-2	Peroxisome assembly factor-2	6p 21	(71)
ARG1	Arginase I	6p 23	(71)
TGFβ1	Transforming growth factor BETA 1	6ql1	(87)
SOD2	Superoxide dismutase 2 mictochondrial	6q25	(71)
IL-6	Interleukin-6	7pl5	(71)
GPRA	G-protein- coupled receptor for asthma susceptibility	7p14	(71; 82; 85; 98)
TCRG	T cell receptor gamma	7p14	(71)
EGFR	Epidermal growth factor receptor precursor	7p11	(71)
PAI-1	Plasminogen activator inhibitor-1 precursor	7q22	(71)
eNOs, NOS3	Nitric-oxide synthase-endothelial	7q36	(71)
NAT2	N-acetyltransferase 2	8p22	(85)
PAF-1	Peroxiome assembly factor-1	8q21	(71)
PTPRD	Protein-tyrosine phosphatase receptor-type delta	9p	(99)
PTGES	Prostaglandin E synthase	9q34	(71)
PTFN	Phosphatase and tensin homolog deleted	10q23.3	(100)
FcεRIβ	β-chain for high-affinity receptor for IgE	11q13	(95)
PHF11	Protein translation	13q14	(101)
IL-4Ra	IL-4 receptor-α, Th mediated cell responses	16	(102; 103)
GPRA	G-protein coupled receptor	7p	(98)
IFNG	Interferon gamma	12q21	(102)
iNOS	Inducible nitric oxide synthase	17q11.2-q12	(71; 72)
INPP4A	Inositol polyphosphatase type 1	2q11.2	(88)
CC16	Clara cell secretory protein	11q13	(78; 85)
STAT6	Signal transducer and activator of transcription 6 Th cell mediated responses	12q13	(71; 102)
CMA1	Mast cell chymase	14q11.2	(74)
SCYA11	Eotaxin	17q21.1-q21.2	(72)
CHIA	Acid mammalian chitinase	1q13-21.3	(76)

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About 50% of asthmatic patients do not respond to drug therapy [104], thus suggesting that genetic factors may be involved with medication reponse and adverse effects. Studies in pharmacogenetics and pharmacogenomics have identified genes responsible for differences in response to pharmacological treatment [104]. For example, corticotrophin-releasing hormone receptor 1 (*CRHR1*) and glucocorticoid-induced transcript 1 gene (*GLCCI1*) modify responses to corticosteroid treatment [105]. A plausible explanation for non-responders to inhaled steroids is a mutation in gene(s) controlling the anti-inflammatory response of steroids. Such observations have triggered interest in personalized treatment of asthma via DNA testing [106]. Nevertheless, genes alone cannot account for the rapid surge in asthma prevalence worldwide, as genetic factors require several generations to develop and asthma is not always inherited, thus implicating environment and lifestyle as contributing factors.

1.4.2 Demographic and lifestyle factors

Race

Race and ethnicity have been linked with asthma, most likely due to genetic variability and differences in exposures related to environmental and lifestyle factors [107]. This is of clinical importance because asthma severity, morbidity, emergency visits and hospitalization rates are higher in black children than in white [108]. More specifically, according to US data for 2010, asthma prevalence was 1.6 times higher in black children and 1.7 times more likely to have at least one asthma attack in the past 12 months as compared to white children [108]. As for emergency department visits, the black/white rate ratio was 4.9 versus 2.6 [108]. And for hospitalization, the rate ratio was 3.3 versus 1.7 respectively. Regarding asthma mortality, black children were 7.1 times more likely to die from asthma than white children [108]. With respect to ventilatory dynamics, lung

volume as measured by FEV_1 is 15% lower in black children, 10% in South Asian children and 6% in children of other mixed ethnicities as compared to Caucasian children [109].

From another point of view, poverty and black race were found to be determinants for higher rates of asthma and emergency visits in children [110]. Black children with asthma in poor famililies were 4.18 times more likely to have ≥ 1 emergency visit, 1.76 times more likely to be hospitalizd than those from higher income families [110]. Parental divorce and large families increased the risk of hospitalization for asthma due to low income which influences parents ability to manage their child's asthma, purchase medication or for medical consultations [110].

Age

Age of onset is an important consideration in respect to the different phenotypes of childhood wheeze. Children with persistent and late onset of wheeze, have greater risk of having persistent asthma into childhood and adulthood [21; 22]. In contrast, wheeze in children under 3 years of age is usually 'transient' caused by respiratory infection and not an indicator of 'true asthma' [111]. Among infants, 20% have wheezing only when they have upper respiratory tract infections and 60% no longer have wheezing by age 6 years [112]. Gender is another important risk factor since asthma is more prevalent in males younger than 13 years, after which it predominates in females [21; 112]. Sex differences in aiway smooth muscle and airway size (diameter) have been noted with lungs being smaller in males than in females during infancy [22] and reversed in adulthood [113]. Females appear to have higher airflow rates (FEV_1) and vital capacity (FVC) than males [114]. A potential explanation is that sex hormones appear to exert regulatory effects on lung development, airway size and physiology [115]. It has been documented that hormonal changes, pregnancy and menstruation cause changes in oestrogen levels and this

fluctuation activates proteins that can produce airway inflammation and impact asthma risk [114].

Family size, birth order and siblings

More than two decades have elapsed since an inverse association was reported between family size, number of siblings and parentally reported prevalence of hay fever and eczema in offspring from two British national cohorts performed in 1970 and 1958 [116; 117]. These observations have been replicated widely demonstrating that as family size increased, the higher the protective effect on asthma development and wheezing later in childhood [118; 119; 120]. In Phase Three of the ISAAC Study comprehensive assessment of questionnaire data for 210,200 children (6-7 years) and 337, 226 children (13-14 years) documented a significant trend for lower prevalence of wheeze in children and adolescents with increasing sibship size ($p< 0.001$) [121]. Children with more siblings ($\geq$ 3) were less likely to have a history of wheeze in both age groups possibly due to early microbe exposure which enhances immunity [57] . However, severe asthma was greater in larger families.

Pregnancy and gestation

Low birth-weight (< 2.5 kg), gestational age (pre-term < 37 weeks), mode of delivery (vaginal or caesarean) are associated with increased risk of development and hospitalization for childhood asthma [122; 123]. Children born earlier than 32 weeks gestation are at risk for impaired lung function at school age [124]. The rate of caeserian delivery has increased in recent decades and has been linked to asthma development and rhinitis among other immune disorders in offspring [125]. Data compiled from records of 2 million children (0-15 years old) in the Danish National Registry from 1977-2012, showed that children delivered by caeserian section had increased incidence rate ratio (IRR) for asthma (aIRR: 1.23 95%CI: 1.21–1.25; $P < 0.0001$) adjusted for age, calendar year, birth weight, parity, gender, season of birth, maternal age and maternal illness. The

outcome did not change when limiting the analysis to children over 5 years old (aIRR:1.16 95%CI: 1.13–1.19; P < 0.001) [125]. In another recent prospective cohort study (Generation R study) that included data from 6, 128 Dutch children, caesarean section was associated with increased risks of early and persistent wheezing up to school age (OR: 1.36 95% CI: 1.06-1.75) and (OR: 1.73, 95% CI: 1.24-2.4) respectively as compared to normal delivery [126]. It has been speculated that the effect of caeserian delivery on asthma is mediated by changes in the microbiome of the newborn [125; 127].

According to the hygiene hypothesis early postnatal period is a critical time for immune and allergy prevention. The gut apart from digestion, accommodates 10^{14} millions of microbes of more than 1000 different species that play a fundamental role in health and immunity [128]. The normal delivery canal exposes the child to a composite microbiome obtained from exposure to the mother‘s birth canal, different from the one encountered during caesarean delivery in an operating theatre [127]. Immediately after birth, there is an enormous influx of bacteria in an infant’s gut, which stimulates the local immune system and the processes that prevent bacteria from ‘infecting’ or ‘invading’ [128]. Infants who develop allergic disease are known to have lower levels of ‘friendly’ bacteria in the first week of life, and higher levels of disease-producing bacteria as well as reduced diversity of gut bacteria. In addition, biomarkers in the blood of newborn’s delivered by caeserian differed from those born vaginally [129]. Lower frequencies and composition of leukocytes, neutrophils, monocytes, natural killer cells in cord blood during the first year of life and higher frequencies of blood dendritic cell antigen-3 dendritic cells, regulatory T cells, CD4 T cells, T cells and B cells were observed in neonates born by caeserian-section than in those born by natural delivery [129]. Likewise, stress hormone production in the foetus is lower which may affect immune maturation. Collectively, this evidence strongly suggests that the pattern of colonisation of bacteria in the first few weeks of life could influence the patterns of immune development in later life and promotion of allergic disease. Another factor that might consequently affect the newborn’s microbiome is the

administration of antibiotic therapy in mothers to reduce the possibility of post-partum infection [125].

Breastfeeding

The benefits of breastfeeding for the infant is unequivocable and extend beyond good nutrition. In a meta-analysis of 117 studies investigating the effect of breastfeeding on childhood asthma, it was found that breastfeeding was inversely associated with asthma ever (OR: 0.78; 95% CI 0.74-0.84), recent asthma (OR: 0.76; 95% CI: 0.67-0.86) and recent wheezing (OR: 0.81; 95%CI: 0.76-0.87) [130]. A dose-response relationship between breast-feeding duration and hospitalization for asthma was observed in Japanese children [131]. Exclusive breast-feeding at 6-7 months of age was associated with reduced hospitalization risk for asthma in children ($OR_{adj:}$ 0·77 , 95 % CI: 0·56, 1·06), controlling for maternal education attainment and smoking habits, sex, day-care attendance and older siblings. In particular, an additional month of breast-feeding was associated with a further decrease in hospitalization risk for asthma by 4% ($OR_{adj:}$ 0·96; 95 %CI: 0·92, 0·99). Kull et al (2004) observed in a birth cohort of 4089 children that exclusive breast-feeding for at least 4 months reduced asthma risk significantly at the age of 4 years (OR: 0.72; 95% CI: 0.53-0.97) [132]. More versus less breastfeeding duration reduced asthma risk in children (5-18 years) was documented in a recent meta-analysis of 29 observational studies performed by Lodge et al, (2015) (OR: 0.90; 95%CI:0.84, 0.97; p<0.0001) [133].

The association between breastfeeding and wheezing, lung function and atopy was evaluated in Phase 2 of the ISAAC study [134]. From cross-sectional studies undertaken in 20 countries it was found that, any breastfeeding was associated with less wheeze in affluent and non-affluent countries for non-atopic wheeze and improved lung function (as reflected by FEV_1) in affluent countries only [134]. The protective effect of breastfeeding may be attributed to the various components of human milk (antigens, immunoglobulins, cytokines, PUFA, chemokines, polyamines) that favour Th1 cell responses leading to the

production of anti-inflammatory cytokines that protect against infections, especially gastrointestinal and enhance the maturation of the immune system, thus protecting against allergy [135].

High Body Mass Index (BMI)

With regards to body weight, during the past 20 years there has been a concurrent rise in obesity and asthma prevalence suggesting a possible link between these two conditions [136]. Childhood asthma/wheeze and obesity, measured by BMI, have been linked in cross-sectional, case–control, and prospective epidemiologic studies [136; 137] . Whether high BMI precludes asthma development or asthma increases the risk of becoming overweight is debatable. A current systematic review of the literature and meta-analysis of 14 case-control studies undertaken in children/adolescents (2-19 years) was conducted by Azizpour et al (2018) to examine the effect of BMI on asthma [138]. Pooled analysis of data from fourteen relevant studies showed significant relationship between overweight/obese and asthma among children and adolescents. Asthma in overweight children/adolescents was 1.64 times more likely than in underweight/normal weight (OR: 1.64; 95%CI:1.13–2.38; p=0.00) and 1.92 times more likely in obese children/adolescents (OR: 1.92; 95% CI: 1.39–2.65; p=0.00). When investigating for sex differences between high BMI and asthma, Egan et al found in a meta-analysis of six cohort studies examining the effect of overweight (BMI $\geq 85^{th}$ percentile) on doctor-diagnosed asthma in children (0-18 years), that the risk for asthma development was significant in boys (RR= 1.41; 95% CI = 1.05, 1.88; p=0.02) but not in girls (p=0.21) [139]. However, the effect of obesity on doctor-diagnosed asthma remained significant in both sexes, (boys: RR = 1.40; 95% CI = 1.01, 1.93; p=0.04) and girls (RR = 1.53; 95% CI = 1.09, 2.14; p=0.01).

Adverse effects of excess bodyweight on asthma in children was documented in the ISAAC Study (Phase 2) [35]. Data from 10, 652 children aged 8-12 years old confirmed a strong relationship between overweight/obese and wheeze in affluent countries.

Specifically, there was an increasing trend between overweight/obese and wheeze in children. BMI was inversely associated with airway obstruction indicator FEV_1/FVC for overweight [ΔFEV_1/ FVC: 20.90, 95%CI: 21.33%-20.47%, and for obesity [ΔFEV_1/FVC: 22.46%, 95%CI: 23.84%-21.07%].

Asthma in overweight and obese patients may constitute a unique asthma phenotype that is more difficult to manage and worsens asthma severity. Research has shown that asthma in overweight/obese children is associated with more daily symptoms, days hospitalized, missed school days, increased use of reliever medication and higher doses required to reach asthma control along with steroid resistance, exercise-related asthma as as well as impaired lung function (↓FEV_1, FVC) and worse asthma control compared to normal weight [140; 141; 142]. Limited evidence is available on the effect of childhood adiposity on lung dynamics documenting contradictory results [143; 144; 145]. To the candidate's knowledge only one cohort has been conducted in Greek children (6-11 years) reporting adverse consequences on lung function (↓FVC, FEV_1, FEV_1/FVC) in overweight/obese children compared to normal weight [146] and further investigation on this issue is merited and will be analysed in Chapter 6 of this thesis.

Sedentary lifestyle/Physical activity

Sedentary behaviour, as measured by television viewing or computer-time, is positively correlated with asthma symptoms and obesity in children. Mitchell et al, (2013) conducted a cross-sectional study which was part of the ISAAC Study (Phase 3), to examine the relationship between BMI, vigorous physical activity, television viewing and the risk of asthma symptoms, rhinoconjunctivitis and eczema [147]. Data from 76, 164 children aged 6-7 years and 201, 370 adolescents aged 13-14 years showed that vigorous physical activity was positively associated with asthma in adolescents, but not in children. Although , television viewing for five or more hours per day was associated with

increased asthma risk and symptoms in children. Similarly, low physical activity in adolescents and children has been correlated to increased risk of asthma onset and higher risk of current asthma or wheezing regardless of BMI [148]. Nevertheless, the benefits of physical activity outweigh asthma risk. Regular physical activity during childhood improves cardiovascular fitness, self-esteem, co-ordination, and bone development. In asthmatic children, the beneficial effects of regular exercise include improved aerobic capacity, asthma control, ability to cope with asthma, better quality of life and reduced hospital admissions, school absenteeism, medication use and fewer medical consultations [149]; [150].

Physical activity is also important in the maintenance and prevention of overweight and obesity in children and adolescents. According to a cross-sectional study involving 700 Greek children aged 10-12 years old, increased physical activity on weekends and vigorous physical activity in boys were negatively associated with being overweight/obese (OR: 0.65; 95%CI: 0.48-0.90 and OR: 0.66; 95%CI: 0.49-0.88, correspondingly) and moderate physical activity was marginally positively associated in girls (OR: 1.28; 95%CI: 0.97-1.69) [151]. The protective effect of regular physical activity on weight status in Greek children is consistent with other studies [152].

Socio-economic status (SES) and parent's education level

Numerous studies have demonstrated that low socio-economic status (SES) is correlated with poor living conditions, dwelling in low residential areas, poverty, lower parental education, emotional and behavioural difficulties, anxiety, depression, poor diet quality, higher BMI, increased sedentary lifestyle in children including lower education level due to absence from school, and poor health [153; 154; 155]. SES may contribute as an aetiologic factor per se or as a proxy for an environmental risk factor (for example passive smoking, pollution, poor housing, poverty, stress) that exacerbates the disease, or as a determinant

of quality healthcare that patients receive and might contribute to psychological behaviour, which impacts on the management of the condition.

Furthermore, SES is a key factor that influences children's quality of life, by affecting family stability, parenting practices and upbringing of children. Low social class and low income have been linked to increased hospitalizations, poor adherence to drug therapy, development of persistent asthma and severity of asthma in children [153; 154]. In a prospective cohort study (Generation R study) undertaken by Hafkamp-de Groen et al, low parental education was associated with wheezing (OR: 1.53, 95%CI: 1.22-1.92) and asthma (OR: 1.66, 95%CI: 1.28-2.16) in children at age 6 years [156]. Children from families with a household income < 2000 € per month or having financial difficulties were also at increased risk of wheezing [(OR: 1.43, 95%CI: 1.10-1.88);(OR: 1.63, 95%CI: 1.18-2.24) respectively] and parental unemployment was associated with asthma (OR:1.95; 95% CI: 1.24-3.07) [156].

Social and environmental disparities exert a cumulative effect with early life exposures to outdoor air pollution, dwelling in poverty-stricken neighborhoods and poor-quality housing due to dampness and mould, increasing asthma risk in children [157; 158; 159]. In contrast to living in rural areas, the higher asthma prevalence and morbidity in children residing in urban areas is mainly due to traffic-related pollutants such as carbon monoxide and nitric oxide [160]. Most likely causing impaired lung growth and subsequent increased risk of poor asthma control and exacerbations in children [161].

Air pollution

Air pollutants are known to exacerbate asthma symptoms by initiation of disease [162]. Environmental pollutants, such as NO, black carbon and polycyclic aromatic hydrocarbons (PAHs) that are formed during incomplete combustion of fossil fuels and oil products carry allergens including pollens, endotoxin and fungal spores [163]. Air

pollutants probably cause oxidative injury to the airways, leading to inflammation, remodelling, and an increased risk of sensitization [163].

Co-morbidities: Rhinitis and sinusitis

A growing body of evidence clearly demonstrates that rhinitis and sinusitis are co-morbidities associated with asthma risk and development that contribute to worse asthma control [164; 165]. Over 80% of asthmatics have rhinitis, and 10%–40% of patients with rhinitis have asthma, suggesting the concept of 'one airway and one disease' [166]. Childhood allergic rhinitis predicted asthma incidence and persistence to middle age [167]. The underlying mechanism being that the upper respiratory system influences lower airways via parallel inflammatory pathways that cause progressive manifestations of asthma symptoms or vice-versa [164; 166]. An immune response involving T-helper 2 cells (Th2) propagates an extended inflammatory process that begins in nasal mucosa and ends in bronchioles and alveoli, particularly in symptomatic asthmatics [166]. It has been shown that treatment of rhinitis can be beneficial to the lower airways, reducing symptoms, emergency visits, hospitalization, severity of bronchial hyperresponsiveness as well as improving patient's quality of life, school and work productivity [168].

1.4.3 Environmental Factors

Exposure to tobacco smoke

The detrimental effect of smoking to respiratory health has been well documented [21]. Maternal smoking during pregnancy and paternal smoking during childhood have been linked to asthma development not only during childhood but as a risk factor for asthma in adolescence [169; 170]. According to a meta-analysis exposure to passive smoking increased the incidence of wheeze and asthma in children up to 18 years by at least 20% [171]. Exposure to pre or post-natal passive smoke was associated with 30% to 70% increased risk of incident wheezing and 21% to 85% increase in incident asthma [171].

Data from the ISAAC Phase 3 Study that included 220,407 children aged 6-7 years from 75 centres in 32 countries and 350,654 adolescents aged 13- 14 years in 118 centres in 53 countries, showed that maternal and paternal smoking were associated with increased risk of asthma symptoms in both age groups [169]. Maternal smoking was associated with higher risk of severe symptoms in children 6-7 years than paternal smoking (maternal: OR: 1.31, 95%CI: 1.18 to 1.46 versus paternal: OR: 1.19, 95%CI: 1.11 to 1.27) and maternal smoking during the first year of the child's life (OR: 1.36, 95%CI: 1.17 to 1.58), although the effect was additive (mother and father smoke: OR: 1.46, 95%CI: 1.34 to 1.59). There was a dose-response effect for current smoking by the mother and father and asthma risk (1-9 cigarettes/day, OR: 1.27; 10-19 cigarettes/day, OR: 1.35; and >20 cigarettes/day, OR: 1.56). The more cigarettes, higher the risk.

Clean living, farm and domestic pets

Progressive modernisation and cleaner living appear to have altered the balance between humans and their friendly gut microbes [128]. The effect of exposure to pets on the respiratory system is controversial. Some epidemiological studies have reported that early exposure to cats or dogs may protect a child against asthma development [172; 173]. In a large nationwide cohort study including 1,011,051 children in Sweden found that dog exposure during the first year of life was associated with decreased risk of asthma in school-aged children (OR: 0.87, 95%CI: 0.81-0.93) and in children ≥ 3 years (HR: 0.90, 95% CI: 0.83-0.99) [173]. In addition, children exposed to female dogs had lower asthma risk compared to male dogs (OR: 0.84, 95% CI: 0.74 -0.95) as well as exposure to two dogs or more had lower risk of asthma than those with one dog only, OR: 0.79, 95% CI: 0.65-0.95) [172]. It has been speculated that sex hormone differences in allergen excretion between male and female dogs may explain this paradox [172]. Attenuation of asthma risk was also observed with farm animals (OR: 0.48, 95% CI: 0.31-0.76) [173] and exposure to cats in early life (HR_{adj}: 0.83, 95% CI: 0.71-0.97) [174]. In comparison, Lombardi et al

found that cat exposure during the first year of life significantly increased the risk of current wheeze [OR: 1.88, 95%CI:1.33-2.68, $p < 0.001$] and current asthma [OR: 1.74, 95% CI: 1.10-2.78, $p < 0.05$] in Italian children at age 7 years [175].

Child care centres

Whether attending child care centres influence asthma development is questionable. A recent meta-analysis demonstrated that early child care attendance protected against asthma in children aged 3 to 5 years, but did not protect children aged ≥6 years from developing asthma [176]. In addition, early child care attendance increased the risk for wheeze in children aged ≤ 2 years but did not increase the risk for wheeze in children aged >2 years. Pooled analysis of 32 studies undertaken from 1964 to January 2017 showed that children who attended child care at any age had higher odds of developing asthma compared with children who did not attend child care (OR: 1.17, 95% CI: 1.01-1.35; I^2 statistic= 80.4%). However, no association was found between asthma in children and adolescents ages 0 to 18 who attended early child care vs children who did not attend child care (OR: 0.94; 95% CI: 0.70-1.27; I^2 statistic= 64.4%). Contrastingly, there was an increased risk for asthma in children and adolescents ages 0 to 18 who attended late child care compared with children who do not attend child care (OR: 1.19; 95% CI: 1.01-1.41; I^2 statistic= 32.5%). The investigators concluded that in children aged ≥ 6 years early child care attendance is not statistically significantly associated with a risk for asthma or wheezing. A limitation of this analysis was high diversity among studies and need for standardized definitions of child care exposure and asthma diagnosis in paediatric patients in order to increase the reliability and validity of the study findings [176].

The protective effect of early exposure to farm and domestic pets, other children in child care centres and large families (> 1 sibling) on childhood asthma development can be explained by the the hygiene hypothesis. This hypothesis is based on the central concept that respiratory infections early in life can protect against allergy development. Increased

exposure to particular viruses, bacteria or parasites help in the maturation of the immune system [57]. On a molecular level, the mechanism behind the 'hygiene hypothesis' is that the immune system of the newborn child is skewed toward Th2 cytokine generation (IL-4, IL-5, IL-6 and IL-13) which are responsible for mediating allergic inflammation and Ig E production [177]. On the other hand, environmental stimuli such as infections, living in rural areas, exposure to animals and other children along with less frequent antibiotic use [178], activate Th1 responses and consequently production of IL-2, interferon (IFN)-g and tumor nectosis factor (TNF-α) that promote cell-mediated immunity. Thus bringing the Th1/Th2 relationship to an appropriate balance [128]. So the lack of microbial burden in early childhood, redirects the immune response towards Th2 dominance which triggers a pro-inflammatory response, believed responsible for initiating asthma onset.

Swimming Pools

With regards to exposure to chlorinated pools on asthma in children, the Avon Longitudinal Study of Parents and Children reported a reduced risk for current asthma in children at 7 years old (OR 0.5: 0.28-0.87) [179]. In fact, swimming was associated with improved lung function and lower risk of asthma symptoms, especially among children with established respiratory conditions. Nevertheless, outdoor chlorinated swimming pool attendance was associated with higher risks of asthma and airway inflammation in adolescence [180] .

1.4.4 Dietary hypothesis

Changing environmental and lifestyle factors rather than genetic susceptibility have most likely driven the rapid increase in asthma prevalence with alterations in eating habits emerging as a promising candidate. The role of diet has clearly established mechanisms in diseases such as cardiovascular disease, type 2 diabetes, and cancer. It is not commonly identified as a causal factor in asthma. Dietary habits of asthma patients are not commonly investigated in clinical practice. Diet might influence susceptibility to allergic diseases,

as it contributes to the 'priming' and regulation of the immune system. Current research in asthma aetiology is based on three dietary hypotheses: anti-oxidant, lipid and vitamin D deficiency [181; 182; 183].

Antioxidant theory

Airway inflammation causes repeated episodes of airway obstruction in asthmatic patients. A byproduct of cellular respiration is the production of oxygen free radicals that activate an inflammatory response [54]. Exposure to exogenous free radicals from allergens such as cigarette smoke and air pollutants add to the oxidant load on lung tissues promoting further inflammation [54].

Excessive ROS production in asthma leads to alteration in key enzymatic as well as nonenzymatic antioxidants such as glutathione, vitamins C and E, β-carotene, uric acid, thioredoxin, superoxide dismutases, catalase, and glutathione peroxidases leading to oxidant-antioxidant imbalance in airways [184] . Oxidant-antioxidant imbalance leads to pathophysiological effects associated with asthma such as vascular permeability, mucus hypersecretion, smooth muscle contraction, and epithelial shedding [184]. The lung has several natural intracellular and extracellular antioxidant scavenging mechanisms, which inhibit reactive oxidant propagation and scavenge oxidative free radicals. However, the functioning of such mechanisms depend on antioxidants provided from dietary sources such as vitamins C, E and A, β-carotene and selenium. According to Seaton, the antioxidant theory proposed that the shift in dietary habits over the years to a diet which is low in antioxidants has led to an increased susceptibility to oxidant attack and airway inflammation, resulting in a rise in childhood asthma [183]. This hypothesis is supported by data from observational studies that have reported an association between a deficiency in vitamin C [185; 186], vitamin E [187], carotenoids [185; 186], selenium as well as reduced glutathione peroxidase [188] and higher prevalence of asthma symptoms and impaired lung function [189] in children.

Sackesen et al, (2008) investigated the anti-oxidant status in asthmatic children and found that apart from a suboptimal level of antioxidants (vitamins C, E, lycopene and β-carotene), non-enzymatic and enzymatic antioxidant systems such as glutathione, glutathione peroxidase and superoxide dismutase were also lower in asthmatic children [(190)]. Molecular studies have revealed that selenium found in seeds, seafood and fish, is necessary for the functioning of a selenium-containing antioxidant enzyme glutathione peroxidase, a free-radical scavenger [(191)]. Glutathione peroxidase effectively reduces hydrogen peroxide (ROS), a cytotoxic byproduct of cellular respiration with the potential to damage proteins, lipids and nucleic acids, to water thereby preventing lipid peroxidation and instability of cell membranes [(191)]. Zinc found in oysters, beef and seafood, is an essential trace element with antioxidant, anti-inflammatory and anti-apoptosis properties that is required by proteins for catalytic, structural and transcriptional functions [(191; 192)]. Zinc deficiency has been reported in Australian asthmatic children compared to non-asthmatics [(193)]. Increased wheeze frequency, asthma severity and low FEV_1 was significantly associated with low labile sputum zinc [(193)].

Other epidemiologic studies in chlldren have highlighted that low dietary intake of magnesium was associated with impairment of pulmonary function as measured by airway flow rates, airway hyperactivity and increased risk of wheezing [(194)]. Good dietary sources of magnesium are found in green leafy vegetables, nuts, wholegrains, milk, meat and starches [(194)].

In comparison, evidence from cross-sectional studies indicate that high intake of vitamin A, C, E, selenium, magnesium and flavonoids were associated with a reduction in asthma symptoms, improvement in pulmonary function and reduced airway inflammation by limiting reactive oxygen species production, inhibiting lipid peroxidation and histamine release [(56; 195; 196)]. More specifically, a high intake (> 40 g/day) of vegetables (tomatoes, eggplants, cucmber, green beans and zucchini), citrus fruits and kiwi fruit, food sources

high in antioxidants and other bioactive molecules have been found to be inversely associated with wheezing outcome in children [197]. Consistent results from the ISAAC study reported that fruit, milk, eggs, cereals, fish ≥ 1-2 times/week and vegetables > once/week were inversely associated with asthma and wheeze [8; 36; 198]. Furthermore, food selection according to the Mediterranean diet was associated with lower prevalence of current wheeze and asthma in children [8]. The Mediterranean diet is a plant diet rich in vitamins, minerals, fibre, antioxidants, bioprotective nutrients and phytochemicals that interact synergistically providing protective effects for a wide spectrum of diseases [199]. Moreover, cereals (whole grain) are rich in vitamins B, D and E, zinc, magnesium, iron, selenium, phenolic acids, and phytic acid; fruits, vegetables, and legumes in vitamins A, B and C, folic acid, iron, zinc, calcium, potassium, carotenoids, magnesium, phytoestrogens, flavonoids; olive oil contains vitamins E, A and K, calcium, iron, magnesium, potassium, phenolic derivatives (hydroxytyrosol, tyrosol, oleuropein, and ligstroside), squalene and oleuropein; nuts and seeds in vegetable protein, vitamins B and E, tocopherols, calcium, magnesium, potassium, phytosterols and phenolic compounds, all of which exhibit high antioxidant activity [200].

In 2015, Papadopoulou et al used data from Phase 2 of the ISAAC Study in which 2023 children aged 9-10 years participated. The purpose of this study was to analyse the effect of antioxidants on asthma and allergic disease in Greek children residing in the cities of Athens and Thessaloniki [56]. Dietary habits were assessed using a FFQ. Antioxidant levels from dietary intake were measured from FFQs using an Anti-oxidant Eating Index (AEI) (AEI score range 0-6) based on pro-antioxidant (vegetables, fruits, fresh juice, fish) and non-antioxidant (meat and burgers) food intake. Higher values of the score suggested an antioxidant diet and lower values, a more saturated fatty diet. According to the investigators, the mean AEI score for the cohort was 4.2 and it was found that the AEI score was inversely associated with lifetime asthma in either city (OR: 0.87, 95%CI: 0.77-

0.99) independent of family history, sensitization, exercise, smoking, breast-feeding, pet and dampness in homes.

Hence the ability of the Mediterranean diet to counteract oxidative stress might have an effect on asthma development and warrants further investigation. The potential of the Mediterranean diet as a therapeutic diet for asthma is critically assessed by the candidate in an extensive systematic review of the literature presented in Chapter 2.3 titled *Papamichael et al, 2017. Does adherence to the Mediterranean dietary pattern reduce asthma symptoms in children? A systematic review of observational studies. Public Health Nutr. 20(15):2722-2734. doi: 10.1017/S1368980017001823.*

Lipid hypothesis

Over the past few decades, changes in dietary habits from the traditional to a Westernized diet have been associated with changes in dietary fat intake. Primarily, increased intake of omega-6 fatty acids, linoleic acid (LA) present in margarines and vegetable oils; saturated fats from butter, fast foods, meat and sweets; and decreased omega-3 fatty acid intake, eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) found in oily fish (tuna, sardines, herring, mackerel, trout and salmon) [(201)]. The composition of omega-3 fatty acids found in common Greek fish and seafood is presented in Table 3.

Table 3 Composition of omega 3 fatty acids found in common Greek fish and seafood

Type of Fish		**Omega 3 fatty acids content per 100g**
English	**Greek Translation**	
Sardines	Σαρδέλα	1.41
Mackerel	Σκουμπρί	1.34
Chubb Mackerel	Kolios	1.34
Salmon (fresh)	Σολομός	1.03
Salmon (smoked)	Σολομός	0.45
Anchovies	Γαύρος	0.80
Trout	Πέστροφα	0.73
Gilthead Seabream	Τσιπούρα	0.48
Streaked Gurnard	Καπόνι	1.35
Saddled	Μελανούρι	0.96
Boque	Γόπα	0.86
Dog fish	Γαλέος	0.84
Swordfish	Ξιφίας	0.75
European Bullhead	Γοβιός	0.68
Smelt	Μαρίδα	0.61
White Bait	Αθερίνα	0.61
European Bass	Λαβράκι	0.59
Calamari	Καλαμάρι	0.48
Annular Sea Bream	Σπάρος	0.48
White Sea Bream	Σαργός	0.48
Flathead Mullet	Κέφαλος	0.32
Crab	Καβούρι	0.32
Scad	Σαφρίδι	0.26
Atlantic Bonito	Παλαμίδα	0.26
Dusky Grouper	Ροφός	0.25
Flounder	Γλώσσα	0.25
Tuna (canned)	Τόνος (κονσέρβας)	0.24
Prawns	Γαρίδα	0.24
Common Pandora	Λιθρίνι	0.22
Cod	Βακαλάος	0.18
Salema	Σάλπα	0.17
Octopus	Χταπόδι	0.16
Crayfish	Καραβίδα	0.14
Eel	Χέλι	0.12
Lobster	Αστακός	0.11
Oysters	Μύδια	0.11
Tuna	Τόνος	0.10

Source: Adapted from Stergiou et al. (2011) Shouting fishes: fishes from the greek seas-biology, fisheries and management. Greece. Pataki

Ziantanos S, AN. S (1993) The fatty acid composition of some important Mediterranean fish species. Fett/Lipid ***95*** *66-69.*

One meal of fatty fish can provide between 1.5 and 3.5 g of these long-chain n-3 PUFAs. And consumption of 1 g fish oil capsule per day provides approximately 300 mg of EPA/DHA [202]. Fish oil owes its potential anti-inflammatory properties to its active ingredients, EPA and DHA.

In European populations it has been estimated that dietary fats constitute 28-42% of the total energy intake as opposed to 20-30% in previous years [203]. The balance between omega 3 fatty acid intake and omega 6 fatty acids is important in the prevention and treatment of chronic diseases including cardiovascular disease, hypertension, diabetes, osteoporosis, cancer, mental health and inflammatory disease [204]. The Western diet is deficient in omega-3 fatty acids with a ratio of omega-6/ omega-3 fatty acids of 10:1 to 20:1 [201] instead of 1:1 [204] as was the case during the Paleolithic era, supporting optimum health and cellular functioning [204].

Omega-3 fatty acid α-linolenic acid (ALA; 18:3ω3) and omega-6 fatty acid linoleic acid (LA; 18:2ω6) are termed essential fatty acids because they cannot be synthesized by humans due to the lack of enzymes for omega-3 desaturation and thus must be derived exogenously from the diet [204]. The difference between these two essential fatty acids is the position of the first double bond, counting from the methyl end of the fatty acid molecule [204]. In the omega-6 fatty acids, the first double bond lies between the 6th and 7th carbon atoms; and for the omega-3 fatty acids, the first double bond between the 3rd and 4th carbon atoms [204]. LA is abundant in nature and is found in plant seeds (except for coconut, cocoa and palm), vegetable oils (corn, sunflower, soybean) and margarines [202; 204]. ALA is found in the chloroplasts of green leafy vegetables and in the seeds of flax (or linseeds), rape, chia, perilla and in walnuts [204]. The increased intake of margarines and vegetable oils in the Western diet is responsible for the increase in linoleic acid intake. ALA and LA are both metabolized to 20 and 22 carbon long-chain fatty acids[204]. Omega-6 fatty acid, LA is the precursor for arachidonic acid (AA) (20:4ω-6) and α-linolenic acid for eicosapentaenoic acid (EPA) (20: 5ω-3) and docosahexaenoic acid (DHA) (22: 6ω-3) [204] (Figure 7).

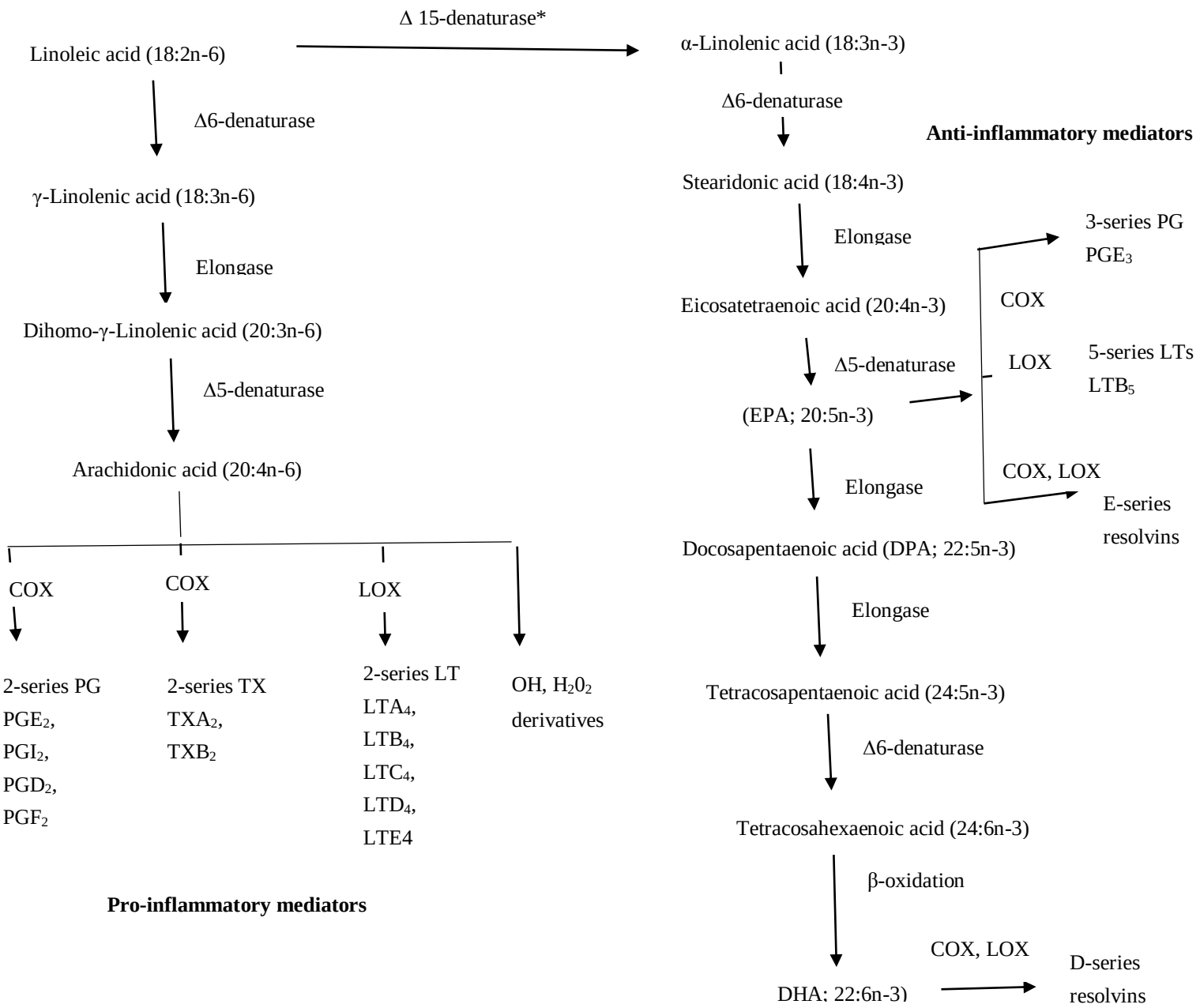

Figure 7 Biosynthesis of omega-6 and omega -3 fatty acids, and eiconasoid derivatives produced from arachidonic acid (AA), Eicosapentaenoic acid (EPA), Docosahexaenoic acid (DHA)

Key: Eicosapentaenoic acid-EPA; Docosapentaenoic acid –DPA; Docosahexaenoic acid - DHA ; PG-Prostaglandins; TX- Thromboxanes; LT- Leukotrienes; COX-cyclooxygenase; LOX- lipoxygenase
*in plants only

Metabolism of omega-6 and omega -3 fatty acids obtained from dietary sources is a complex process involving several enzymes of desaturation, elongation and β-oxidation producing the final products of EPA, DHA as well as their eicosanoid derivatives [201; 204].

Source: Modified from:

Patterson et al, 2012. Health Implications of high dietary omega-6 polyunsaturated fatty acids. J Nutr Metab, 2012, 1-16. doi: 10.1155/2012/539426 according to the Creative Commons Attribution License.

Calder, 2011. Fatty acids and inflammation: The cutting edge between food and pharma. Eur J Pharmacol 668 (2011) S50–S58. doi: 10.1016/j.ejphar.2011.05.085. Copyright permission granted from the author.

The lipid hypothesis proposed that the imbalance in omega-6: omega-3 fatty acid ratio has contributed to the increase in allergic diseases including asthma [181]. The theory is based on the hypothesis that the high intake of omega-6 fatty acids (LA) in the Western diet and low intake of omega-3 fatty acids, have shifted the physiological state to one that is pro-inflammatory by favouring the production of pro-inflammatory eicosanoids (2-series and 4-series) and subsequent promotion of Th2 response, stimulation of IgE and the development of disease [181]. Conversely, a high intake of omega-3 fatty acids (EPA, DHA) give rise to anti-inflammatory eicosanoids (3-series and 5 series) along with inflammation-resolving molecules (maresins, resolvins and protectins), thus preventing disease [202].

The physiological effects of omega-6 fatty acids derived eicosanoids 2-series prostaglandins (PGD_2, PGE_2, PGF_2), 2-series thromboxanes (TXA_2, TXB_2) and 4-series leukotrienes (LTA_4, LTB_4, LTC_4, LTD_4, LTE_4) include bronchoconstriction, vasoconstriction, activation of eosinophils, chemotaxis and leukocytes, increased production of IL-6, accelerated release of reactive oxygen species, hydroxy and hydroperoxide derivatives [201]. In contrast, anti-inflammatory effects of omega-3 fatty

acids EPA and DHA derived eicosanoids 3–series prostaglandins (PGE_3), 3-series thromboxanes (TXA_3) and 5-series leukotrienes (LTB_5) include antirrhythmic, anti-aggregation, and anti-apoptosis as well as downregulation of leukocytes and decrease in oxidative stress by the action of EPA and DHA derived E-series resolvins, lipoxins, D-series protectins [(201)].

Therefore, the coinciding increase in the incidence of diseases with an inflammatory component including cardiovascular, obesity and respiratory disease [(204)] suggest that the decrease in omega-3 fatty acid intake and asthma prevalence could be interrelated. Based on the anti-inflammatory and immunomodulating effects of marine omega-3 fatty acids, it has been speculated that supplementation of the diet with these fatty acids may have therapeutic value in childhood.The latest evidence on the potential of omega-3 fatty acids as an adjunct therapy for asthma development in children is investigated by the candidate in the manuscript *Papamichael et al, 2018. The role of fish intake on asthma in children: A meta-analysis of observational studies. Pediatr Allergy Immunol 29, 350-360* presented in Chapter 3.2.

1.4.5 Vitamin D and asthma

The role of vitamin D in asthma pathophysiology is unclear and this has received particular attention in recent years. Meta-analyses of data from observational studies have confirmed that vitamin D deficiency [(25(OH)D < 20 ng/ml] and insufficiency [25(OH)D 20-30 ng/ml] is more frequent among asthmatics than in non-asthmatics [(205)]. Observational studies suggest that vitamin D deficiency could be related to asthma development and disease severity in children. In asthmatic children decreased levels of serum 25(OH)D were correlated with increased asthma prevalence [(206; 207; 208)], exacerbations [(209; 210; 211)], hospitalization [(212; 213)], emergency visits [(213; 214)], airway

hyperresponsiveness [212; 215], airway remodelling [211], respiratory infections [213; 216], medication use [209; 210; 211; 217], along with declined lung function [206; 208; 209; 217; 218] and asthma control [211; 213; 218; 219]. As for lung function, a dose-response effect was observed between increasing concentrations of 25(OH)D and predicted forced expiratory volume in 1 second (FEV_1), forced vital capacity (FVC) and FEV_1/FVC [206; 211; 213; 217; 218] measurements.

Han et al, (2017) conducted a study using data from the cross-sectional U.S National Health and Nutrition Examination Survey (NHANES) from 2001-2010 to examine vitamin D insufficiency and current asthma or wheeze in 10, 860 children (6-17 years) and lung function in a subset of participants [220]. Spirometry was used to assess pulmonary function and serum vitamin D insufficiency was defined as 25(OH)D level <30ng/mL. After stratifying data, results showed that vitamin D insufficiency was associated with 'current asthma' (OR: 1.35, 95%CI: 1.11-1.64; p<0.001) and 'current wheeze' (OR: 1.24, 95%CI: 1.02-1.52; p= 0.03) in children. In the multivariate model adjusted for confounders of age, sex, BMI, household income, race/ethnicity, cigarette smoke (measured by serum cotinine) and C-reactive protein, 25(OH)D < 30 nm/mL was associated with a decrement in FEV_1 [β= -110.17; 95%CI: -146 to -75.54 ml; p< 0.01] and FVC [β= - 129.13; 95%CI:-176.09 to -82.10 ml); p<0.01] in children.

The adverse effect of vitamin D deficiency on asthma morbidity in children has been demonstrated in other studies. A 4-year longitudinal study was conducted by Brehm et al to establish the causal relation between plasma vitamin D levels and asthma exacerbations in 1024 North American asthmatic children (7-11 years old) with mild to moderate asthma [214]. In this study 25(OH)D levels <30 ng/mL had 1.5 times increased risk of asthma exacerbations (OR: 1.5; 95% CI: 1.1-1.9). Comparable results were documented by Brehm (2012) in a cross-sectional study of 560 Peurto Rican children aged 6-14 years with asthma [209]. Children with vitamin D insufficiency <30 ng/mL were 2.6 times more likely to develop asthma exacerbations (OR: 2.6; 95% CI: 1.5-4.9).

With regards to vitamin D supplementation, clinical trials have reported a protective influence of vitamin D supplementation among asthmatic patients. According to a recent meta-analysis of 7 clinical trials involving 297 children aged 2-11 years old and 658 adults, vitamin D supplementation (400-4000 IU) reduced the rate of asthma exacerbations among patients requiring cortico-steroid treatment (adjusted incidence rate ratio [aIRR] 0·74, 95% CI 0·56–0·97; p=0·03) [221]. Subgroup analyses showed a protective effect on rate of asthma exacerbations treated with systemic corticosteroids in patients with baseline 25(OH)D < 25 nmol/L (aIRR 0·33, 0·11–0·98; p=0·046) but not in participants with higher baseline 25(OH)D levels (aIRR 0·77, 0·58–1·03; p=0·08).

A parallel, double-blind, randomized controlled trial of 6-months duration was undertaken on 48 asthmatic Polish children (5-18 years old) [222]. The purpose of this study was to investigate the effect of steroid (inhaled budesonide) with vitamin D supplementation (500 IU) on asthma control. At the end of the study it was observed that children taking steroids with low vitamin D levels had 8 times higher risk of asthma exacerbation compared to children with steroids and stable or increased vitamin D levels (odds ratio, 8.6; 95% CI, 2.1-34.6). A significant linear relationship was observed between vitamin D level and asthma control (r= -0.372, p=0.009) with low baseline vitamin D levels being associated with severe clinical manifestations of asthma.

Genetics studies mapping the Vitamin D Receptor (VDR) gene in animal models of asthma suggest that VDR polymorphism is linked with expression of asthma [223]. In humans, Poon et al, (2004) compared VDR genetic variants between members of a family-based cohort (223 families of 1,139 individuals) with and without asthma. Their analysis found significant association between six polymorphisms in the VDR gene and clinical diagnosis of asthma [224].

Vitamin D mode of action

Vitamin D is a fat-soluble vitamin that cannot be synthesized by the body but is obtained via skin exposure to the sun and from dietary sources (such as fatty fish, cod liver oil, egg yolk, meat, kidney and liver), fortified foods (milk products, breakfast cereals and margarine) and vitamin supplements [(225)]. Vitamin D concentration of common foods are shown in Table 4.

Table 4 Dietary and pharmaceutical sources of vitamins D2 and D3

Source	Portion size	Vitamin D Content	References
Salmon			
Fresh wild	3.5 oz	600-1000 IU vit D3	(226)
Fresh farmed	3.5 oz	100-250 IU vit D3	(226)
Canned	3.5 oz	300-600 IU vit D3	(226)
Swordfish	3oz	566	(227)
Sardines canned	3.5.oz	300 IU D3	(226)
Mackerel canned	3.5.oz	250 IU D3	(226)
Tuna canned	3.6 oz	230 IU D3	(226)
Cod liver oil	1 teas	400-1000 D3	(226)
Shitake mushrooms			
fresh	3.5	100 IU D2	(226)
Sun-dried	3.5	1600 IU D2	(226)
Egg yolk	1	20 IU D3 or D2	(226)
Liver beef	3 oz	42 IU	
Exposure to sunlight UV (B) radiation (0.5 minimal erythemal dose)	5-10 minutes sun exposure	3000 IU vit D3	226); (227)
Fortified foods			
Fortified milk, nonfat reduced fat, whole	8 oz	100-125 IU D3	(226)
Fortified orange juice	8oz	100 IU D3	(226)
Infant formulas	8 oz	100 IU D3	(226)
Fortified yogurts	8 oz	100 IU D3	(226)
Fortified butter	3.5 oz	50 IU D3	(226)
Fortified margarine	3.5 oz	430 IU D3	(226)
Fortified cheeses	3 oz	100 IU D3	(226)
Fortified breakfast cereals	1 serving	100 IU D3	(226)
Supplements			
Vitamin D2 (ergocalciferol)	1 capsule	50,000IU	(226)
Multivitamin		400 IU D2 or D3	(226)
Vit D3		400,800,1000, 2000IU	(226)

Data elaborated from: Holick, 2007. Vitamin D deficiency. N Engl J Med 57, 266-281. USDA, 2011. USDA National Nutrient Database for Standard Reference. Available from https://ndb.nal.usda.gov/ndb/.

Vitamin D is produced endogenously by the action of ultraviolent rays on the skin that triggers vitamin D synthesis [226]. However, vitamin D obtained from the sun and dietary sources is biologically inert and must undergo two hydroxylations in the body for activation [226]. Vitamin D3 is obtained from ambient UV radiation (290-315 nm) exposure of the skin by converting 7-dehydrocholesterol in the skin via an isomerization process, as well as from dietary intake of vitamin-D3 rich foods, fortified foods and vitamin supplements [226]. Vitamin D2 is plant derived and synthesized during UV radiation of ergosterol. Both vitamins are converted in the liver to 25(OH)D by the action of 25-hydroxylases. Thereafter, 1α-hydroxylase in the kidneys convert 25(OH)D to the biologically active form 1,25-dihydroxyvitamin D [1,25(OH)2D] also known as calcitriol. Intracellularly, 1,25-OH-vitamin D3 binds to its receptor (VDR) in the cytoplasm and is translocated to the nucleus where it is able to activate or repress target gene transcription involved in calcium metabolism, proliferation, differentiation, cell apoptosis, immunity and in respiratory health [226; 228]. Vitamin D metabolism is illustrated in (Figure 8).

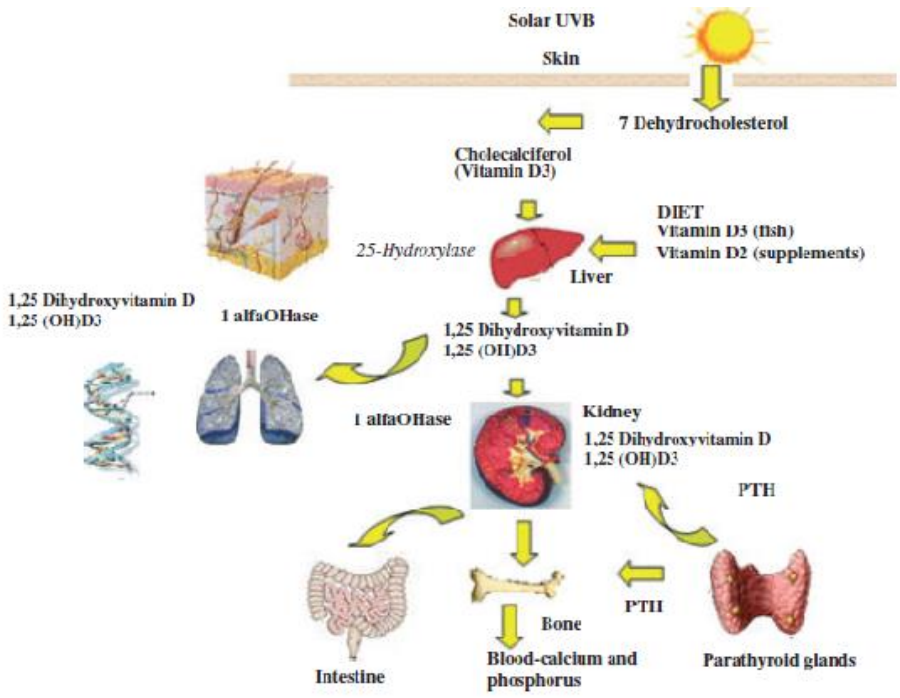

Figure 8 Diagram of vitamin D metabolism

Source: Permission granted from co-author for image from Bozzetto S, Carraro S, Giordano G, Boner A, Baraldi E. Asthma, allergy, and respiratory infections: the vitamin D hypothesis. Allergy 2012; 67:10–17. doi: 10.1111/j.1398-9995.2011.02711.x.

There are multiple potential mechanisms by which vitamin D status might influence pulmonary function and asthma via effects on lung development, immunomodulation, airway smooth muscle, airway obstruction, hyperresponsiveness, protection from respiratory infections that can trigger an asthmatic attack as well as enhancing asthma therapy [(228)]. Vitamin D by binding and activating the vitamin D receptor stimulates innate and adaptive immune response [(229; 230)] (Figure 9). 1,25(OH)2D3 stimulates innate immune responses by enhancing the chemotactic and phagocytotic responses of macrophages as well as the production of antimicrobial proteins such as cathelicidin. On the other hand, 1,25(OH)2D3 also modulates adaptive immunity. 1,25(OH)2D3 inhibits the surface expression of MHC-II-complex antigen and of co-stimulatory molecules, in addition to production of the cytokines IL-12 and IL-23. Thus indirectly shifting the polarization of T cells from a Th1 and Th17 phenotype towards a Th2 phenotype. 1,25(OH)2D3 also directly affects T cell responses, by inhibiting the production of Th1 cytokines (IL-2 and IFN-g), Th17 cytokines (IL-17 and IL-21), and by stimulating Th2 cytokine production (IL-4). Moreover, Treg cell development is favoured via modulation of dentritic cells (DCs) and by directly targeting T cells. The increased production of regulatory T cells (specifically CD4+ Foxp3+Tregs) are responsible for the expression of anti-inflammatory IL-10 and TGFβ that enhance tolerogenity to allergen exposure and suppression of IL4, IL-5 and IL-13 from Th2 cells [(230)]. Finally, 1,25(OH)2D3 blocks plasma-cell differentiation, immunoglobin secretion (IgG, IgE and IgM), B-cell proliferation and induces B cell apoptosis, consequently preventing the onset of asthma symptoms [(229; 230)].

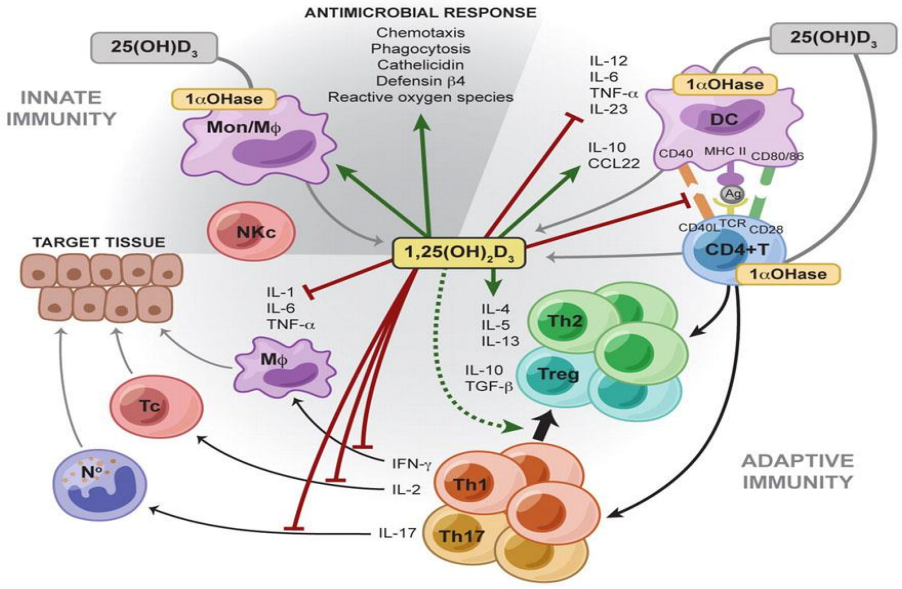

Figure 9 Action of vitamin D on the immune system

Figure 9 illustrates that vitamin D is a potent modulator of the immune-inflammatory response via mediating a shift from Th1 to Th2 phenotype; inhibition of Th1,Th9, Th17, pro-inflammatory cytokines (IL17, 1L13) and promotion of Th2, Tregs, anti-inflammatory cytokines IL4, IL5, IL10, phagocytic activity and anti-microbial peptide cathelicidin counteracting the production of IgE from B cells and therefore deterrence of asthma symptomology and airway remodelling.

Source: Permission granted from co-author for reproduction from Baeke et al, 2010. Vitamin D insufficiency: Implications for immune system. Pediatr Nephrol 25(9):1597-606. doi: 10.1007/s00467-010-1452-y.

One of the hallmarks of asthma pathogenesis is airway narrowing due to smooth muscle constriction and mucus secretion. Vitamin D is able to act on airway smooth muscle by inhibiting airway smooth muscle cell proliferation, down regulation of expression of pro-inflammatory cytokines (TNF-α, TGF-β, Chemokine ligand 5 (CCL5)), matrix metalloproteinases (MMP 9 and ADAM33 modulators of airway modelling) as well as

mucus secretion [230]. With respect to tissue repair, vitamin D significantly reduces prostaglandin E2 (PGE2) production by human lung fibroblasts and stimulates PGE2 degradation which modulates fibroblast tissue repair mechanism [230]. Thus, suggesting that sufficient serum vitamin D levels are important in the prevention and treatment of damaged structural changes in airways.

Another interesting mechanism of vitamin D is its ability to modulate the response to asthma therapy by inducing the production of IL-10, a potent anti-inflammatory cytokine from regulatory T-cells [217; 231; 232]. Two studies have documented an inverse relationship between vitamin D levels in asthmatic children and use of steroids and anti-leukotriene therapy [212; 217].

1.4.6 Hypovitaminosis D in Greek children

Reports from across the world indicate that hypovitaminosis D is widespread even in sun-replete Mediterranean countries and is re-emerging as a major health problem globally [233]. More specifically, in Greece the prevalence of Vitamin D deficiency in children and adolescents is high [234]. In a study performed by Lapatsanis et al (2005) that included 178 healthy Greek children (3-18 years), 47% of children aged 15-18 years had low plasma 25-hydroxy vitamin D (25(OH)D) < 10 ng/ml in winter and > 10 ng/ml in summer months [234].

Convincing evidence from a cross-sectional epidemiological study, the Healthy Growth Study (HGS) that comprised of 1100 school-children aged 9-13 years, reported that 100% of children had vitamin D intake below the estimated average requirement [EAR 1-13 yrs 400 I.U or 10 mcg] [235; 236]. Possible reasons for vitamin D deficiency in Greek children are sun avoidance due to cancer risk, pigmented skin, increased time indoors, inadequate dietary intake, low use of dietary supplements and lack of mandatory food fortification policies [226]. Increased consumption of fatty fish, fortified foods such as breakfast cereals and milk could considerably increase the likelihood of meeting nutrient intake

recommendations for vitamin D [236]. Another factor is individual variability in absorption, metabolism, increased requirement and excretion of this nutrient [226; 235].

According to data from the Greek Childhood Obesity (GRECO) study which involved 4786 school children aged (10-12 years old), 36% of children did not consume fish once a week, a major source of vitamin D in the diet [237]. Similar findings were documented from the national health program for children (EYZHN) that assessed approximately 1,246, 135 children and adolescents over a 5-year period. The aim of this program was to ensure the healthy development of all children and adolescents through the adoption of healthy eating habits and physical activity. Assessment of dietary habits showed that 50% of children consumed fish less than 2-3 times per week [238]. Conversely, in Nordic countries, vitamin D levels in children are higher > 25 nmol/l (10 ng/mL) as a result of food fortification, cod liver oil supplementation and habitual fatty fish intake [239].

In summary, research shows that there could be a link between widespread vitamin D deficiency found today and the growing incidence of asthma. The anti-inflammatory and immunomodulatory effects of vitamin D may potentially be important in altering asthma pathogenesis and severity of disease. Nervetheless there is a scarcity of randomized controlled trials (RCTs) to demonstrate a causal role. To the candidate's knowledge no clinical trial has been performed to investigate the effect of vitamin D status on lung function and asthma in children consuming fatty fish, a dietary source of vitamin D. Most of the evidence documenting preventive effects is from studies examining the impact of vitamin D supplementation on asthma [221].

Therefore it was considered worthwhile to examine the effect of plasma vitamin D status on lung function and asthma control in children suffering with 'mild asthma' following a dietary intervention in the present thesis. The findings of this sub-analysis is outlined in Chapter 6.6.3.

1.4.7 Salt intake and asthma

With respect to salt intake, overconsumption and processed foods high in sodium, cause adverse health effects for a number of diseases including asthma [240; 241]. Studies have documented a higher prevalence of asthma in children consuming a diet high in salt [194; 240; 241]. A dose-response effect was observed between salty-snack intake and respiratory symptoms in asthmatic children. Arvaniti et al, (2013) used data from the the PANACEA study (Physical Activity, Nutrition and Allergies in Children Examined in Athens) which was a cross-sectional study of 700 Greek children aged 10-12 years to evaluate the association between salty-snack eating and TV/video-game viewing with asthma in children [240]. The PANACEA FFQ was used to gather information on the frequency of foods and beverages habitually consumed by children in Greece. Salty snack intake was represented by consumption of hamburgers, pizza, hot dog, toast, cheese pie, potato crisps and popcorn. Overall dietary habits, diet quality and Mediterranean diet compliance was assessed using the Mediterranean dietary quality index for children and adolescents (KIDMED Index) [242]. The KIDMED test was a questionnaire designed specifically to assess adherence to the Mediterranean dietary pattern in Spanish children and adolescents participating in the ENKID study [242]. This 16-item questionnaire was based on the characteristics of the Mediterranean dietary pattern. A score of +1 was assigned for eating behaviours that sustain the Mediterranean diet and -1 for those that are contradictory. The final score was the sum of the scores for each of the 16 questions which ranged from 0-12 [242]. The higher the score, the better the adherence to the Mediterranean diet. According to the investigators data analysis showed that 48% of children consumed salty snacks and about 1/3 almost every second day. The analysis showed that in children reporting frequent intake of salty snacks (> once/week) were 1.26 times more likely to develop asthma symptoms. The association of salty-snacking (≥ once/week) and asthma was 1.48 times greater in children who watched TV or played video games > 2 times/day. After stratification by hours of TV/video game viewing > 2 hours/day, in the adjusted

analysis controlling for age, sex, BMI, physical activity, parental education, and KIDMED score, salty-snacking ≥ 3 times/week was associated with 5.8 times higher likelihood of having asthma symptoms (95%CI: 1.03-32.61) compared to never or rare consumption. In comparison, high Mediterranean diet adherence (KIDMED score 8-12) was associated with lower likelihood of asthma. Moreover, a unit increase in the KIDMED score was associated with lower likelihood of asthma, irrespective of hours of TV/video-game viewing (OR: 0.84, 95%CI: 0.72-0.96) [(240)].

This study highlighted that adherence to a low-sodium Mediterranean diet was independently associated with lower likelihood of asthma symptoms irrespective of number of hours spent TV/video game viewing.

1.5 Asthma assessment in children

According to the Global Initiative for Asthma (GINA), assessment of asthma is based on the presence of symptoms (day and night), medication use and lung function [(243)]. By consensus, asthma severity is established on the intensity of treatment needed to acquire good control of symptoms [(243)]. Mild asthma is defined as 'well-controlled' asthma with low intensity treatment such as low-dose inhaled corticosteroids (ICS), leukotriene modifiers or cromones [(243)]. In contrast, severe asthma requires high intensity treatment to maintain good control. Several diagnostic tools invasive and non-invasive methods such as spirometry, exhaled nitric oxide (NO) analyzers, have been developed in assessing and managing asthma in patients [(244)] (Table 5).

Table 5 Tools available for monitoring paediatric asthma based on age

TOOLS	0-2 years	2-4 years	4-6 years	>6 years
Symptoms	√	√	√	√
C-ACT/ACT			√	√
ACQ				√
Exacerbations	√	√	√	√
Flow volume/ BDR			√	√
Peak Expiratory Flow			√	√
AHR			√	√
FeNO	√	√	√	√
Induced Sputum				√
EBC			√	√

Key: C-ACT: Child Asthma Control Test. ACT: Asthma Control Test. BDR Bronchodilatory response. PEF: peak expiratory flow. AHR: Airway hyperresponsiveness; FeNO: Fractional exhaled Nitric Oxide ; EBC: exhaled breathe condensate; √ -tool applicable for this age group

Source: Permission granted from the publishers European Respiratory Society for use of the table from Pinenburg et al, 2015. Monitoring asthma in children. Eur Respir J 45: 906-25. doi: 10.1183/09031936.00088814.

1.5.1 Asthma control in children

The goal of asthma management is to control symptoms, prevent exacerbations and need for medical care, be able to perform daily activities without restrictions, lead good quality of life as well as to achieve optimal lung function with minimal medication use and side effects from treatment [(12)]. Concepts of asthma severity and control are useful in evaluation and maintenance of control and in following patients' progress with treatment [(20)]. Asthma control in children is described in terms of manifestation of symptoms, which can vary in frequency and intensity, limitation in daily activities, use of reliever medication and risk of exacerbations [(12)] (Table 6). This is significant because poor

symptom control is strongly associated with increased risk of asthma exacerbations, hospital admissions and poor quality of life [12; 20].

Table 6 Levels of Asthma Control

Domain	Component	Level of Control			
Impairment		Complete	Good	Partial	None
	Symptoms				
	Daytime	None	≤ 2x/wk	>2x/wk	Continuous
	Night/awakenings	None	≤1x/mth	>1x/mth	Weekly
	Need for rescue medication	None	≤ 2x/wk	>2/wk	Daily
	Limitation of activities	None	None	Some	Extreme
	Lung function*-FEV_1, PEF	> 80%	≥80%	60-80%	< 60%
Risk	Exacerbations (per year)	0	1	2	>2
	Medication side effects	None		Variable	

Key: FEV_1 –Forced expiratory volume in 1 second; PEF-Peak Expiratory Flow; wk-week, mth-month

* Predicted/or personal best

Source: Permission granted from the author to reproduce table from Papadopoulos et al, 2012. International consensus on (ICON) pediatric asthma. Allergy 2012; 67: 976–997. doi: 10.1111/j.1398-9995.2012.02865.x.

Population studies have shown that despite the availability of effective drug therapy, in Europe only one in 20 children have adequate asthma control according to GINA guidelines [245]. There is evidence of underuse of inhaled corticosteroids in asthmatic children and overuse of rescue medication (short-acting β2 agonists) [245]. Both parents and physicians tend to overestimate asthma control [245]. This highlights the need for a simple and quick diagnostic tool that effectively evaluates asthma control in children.

1.5.2 Asthma –related quality of life

Asthma effects the child, physically, psychologically and socially [246]. Uncontrolled asthma increases risk of exacerbations, need for medication, hospital admissions, causes an economic burden and decreases quality of life for the patient and family [247]. Children with asthma are distressed by symptoms (shortness of breath, wheezing and cough) and they are often limited in their daily activities [248]. Moreover, an asthma attack is frightening, upsetting, frustrating and at times the child may express anger [246]. Asthmatic children often feel different from their friends and isolated because they cannot participate in activities that might trigger an asthma attack [249]. Assessment of symptoms and quality of life are not only important in evaluating the efficacy of a therapeutic intervention but also useful in providing valuable information about the disease and its effects on the child and family's daily lives.

1.5.3 Spirometry

In primary care, asthma is monitored by routine clinical follow-ups on at least an annual basis. Medical check-ups usually include physical examination, assessment of the patient's symptom score (via a validated questionnaire), exacerbations, corticosteroid use, absenteeism from school or work, growth in children, inhaler technique and lung function assessed by spiromentry [250]. Evaluation of lung function is important in diagnosing, monitoring asthma symptoms and evaluating the effectiveness of asthma therapy on pulmonary function [111]. The most common tests available for assessing lung function are Peak Expiratory Flow measurements (PEF) and Spirometry. Peak Expiratory Flow meters may be used by some to monitor respiratory flow in children, but are inaccurate and difficult for children to perform properly [111]. On the other hand, spirometry is the gold standard of pulmonary function tests in children over 6 years old [109; 111]. It is a non-invasive test of pulmonary function that assesses the mechanical properties of the

pulmonary system by measuring the dynamics of lung volume and capacities [251]. This test is important in diagnosing, measuring the effect and severity of disease, monitoring its course or to evaluate the response of therapy on pulmonary function [111; 251]. Spirometry tests how an individual inhales or exhales volumes of air as a function of time [251]. Dynamic lung volumes are measured at the mouth from registration of forced ventilatory manoeuvres which are recorded on a volume-time plot and flow-volume loop [111] [251] that display amount of air inhaled and exhaled. The plot begins with normal tidal breathing, then the patient inhales air to fill the lungs, followed by expiration made as forcefully and completely as possible [251] (Figure 10, Figure 11).

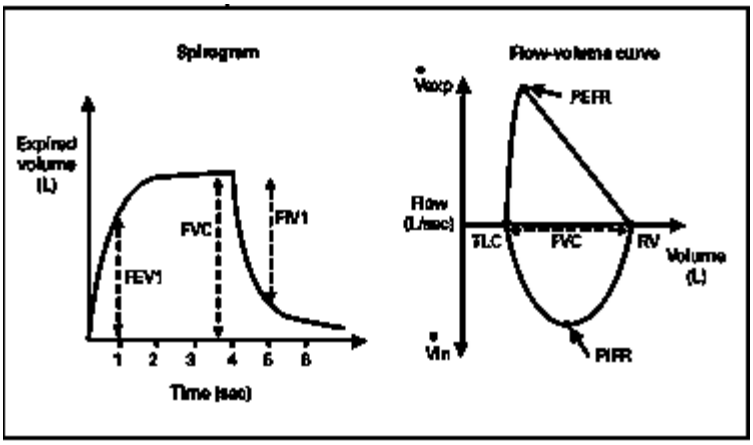

Figure 10 Normal spirogram and Flow-Volume curve showing conventional pulmonary measurements

Key: FEV_1 – Forced Expiratory Volume in 1 second; Forced Inspiratory volume in 1 second; FVC Forced Vital Capacity; PEFR- Peak Expiratory Flow Rate; IRV- Inspiratory Residual Volume; Vt- tidal volume; FVC- Forced Vital Capacity; TLC-Total Lung Capacity; RV-Residual Volume; PIFR- Peak Inspiratory Flow Rate; ERV- Expiratory Residual Volume.

Source: Reproduced with permission from The Royal Australian College of General Practitioners from: Pierce R. Spirometry: An essential clinical measurement. Aust Fam Physician 2005;34(7):535–39.

Available at https://www.racgp.org.au/afpbackissues/2005/200507/200507pierce.pdf

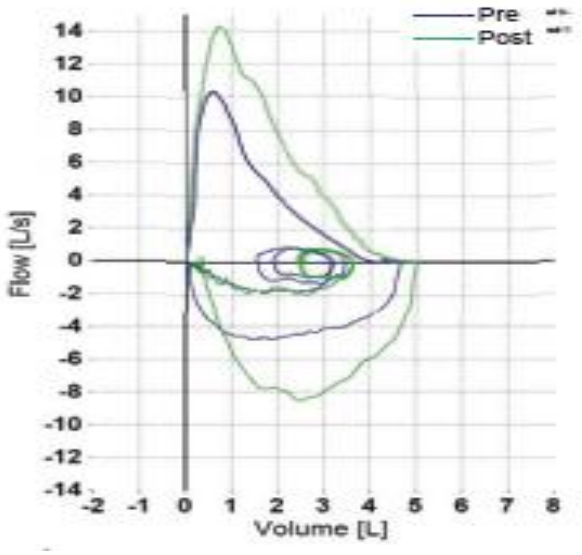

Figure 11 Flow -Volume plot

Figure 11 shows inspiration and expiration volumes pre (blue line) and post bronchodilator (green line) administration. The loops above the volume axis represent exhalation whereas below inhalation. In this figure it is apparent that post bronchodilator administration, the green loop has a higher peak than the loop pre-bronchodilator use (blue), indicating an improvement in airway conduction thus confirming the presence of asthma in the patient.

Source: Reproduced from Coates et al, 2013. Spirometry in primary care. Can Respir J, 20(1): 13-22 under Creative Commons Attribution License.

Normal flow-volume curves appear like a sail that rises sharply to a peak and then descends to an angle of 45° [(252)] (Figure 12). A concave curve indicates mild to moderate airway obstruction and an elongated tail indicates severe obstruction. A normal volume-time curve rises sharply and reaches a plateau. A slow gradual rise and no definite plateau indicates moderate to severe airway obstruction. A small flow-volume curve suggests a restrictive pattern. In young children the flow-volume curve has a convex shape due to rapid emptying of large airways compared with smaller lung volume, which becomes more linear as the child grows [(252)].

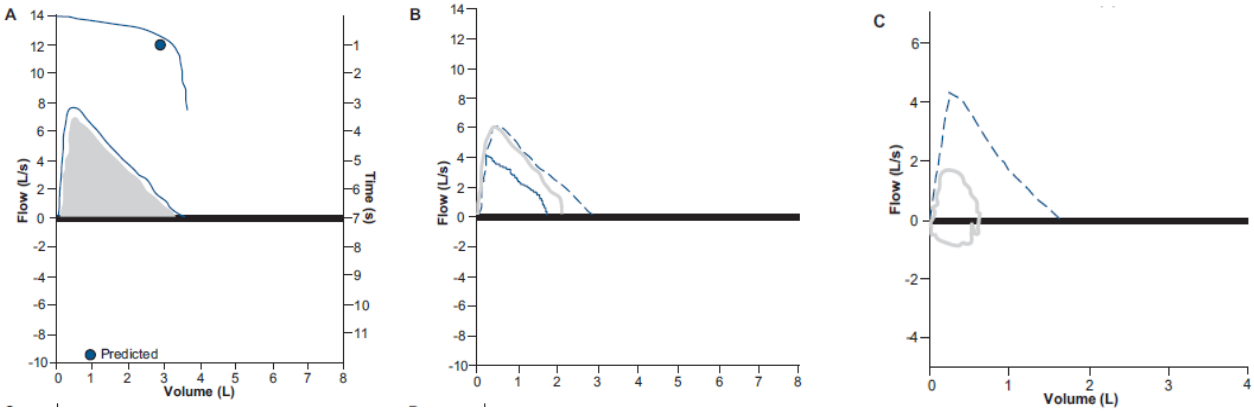

Figure 12 Ventilatory pattern identified from spirometry curves

In Figure 12, diagram A illustrates normal flow-volume curve (lower triangle) and volume-time curve (upper blue line). The grey shaded area represents the predicted curve and the blue line the actual measurement. Diagram B shows flow-volume curve suggestive of mild-moderate airway obstruction, with the grey line indicating the predictive curve, blue line the actual measurement and the blue dotted line, post-bronchodilation. And in diagram C, the flow-volume curve depicts the restrictive pattern. The blue dotted line is the predicted curve and the grey line the actual measurement.

Source: Permission granted for reproduction of image from Springer. Available in Jat, 2013. Prim Care Respir J 2013; 22(2): 221-229. doi: 10.4104/pcrj.2013.00042.

Spirometry variables

Conventional spirometry measurements are the Forced Expiratory Volume in one second (FEV_1), Forced Vital Capacity (FVC), the ratio of these two volumes (FEV_1/FVC), Peak Expiratory Flow (PEF) and mean expiratory flow between 25% and 75% of FVC ($FEF_{25-75\%}$) [111]. FEV_1 is the most useful index from all variables. It exhibits an almost linear relationship to the status of lung function. Also, it is an important component of assessment of asthma control and a predictor of future risk as reduced lung function is

associated with poorer asthma outcomes [253]. Measurements of FEV_1 and FVC equal to or above 80% predicted indicate normal pulmonary function in children [12] and reflect large airway calibre [111]. In comparison, $FEF_{25-75\%}$ reflects small airway conduction and is a sensitive indicator of airway obstruction in children [111]. Values of $FEF_{25-75\%}$ less than 80% suggest poor conduction and less than 60% obstructive pattern [111; 254]. The ratio of airway flow to lung volume (FEV_1/FVC) distinguishes pulmonary obstruction from restrictive disorders [111] and values above 80% are considered to be normal in healthy children [111]. The change in FEV_1 is important in interpreting the bronchodilation test which involves administering a bronchodilator prior to spirometry evaluation in order to diagnose asthma. Clinical significance is considered to be an increase in $FEV_1 \geq 12\%$ or 15-25% in $FEF_{25-75\%}$ post-bronchodilator administration [111]. This suggests reversibility of airway limitation consistent with asthma [111]. FEV_1 is superior in diagnosing the severity of obstruction as compared to Peak Expiratory Flow (PEF) which underestimates obstructive processes [111]. In the case of asthmatic children exhibiting normal FEV_1 values and $FEF_{25-75\%}$ parameter < 65-67% predicted, is consistent with airway dysfunction [111].

Reference values

As all biological parameters, lung function data follow a normal distribution or may be transformed to a normal distribution[255]. Assuming that data follow a normal distribution, values within 2 standard deviations (SD) of the mean value represent that 95% of the population are considered to be normal, and that 5% of the population (2.5% above and below 2 SDs) are abnormal [255]. For spirometry variables, the lower limit (LLN) is defined as 5^{th} percentile, that is the lower 5% of the population [255].

In assessment of pulmonary function, patients undergo spirometry testing pre and post bronchodilator administration. Abnormalities in a spirometry test are identified by the shape of the flow-volume and volume-time curves [252] and by comparing the values of

test parameters to reference values for FEV_1, FVC, FEV_1/FVC from population studies of healthy children without respiratory disease [255] along with percentage change after the bronchodilator test. The Global Lung Function Initiative (GLI-2012), recommend that spirometry values developed by Quanjer et al, (2012) for 3-95 years old be used [256]. These equations were developed from > 74, 000 spirometry measurements in a healthy population of 3-95 years from over 70 centres worldwide. The GLI-2012 equations have been adopted in several populations and seem to be appropriate for a wide age range including the transition from childhood to adulthood and almost for all ethnic groups. Differences in spirometry values may exist between different ethnic groups. Caucasians have been known to have higher spirometry values than Asians [111]. Slight differences may exist due to differences in chest wall dimensions, environment, socioeconomic factors, age, height and puberty [109; 111]. However, reference values for healthy Greek children have been developed and are validated for children 6-18 years and 115-194 cm in height [109]. These equations are comparable to those developed by other authors and are recommended for use in Greek children and adolescents and are applicable for Balkan populations [109].

Interpretation of spirometry and asthma severity

In children the severity of airway obstruction is graded according to FEV_1 percentage predicted [252]. A value of FEV_1 predicted < 100% to 80% indicates 'mild' airway obstruction; FEV_1 < 80% to 50% 'moderate'; FEV_1 < 50% to 30% severe and FEV_1 < 30% very severe [252]. A significant relationship exists between FEV_1 % and asthma symptoms and severe asthma exacerbations requiring oral steroids, hospitalization and medical help. In addition, FEV_1 has been used to classify mild, moderate and severe persistent asthma having FEV_1 > 80%, >60% to <80% and ≤ 60% predicted respectively [252] (Table 7).

Table 7 Assessment of severity of airflow obstruction according to FEV_1% predicted

% FEV_1 predicted	Category*
≥80	Mild
50-79	Moderate
30-49	Severe
<30	Very Severe

*post-bronchodilator

Source: Modified from Moore, 2012. Spirometry: Step by step. Breathe 2012 8:232-240. doi: 10.1183/20734735.0021711

1.5.4 Fractional exhaled Nitric Oxide (FeNO)

Nitric Oxide and airway inflammation

Asthma is a chronic inflammatory disorder in the lungs characterized by eosinophilic and neutrophilic infiltration of the airways [257; 258]. Eosinophilic asthma is a distinct phenotype of asthma associated with a rise in nitric oxide (NO) in exhaled breath which may respond to treatment with corticosteroids, while neutrophilic generally does not [232; 233]. In asthma pathophysiology, nitric oxide is secreted by the conducting airways and alveoli of the lungs during expiration and acts as a vasodilator, bronchodilator, neurotransmitter and inflammatory mediator in the lungs and airways [257; 259]. Studies using NO sampling with bronchoscopy from trachea and bronchi indicate that NO levels recorded at the mouth are analogous to NO derived from lower airways [258]. NO is generated through the conversion of L-arginine to L-citrulline by the action of nitric oxide synthase (NOS) [259]. Inflammation of the airways causes an increase in the expression of NO synthase type 2 and subsequent rise in NO [259]. In asthmatics, FeNO levels are elevated as a result of eosinophilic airway which decline in response to anti-inflammatory

treatment [259]. Studies have shown that FeNO levels are significantly correlated with the intensity of clinical symptoms, bronchial hyper-responsiveness, bronchodilator response, serum Ig E and percentage of eosinophils in the bronchoalveolar lavage [259]. Effective asthma treatment with inhaled corticosteroids lower FeNO levels in patients [259]. Therefore, measuring FeNO in the breath provides supplemental information for the diagnosis and management of asthma in a simple and non-invasive manner.

There are a number of factors that affect exhaled NO (eNO) levels which must be taken into account when interpreting the results of FeNO assessment in clinical practice. Exhalation flow rate, presence of atopy, environmental NO, respiratory infections, a diet rich in nitrates, caffeinated drinks, exercise, atopic rhinitis, exacerbations and menstruation increase exhaled NO levels [258; 259]. Nitrate-rich foods such as strawberries, currents, raspberries, cherries, apples, lettuce, beets, carrots, green beans, spinach, parsley, cabbage, radishes, celery and collard greens which collect nitrates from soil may cause up to 60% increase in FeNO levels [258]. In addition, NO levels are higher in the nasal cavities, so it is important to prevent the contamination of the measured exhaled NO with the nasal NO [258]. This is achieved by exhaling against resistance, which pushes the soft palate upwards sealing the nasal cavity from the oropharynx [258]. Holding one's breathe also increases the exhaled NO by the accumulation of gas in airways. In comparison, NO levels may decrease due to spirometry and anti-inflammatory treatment using corticosteroids that have the potential to reduce NO levels rapidly from 48 hours to one week after initiation of treatment and is dose-dependent [258]. Therefore, NO examination should be performed before spirometry [258]. Anthropometric measurements age, height, BMI and demographic characteristics, ethnicity and gender influence FeNO levels. Studies have shown that in patients with very high BMI, NO is falsely low [258]. Males have been shown to have 17-25% higher NO than females [258].

Fractional exhaled Nitric Oxide (FeNO) testing

The management of asthma is based on the suppression of symptoms and on the resolution of airway obstruction evaluated by functional tests, namely spirometry [111]. Treatment and monitoring guided by symptoms, scores or lung function parameters are not always accurate markers of asthma severity and may not reflect the underlying inflammatory process [111]. Airway inflammation can continue to be present even in well-controlled mild asthmatic patients, suggesting that monitoring symptoms, use of questionnaires and pulmonary function may not be sensitive enough to indicate whether inflammation has been resolved [111; 260].

Eosinophilic airway inflammation can be measured by a number of biomarkers: FeNO, cytological examination of sputum, bronchoalveolar lavage, determination of serum interleukin concentration (IL 5, 10, 13, IFNγ) and analysis of the exhaled breath condensate (EBC) [244; 260]. These techniques are useful in clinically stable patients for drug dose adjustment and definition of treatment duration in order to prevent bronchial remodelling [244]. EBC is the less invasive technique for monitoring inflammation, however cost of equipment and the collection procedure in young children may be more difficult due to the lower level of co-operation and smaller amounts of EBC [244]. Induced sputum examination is impractical, since it is difficult for children to cough up enough sputum for analysis. And use of saline to provoke sputum production may provoke bronchospasms and excessive secretions in patients often requiring bronchodilators [261]. In the case of serum interleukin analysis, often parents are reluctant when it comes to blood sampling in children [261]. Contrastingly, FeNO is recognized as a reliable robust surrogate marker of eosinophilic airway inflammation [261]. Changes in eNO are useful in predicting asthma exacerbations, loss of control and in monitoring the response to treatment [259]. An advantage of FeNO analysis, apart from being non-invasive is that, it is simple, easily executable, requires only 2-3 minutes, is low in patient burden and results

are obtained instantly [257]. FeNO testing has been approved by the ATS/ERS and the National Institute of Health for Health and Care Excellence (NICE) as a biomarker of inflammation for asthma monitoring, predictor of disease exacerbation, response/and or patient compliance to ICS therapy and as a guide for medication adjustment [257; 262]. As in spirometry, this method is indicative for children at least 5 years of age [257].

Fractional exhaled nitric oxide analyzers

Exhaled NO is usually measured by chemiluminescence or electrochemical sensing [259]. The chemiluminescence method was first used for detection of exhaled NO in the breath of humans and became the gold standard for FeNO measurements [259]. However, the chemiluminescence-based NO analyzer is expensive and not portable [259]. Alternatively, NO analyzers with electrochemical sensors are hand held devices and relatively cheaper than those based on chemiluminescence [259]. Since there is good agreement in FeNO levels between both devices, the use of hand held devices has become increasingly popular in clinical practice [259]. There are three eNO analyzers accredited by the ATS/ERS and NICE [257; 262] in measuring exhaled fractional nitric oxide: NIOX VERO and NIOX MINO (Aerocrine Ltd. USA) [263] and NO Breath (Bedfont Scientific Ltd, UK) [264]. All three are accurate, reliable, comparable and most importantly, cost-effective [257; 262]. According to NICE guidelines, NO Breath (Bedfont Scientific Ltd) is a diagnostic monitoring tool that measures exhaled nitric oxide produced by airway inflammation [262]. The reading is presented in parts per billion (ppb) and is directly related to the severity of airway inflammation. In order to produce valid results, "NO breath" requires 10 seconds of exhalation in children [262].

Interpretation of FeNO

The 2011 ATS clinical practice guidelines for the interpretation of FeNO, recommend low FeNO values < 20ppb in children represent absence of bronchial eosinophilic inflammation, unlikely response to corticosteroid therapy and presence of non-

eosinophilic inflammation [257] (Table 8). Intermediate FeNO levels of 20-35 ppb warrant cautious interpretation and additional factors such as age, atopy and infection should be taken into account [257; 259]. Under such circumstances, repeated measurements are required to enable the physician to verify that the increase in NO is the result of atopic asthma. FeNO levels higher than 35 ppb in children strongly suggest airway eosinophilia, ongoing asthma symptoms and continued exposure to an allergen [257]. In symptomatic patients, FeNO > 35 ppb signifies poor adherence to ICS therapy along with better clinical improvement with corticosteroid treatment [257].

Table 8 Interpreting FeNO values in children using portable NO Breath FeNO Monitor

FeNO Levels (ppb)	**LOW** **< 20 ppb**	**INTERMEDIATE** **20-35 ppb**	**HIGH** **>35 ppb or FeNO > 40% from previously stable levels**
Airway Inflammation	UNLIKELY	Present MILD	Present SIGNIFICANT
Asymptomatic	• Implies adequate dose and good adherence to anti-inflammatory therapy. • ICS dose may possibly be reduced (repeat FeNO after 4 weeks to confirm. • If it remains low, relapse is unlikely	• Adequate ICS dosing • Good adherence • Monitor change in FeNO	• ICS withdrawal or dose reduction may result in relapse • Poor compliance or inhaler use
Symptomatic: • Cough • Wheeze • Shortness of breath during past 6 weeks	*Possibly due to alternative diagnosis:* • non-allergic asthma • vocal cord dysfunction • anxiety hyperventilation • bronchiectasis • cardiac disease • rhinosinusitis • GERD Unlikely to benefit from increase in inhaled corticosteroids	• Persistent allergen exposure • Inadequate ICS dose • Poor compliance • Steroid resistance	• Persistent allergen exposure • Poor adherence or inhaler use • Inadequate ICS dose • Risk for exacerbation • Steroid resistance due to: Allergic/or atopic asthma, or allergic bronchitis

Source: Adapted from Bedfont Scientific Limited. Available from https://www.bedfont.com/file/2885-MKT140_NObreath%20catalogue_iss%2013.pdf

In a study involving children who stopped ICS treatment for a month, having FeNO higher than 49 ppm, showed 93% specificity and 71% sensitivity for asthma relapse [265]. In another paediatric study where medication dose was reduced by 50% every 8 weeks, a FeNO of at least 22 ppb was associated with an odds ratio of 10.4 risk for an exacerbation [266]. Hence, a 60% rise in FeNO in asthmatic children after medication cessation would strongly indicate deterioration of lung function and likelihood of asthma exacerbation [258]. It is recommended that personal best values of FeNO using individual cut-offs give a more precise clinical picture of the patient than using normal values via predictive equations [261].

1.5.5 Pulse Oximetry

One of the symptoms described by asthmatic patients is 'shortness of breath' due to a state of hypoxia associated with asthma [267]. In daily paediatric practice monitoring of hypoxemia associated with respiratory disease is an important diagnostic tool of asthma status. Pulse oximetry is a simple, non-invasive method for the estimation of haemoglobin oxygen saturation ($Sp0_2$) in the assessment of hypoxia associated with asthma in children [267]. Oxygen saturation is a sensitive indicator to ventilation/perfusion mismatch that occurs in exacerbations of asthma. In healthy children haemoglobin oxygen saturation should range between 95 to 100%. $Sp0_2 < 92\%$ was associated with increased respiratory distress [267], severity of asthma exacerbation and 6.3% fold greater relative risk for requiring additional treatment [268]. Normal heart rate in children aged (6-12 years old) is defined from 75-118 beats/minute [269].

1.6 Management of asthma

Pharmacotherapy remains the cornerstone of asthma management [20; 243]. The aim of asthma therapy is to control symptoms using the least possible medications and reduce the risk for future morbidity [20]. Treatment depends on the severity and frequency of symptoms. Mode of administration of asthma therapy is usually by use of an inhaler (with aero-chamber for children < 5 years) or nebulizer [20].

1.6.1 Asthma Therapy

Current asthma therapy can be classified to two pharmacological categories, namely quick-relievers (or rescue medication) and long-term acting (Figure 13).

1. **Rescue medication**: Short-acting β_2 adrenergic agonists (SABA) deliver rapid relief of asthma symptoms (cough, wheeze and dyspnoea) via bronchodilation. Use of inhaled SABA such as 'salbutamol', is recommended as the first-line reliever therapy for an asthma attack in children or before exercise [270]. SABAs are used as 'needed' and have a rapid effect on airway smooth muscle. An advantage of SABAs is that they are user-friendly. Possible side-effects include tachycardia, trembling of fingers/hands, nervous tension, hyperactivity, headaches and muscle cramps in susceptible children that usually last a few hours and subside over a period of weeks [270] . Example of SABA is '*Aerolin'*.
2. **Long-term asthma control**: Long–acting therapy is targeted on reducing airway inflammation and is taken daily to prevent symptoms and exacerbations [20]. There are three main classes of long-term acting medications for asthma in children:

a) **Inhaled corticosteroids (ICS):** ICS are better known as anti-inflammatory drugs that are used as daily controller medications in persistent asthma. There is strong evidence that ICS improve symptoms and lung function, decrease need for additional mediation, reduce rate of asthma exacerbations and hospitalization in children [20]. Due to their

pleiotropic anti-inflammatory effects, initiation of ICS therapy constitutes the first step of regular on-going treatment [20]. Unlike oral steroids, ICS have low risk of side-effects which include hoarse voice and oral thrush that can be prevented by using an aerochamber or rinsing the mouth with water [270].

Examples of ICS commonly prescribed for children are: Fluticasone (‘*Flixotide’*), Beclomethasone dipropionate (‘*Becotide’*) and Budesonide (‘*Pulmicort’*).

b) **Oral and intravenous corticosteroids**: In the case of severe asthma or ‘resistant asthma’ which do not respond to usual asthma therapy, oral and intravenous corticosteroids are administered to relieve airway inflammation and have anti-allergy and immune-modulating effects [20]. Regarding long-term use of high dose oral corticosteroids, robust evidence indicates adverse effects such as moon-face, weight gain, centripetal redistribution of fat, muscle-wasting, acne, bruising, thinning of the skin, stretch marks, decreased bone density (osteopenia) and growth velocity, predispose diabetes or cause hypertension, cataract formation, glaucoma and adrenal suppression [270];[271]. However, this sub-group of children is relatively small and high doses of ICS may be potentially lifesaving which outweighs the small risk of side-effects [270]. Systemic adverse effects are rare when normal-dose ICS are prescribed [270]. A Cochrane review suggested that daily low to medium doses of ICS was associated with a 0.48 cm reduction in linear growth velocity in children with persistent asthma and 0.61 cm change from baseline in height during the first year of corticosteroid treatment [272]. With respect to bone health, no detrimental effect was observed with low-medium ICS use but with high doses in susceptible children [273]. Examples of oral corticosteroid are ‘*Medrol’ and ‘Prezelon’*.

c) **Leukotriene receptor antagonists**: During an asthma attack, leukotrienes cause narrowing and oedema of airways. Leukotriene receptor antagonists such as montelukast are effective in improving lung function and symptoms up to 24 hours as well as in preventing exacerbations in children [20]. According to GINA guidelines leukotriene receptor antagonists are second-choice to ICS therapy [12]. Evidence

suggests that montelukast is particularly effective in exercise-induced asthma. Systemic effects of montelukast include nightmares, disturbed sleep, behaviour and mood changes [270]. Examples of montelukast are '*Singulair*', '*Modulair', 'Apilone' and 'Miralust'* which are available as tablets.

d) **Long-acting β_2 adrenergic agonists (LABA):** Long-acting β_2 adrenergic agonists (LABA) such as salmeterol and formoterol, have long-lasting bronchodilator action especially in children over 5 years [12]. ICS-LABA combinations are effective in ameliorating asthma symptoms in older children and are recommended as 'add-on' treatment [12]. LABA have similar side-effects as SABA but are less pronounced [270]. Examples of LABA are '*Serevent'*, '*Foradil*'and '*Formopen'*.

3. **Combination therapy:** A combination of inhaled corticosteroid (e.g fluticasone) and long-acting beta agonist (e.g salmeterol) are used for maintenance and reliever medication in patients with moderate to severe asthma. These medications contain two active ingredients that target inflammation and bronchodilation in one inhaler. Examples include '*Seretide' and 'Symbicort' (*Budesonide-Formoterol*)*.

Asthma Medication					
SABA	**Corticosteroids**	**Corticosteroids Oral**	**Anti-Leukotrienes**	**LABA**	**Combination Therapy**
					
Salbutamol	**Fluticasone**	**Prednisolone**	**Montelukast**	**Salmeterol**	**Budesonide + Formoterol**
Aerolin Inhaler (100 µg)	Flixotide Inhaler (125 µg)	Medrol (4mg)	Singulair Tablets (10mg)	Serevent Inhaler (25µg)	Seretide Inhaler (125 µg)

Figure 13 Medication commonly used to treat asthma in children

Source: Galinos, 2019. Pharmaceutical catalogue. Available from https://www.galinos.gr

1.6.2 Rhinitis Therapy

Over 80% of asthmatics have rhinitis, and 10%–40% of patients with rhinitis have asthma, suggesting a relationship between the two conditions via common inflammatory pathways in airways [166]. Rhinitis may aggravate asthma in children causing poorer asthma control, increased risk of emergency visits, hospitalization, impaired sleep and cognitive function, irritability, fatigue, school and work absenteeism, reduced quality of life, including higher healthcare costs [168; 253]. Therefore, it is imperative that symptoms of rhinitis are monitored and treated in order to reduce asthma burden, improve quality of life, work and school performance [165; 168]. The goal of medication is to control symptoms of nasal itching, sneezing, rhinorrhea, and nasal obstruction. Common medications used in children include: antihistamines, oral leukotriene antagonists, and intranasal corticosteroids, the latter being considered the most efficacious drug [166; 168]. Corticosteroids used to treat allergic rhinitis in children include 'Soldesanil','Nasonex', 'Pulmicort' and 'Mometasone' (Figure 14).

Rhinitis Medication	
Antihistamine	**Corticosteroids**
	
Desloratodinum Aerius (5mg) Syrup/Tablets	**Budesonide** Pulmicort Nasal Spray

Figure 14 Medication used to treat rhinitis in children

Source: Galinos, 2019. Pharmaceutical catalogue. Available from https://www.galinos

1.7 Novel approaches to asthma diagnosis

1.7.1 Metabolomics and Asthma

Asthma has been defined as a syndrome of heterogeneous diseases with variable clinical symptoms [12]. The diagnosis of asthma in children is challenging and relies on a combination of clinical factors, patient history, lung function and response to asthma therapy. No single test is diagnostic alone. The identification of measurable objective biomarkers in biological fluids to predict asthma and therapeutic response is of great clinical importance. Asthma-specific biomarkers could guide diagnosis, phenotyping and management of this condition as well as be useful in the implementation of targeted personalized therapy. In nutritional epidemiology, biomarkers are often used to evaluate nutrient intake of an individual's diet, assess the validity of dietary intake recorded in dietary assessment tools (FFQs), and as a method of assessing dietary change and monitoring patient's compliance in dietary intervention studies [274].

Metabolomics, the systematic analysis of small molecules, including carbohydrates, amino acids, organic acids, nucleotides and lipids, has been used to identify new biomarkers and novel pathogenic pathways for a number of complex chronic diseases including asthma [275; 276; 277]. It is based on the theory that abnormal concentrations of particular metabolites trigger the onset of disease. Asthma, a disease with a gene-environment aetiology is suitable for the application of metabolomics because it has the potential to capture the history of the cellular response to past exposures [275]. The availability of non-invasive methods to study and monitor disease inflammation is relevant especially in childhood asthma.

Given that there is a paucity of metabolomics studies investigating asthma in Greek children, the purpose of using this innovative technique in this PhD project was to better understand the metabolic pathways involved in the pathophysiology of paediatric asthma, determine imbalances which can then be treated using tailored nutrition therapy.

SYNOPSIS

In Chapter 1 a brief description was given of what is known regarding asthma prevalence aetiology, pathophysiology and management in children. Based on evidence from the literature, paediatric asthma continues to rise globally and is a major public health concern due to increased morbidity, decreased quality of life and economic burden. To date, there is no cure for asthma. Symptoms are controlled only by medication which on a long-term basis may cause side-effects. There is general consensus that asthma is caused by genetic and environmental factors that trigger an immune response promoting chronic inflammation in the lungs and manifestation of symptoms. It has been postulated that diet may influence asthma development and onset in children. Animal studies suggest an anti-inflammatory role of omega-3 fatty acids in respiratory disease. However, the role of dietary omega-3 fatty acids as fatty fish in childhood asthma is yet to be elucidated. With respect to dietary patterns, epidemiological studies suggest a protective effect of adherence to the Mediterranean diet in chronic disease. There is little published evidence to date evaluating the link between adherence to a Mediterranean diet and childhood asthma.

CHAPTER 2 Mediterranean diet and childhood asthma

2.1 The Traditional Greek Mediterranean Diet

The word 'Mediterranean' is derived from the Latin word 'mediterraneus' meaning 'in the middle of the earth' or between islands (medius- middle, between and terra – land, earth) (Oxford, 2019) [278]. This is based on the sea's position between the continent of Africa and Europe. Countries bordering the Mediterranean sea are illustrated in Figure 15. Similarly, the Greek translation for Mediterranean, 'mesogeios' (Μεσόγειος) from 'meso' (μέσο) meaning 'middle' and 'geios' (γή), land or earth.

Figure 15 Map of countries surrounding the Mediterranean basin from antiquity to the present

Source: Adapted from https://en.wikipedia.org/wiki/List_of_Mediterranean_countries under Creative Commons Attribution License.

Mediterranean dietary patterns have developed over the past 5000 years spreading from the Fertile Crescent and influenced by the conquests of many different civilizations, dietary rules of the three main religions (Judaism, Christianity, and Islam), and continuous interactions and exchanges inside and outside the region [3]. As a result, the Mediterranean

diet is an expression of different food cultures present in the Mediterranean region, with diverse food consumption and production patterns. It must be emphasized that there is not one single Mediterranean diet, but rather a number of variations adapted to individual country's cultures. Therefore, the Mediterranean diet is more than just a defined diet, but it represents various cultural expressions of different Mediterranean food cultures and lifestyles [3].

Interest in the Mediterranean dietary pattern stemmed from the infamous pioneer study "Seven Countries Study" launched by Ancel Keys in 1950s which involved 12, 763 men 40-59 years enrolled in 16 sub-cohorts in Greece, Italy, Yugoslavia, Japan, Netherlands and United States [279; 280]. Over a period of 30 years, Keys and collaborators examined the relationship between diet, cholesterol and cardiovascular disease [279; 280]. The results of this study showed that Cretans had lower rates of coronary heart disease and overall mortality not only because they consumed diets low in saturated fat but followed a *lifestyle* which added to these beneficial effects. After 25 years of prospective cohort studies, Keys reported that the Mediterranean diet is characterized by various food models influenced by different cultures, histories and religions. In the Greek language the word "diaita" means way of life as opposed to a restrictive diet as is commonly thought [281].

Since then, numerous studies have explored and clearly demonstrated that greater adherence to the Mediterranean diet is associated with reduced risk for overall mortality, cardiovascular disease, cancer incidence, neurodegenerative diseases and diabetes as well as longeveity [282]. However, the beneficial effects of Mediterranean diet adherence for other diseases (Obesity, dyslipidemia, stroke, metabolic syndrome, cognitive impairment, dementia and depression) are less clear [282; 283; 284; 285; 286].

A recent umbrella review was performed by Dinu et al (2018), estimating the association between Mediterranean diet adherence and 37 different health outcomes including overall mortality, cardiovascular disease, cancer, cognitive and metabolic disorders along with

inflammation [282]. Overall, pooled analysis of data from 12,800,000 subjects in observational studies and RCTs supported the concept that greater adherence to the Mediterranean diet reduced chronic disease risk and overall mortality. To date, the scientific literature has identified robust evidence for overall mortality, cardiovascular disease, myocardial infarction, cancer incidence/mortality, breast cancer, neurodegenerative disease, cognitive impairment, Alzheimer's diseases, dementia and diabetes [282]. On the other hand, the grade of evidence for overall cancer mortality, colorectal, gastric pancreatic, liver and respiratory cancers, depression, anthropometrical, metabolic and inflammation was suggestive (weak) [282]. As for stroke, blood pressure and metabolic parameters mixed results were observed, depending on study design. No association was found between Mediterranean diet adherence and bladder, endometrial, ovarian cancers, heart failure and major cardiovascular events in meta-analyses of observational and RCTs respectively. Plausible reasons for the null findings observed could be the limited number of studies and evidence, high heterogeneity among study designs therefore comparing studies is difficult. Additionally, small sample sizes may have limited statistical power to detect associations, as well as indices used to measure Mediterranean diet adherence. The investigators concluded that there is need for more well-designed RCTs with uniform methodologies to clarify the Mediterranean diet effects on these health outcomes [282].

Although, the robust evidence on the health benefits of the Mediterranean dietary pattern in chronic disease, little is known of the respiratory effects in inflammatory diseases such as asthma [287; 288]. A randomized controlled trial (RCT) involving 38 adults with asthma, showed small but insignificant improvements in asthma related quality of life and lung function (FEV_1, FVC) in those patients with high adherence to the Mediterraean diet intervention compared to the control group [288]. No change was observed in asthma control or inflammatory markers, although the intervention substantially improved dietary behaviour in asthmatic adults [288]. In comparison, a cross-sectional study of 174

asthmatics reported that high adherence to the Mediterranean diet improved the likelihood of asthma control [287]. Nonetheless, a major limitation of this study is the cross-sectional design which lacks the ability to determine a cause-effect relationship. Even though this study provides a beneficial link between Mediterranean diet adherence and adult asthma control, more prospective studies are needed to confirm whether changing diets towards a Mediterranean dietary regime indeed improves asthma outcome in adults. On the topic of the role of the Mediterranean diet in paediatric asthma, this will be explored in a published systematic review written by the candidate presented in Chapter 2.3.

The Mediterranean dietary pattern can be best described as a way of life, not only eating habits, commonly practised in the olive-growing countries surrounding the Mediterranean basin during the late 1950s and early 1960s after the Second World War [4; 200]. It reflected dietary habits typical of Crete, the rest of Greece and Southern Italy [4]. The composition of this diet was based on climatic conditions, poverty and hardship during that era rather than by wisdom. The Traditional Mediterranean diet is primarily a moderate carbohydrate, plant-based diet characterized by high content of fresh seasonal fruits, vegetables, salads, wild greens, cereals, legumes, nuts, seeds, olives, virgin olive oil and moderate intake of fish, dairy products, poultry, wine (usually with meals) and low intake of red meat [4; 200] (Figure 16). A wide variety of food sources rich in antioxidants, optimal omega-6:omega-3 fatty acid ratio, mono-unsaturated fatty acids, micronutrients and vitamins that have been linked to good health and well-being [199]. One of the common features in all of the Mediterranean countries is the use of olive oil as the primary source of fat and abundance of fruits and vegetables, garlic, onions and herbs as condiments. It is the high content of vegetables and salad greens that promote the use of olive oil as a dressing [200]. The total fat content of this dietary pattern is approximately 40% of the total energy intake in Greece and 30% in Italy [200]. In general, the high intake of extra virgin olive oil contributes to the high ratio of monounsaturated to saturated fats in Mediterranean countries than in other parts of the world with saturated fat comprising

about 7-8% [200]. To this day, the olive tree is regarded to be sacred, a symbol of eternity and its oils to possess therapeutic properties which are believed to promote good health and longevity. Other features associated with this dietary pattern are daily physical activity, afternoon 'nap' and the social aspect of mealtimes [200].

The Traditional Mediterranean diet consists of the following components:

- High intake and variety of plant foods including fruits, salads, vegetables including wild greens, bread, cereals, potatoes, legumes, nuts, seeds
- Minimally processed, seasonally fresh and locally grown foods
- Fresh fruits as the typical daily dessert with sweets consisting of nuts, sesame seeds, olive oils and honey consumed during feast days
- Moderate intake of fermented dairy products (mainly as cheese and yogurt)
- Moderate consumption of fish, seafood and poultry
- Use of olive oil as the main source of dietary fat and olives accompanying meals.
- 0-4 eggs consumed per week
- Low intake of red meat
- Moderate intake of wine, generally with meals
- High monounsaturated : saturated fat ratio [4]

In the Traditional Greek Mediterranean diet meals always comprise large quantities of whole-grain bread, olives, salads, herbs, vegetables, wild greens ('*horta*' in Greek), legumes and extra virgin olive oil [200]. Intake of yogurt is high, milk moderate and cheese is consumed in smaller amounts except for feta cheese which accompanies legumes, salads and vegetable stews (*'ladera'*). Meat being expensive is consumed less and fish consumption is determined by proximity to coastal areas [200]. Greeks enjoy food, live to eat and eat to live. Mealtimes for Greek families is a social event, when meals accompanied by a bottle of good red wine are shared among family and friends. Contrastingly, the Italian variation of the Mediterranean diet is characterized by higher consumption of pasta, while fish intake is higher in Spain [200].

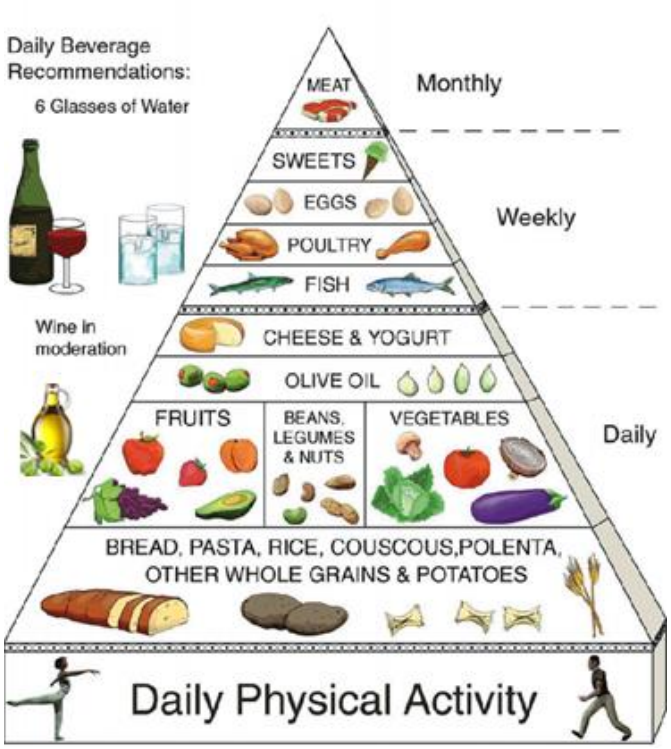

Figure 16 Traditional Mediterranean Diet Pyramid

Figure 16 depicts the Traditional Mediterranean diet pyramid. The purpose of this pyramid is to act as a guide on healthy food choices for the healthy adult population and indicates frequency of consumption rather than specific weights of foods and calorie intake.

Source: Adapted from 2000 Oldways Preservation & Exchange Trust. Available from http://www.oldwaysspt.org/med_pyramid.htm

What is so unique about the Greek Mediterranean diet? Simopoulos explained in the manuscript published in J Nutrition (2001), that the combination of food groups in the aforementioned dietary pattern provide a high intake of fibre, vitamins, antioxidants (such as β-carotene, tocopherols, polyphenols, flavonoids, carotenoids, glutathione, reservetrol), minerals (calcium, magnesium, potassium, magnesium, selenium), vitamins C, D, and E, optimal omega 6: omega 3 ratio (1-2:1) and is low in saturated fats [(199)].

2.1.1 The Mediterranean diet pyramid today

In November, 2010, the Mediterranean diet was recognized as a sustainable dietary pattern that has been ascribed to the list of Intangible Cultural Heritage of UNESCO and as a cultural monument of Greece, Italy, Spain and Morocco (Decision 5.COM 6.41) [289]. Nevertheless, today there is a trend towards abandonment of this healthy eating model by countries surrounding the Mediterranean basin attributed to the widespread dissemination of Western economy, urbanization along with globalization of food production and consumption [290]. Overwhelming evidence from observational studies indicate that Mediterranean diet adherence in children and adolescents is declining in Mediterranean countries with a parallel shift towards a Western dietary pattern [5]. Low Mediterranean diet adherence (4.2%) as evaluated by the KIDMED index was documented in children and adolescents in Spain [242], 46.8% for children in Greece [237], 33% in Italy [291] and 37% in Cypriot children [292]. Nonetheless, the Traditional Greek Mediterranean dietary pattern is still promoted by the Hellenic Ministry of Health and Welfare as a guide for healthy eating for children, adolescents and adults [293; 294]. It continues to be an important part of Greek culture as well as religious customs practised by the Greek people and is handed down as a legacy to the next generation [293; 294].

In order to address these issues of nutrition transition due to societal changes, in 2010, the Mediterranean Diet Foundation together with the Forum on Mediterranean Food Cultures designed a new pyramid that is based on the key principles of the Mediterranean diet as well as indicates the relative proportions, frequency of consumption and suggested portion servings of the main food groups that constitute this dietary pattern [290]. The pyramid emphasizes daily, weekly and occasional dietary guidelines in order to follow a healthy and balanced diet. As in the original pyramid, food that should sustain the diet is at the base of the pyramid and in the upper levels foods to be eaten in moderation. Adequate hydration, the importance of drinking water during the day is also emphasized.

Social and cultural elements characteristic of the Mediterranean way of life have been incorporated into the pyramid, such as regular physical activity and adequate rest.

2.2 Dietary patterns vs individual foods and nutrients

Previous research examining the effect of individual nutrients on respiratory function and asthma in children have documented mixed results and failed to explain the rise in childhood asthma [(295)]. Moreover, such studies did not take into account the interaction or synergistic effect between nutrients or food groups when consumed together rather than alone [(296)]. During a meal people consume a variety of foods and nutrients and a dietary pattern may represent a better picture of a person's true diet [(296)]. Under this overall concept of dietary assessment, a link between Mediterranean diet adherence and childhood asthma is revealed in the published manuscript authored by the candidate in Chapter 2.3 titled *Papamichael et al, 2017. Does adherence to the Mediterranean dietary pattern reduce asthma symptoms in children? A systematic review of observational studies. Public Health Nutr 20(15), 2722–2734. doi: 10.1017/S1368980017001823. Epub 2017 Aug 14.*

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2.3 Does adherence to the Mediterranean dietary pattern reduce asthma symptoms in children? A systematic review of observational studies

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Review Article

Does adherence to the Mediterranean dietary pattern reduce asthma symptoms in children? A systematic review of observational studies

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Abstract

Objective: The purpose of the present systematic review was to synthesize evidence from the literature to assess efficacy of the Mediterranean dietary pattern in childhood asthma.
Design/Setting: A systematic search of six databases, three clinical trial registries and hand-search of peer-reviewed articles was conducted up to 29 October 2016. Inclusion criteria included exposure to a Mediterranean dietary pattern, measurement of asthma symptoms and study population of children aged <18 years. Quality assessment was conducted. Due to significant heterogeneity, meta-analysis was not feasible.
Results: Of the 436 articles identified, after removal of duplicates and based on inclusion criteria, fifteen observational studies conducted in Mediterranean and non-Mediterranean countries were relevant. No randomized controlled trials were retrieved. Twelve studies reported an inverse association between adherence to a Mediterranean dietary pattern and asthma in children, two studies showed no association and one study showed an increase in asthma symptoms. In fourteen out of fifteen studies, quality assessment checks revealed good reliability and validity among study methodologies.
Conclusions: The current systematic review revealed a consistent inverse relationship (protective) between a Mediterranean dietary pattern and asthma in children. Future well-designed randomized controlled trials are needed to provide solid evidence. Nevertheless, the existing level of evidence adds to the public health message relating to the beneficial effects of a Mediterranean-type diet in children suffering with asthma.

Keywords
Mediterranean dietary pattern
Mediterranean diet
Asthma
Child
Nutrition

Asthma remains a global public health problem of epic proportions, especially in children and adolescents. Asthma is a complex respiratory disorder characterized by symptoms that include wheezing, chest tightness, coughing (especially at night) and breathlessness resulting from air flow obstruction triggered by the interaction of genetic and environmental factors [1,2].

Diet has been implicated as one of the environmental factors contributing to the pathogenesis of this disease[3,4]. In many Westernized countries today, dietary intakes are generally low in fruit, vegetables, wholegrain cereals and fish, and high in fast foods, sweets and salty snacks that are high in saturated fat, sugar and salt and low in fibre and antioxidants. Earlier studies suggested that the low prevalence of asthma symptoms in children from Mediterranean countries compared with English-speaking countries is possibly due to the different dietary patterns of these regions[5,6].

The traditional Mediterranean diet is a collection of eating habits traditionally followed by people in countries around the Mediterranean basin during the early 1960s[7,8]. The main characteristics of traditional Mediterranean diets are that all foods consumed are minimally processed, seasonally fresh and locally grown. Furthermore, this pattern is characterized by high intakes of fruits, vegetables, wholegrain cereals, legumes and nuts, low to moderate consumption of dairy products (mainly as cheese and yoghurt), fish, poultry and red wine (usually

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with meals), fewer than four eggs weekly, small amounts of red meat and liberal use of olive oil as the primary source of added fat [9] Consequently, this pattern is low in SFA, high in MUFA and n-3 fatty acids, and rich in fibre, vitamins D and E, Mg and antioxidants[8,9].

Most studies on diet and asthma have focused on individual nutrients or food groups and results have been inconsistent.A limitation of these studies is that they fail to take account of the interaction or synergistic effect between dietary components [10]. People consume a variety of foods and food groups in a meal, rather than individual nutrients or food groups, and for this reason it has been stated that dietary patterns represent a better picture of the true diet and nutrient intakes of a given population [11,12]. Hence, it may be more appropriate to better understand the effect of a dietary pattern such as the Mediterranean diet, rather than specific foods or nutrients, on childhood asthma.

Few studies have investigated the association of dietary patterns with childhood asthma and most published reviews are narrative and reflect an opinion rather than an objective analysis of the evidence. To date, only one systematic review and meta-analysis of eight observational studies up to 2013 has been published which examined the influence of the Mediterranean diet on childhood asthma [13]. Results of the meta-analysis showed that adherence to a Mediterranean diet during childhood might protect against 'asthma ever' and 'current wheeze'. Therefore, the purpose of the present systematic review was to conduct an up-to-date extensive databasesearch to collate and analyse the literature on the effectiveness of a Mediterranean dietary pattern in childhood asthma. The review will add further to the evidence base that links the Mediterranean diet with lower asthma symptoms in children.

Methods

Literature search/data collection

The current systematic review was prepared and reported according to PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analyses) guidelines [14]. Relevant studies were identified by systematic search from PUBMED, MEDLINE, EMBASE, Cochrane Library, EBSCO (Ovid) and SCOPUS databases for articles published up to 29 October 2016 and extended back to 1946. Furthermore, in an effort to capture all published studies from a variety ofinternational journals, no restrictions were applied on language, age or publication dates. Additional studies were sought from conference proceedings as well as clinical trial registries (international, European, Australia and New Zealand) in order to identify published/unpublished trials focusing on the Mediterranean dietary pattern and asthma in children. Other citations were identified by hand-searching the reference lists of potential articles. Finally, cross-checks were done to compare relevant articles in our review with previous reviews and systematic reviews.

Search terms were as follows: ['Mediterranean dietary pattern' OR 'Mediterranean diet' OR 'Mediterranean-type diet' OR 'Mediterranean food pattern' OR 'dietary pattern' AND 'asthma' AND 'children'] OR ['Mediterranean dietary pattern' OR 'Mediterranean diet' OR 'Mediterranean-type diet' OR 'Mediterranean food pattern' OR 'dietary pattern' AND 'childhood AND asthma']. The full search strategy is provided in Appendices 1 to 4.

Study eligibility criteria

Type of studies

Since no randomized controlled trials were identified the literature search included 'all study types', primarily observational studies (intervention, case–control, cohorts and cross-sectional) investigating Mediterranean diet as the primary exposure variable associated with asthma as the outcome in the child population.

Outcomes of interest

Primary outcomes were asthma symptoms, risk and prevalence.

Inclusion criteria

Inclusion criteria were based on PICO (population, intervention, comparator, outcomes) [15]. A study was included when the population under investigation was 'children', 'Mediterranean diet' was the intervention and 'asthma symptoms' was the outcome. A publication was considered for the systematic review if it focused on human subjects, was published in English or any other language with an English translation available, and the dietary intervention was described in sufficient detail, highlighting the main components of the dietary pattern and being evaluated by a questionnaire or diet history. Regarding assessment of adherence to the Mediterranean dietary pattern, only studies using a score and results presented as odds ratio or relative risk and corresponding 95% confidence interval were considered. Composite outcomes (e.g. allergy) were eligible when diet was part of a multifaceted intervention and results on the Mediterranean diet and asthma in children could be separated.

Exclusion criteria

Exclusion criteria were based on PICO characteristics and reasons for study exclusion were summarized.Types of studies not included were: reviews, systematic reviews, editorials, comments, letters, dissertations, newspapers, case studies and animal studies, as well as those with no abstract, full text or English translation available. Studies conducted in adults, infants and pregnant women were not the population under investigation. In addition, interventions or risk factors other than Mediterranean diet such as supplementation, specific nutrients, intake of single food groups, other dietary patterns (e.g. Western), probiotics, medication, pollution, urban environment, obesity and

BMI, as well as studies focusing on maternal nutrition or the role of genetics in asthma,were excluded. Also deemed inappropriate were studies investigating the role of diet in relation to other outcomes,for example dermatitis, eczema, CVD, rheumatoid arthritis,obstructive lung disease, metabolic syndrome, cognitive function, or growth and development.

Selection of studies

The titles and abstracts of all articles identified were reviewed by two authors (M.M.P., C.I.) to assess eligibility and duplicates removed. Papers were screened for relevance based on the information contained in titles and abstracts. Potentially eligible abstracts were selected for full-text reading provided that all inclusion criteria were satisfied. Also, when there was insufficient information in the abstract to warrant exclusion of the article, the full text was retrieved. Finally, full-text articles satisfying inclusion criteria were reviewed by both reviewers and details were extracted. Discrepancies were resolved by discussion and consensus that led to agreement.

Quality assessment

Two authors (M.M.P., N.H.S.) assessed the quality of each study independently using a validated quality assessment tool modified from Zaza et al.[16]. The quality assessment tool included a checklist of: description of the study design; description of the study population and how it was selected; how the exposure (Mediterranean diet score) and outcome (asthma in children) were measured; whether the measurements were valid and reliable; the appropriateness of the statistical analysis to answer the research question; and finally how each study controlled for potential bias (i.e. recall bias, measurement bias) or any potential bias that may have been missed. Results were evaluated as the percentage of how many points each study scored out of the maximum number of points it could have achieved, as not every study had the same maximum score due to factors such as different study design or statistical analysis. Finally, scores from both authors were averaged and became the final score. We used a score of 70 % or above to define high study quality and a score below 70 % as low study quality. Any disagreements that arose between the authors were resolved through discussion or with a third author (B.E.).

Data extraction

The data extracted from relevant studies included specific details about the author, year of publication, study design, study name, geographic area, sample size, age of target population, Mediterranean dietary assessment tool, outcome measure, exposure estimate, confounders and main findings. Where available, data analysis results were reported as odds ratios and 95% confidence intervals, with P- value at 5% significance level.

Results

Electronic search

The database searches identified a total of 435 potential articles, of which 318 original articles remained after removal of duplicates (Fig. 1). One citation was found by cross-checking of reference lists. No trials (published or unpublished) were identified from international clinical trial registries. Of the remaining eligible articles, 319 were screened by two authors (M.M.P., C.I.) independently scanning titles and abstracts. A total of 270 articles were excluded as they did not examine the specific exposure (Mediterranean diet) in relation to the outcome (asthma symptoms) in the study population (children) or satisfy the publication type (original article) (not outcome, n 117; not intervention, n 20; not study population, n 9; no abstract, n 16; reviews, n 107; no English translation, n 1), leaving forty-nine full-text articles to be read for relevance. Of the remaining forty-nine potential studies, thirty-four full texts were considered inappropriate based on the exclusion criteria (not study population, n 6; not intervention, n 12; not study type, n 11; no abstract or full text available, n 5), leaving a total of fifteen studies relevant to the topic. Specifically, eleven cross-sectional, one intervention (with no control group), one case– control and two cohort studies investigating the association between children's adherence to a Mediterranean dietary pattern and asthma[17–31].

Study characteristics

The database search revealed fifteen original studies investigating children's adherence to the Mediterranean dietary pattern and asthma (Tables 1 and 2). Ten of these studies were conducted in Mediterranean regions including Greece (n 4) [18,19,22,27], Spain (n 5) [20,21,23,25,26] and Turkey (n 1) [17]; four were conducted in non-Mediterranean countries, namely Mexico (n 2) [24,30], Peru (n 1) [29] and Brazil (n 1) [31]; and the ISAAC (International Study on Allergies and Asthma in Childhood)[28] involved twenty countries globally. Collectively, 103 248 children and adolescents aged 1 to 19 years participated, with sample sizes ranging from 104 to 50 004. In all these studies children's dietary intake was evaluated using an FFQ that was either self-administered or completed by parents. Regarding assessment of asthma symptoms, thirteen studies [17–28,31] used the ISAAC respiratory questionnaire [33] and two studies used spirometry [29,30]. A variety of outcomes were measured which included: prevalence of asthma, wheeze, exercise wheeze, any asthma symptoms, night-time cough, sleep disturbance due to wheeze, persistent asthma, atopic asthma, current severe asthma, spirometry (forced expiratory volume 1, forced vital capacity), bronchial inflammation, medication use, number of episodes/attacks, hospital admissions, severity of attacks, asthma control, asthma severity and bronchial hyper-responsiveness. All studies, except for one [20], adjusted for confounding factors although the

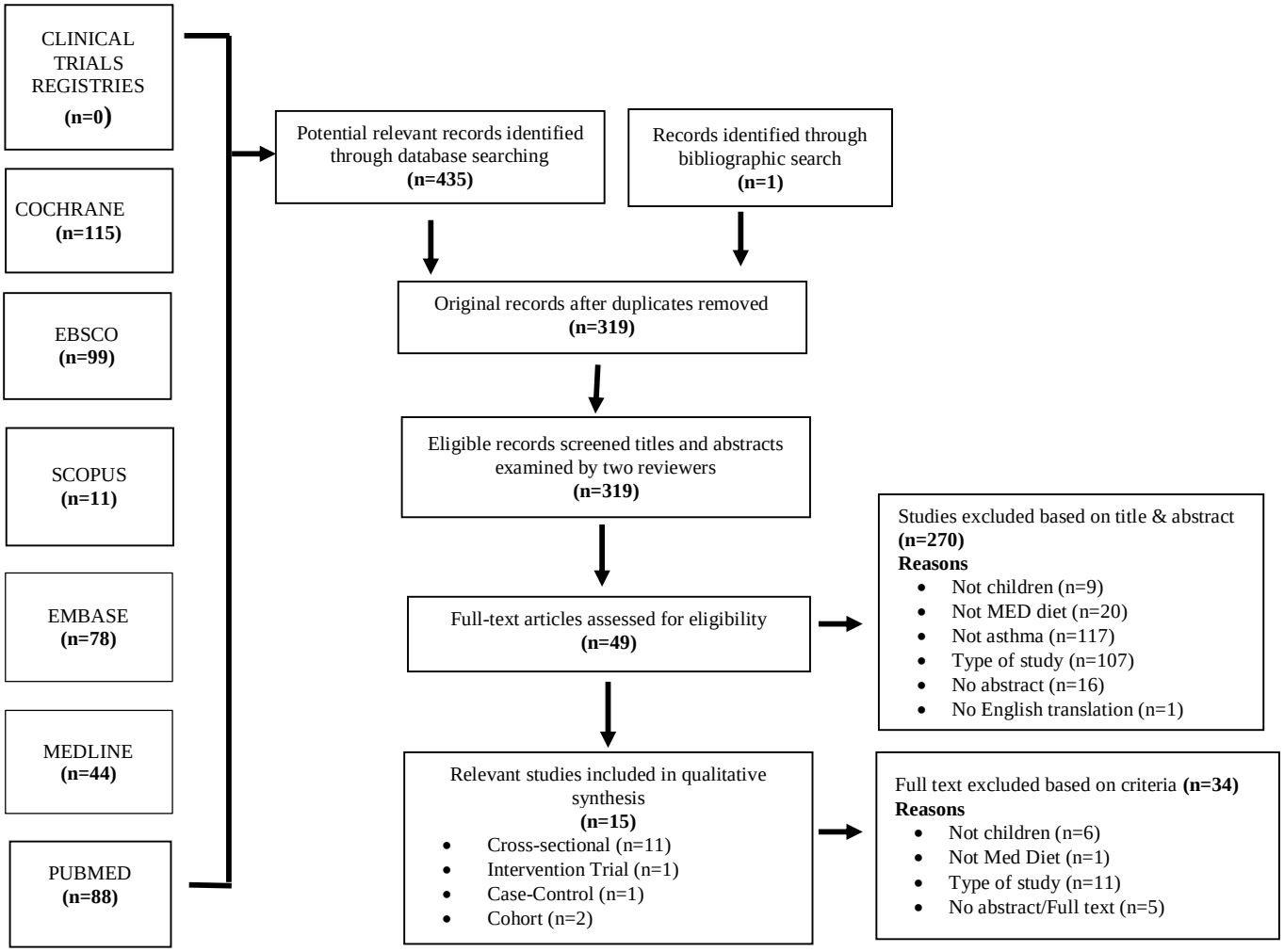

Fig.1 Flowchart detailing the study search for the present systematic review on the Mediterranean dietary pattern (Med diet) and childhood

confounding variables differed between studies. Children's adherence to the Mediterranean dietary pattern was assessed using three indices. Three studies[22,24,30] used the original Mediterranean diet score developed by Trichopoulou et al.[34], seven studies[17,21,25,26,28,29,31] used the scoring system by Psaltopoulou et al.[35] and six studies[18,19,20,22,23,27] used the KIDMED index developed by Serra-Majem et al.[36]. In the studies that used the scoring system based on Psaltopoulou, dietary intake was further categorized into two eating patterns: (i) a 'Pro-Mediterranean' pattern which correlated with intakes of fruit, vegetables, fish, cereals, pasta, rice and potatoes; and ii) an 'Anti-Mediterranean' pattern as intakes of milk, meat, eggs, fast foods, soft drinks and butter[17,21,25,26,28,29,31].

In six of these studies [17,21,25,26,28,29] a score was allocated according to frequency of intake, whereas in the study undertaken by Silveira et al., adherence to the Mediterranean dietary pattern was measured qualitatively. Intake of at least five foods in each dietary pattern at a frequency of at least 3 times/week was classified as 'yes'[31].

Summarizing the findings of the literature search, the majority of studies reported a beneficial effect of adherence to a Mediterranean dietary pattern on asthma in children. Overall, twelve of these studies reported an inverse association between children's adherence to the Mediterranean diet and asthma symptoms [18–25,27–30] and/or improvement in lung function[20,30], although the results of two studies were not statistically significant[22,23]. On the other hand, two studies docu-mented no association between Mediterranean diet and asthma symptoms[17,31] and one an increase in asthma symptoms[26].

Quality assessment

After assessment of the quality of each study included the averaged quality score was 79 %, the highest score was

Table 1 Characteristics of cross-sectional studies included in the Systematic Review

Mediterranean diet during childhood							
Author/Year/ Name of study/ Location/ Study design	Sample size	Age (years)	Exposure	Tool used to evaluate adherence to Mediterranean Diet	Asthma outcome measured	Exposure estimate	Confounders	Findings
Antonogeorgios et al [18], 2014, PANACEA study, Greece Cross-sectional	1125	10-12	Med diet	KIDMED INDEX [2] Score 0-16 <8 improvement needed ≥ 8 optimal	• Current asthma • Ever-had asthma	Path Analysis Standardized beta= - 0.224, p=0.02	Age, gender, parental, atopy	High adherence to Med diet inversely related to asthma symptoms
Arvaniti et al.[19], 2011, PANACEA study, Greece Cross-sectional	700	10-12	Med diet	KIDMED INDEX [2] 0-3 poor 4-7 average 8-12 good	• Overall lifetime prevalence asthma, • Ever had wheeze • Exercise wheeze • Night cough • Ever had diagnosed wheeze • Any asthma symptoms	• Prevalence asthma symptoms (OR:0.86; 95%CI: 0.75-0.98)	Age, sex, BMI, physical activity, energy intake	High adherence to Med diet inversely associated with "ever had wheeze" (high v low adherence) (8 % v 29 %) (p=0.001); "exercise wheeze" (0% v 11%) (p=0.004); "ever-had diagnosed wheeze" (3% v`6%) (p=0.002); and "any asthma" (5% v 34%) (p<0.001). Med diet consumers had 89% lower likelihood of having asthma symptoms and 48% of having diagnosed asthma compared to non-consumers. 1 unit increase in score associated with 14% lower likelihood of having asthma symptoms (OR: 0.86; 95%CI: 0.75-0.98)
Grigoropoulou et al [27] 2011 PANACEA study, Greece Cross-sectional	1125	10-12	Med diet	KIDMED INDEX [2] ≤3 very low 4-7 improvement needed ≥ 8 optimal	• Any asthma symptoms ever • Night cough • Exercise wheeze • Wheeze limiting speech • Wheeze disturb sleep	• Ever had wheeze (OR:0.88;95%CI:0.78-0.98) • Exercise wheeze (OR: 0.79;95% CI:0.6-0.93) • Lower likelihood asthma in urban (OR:0.81; 95% CI:0.73-0.91) and rural areas (OR: 0.87, 95% CI: 0.75-1)	Urban /rural, sex, BMI, energy intake, physical activity	High adherence to Med diet was inversely associated with 'ever had wheeze' and 'exercise wheeze'. 1 unit increase in KIDMED score, 16% lower likelihood of asthma symptoms in urban and rural areas.
Nagel et al [28], 2010, In 20 countries Cross-sectional	50,004	8-12	Med diet	Med diet score based on food frequency consumption in FFQ	• Current wheeze • Asthma ever • BHR	• Current wheeze (OR:0.97; 95% CI:0.94-0.99) • Asthma ever (OR:0.95; 95% CI:0.92-0.99)	Gender, age, tobacco smoke, no. of siblings, exercise, atopy, maternal education, parental atopic & non-atopic wheeze	High adherence to Med diet lower prevalence of "current wheezing" and "asthma ever" (p-trend=0.03)
Castro-Rodriguez et al, 2008; Spain [21] Cross-sectional	1784	3-4	Med diet	Med Diet Score[4] (Range 14-36) Pro-Med/Anti-Med dietary pattern	• Current wheezing	• Current wheeze (OR: 0.54; 95%CI 0.33-0.88) p=0.014	Age, birth-weight, livestock during pregnancy, delivery by caesarean, antibiotics during first year life, acetaminophen use during past 12 months, rhino-conjunctivitis, dermatitis, paternal/ maternal asthma, maternal age, maternal educational level, current parent/ maternal smoking, vigorous physical activity, cats in the last 12 months	High adherence to Med diet lower risk of 'current wheezing'
de Batlle et al [24], 2008, Mexico Cross-sectional	1476	6-7	Med diet	Med Diet Score [1] (Range 0-8) Mean score 3.7 Ist tertile: 0-3 2nd tertile: 4 3rd tertile: 5-8	• Asthma ever • Wheezing ever • Current wheezing	• Asthma ever (OR:0.6;95% CI:0.40-0.91) p-trend = 0.034 • Wheezing ever (OR: 0.64; 95% CI:0.47-0.87) p- trend = 0.001	Gender, birth weight, BMI, number of siblings (older/younger), breast-feeding, parental educational level, children's exercise, smoking exposure during pregnancy, parental asthma and atopy, pets during pregnancy, pet at home during first year of life, living on a farm during pregnancy, premature birth, respiratory infections during early life, body changes since 4 years old., type of fuel for cooking, sleeping alone, mould/dampness at home.	High adherence to Med diet inversely associated with asthma and wheezing.

Author/Year/ Name of study/ Location/ Study design	Sample size	Age (years)	Exposure	Tool used to evaluate adherence to Mediterranean Diet	Asthma outcome measured	Exposure estimate	Confounders	Findings
Garcia-Marcos et al, 2007, Spain [25] Cross-sectional	20 106	6-7	Med diet	Med Diet Score [3] (median score 13) (Range 4-20) Pro-Med/Anti-Med pattern	• Current severe asthma (CSA) • Current occasional asthma (COA)	Girls: • CSA:OR:0.9 (95% CI:0.82-0.98) Boys: • CSA:OR:0.98 (95%CI:0.91-1.06)	Obesity, maternal smoking, number of siblings, exercise	High adherence to Med diet decreased 'CSA' in girls. 1 unit increase in score 10% protective effect. No statistically significant result found for 'COA'.
Chatzi et al, 2007 Crete, Greece [22] Cross-sectional	690	7-18	Med diet	KIDMED INDEX [2] ≤3 very low 4-7 improvement needed ≥ 8 optimal Med Diet Score[1] (Range 0-8)	• Wheezing ever • Current wheezing • Wheezing ever with atopy • Nocturnal dry cough	• Wheezing ever OR:0.67(95% CI:0.34-1.32), p=0.229 • Current wheezing OR:0.64(95%CI:0.2-2.05); p=0.564 • Nocturnal dry cough OR:0.49 (95%CI:0.23-0.96) p=0.095 • Wheezing ever with atopy OR:0.53 (95%CI:0.16-1.80) P=0.417	Age, gender, BMI, parental asthma, number of older siblings	High adherence to Med diet beneficial effect on asthma. BUT not statistically significant
Akcay et al 2014, Turkey[17] Cross-sectional	9991	13-14	Med diet	Med Diet Score[3] Median (Range 0-22) score 12.5 Pro-Med/Anti-Med dietary pattern	• Wheeze ever, wheeze in last 12 months • Doctor-diagnosed asthma • Severe attacks of wheeze • Exercise wheeze • Night cough • Sleep disturbed by wheeze	-	Gender, family atopy, television viewing, paracetamol use, number of siblings at home, born in Istanbul, time lived in Istanbul, education level of mother/father, parent's/guardian, smoking, tonsillectomy, adenoidectomy, pets at home (cat, dog, fish, bird).	No significant difference between Med Diet scores and asthma in asthmatic and non-asthmatic children p=0.85.
Silviera et al, 2015, Brazil [31] Cross-sectional	394 (268 persistent-asthma, 126 control)	3-12	Med diet	No score Qualitative (yes/no): Pro-Med/Contra-Med dietary pattern [5]	• Persistent asthma: (mild, moderate, severe) • Intermittent asthma	• Pro-Med dietary pattern OR: 1.20 (95% CI 0.78-1.86) p=0.40 • Contra-Med dietary pattern • OR: 0.82 (95% CI 0.53-1.27) p=0.38	Maternal smoking during pregnancy, preterm-birth, gender, skin colour, maternal schooling, income, paternal schooling and smoking during pregnancy, allergens in home, gestational age, birth-weight, family history of allergic rhinitis, exposure to passive smoking, dietary variable, obesity	No association was found between 'Pro-Med' dietary pattern and asthma severity in both groups (intermittent/persistent) of children 3-12 years
Gonzales-Barcala et al [26] 2010; Spain Cross-Sectional	14,700	6-7 & 13-14	Med diet	Med Diet Score [3] Mean score value: 13.1 in 6-7 years 12 in 13-14 years Pro-Med/Anti-Med dietary pattern	• Asthma ever • Current asthma • Severe asthma • Exercise-induced asthma	• Severe asthma OR:2.26 (95%CI 1.21-4.22)	Parental smoking, BMI, maternal education	Adherence to Med diet higher risk of 'severe asthma' in 6-7 year old girls. No significant association for other asthma categories in girls or boys in both age groups

Med diet, Mediterranean diet; Pro-Med, Pro-Mediterranean dietary pattern; Anti-Med, Anti-Mediterranean dietary pattern; Contra-Med, Contra-Mediterranean dietary pattern; BMI, Body Mass Index.

[1] Mediterranean diet score based on the score developed by Trichopoulou et al [34]

[2] KIDMED index, a Mediterranean diet quality index evaluating food habits in Spanish children developed by Serra_Majem et al [36]

[3] Mediterranean diet score developed by Garcia-Marcos et al [25] and based on the scoring system of Psaltopoulou et al [35])

[4] Mediterranean diet score developed by Castro-Rodriguez [21] also based on the scoring system of Psaltopoulou [35]

[5] Mediterranean dietary pattern developed by Gonzalez-Barcala et al [26]

Table 2. Characteristics of Cohort and Case-control studies in the Systematic Review

Study Author/Year/Location/ Study design	Sample size	Duration of study	Age (years)	Exposure	Tool used to evaluate adherence to Mediterranean Diet	Asthma outcome measured	Exposure estimate	Confounders	Findings
Calatayud-Saez et al [20] ,2016 Spain Prospective Intervention (no control group)	104 asthmatic children	Follow-up at 1 year	1-5	Med diet	KIDMED INDEX[2]	• Wheezing • Cough • BHR • Episodes • Medication use • Antibiotics • Infections • Hospital/emergency admissions • Intensity of attacks	**Corticosteroid therapy:** Pre (1 yr before): (3.92± 1.61) Post (1 yr after): (1.11 ± 1.09) **Bronchodilator therapy:** Pre: (4.14 ± 1.61) Post:(1.11± 1.11) **Symptomatic treatment**: Pre: (7.5 ± 2.88) Post: (3.4 ± 1.24)	-	Adherence to Med diet resulted in improvement in lung function (BHR) (p<0.001), decrease in episodes, bronchodilators and corticosteroids use (p=0.001). Also decrease in hospital admissions, infections, and respiratory infections in asthmatic children. 32.2% of patients remained "free of crisis, 35.3% had one attack during the year, 24.9% 2 episodes compared to 4.73 episodes (average) during the past year.
Chatzi et al [23], 2008 Spain Cohort	460	Follow-up at 6 years	6.5	Med diet	KIDMED INDEX [2] ≤3 low quality 4-6 medium ≥8 optimal	• Persistent wheeze • Atopic wheeze • Atopy	• Persistent wheeze: OR: 0.46 (95% CI: 0.1-2.17) P= 0.529 • Atopic wheeze: OR: 0.64 (95% CI: 0.1-4.06) P=0.902 • Atopy: OR:0.49 (95% CI:0.18-1.32) p=0.213	Gender, paternal asthma; maternal age at pregnancy, maternal asthma atopy, social class, education, smoking and supplement use during pregnancy. Breast-feeding, birth weight and order, gestational age, number of siblings, lower track respiratory infection at age 1., BMI at 6.5 years.	High adherence to Med diet inversely related to wheeze. BUT not statistically significant p>0.05
Romieu et al [30] ,2009 Mexico Cohort	208 (158 asthmatics, 50 control)	22 weeks	6-14	Med diet	Med Diet Score [1] (Range 1-8)	• IL-8, FEV_1, FVC • eNO • EBC • Asthma (mild, moderate, persistent)	-	Air pollution, gender, BMI, climate, age, temperature, corticoid use, (SES- maternal education, school type), outdoor activities, atopic status, exposure to tobacco smoke, use of allergy medicine, season, vitamin supplementation	Med diet improved lung function and decreased inflammation in asthmatic children. Med diet score positively related to lung function (p trend<0.05). High adherence to Mediterranean diet increased FEV_1 by 15.3% & FVC by 16.3% in asthmatic children (p<0.12) compared to non-asthmatics (p- trend<0.06). No statistical significant association observed for non-asthmatics p>0.05.
Rice et al [29], 2015 Peru Case-Control	383 (287 asthmatic, 96 control)	-	9-19	Med diet	Med Diet Score[4] (range 0-22) Median=15 Pro-Mediterranean Anti-Mediterranean	• Current asthma • Asthma control • FEV_1	• Asthma OR adj:0.55 (95% CI: 0.33-0.92), p=0.02 • Asthma in children with educated mothers OR adj: 0.31 (95% CI 0.14- 0.71), p<0.01	Age, sex, BMI, maternal education	Med diet was inversely associated with asthma especially in children with educated mothers, but did not improve asthma severity.Med diet scores were not associated with asthma control (p=0.3) or FEV_1 (p=0.24) in children

Med diet, Mediterranean diet; BMI, Body Mass Index; IL-8, inflammatory marker; FEV_1, Forced Expiratory Volume in 1 sec, FVC, Forced Vital capacity; BHR, Bronchial hyperresponsiveness; eNO, Exhaled Nitric Oxide level; EBC, Exhaled Breathe Condensate; AOR, Adjusted Odds Ratio

[1]Mediterranean diet score based on the score developed by Trichopoulou et al [34].

[2]KIDMED index, a Mediterranean diet quality index evaluating food habits in Spanish children developed by Serra-Majem et al [36]

[4] Mediterranean diet score developed by Castro-Rodriguez et al [21] also based on the scoring system of Psaltopoulou [35]

88 % and lowest score was 64 % (Table 3). Almost all studies (14/15) were of high quality [17–19,20–31] as they described the study population, measured the exposure and outcome clearly, used appropriate statistical analysis and considered bias in the analysis or discussed any potential bias. The only low-quality study (Calatayud-Saez et al.) suffered from inappropriate statistical analysis and lack of consideration of potential bias such as non-response and dropout bias that led to unreliable results and ambiguity in study population characteristics which made it difficult to compare the results against other studies [20]. Due to heterogeneity among study methodologies, namely differences between age, exposure measurements and outcome measures, meta-analysis of the fifteen observational studies was deemed inappropriate.

Discussion

The current systematic review identified recent evidence suggesting that adherence to a Mediterranean dietary pattern is inversely related to, and provides a potentially protective effect against, asthma symptoms in children residing in Mediterranean and non-Mediterranean regions[18–25,27–30]. The majority of studies documented a reduction in wheeze[19,21–24,27,28], nocturnal cough[22], exercise wheeze[19,27]) asthma episodes[20], hospital admissions[20], medication use[20] and improvement in lung function[20,30]. A one-unit increase in the KIDMED score was associated with a 14–16 % lower likelihood of having asthma symptoms [19,27] irrespective of potential confounders.

Our qualitative synthesis showed that for ten out of the twelve observational studies documenting a protective effect, statistically significant findings were observed for high adherence to the Mediterranean dietary pattern and asthma symptoms even though heterogeneity existed among study methodologies, namely Mediterranean diet scores, cut-off points, FFQ and asthma outcomes[18,20,21,24,25,27–30,32]. These findings coincide with two earlier systematic reviews [13,37] and one meta-analysis[13] investigating the role of the Mediterranean diet in relation to childhood asthma that was performed on seven observational studies (cross-sectional)[19,21–25,28] included in our manuscript. Both systematic reviews documented that despite the heterogeneity and inherent limitations of cross-sectional studies included, the evidence is suggestive of a protective effect between the Mediterranean diet and asthma in children, particularly in Mediterranean areas[13].

A possible explanation for the beneficial/prophylactic effects observed is that the Mediterranean diet is characterized by low consumption of red meat and saturated fats, high intakes of fruit, vegetables, wholegrain cereals, legumes and fish, and abundance of olive oil, which are rich in antioxidants (vitamins A, C and E, β-carotene, polyphenols, glutathione, lycopene, flavonoids), micronutrients (Mg, Se, Zn) and vitamin D[9,12]. These bioactive compounds may prevent or limit inflammatory responses in the airways by reducing reactive oxygen species and inhibiting lipid peroxidation, thus reducing asthma symptoms. In addition, the high content of long-chain n-3 PUFA found in fish triggers the production of EPA-derived eicosanoids which have anti-inflammatory effects and influence the differentiation of T-lymphocytes modulating immune responses, thereby also improving pulmonary function and decreasing asthma symptoms [38].

In contrast, two studies reported no association [17,31] and one an adverse effect [26] of adherence to a Medi-terranean dietary pattern in asthmatic children. In the study undertaken by Gonzales Barcala et al.[26], adherence to the Mediterranean diet was associated with an increase in asthma symptoms in children with ‘severe asthma’. This outcome may have been due to a reverse-causal effect. It is well established that the family environment, particu-larly parents, plays a major role in the dietary habits of young children [39,40]. More specifically, in those families with children suffering from severe asthma, parents may improve the quality of the child’s diet in an effort to improve overall health. No association was reported by Silveira et al.[31] in the study examining the impact of adherence to a Mediterranean diet in children with persistent and intermittent asthma. A possible explanation may be the coexistence of other environmental factors not analysed in their study that could modify the prevalence or severity of asthma. Possible limitations of that study are the small sample size and no tool such as the Asthma Control Questionnaire was used to evaluate asthma severity. Also, mild, moderate and severe persistent asthma were categorized collectively as ‘persistent asthma’ rather than being assessed separately, which may have led to some effects being overlooked. Medication remains the cornerstone of asthma management. Perhaps medication use might have masked any beneficial effects of the Mediterranean diet. Regarding the study undertaken by Akcay et al .[17] in adolescents, no association was reported between adherence to the Mediterranean diet and asthma symptoms. Adolescence is a period when youth defy authority, especially parental advice, gain autonomy and are influenced by peer-group pressure. Adolescents are known to practise poor dietary habits including high intakes of fast foods, sweets and soft drinks, which are rich sources of saturated fats, sugar and salt, and have low intakes of nutritious foods such as fruit, vegetables, cereals and fish that are high in fibre, antioxidants and n-3 fatty acids[41,42]. These poor dietary habits may have concealed the beneficial effects of adherence to a Mediterranean dietary pattern, hence explaining why no association was documented. In addition, Akcay et al. mentioned that adolescents had a high consumption of pickled and salted foods. Research has shown that a high intake of salt is associated with asthma symptoms [32,43]. In addition, adole scents have been known to misreport dietary intake [44,45].

Table 3. Summary table of quality assessment* of relevant studies included in the present systematic review on the Mediterranean dietary pattern and childhood asthma

Study	Reviewers	Study design	Population density	Population Description	Time points	Outcome measures	Eligibility criteria	Exposure measures		Outcome measures		Statistical test suitable	Adjustment for confounders	Bias discussed	Bias not covered	Overall average score (%)
								Validity	Reliability	Validity	Reliability					
Calatayud-Saez et al [20], 2015	NHS/MMP	✓	✓	☒	✓	✓	✓	✓	✓	✓	✓	✓	☒	✓	☒	64
Antonogeorgios et al [18], 2014	NHS/MMP	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	75
Arvaniti et al [19], 2011	NHS/MMP	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	79
Grigoropoulou et al [27], 2011	NHS/MMP	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	82
Nagel et al [28], 2010	NHS/MMP	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	86
Garcia-Marcos et al [25], 2007	NHS/MMP	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	74
Castro-Rodriguez et al [21], 2008	NHS/MMP	✓	✓	✓	☒	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	79
Chatzi et al [23], 2008	NHS/MMP	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	88
Chatzi et al [22], 2007	NHS/MMP	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	79
de Batlle et al [24], 2008	NHS/MMP	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	75
Romieu et al [30], 2009	NHS/MMP	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	88
Gonzales-Barcala et al [26], 2010	NHS/MMP	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	77
Rice et al [29], 2015	NHS/MMP	✓	✓	✓	☒	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	82
Ackay et al [17], 2014	NHS/MMP	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	75
Silviera et al [31],2015	NHS/MMP	✓	☒	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	81

✓: assessment criteria satisfied ; ☒: assessment criteria not satisfied

*Using validated quality assessment tools modified from Zaza et al[16]

Strengths/limitations of studies reviewed

Limitations must be considered in the present systematic review, some of which may be due to gaps in the literature. There was heterogeneity in study design, age groups considered, time periods of exposure, sample population (asthmatics v. non-asthmatics), outcome measures, outcome parameters and associations studied, thus limiting the possibility of drawing strong and consistent conclusions. The majority of studies reviewed were cross-sectional and hence a cause–effect relationship cannot be determined; however, hypotheses might be suggested for further exploration in more robust clinical trials. Diversity in results documented may be due to differences in design of FFQ used to collect information on the dietary habits of children; for example, number of food items included and frequency of food consumption categories. Some FFQ were not validated [17,22,29], however research has shown that FFQ produce valid and reproducible estimates of dietary intake of children and adolescents [46]. Another weakness in these studies is that dietary questionnaires were self-administered or completed by parents, which may have led to recall or information bias. On the other hand, research often uses parents as proxy reporters for children's dietary intake [47] and parental report of children's fruit and vegetable intake is an accurate estimate [48]. Regarding assessment of asthma outcome, respiratory function was evaluated using a questionnaire and by parent report of symptoms. This method is inferior to the use of pulmonary function tests such as spirometry [49]. In studies undertaken in non- English speaking countries, the ISAAC questionnaire had been translated but not validated for that specific population [17,18,22]. Cultural differences cannot be excluded in the assessment of asthma symptoms and report of wheezing, as was delineated by the differences in asthma outcome in the fifteen studies analysed. It is well recognized that, in children younger than 3 years, wheezing is often transient due to respiratory infection and diminishes as the child grows older[49,50]. Regarding differences in Mediterranean dietary scores used to assess adherence, not all scores have been developed for use in children and adolescents [34,35].

A strength is the extensive literature search focusing exclusively on the Mediterranean dietary pattern as opposed to food groups or nutrients, although publication bias cannot be dismissed. Nevertheless, the studies reviewed were of high quality. In comparison to studies investigating the effect of nutrients or single food groups, the use of a Mediterranean diet score takes account of the synergistic effect or interactions between foods and nutrients [12], as dietary scores are designed to reflect the whole dietary pattern. In addition, the use of scores improves the statistical power as compared with single nutrients or food groups that might account for small effects. The present systematic review highlights the need for future clinical trials investigating the effect of a Mediterranean dietary pattern on asthma symptoms in order to verify the promising findings documented in the literature. To enhance validity and reliability of the results published, there is a need for homogeneity between study methodologies and statistical tests in evaluation of the data compiled.

Conclusion

In conclusion, the present systematic review assessed evidence for the effectiveness of a Mediterranean dietary pattern in childhood asthma. Although the available evidence is limited, it is nevertheless supportive that adherence to a Mediterranean dietary pattern may reduce asthma symptoms in children. However, well-designed randomized controlled trials are warranted to confirm the prophylactic effects of this dietary pattern. The present findings have important public health implications because they suggest a non-pharmacological means for preventing childhood asthma.

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Appendix 1

Websites accessed
PUBMED:
http://www.ncbi.nlm.nih.gov/ (accessed 29 October 2016).
MEDLINE:
http://www.ovidsp.tx.ovid.com (accessed 29 October 2016).
EMBASE:
http://www.ovidsp.tx.ovid.com (accessed 29 October 2016).
Cochrane Central Registry of Controlled trials:
http://onlinelibrary.wiley.com (accessed 29 October 2016).
EBSCO (CINAHL):
http://www.web.b.ebxco.host.com (accessed 29 October 2016). SCOPUS: https://www-scopus-com (accessed 29 October 2016).
International clinical trials registry:
http://www.clinicaltrials.gov (accessed 29 October 2016).
Australian & New Zealand Clinical Trials Registry (ANZCTR):
http://www.anzctr.org.au (accessed 29 October 2016).
European Clinical Trials Registry:
https://www.clinicaltrialsregister.eu (accessed 29 October 2016).

Appendix 2

Details of PUBMED database search: Medical Subject Headings (MESH) terms

(Mediterranean [All Fields] AND (‘diet’ [MeSH Terms] OR ‘diet’ [All Fields] OR ‘dietary’ [All Fields]) AND pattern [AllFields]) OR (‘diet, mediterranean’ [MeSH Terms] OR (‘diet’[All Fields] AND ‘mediterranean’ [All Fields]) OR ‘mediterraneandiet’ [All Fields] OR (‘mediterranean’ [All Fields]AND ‘diet’ [All Fields])) OR (Mediterranean-type [All Fields]AND (‘diet’ [MeSH Terms] OR ‘diet’ [All Fields])) OR(Mediterranean [All Fields] AND (‘food’ [MeSH Terms] OR‘food’ [All Fields]) AND pattern [All Fields]) OR ((‘diet’[MeSH Terms] OR ‘diet ’[All Fields] OR ‘dietary’ [All Fields]) AND pattern [All Fields])AND(‘asthma’[MeSH Terms] OR‘‘child’[All Fields] OR ‘children’ [All Fields])) OR ((((Mediterranean[All Fields] AND (‘diet’ [MeSH Terms] OR ‘diet’ [All Fields]OR ‘dietary’ [All Fields]) AND pattern [All Fields]) OR (‘diet,Mediterranean’ [MeSH Terms] OR (‘diet’ [All Fields] AND ‘mediterranean’ [All Fields]) OR ‘mediterranean diet’ [All Fields] OR (‘mediterranean’ [All Fields] AND ‘diet’ [All Fields])) OR (Mediterranean-type [All Fields] AND (‘diet’ [MeSH Terms] OR ‘diet’ [All Fields])) OR (Mediterranean [All Fields] AND (‘food’ [MeSH Terms] OR ‘food’ [All Fields]) AND pattern [All Fields]) OR ((‘diet’ [MeSH Terms] OR ‘diet’ [All Fields] OR ‘dietary’ [All Fields]) AND pattern [All Fields])) AND (‘Childhood’ [Journal] OR ‘childhood’ [All Fields])) AND (‘asthma’ [MeSH Terms] OR ‘asthma’ [All Fields])) Sort by: Relevance

Appendix 3

PUBMED database search details

Search no.	PUBMED search details	No. of articles retrieved
3	Search ((Mediterranean dietary pattern OR Mediterranean diet OR Mediterranean-type diet OR Mediterranean food pattern OR dietary pattern AND asthma AND children)) OR (Mediterranean dietary pattern OR Mediterranean diet OR Mediterranean-type diet OR Mediterranean food pattern OR dietary pattern AND childhood AND asthma)	88
2	Search (((Mediterranean dietary patterr OR Mediterranean diet OR Mediterranean-type diet OR Mediterranean food pattern OR dietary pattern)) AND childhood) AND asthma	49
1	Search (Mediterranean dietary pattern OR Mediterranean diet OR Mediterranean-type diet OR Mediterranean food pattern OR dietary pattern AND asthma AND children)	83

Articles retrieved from https://www.ncbi.nlm.nih.gov/pubmed (accessed 29 October 2016)

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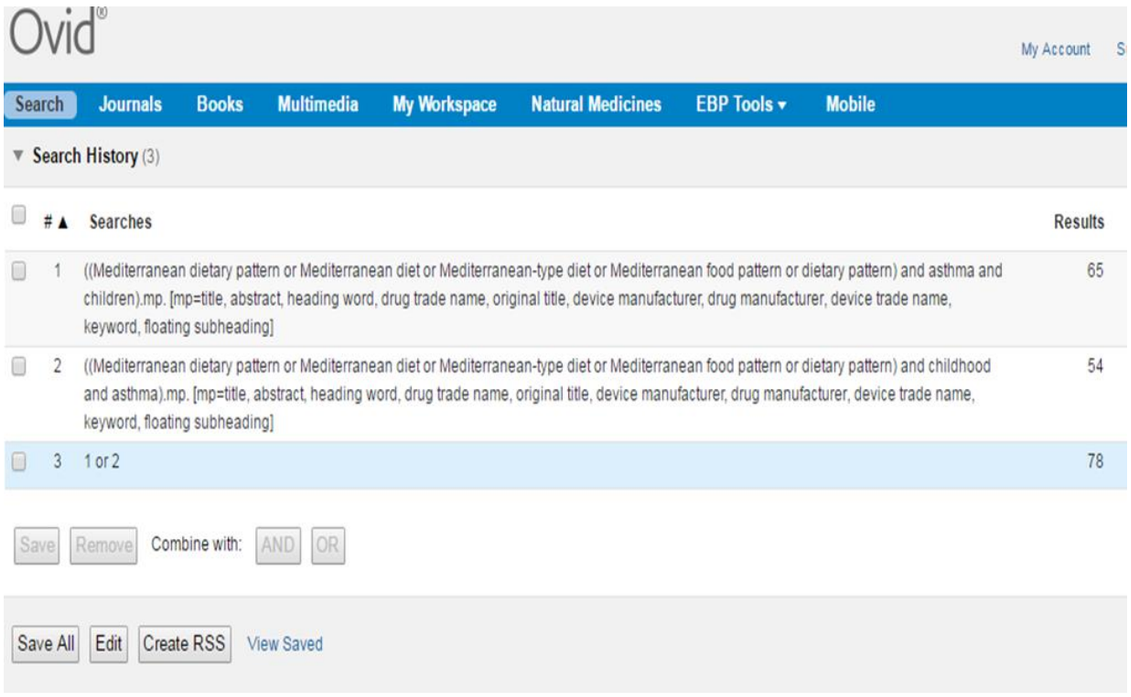
Appendix 4

Screenshot of EMBASE database search



SYNOPSIS

Chapter one gave a brief overview of the problem of asthma prevalence in children, pathophysiology and management of this disease. In spite of the conflicting views on the influence of diet on asthma, there is strong evidence showing that specific foods or nutrients can either trigger asthma onset or confer added protection through anti-oxidant, anti-inflammatory and immunomodulating properties. Nevertheless, traditional analysis in nutritional epidemiology examining the relationship between risk of disease and single, few nutrients or individual food groups have failed to reveal potential associations between single foods and asthma prevalence. Conceptually, dietary patterns represent a broader picture of food and nutrient intake, and thus may be more predictive of disease risk. The Mediterranean dietary pattern is a moderate carbohydrate, plant-based diet rich in a variety of fresh seasonal fruit, vegetables, wholegrain cereals, legumes, virgin-olive oil, wild greens; moderate intake of fish, dairy products, nuts and low intake of red meat. This healthy eating pattern can be described as a cocktail of bioactive compounds including anti-oxidants polyphenols, reservetrol, carotenoids, vitamins C and E, β-carotene, glutathione, vitamin D, omega-3 fatty acids, mono-unsaturated fatty acids, ideal omega- 6 to omega-3 fatty acid ratio that interact synergistically providing health benefits. Scientific evidence predict that the benefits of the Mediterranean diet in respiratory diseases are owing to its anti-inflammatory and anti-oxidant components, but intervention studies undertaken in children are non-existant. The questions raised regarding the impact of the Mediterranean diet in asthmatic children were answered in a recently published manuscript authored by the candidate. Evidence from the literature complied in a systematic review revealed a consistent inverse relationship (protective) between a Mediterranean dietary pattern and asthma in children in Mediterranean and non-Mediterranean regions. Future well-designed randomized controlled trials are needed to provide solid evidence.

CHAPTER 3 Omega-3 Fatty Acids: One of the secrets of the Greek Mediterranean Diet?

It has been proposed that the modern diet deficient in omega-3 fatty acids intake and high in omega-6 fatty acids might have contributed to the increase in allergic respiratory diseases including asthma. Based on the anti-inflammatory and immunomodulating effects of marine omega-3 fatty acids, it has been speculated that supplementation of the diet with these fatty acids may have therapeutic value in childhood asthma. In Chapter 3.2, the latest data on the potential of dietary omega-3 fatty acids as an adjunct therapy for asthma in children was synthesized, critically reviewed and further investigated in a meta-analysis authored by the candidate in a recent publication, *Papamichael et al, 2018. The role of fish intake on asthma in children: A meta-analysis of observational studies. Pediatr Allergy Immunol 29, 350-360.*

Pediatr Allergy Immunol. 2018 Jun;29(4):350-360. doi: 10.1111/pai.12889.

3.1 The role of fish intake on asthma in children: A meta-analysis of observational studies

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ORIGINAL ARTICLE

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The role of fish intake on asthma in children: A meta-analysis of observational studies

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Abstract

Background: The evidence is mixed on the use of long chain Omega-3 fatty acids in the prevention and management of childhood asthma.

Methods: We conducted a systematic search and meta-analysis investigating the role of fish intake, the main dietary source of long chain omega-3 fatty acids, on asthma in children.

Results: A total of 1119 publications were identified. Twenty-three studies on fish intake in association with childhood asthma were included in the final review. In 15 of 23 studies, early introduction of fish (6-9 months) and regular consumption (at least once a week) improved asthma symptoms and reduced risk in children 0--14 years as compared to no fish consumption; 6 of 23 showed no effect and 2 of 23 studies suggest adverse effects. Meta--analysis revealed an overall "beneficial effect" for "all fish" intake on "current asthma" [OR: 0.75; 95%CI: 0.60--0.95] and "current wheeze" [OR: 0.62; 95%CI: 0.48--0.80] in children up to 4.5 years old. An overall protective effect of "fatty fish" intake as compared to "no fish" intake in children 8-14 years old was also observed [OR: 0.35; 95%CI: 0.18--0.67].

Conclusion: This meta--analysis suggests that introduction of fish early in life (6--9 months) and regular consumption of all fish (at least once a week) reduces asthma and wheeze in children up to 4.5 years old, while fatty fish intake may be beneficial in older children. Future well--designed clinical trials are recommended to confirm the promising findings documented in this literature analysis.

KEYWORDS

asthma, children, fish, nutrition, oily fish, Omega-3 fatty acids

1 | INTRODUCTION

Despite advances in pharmacotherapy, globally, the asthma epidemic in children is a major public health problem as it continues to rise. Asthma is associated with morbidity, substantial healthcare costs and decrease d quality of life. [1,2] There is no cure for asthma; symptoms can only be controlled by medication which on a long term basis may cause side effects. Therefore, identifying potential non-pharmacological interventions which improve management of asthma is of great public health significance.

The modern diet is low in fruits, vegetables, fish and high in refined grains, red meat and fast food [3] which may have contributed to the high prevalence of asthma in Westernized countries. [1] As a result today's diet is deficient in anti-inflammatory nutrients (from fruit/vegetables) and long- chain Omega- 3 fatty acids (LC n- 3 PUFA) (obtained from fish) and high in Omega- 6 fatty acids mainly from processed fats and oils such as margarine and vegetable oils. [3,4] It is believed that the change in anti-inflammatory status, fatty acid composition, increase in Omega- 6: Omega- 3 fatty acid ratio is associated with an increase in oxidative stress

and airway inflammation promoting the development and symptoms of asthma. [5,6] .

There has been considerable interest in the potential therapeutic and protective properties of Omega- 3 fatty acids (EPA/DHA) in the pathogenesis of asthma due to anti-inflammatory and immune-modulating properties. [4,7] To date, the evidence for the beneficial effects of Omega- 3 fatty acid intake in asthma is controversial and intervention trials investigating the impact of fish or fish oil supplementation in children are lacking. [8] Most of the epidemiological evidence which gave rise to the hypothesis that marine Omega-3 fatty acids may have a protective effect is based on studies documenting that regular consumption of fish has a prophylactic effect. [9-11] In addition, many systematic reviews have investigated the impact of Omega- 3 fatty acid intake during pregnancy on allergy outcome in offspring suggesting a beneficial effect. [12-14] Little is known about fish intake on asthma during childhood (post- infancy) and this warrants further investigation.

The purpose of this study is to conduct an up- to- date systematic search, qualitatively synthesize the available evidence and perform a meta- analysis to determine the role of fish (lean and fatty) on childhood asthma. The impact of fish intake during pregnancy is beyond the scope of this review.

2 | METHODS

This review was conducted according to PRISMA guidelines [15] (Data S4).

2.1 | Literature search strategy

We conducted a comprehensive literature search of publications up to July 2017 using the following bibliographic databases: Cochrane Central Register of Controlled Trials, MEDLINE, PubMed, CINAHL (EBSCO), SCOPUS and EMBASE. No restrictions were applied on language or publication dates. Supplementary studies were sought from conference proceedings as well as clinical trials registries (International, European, Australia and New Zealand) in order to identify published/ unpublished trials. Other potential relevant citations were identified by hand search of the reference lists of relevant articles, reviews, systematic reviews and meta- analysis. Databases were searched for relevant publications, by a two-step search strategy using the following search terms:

Search 1: ["fish OR fatty fish OR oily fish OR omega 3 fatty acids OR n- 3 long chain polyunsaturated fatty acids AND "childhood asthma"]

Search 2: ["fish OR fatty fish OR oily fish OR omega 3 fatty acids OR n- 3 long chain polyunsaturated fatty acids AND "children" AND "asthma"].

The final search was a combination of both searches using the term "OR." The full search strategy is provided in (Box S1).

2.2 | Study eligibility criteria

2.2.1 | Inclusion criteria

Inclusion criteria was based on Participants, Intervention, Comparator and Outcomes (PICO). [16]

2.2.2 | Type of participants

This systematic review considered publications that included children younger than 18 years old.

2.2.3 | Type of intervention

Publications reporting the effect of fish as the primary exposure measurement.

2.3 | Outcome measures

Primary outcomes of interest were all asthma symptoms (wheeze, dyspnoea, shortness of breath) and prevalence or incidence of asthma.

A publication was considered when exposures were measured and the presence of asthma was based on the manifestation of symptoms, pulmonary tests, or doctor- diagnosis. [17] In addition, studies examining the effect of Omega- 3 fatty acid intake on atopy in children were considered relevant when results on fish intake and asthma outcome in children could be separated.

2.4 | Type of studies

We considered experimental and epidemiological study designs including randomized controlled trials (RCTs), non- randomized controlled trials, before and after intervention studies, cohort, case-control and cross- sectional studies.

2.5 | Exclusion criteria

Exclusion criteria were based on PICO characteristics and reasons for exclusion are summarized in (Figure 1). Publications not included were as follows: reviews, systematic reviews, editorials, comments, letters, case studies, animal studies and those with no abstract, full text or English translation available. Interventions or risk factors other than fish consumption such as: formula supplementation, dietary patterns, food groups, individual nutrients, fish oil, obesity, dust mites, as well as studies focusing on maternal diet or asthma genotypes, were considered to be irrelevant.

2.6 | Selection of studies

MP and CI initially screened the titles, abstracts, or descriptors of all publications retrieved by the search and duplicates removed

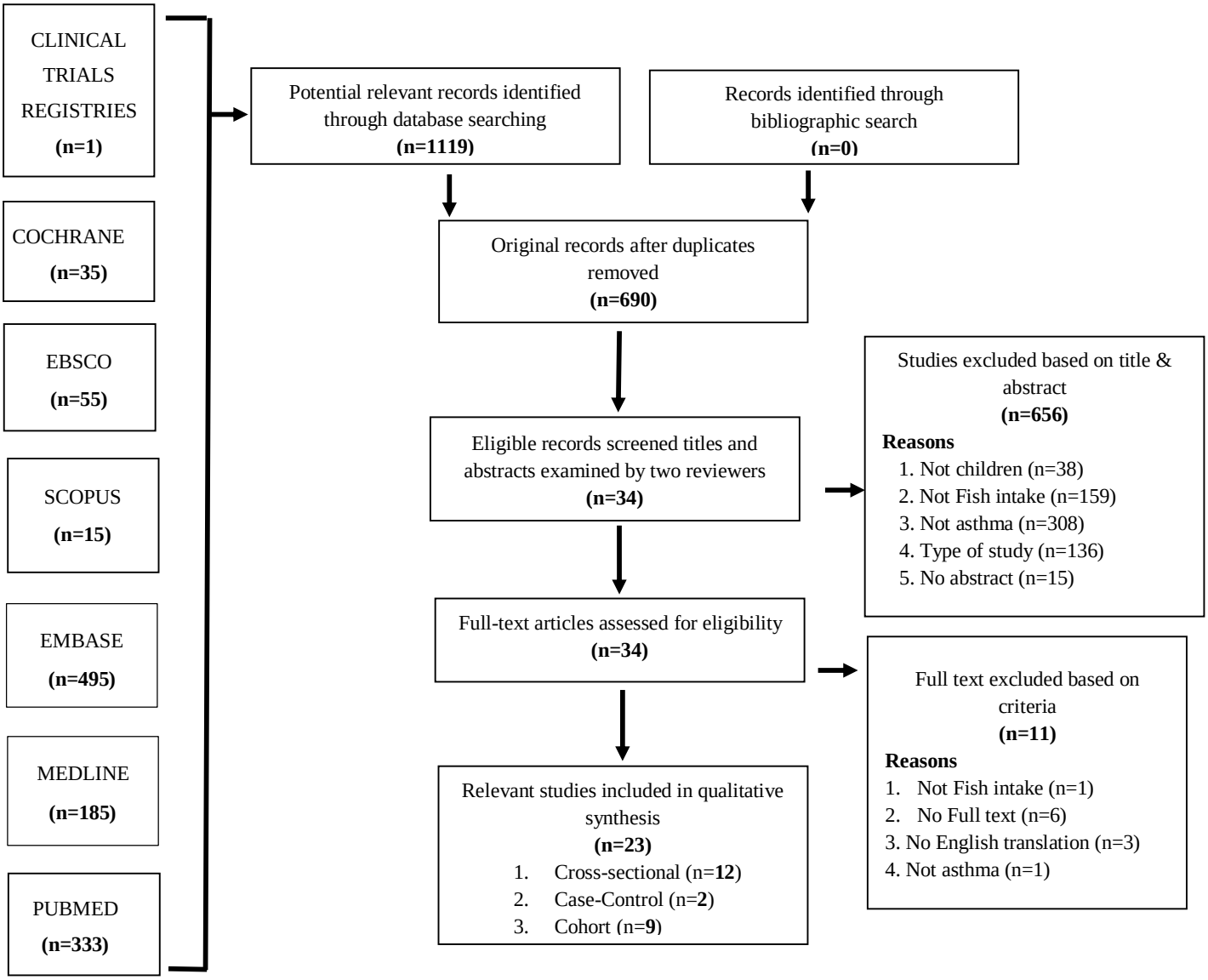

FIGURE 1 Flow chart of search detail

When there was insufficient information in the abstract to warrant exclusion of the article, the full text of the article was retrieved. Full- text papers were then independently appraised by both reviewers for inclusion and details extracted. Discrepancies were resolved by discussion and consensus that led to agreement. The search was supplemented by cross-checking reference and bibliographies of relevant publications, systematic reviews and reviews.

2.7 | Quality assessment and risk of bias

SKS and MP independently rated all publications included in the meta- analysis using a validated quality assessment tool according to Zaza et al [18] (Data S2). The scientific soundness was rated using [24] validity questions with possible responses of "yes" or "no." A value of one was assigned to "yes" and zero to "no." The final score was presented as the percentage of total (maximum) probable scores for each study. Any discrepancies were addressed with mutual agreement between the authors or with a third reviewer (BE). Finally, the scores from both evaluators were averaged and presented as the final score. The observed median study quality score was used as the dividing point between low quality and high quality.[19] We set the benchmark of 70% and above as a high- quality study while below 70% as low quality.

2.8 | Data extraction

Data extracted included authors, year of publication, study name, study design, geographic location, sample size, age range of target population, follow-up, dietary exposure, outcome

measures, dietary and respiratory assessment tools, confounders, main findings (risk estimates along with 95% CI and *P* - values).

2.9 | Data synthesis and statistical analysis

Study outcomes were synthesized and categorized into two groups based on the effect on asthma symptoms (improvement or no improvement). Quantitative data were pooled in statistical meta-analysis software using STATA software version 14.1 (Stata Corp, Texas). For the purpose of the meta- analyses, publications assessing "all" types of fish and "fatty fish" as the exposures and regular fish intake, at least once a week were deemed appropriate. Studies were included in the meta-analysis when effect size was expressed as odds ratio (OR) and their 95% confidence intervals (95%CI) were reported or could be calculated. Eligible publications were categorized into two group based on the common outcome of interest "current asthma" or "current wheeze" as defined by ISAAC. [20]

For the purpose of the meta- analysis and due to heterogeneity among study designs, subgroup analyses were conducted based on study design, age range, follow- up and type of fish consumed. Cut- offs for age were defined based on children's age range in publications.

Two assessments were conducted for each outcome. One was a combination of all study designs with "all fish or 'fatty fish exposure"and the other according to study design and similar age group.

Heterogeneity was assessed using the standard Chi-square test and I^2 statistic [21]. Considerable heterogeneity was considered to be an I^2 value >75% [21] and statistical significance at the 5% level. [22] We applied the random- effects model to estimate the pooled ORs and 95% CIs for "current asthma and 'current wheeze'.

. 3 | RESULTS

3.1 | Electronic search

The literature search identified a total of 1119 potential publications of which 690 remained, after removal of duplicates (Figure 1). No additional citations were found by cross- checking of reference lists. Only one ongoing RCT was identified from international clinical trial registries. Two reviewers (MP, CI) independently screened the 690 eligible papers by scanning titles and abstracts. A total of 656 were excluded based on publication type (136), no abstract (15) or on PICO criteria as they did not examine the effect of fish intake (160) in association with asthma symptoms (308) in children, (37) leaving 34 full- text articles to be assessed for eligibility. Of the remaining 34 potential studies, 11 full texts were deemed to be inappropriate (1 not intervention, 1 not asthma, 6 no full text available and 3 no English translation), leaving a total of 23 relevant studies. Specifically, 12 cross- sectional, [10,23-33] 2 case- control [34,35] and 9 cohort studies [11,36-43] examining the effect of fish consumption on asthma symptoms in children.

3.2 | Study characteristics

The database search identified 23 studies conducted from 1992 to July 2017 examining the effect of fish consumption on asthma in children. Two were undertaken in Australia, [33,34] Japan, [28,32] Spain [10,25]and Finland [35,41] ; three in Norway [38,40,42] and the Netherlands [31,37,43] :four in Sweden 11,27,36,39 ; one in China, [30] Italy, [26] France, [23] Central Europe [29] and the ISAAC study which involved 20 countries globally. [24] Collectively, 163 744 children and adolescents up to 15 years old were investigated with sample sizes ranging from 138 to 50 004. A variety of assessment methods were used to assess dietary intake in children. Twenty studies evaluated fish intake using a number of different questionnaires [10,11,23,24,26,27,29-34,36-43] ranging from one question to questionnaires consisting of limited food groups/items and detailed Food Frequency questionnaires (FFQ) comprising of a wide variety of foods that capture the entire dietary pattern, while two studies used a 3-day food record [25,35] and one, a diet history questionnaire. [28]

In all studies, consumption of "all types" of fish (lean and fatty) were investigated, while in six studies the effect of fatty fish intake on asthma symptoms was measured. [28,34,36-38,42] Asthma was diagnosed using a questionnaire, [10,23-32,34-39,41,43] parent- report, [42] doctor- diagnosed [11,37,40] and in one study by bronchial hyperresponsiveness (BHR). [33] The characteristics of included studies are shown in (Table S1). Overview of the literature search will be presented based on study design and age group.

3.2.1 Cohort studies: 0- 12 years of age

.The nine cohort studies identified, investigated the effect of early fish intake on asthma in 33, 673 children from birth until 12 years of age. [11,36-43] Seven studies revealed that early introduction and regular consumption of "all fish" were associated with a reduction in asthma symptoms up to 12 years of age [11,36-41] and two showed no effect. [42,43]

Two studies reported that early introduction of "all fish" (at 6-9 months of age) and regular consumption (≥1/week) of "all fish" [37] and "fatty fish" [38] reduced asthma incidence (OR c : 0.72; 95%CI: 0.55- 0.93), [38] prevalence of wheeze (OR: 0.64; 95% CI: 0.43- 0.94) [37] and use of medication (OR: 0.75; 95%CI: 0.58-0.96) [38] in children up to 4 years of age. [37,38] Conversely, Kiefte-de- Jong et al noted that no fish during the first 12 months of life and introduction of "all fish" between 0 and 6 months was associated with an increase in prevalence of wheezing [(OR: 1.57; 95%CI: 1.07- 2.31), (OR:1.53; 95%CI: 1.07-2.19)], respectively, at 48 months as compared to a reduction in the prevalence of wheezing with fish introduction at age 6-12 months (OR: 0.64; 95% CI: 0.43- 0.94).

Beneficial effects of "all fish" intake were also documented in children during the first 5 years of life. Fish consumption before 9 months had a protective effect for recurrent wheezing (OR: 0.6; 95% CI 0.4- 0.8), [39] multiple trigger wheeze (OR: 0.6; 95%CI:0.3- 0.99), [39] episodic viral wheeze (OR:0.6; 95%CI:0.4-0.99), [39] asthma risk (OR: 0.73; 95%CI: 0.55- 0.97), 11 (OR: 0.84; 95% CI = 0.57- 1.22), [40] all asthma ($P < .001$) [41] and atopic asthma ($P < .05$) [41] up to 5 years as fish intake increased from two to three times per month (OR adj = 0.82 95% CI: 0.54- 1.29) to once a week (OR adj = 0.66, 95% CI: 0.43- 1.01) and at least

once a week (OR adj = 0.55; 95% CI 0.34- 0.87; p trend = 0.003) [11] a dose response was observed.

A Swedish cohort which involved 3285 children with follow-up till 12 years of age, regular "all fish" intake (≥2- 3 times/month) at age of 1 year was associated with reduced risk of prevalence (OR adj :0.71; 95%CI: 0.57- 0.87) and incidence (OR adj : 0.80; 95%CI: 0.65- 0.98) of asthma up to age 12 years, after adjusting for confounders of parental history of allergic disease, sex, maternal smoking during pregnancy. [36] However, no significant association was observed between fish intake at age 8 years and incidence of asthma at 12 years (p trend = 0.303). [36] This finding is in agreement with two other cohorts that documented no relationship among early, late or long term intake of any kind of fish on asthma in children at ages 2 [42] (*P* = .16) and 8 years. [43] However, Willers et al [43] did report that early fish intake was inversely associated with BHR in children at age 8 years old.

3.2.2| Case- control studies: 5- 15 years of age

A beneficial effect of fish intake on asthma in children was observed in 2 case- control studies. [34,35] In a nested- cohort study which was a subgroup of the Finnish DIPP study, 182 children with asthma and 728 controls participated and were observed at 3, 6, 12 months and thereby annually up to 6 years. [35] Overall, early introduction of "all fish" and fish products (6- 8 months) was associated with a reduction in "all asthma" risk (OR adj : 0.87; 95%CI: 0.77- 0.98) in children at 6 years adjusting for confounders. Another case-control study investigating the impact of fish intake on asthma symptoms in 584 children aged 8- 11 years also reported a protective effect. [34] After adjusting for confounders, children who ate fresh, "fatty fish" at least once a week had a significantly reduced risk of current asthma (OR adj : 0.26; 95%CI: 0.09- 0.72) as compared to "any fresh" fish (OR adj : 0.52; 95% CI: 0.24- 1.15) and "non- fatty" fish (OR adj : 0.68; 95% CI: 0.3- 1.54) consumers. No reduction in asthma risk was observed with canned fish consumption.

3.2.3| Cross- sectional studies: 0- 15 years

The literature search retrieved 12 cross- sectional studies undertaken in 128 577 children aged 0- 15 years. [10,23-33] Five studies observed that regular "all fish" intake (>1/week) was associated with a reduction in lifetime prevalence of asthma (OR adj : 0.92; 95%CI: 0.78- 1.08), [24] current wheeze [(OR adj : 0.85; 95%CI:0.74- 0.97) 24 ; (OR: 0.44; 95%CI: 0.21- 0.93)], [31] current wheeze with atopy positive (OR adj : 0.51; 95%CI: 0.32- 0.81), [24] doctor- diagnosed asthma (OR: 0.54; 95%CI: 0.35- 0.84), [27] night- time breathlessness (OR: 0.36; 95%CI:0.17- 0.78), [27] current asthma (OR: 0.51; 95%CI: 0.31- 0.84), [27] (OR: 0.34; 95%CI: 0.13- 0.85), [31] past- year wheeze (OR adj: 0.75; 95%CI: 0.53- 0.93), [23] past- year wheeze and atopy negative (OR: 0.61; 95%CI: 0.43- 0.87), [23] atopic asthma with BHR (OR: 0.12; 95%CI: 0.02- 0.66), [31] atopic wheeze with BHR (OR: 0.15; 95%CI: 0.03- 0.63) [31] and BHR (OR: 0.35; 95%CI: 0.1- 0.9). [33] While one study [29] reported that irregular fish intake (<1/month) was associated with an increase in "persistent cough" (OR: 1.18; 95%CI: 1.04- 1.34), "wheeze ever" (OR: 1.14; 95%CI: 1.03- 1.25) and "current wheeze" (OR: 1.21; 95%CI: 1.06- 1.39). A recent study undertaken by Xu et al, [30] including 13, 877 children, 0- 14 years old, observed that fish and shrimp intake triggered asthma episodes by 14% ($P < .05$), although estimated effect size was lacking. Adverse effects of "all fish" intake were also reported in a Japanese study of 1673 asthmatic children and 22 109 controls aged 6- 15 years. 32 After adjustment for confounders (age, gender, parental asthma), a statistically significant higher prevalence of asthma was observed in children consuming fish 1- 2 times/week (OR adj : 1.117; 95%CI: 1.005- 1.241) and more than or equal to 3- 4 times/week (OR adj : 1.319; 95%CI: 0.896- 1.943) than among those who consumed no fish (OR adj : 1.039; 95%CI: 0.785- 1.376).

3.2.4 | Quality assessment

Twenty-one of 23 studies were rated as "high quality." The average quality score was 82.1% with the highest score 100% and the lowest score 64% (Table 1). Most of the cohort studies included in the meta- analysis were of high quality, 8 of 9 studies scored above 90%. The studies that were rated lower quality were due to their failure to control for potential confounders and address possible biases. Using *t* test, we checked if there were any differences in the quality score for the selected studies between two reviewers and found no major difference in average score (*P*-value = .33).

3.3 | Meta- analysis

A total of nineteen studies were used in the meta-analysis.[10,11,23-29,31,32,34-37,39,40,42,43]

3.3.1| Current asthma

Assessment of cohort studies alone [11,40,42] revealed a statistical significant effect [21] of "all fish" intake on "current asthma" in children 0- 4 years (I^2 = 11.5%; P = .32; (OR: 0.75; 95% CI: 0.60- 0.95). Overall, a 25% reduction in "current asthma" was observed with "all fish" intake in children up to 4 years old (Figure 2).

3.3.2| Current wheeze

A pooled effect for current wheeze and "All fish" intake was found in cohort studies regarding children up to 4.5 years [I^2 = 0%, P = .809; (OR: 0.62; 95%CI: 0.48- 0.80)] (Figure 3). A 38% reduction in "current wheeze" was observed with "All fish" intake in children up to 4.5 years old.

3.3.3 | Fatty fish versus No fatty fish

When analysing the effect of "Fatty fish" intake in children aged 8- 14 years [28,34] for "current asthma" in the combined sub-analysis, an overall effect was observed [I^2 = 0%, P - value = .481; (OR: 0.35; 95%CI: 0.18- 0.67] (Figure 4). Fatty fish intake reduced "current asthma" in children aged 8- 14 years by 65% as compared to "no fish" intake.

In contrast, no association was observed for "all fish" intake on "current asthma" in children aged (2-15 years) when combining all study designs [11,24,25,27,28,31,32,34-36,40,42,43] (Figure S1) or in subanalysis based on study designs separately (Figure S2). No pooled age specific effects were also found for "current wheeze" and"All fish" intake in children (0-13 years) irrespectiveof study design (Figures S3-S5).

Table 1. Summary table of Quality Assessment of relevant studies in this systematic review (Zaza)[18].

Study	Reviewers	Study design	Population Density	Population Description	Time points	Entire sampling or Probability sampling	Eligibility Criteria	Exposure Measures		Outcome Measures		Statistical Test Suitable	Adjustment For Confounders	Bias Discussed	Bias not covered	Overall Average Score (%)
								Validity	Reliability	Validity	Reliability					
Peat et al [33], 1992	SKS/MP	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	×	×	67.5%
Hodge et al[34], 1996	SKS/MP	✓	✓	✓	✓	×	✓	✓	✓	✓	✓	✓	✓	×	×	70.5%
Takemura et al[32], 2002	SKS/MP	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	×	83.0%
Farchi et al[26], 2003	SKS/MP	✓	✓	✓	✓	×	✓	✓	✓	✓	✓	✓	✓	✓	×	73.0%
Nafstad et al[40], 2003	SKS/MP	✓	×	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	×	91.0%
Kim et al[27], 2005	SKS/MP	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	×	79.5%
Kull et al[11], 2006	SKS/MP	✓	×	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	×	95.0%
Tabak et al[31], 2006	SKS/MP	✓	×	✓	✓	×	✓	✓	✓	✓	✓	✓	✓	✓	×	75.0%
Chatzi et al[10], 2007	SKS/MP	✓	×	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	×	82.0%
Antova et al[29], 2003	SKS/MP	✓	✓	✓	✓	×	✓	✓	✓	✓	✓	✓	✓	✓	×	75.0%
Nagel et al[24], 2010	SKS/MP	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	×	83.0%
Oien et al[42], 2010	SKS/MP	✓	×	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	×	95.0%
Rodriguez et al[25], 2010	SKS/MP	✓	×	✓	×	×	✓	✓	✓	✓	✓	✓	✓	×	×	73.0%
Goksor et al[39], 2011	SKS/MP	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	×	91.0%
Willers et al[43], 2011	SKS/MP	✓	×	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	×	93.0%
Kunitsugu et al[28], 2012	SKS/MP	✓	×	✓	✓	×	✓	✓	✓	✓	✓	✓	✓	✓	×	73.0%
Kiefte de Jong et al[37] 2012	SKS/MP	✓	×	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	×	95.0%
Dotterund et al[38], 2013	SKS/MP	✓	✓	✓	✓	✓	×	✓	✓	✓	✓	✓	✓	×	×	80.5%
Magnusson et al[36], 2013	SKS/MP	✓	×	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	×	×	91.0%
Nwaru et al[41], 2013	SKS/MP	✓	×	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	97.5%
Lumia et al[35], 2015	SKS/MP	✓	×	✓	✓	×	✓	✓	✓	✓	✓	✓	✓	×	×	78.0%
Saadeh et al[23], 2015	SKS/MP	✓	×	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	×	82.0%
Xu et al[30], 2016	SKS/MP	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	✓	×	✓	×	64.0%

Key: ✓: criteria satisfied ; × : criteria not satisfied.

The same trend was established for “Fatty fish” intake vs “current asthma” in children (0- 14 years) in the combined sub-analysis (Figure S6).

4 | DISCUSSION

The aim of this qualitative synthesis and meta- analysis was to clarify the role of fish intake on different asthma outcomes in children. Although the meta- analysis produced mixed results, three important findings were highlighted. Firstly, early introduction of “all fish” intake during infancy (6- 9 months of age) and regular consumption (at least once/week) of “all fish” reduced “current asthma” by 25% and “current wheeze” by 38% in children up to 4.5 years old. [11,37,39,40,42] While “fatty fish” intake seems to confer an overall “prophylactic effect” in older children 8- 14 years and reduced “current asthma” by 65%. [28,34]

Summarizing the results of the database search, in 15/23 studies a protective effect was observed between fish consumption on asthma symptoms in children, [11,23,24,27,29,31,33-41] 6/23 no effect [10,25,26,28,42,43] and 2/23 an adverse effect. [30,32] Two important results were highlighted in these observational studies. Early introduction during the first year of life (between 6 and 9 months) and regular consumption of fish (at least once a week) decreased risk, prevalence and asthma symptoms in children up to 14 years of age. This finding is in accordance with other observational studies that have found early introduction and frequent consumption of fish were associated with decreased asthma symptoms and improvement in pulmonary function in children and adolescents. [9,44-4]

Our meta- analysis of nineteen 19 publications showed an overall beneficial effect of “all fish” intake on “current asthma” and “current wheeze” in children up to 4.5 years old in five cohort studies alone [11,37,39,40,42] and of “fatty fish” intake on “current asthma” in 8- 14 year olds. [28,34] The prophylactic effects documented in the cohort studies for young children up to 4.5 years old may be explained from research on biological mechanisms that have shown that, the development and maturation of the immune system starts early in foetal life continue through infancy and early childhood. [47,48] Infancy is a period in which the immune cells have an increased vulnerability and susceptibility to environmental exposures such as diet. A diet rich in Omega- 3 fatty acids and lower Omega- 6: Omega- 3 fatty acid ratio may result in an increased incorporation of EPA and DHA into cell membranes at the expense of arachidonic acid. More EPA and DHA in the cell membrane result in decreased production of arachidonic acid- derived pro-inflammatory eicosanoids. EPA and DHA act as substrates for COX and LOX enzymes producing anti- inflammatory eicosanoids, protectins, resolvins and maresins that appears to exert anti- inflammatory and inflammatory resolving actions. [49] Apart from anti- inflammatory properties, Omega- 3 fatty acids are able to modulate immune responses by promoting Th 1 cell generation, thereby reducing airway inflammation, improving pulmonary function and decreasing asthma symptoms. [13]

Additional protective effects on asthma symptoms were observed in two studies examining the effect of “fatty fish” as opposed to “all fish” in children aged 8- 11 years old. [34,38] Hodge et al [34] reported a 25% reduction in “current asthma” when fatty fish was consumed as compared to lean or no fish in children. Furthermore, no reduction in asthma risk was observed with the consumption of canned and processed fish. Perhaps, processing and food additives may alter the biological activity of Omega- 3 fatty acids in fish oils. Dotterud et al [38] reported a 32% reduction in incidence of asthma with fatty fish consumption in infants at 2 years old, after adjusting for confounders. A beneficial effect of fatty fish intake was also noted in the studies undertaken by Kunitsugu and Oien, although results were not statistically significant, most probably explained by heterogeneity between study designs and small sample size. [28,42]Our meta- analysis confirmed an overall “prophylactic” effect of fatty fish intake in older children. [28,34]. A 65% reduction in “current asthma” was observed in children aged 8- 14 years. [28,34]

The findings of this meta- analysis highlight that the type of fish consumed whether fatty or lean may matter. Fatty fish as compared to lean fish is a rich source of Omega- 3 fatty acids which are able to counteract the action of Omega- 6 fatty acids metabolites by down regulating pro- inflammatory and immunological pathways, thereby preventing asthma development. [50,51] Another possible explanation why fish consumption reduced asthma risk in children as compared to null effects reported from fish oil supplementation during childhood [52] is that regular fish consumption may be a proxy for other healthy lifestyle habits that might promote the beneficial effect. In addition, fresh fish might have better bioavailability and absorption rate as opposed to fish oil. And apart from EPA/DHA, fresh fish contains other bioactive molecules such as selenium, iodine, vitamin D, potassium and B- vitamins that might interact synergistically providing these prophylactic properties. [53]

On the other hand, our meta- analysis showed no statistical significant results for 14 studies (10 cross- sectional, 2 cohorts and 2 case- controls) on “current asthma” or “current wheeze” in children 6- 15 years old. [10,23-29,31,32,3] A possible reason for the null effects observed might be due to heterogeneity among study methodologies which included: population differences, sample size, children’s age, gender, asthma definition, exposure and outcome measures, possible confounders, design of dietary questionnaires and food frequency categories, amount or typeof fish consumed (fatty versus lean), quantity EPA/DHA, fish consumption patterns since nutritional guidelines may vary in each country and food preparation methods. Use of invalidated and self- administered food frequency questionnaires, 3- day diet history for the recording of dietary intake require education and expertise are prone to information bias and response errors. [43,28]Even though data on habitual diet were collected retrospectively from parents, in young children, it is well- established that parents provide reliable information on children’s diet. [54] Misclassification of asthma may have occurred in young children, since the diagnosis of asthma is uncertain in children younger than 5 years and wheezing is often the result of respiratory infection. [55,56] Furthermore, spirometry which is considered to be the “gold standard” of pulmonary function testing in the diagnosis of asthma, cannot be performed efficiently in children younger than 5 years and diagnosis of asthma in young children is based on parents’ report of symptoms. [55] In addition, parental allergic disease and early onset of allergic disease in children may cause parents to delay introduction or to avoid fish in the child‘s diet, thus influencing asthma outcome.

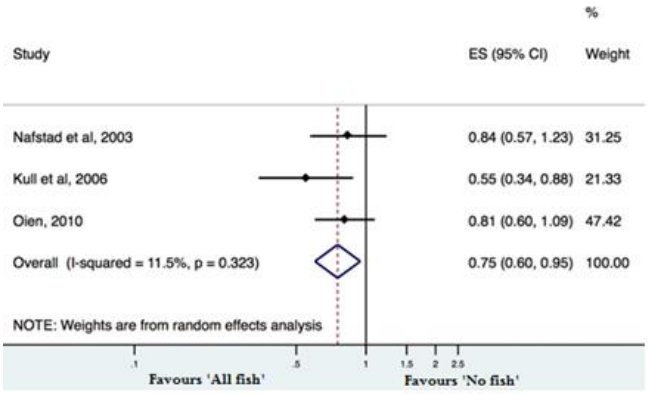

FIGURE 2 Forest plot of cohort studies children (0- 4 years old) for "All fish" intake versus "No fish" and outcome "current asthma" [Colour figure can be viewed at wileyonlinelibrary.com]

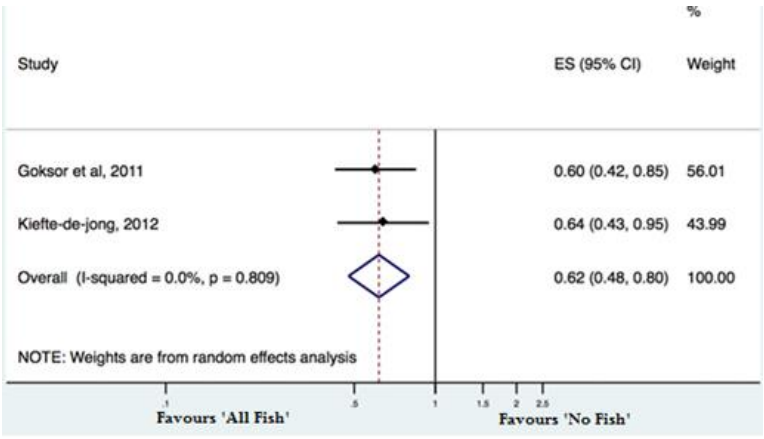

FIGURE 3 Forest plot of cohorts comparing the effect of "All fish" intake versus "No fish" on "current wheeze" in children aged 0-4.5 years.[Colour figure can be viewed at wileyonlinelibrary.com]

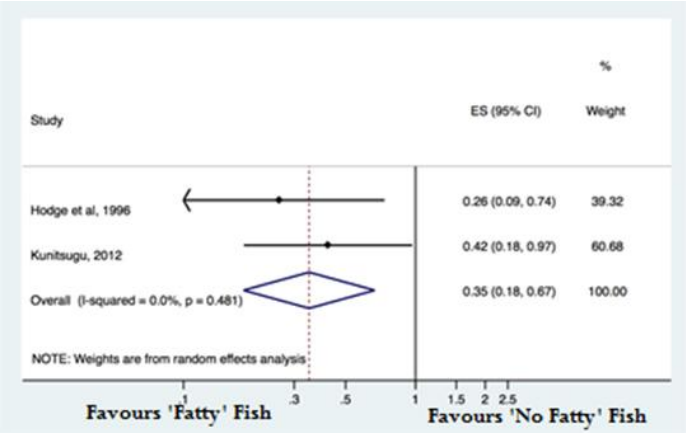

FIGURE 4 Forest plot in the combined analysis comparing "Fatty Fish" intake versus "No Fatty Fish" in children (8-14 years) for "current asthma" [Colour figure can be viewed at wileyonlinelibrary.com]

Two cross- sectional studies documented increased risk of asthma prevalence in children consuming fish in Japan and China. [30,32] Today, the eating patterns of Japanese and Chinese children have changed to a more Westernized diet. [57,58] Processed [23,59] and pickled foods [60] as well as a high salt intake [61,62] have been associated with an increase in bronchial hyperresponsiveness and asthma symptoms in children..
In both studies confounding factors such as overweight, socio-economic level, maternal education, type of fish consumed (lean vs fatty), poor adherence to asthma therapy were not included in regression models and may have contributed to the adverse effects.

One of the strengths of this systematic review is the extensive literature search and large number of recent high- quality publications included which increases the power of the analysis. There are very few meta- analysis in the literature that examine fish intake and asthma in children (Yang, 2013; Zhang, 2017) [14,63] and recent reviews (Best, 2016; Miles, 2017) [12,13] address Omega- 3 fatty acid intake during pregnancy. Our meta-analysis focuses exclusively on children and adolescents and adds to the existing evidence. Another strong point is the inclusion of cohort studies in the meta- analysis which are considered robust and provide strong scientific evidence because they measure events in temporal sequence thereby distinguishing causes from effects. [64] Another drawback might be publication bias where studies reporting no association between fish intake and asthma may not be published by periodicals.

5 | CONCLUSION

We conducted an up- to- date systematic search to determine the impact of fish intake in childhood asthma. This meta-analysis suggests that introduction of fish early in life (6- 9 months) and regular consumption of all fish (at least once a week) reduces asthma and wheeze in children up to 4.5 years old, while fatty fish intake may be beneficial in older children. Future well- designed clinical trials are recommended to confirm the promising findings documented in this literature analysis.

CONFLICT OF INTEREST

The authors declare no potential conflicts of interest.

AUTHOR CONTRIBUTION

MP and CI jointly conducted searches, assessed inclusion, extracted data and assessed validity. MP drafted the manuscript and conducted the meta- analysis. BE and SKS made substantial contributions to analysis and interpretation of data quality. All authors critically reviewed the manuscript and approved the final version as submitted.

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Websites:

Australian and New Zealand Clinical trial registry : http://www.anzctr. org.au/trialSearch.aspx
International Clinical trials registry : http://www.clinicaltrials.gov
European Union clinical trials registry
http://www.clinicaltrialsregister

SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.

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3.2 Fish consumption in Greece

Moderate fish intake is one of the criteria of the Greek Mediterranean diet pyramid [297]. According to recent data from the Hellenic Statistical Society (ELISTAT) in the Household Budget Survey 2016, there seems to be a progressive decline in fish intake in Greek families [298]. Between 2016 to 2017, the average monthly fish intake recorded decreased by 2.7%. While the monthly average expenditure for fish was only 7.2% in 2017 (approximately 20.74 €/ month) and 7.1% in 2016 (20.33 €/ month). On average, only 2.70 kg of fish was consumed per month in both 2017 and 2016 [298].

This data corresponds with evidence from previous cross-sectional studies examining dietary habits of Greek children and adolescents that have reported low adherence to the Mediterranean diet [237; 299; 300] with fish intake being a key component of this dietary pattern. From the Greek Childhood Obesity (GRECO) study that comprised of 4786 children 10-12 years old, only 4.3% of children had optimal Mediterranean diet adherence [237] and 36% of children consumed fish less than once a week [237].

The decrement in fish intake has also been documented by the Hellenic Fishing Association in a newspaper article published in Taxydromos (2014) [301]. According to the article, during the period of 2011-2012, Greece produced 140,000 tonnes of cultured gilthead seabream and seabass, valued at €560 million, with exports surpassing those for olive oil. Surprisingly, mean fish intake for Greeks was 18 kilos per year as compared to 25 kilos per year by Europeans [301]. The decrease in fish consumption patterns have been strongly noted in two large fishing regions of Greece, namely Magnesia and Volos [302]. In 2016, there was a 40% reduction in fish intake by consumers as compared to data pre-economic crisis, due to the high cost of fresh fish. Before the recession, families purchased and consumed fish three times a week as compared to once a week today, with the majority of consumers choosing the cheaper types of fish such as anchovies and sardines, averaging at 5 € per kilo. Nonetheless, small fish are nutritious and rich sources of omega 3 fatty acids [303; 304].

SYNOPSIS

In Chapter 1, studies undertaken by Simopoulos and Calder suggest beneficial effects of omega-3 fatty acids in inflammatory diseases such as CVD and rheumatoid arthritis, but their efficacy with respect to asthma is inconsistent [199; 202]. To the candidate's knowledge no clinical trial has yet been conducted investigating the effects of fish, specifically fatty fish consumption on pulmonary function in asthmatic children. Most of the epidemiological evidence which gave rise to the hypothesis that marine omega-3 fatty acids might have a protective effect in asthma is based on cross-sectional studies documenting that regular consumption of fish had an inverse relationship on asthma incidence and wheeze [305; 306]. A number of systematic reviews have investigated the impact of omega-3 fatty acid intake during pregnancy on allergy outcome in offspring suggesting a beneficial effect [307; 308; 309]. However, little is known about fish intake on asthma post-infancy. Given the gap, a qualitative and quantitative analysis of the literature was performed and and discussed by the candidate in a recent publication. In summary, meta-analysis of the literature revealed encouraging evidence on the protective effect of regular fish intake (≥ once/week) on asthma and wheeze in young children up to 4.5 years and fatty fish in older children (8-14 years). It is crucial that future well-designed clinical trials examining the impact of fatty fish consumption on pulmonary function in asthmatic children are conducted to establish a cause-effect relationship and to confirm these promising findings before omega-3 fatty acids can be recommended as a diet therapy for childhood asthma.

CHAPTER 4 Aims and hypotheses

Aims & Hypotheses

The aim of this PhD thesis was to examine the impact of fatty fish consumption in the context of a Mediterranean diet on asthma symptoms and pulmonary function in children with asthma compared to standard care via pharmacological therapy. Children were included in this study if they were 5-12 years old with physician diagnosed mild-asthma based on GINA guidelines and willing to consume fatty fish within the context of the Mediterranean diet for a period of six months. According to GINA, 'mild asthma' is considered to be 'well-controlled' asthma with day symptoms and need for reliever medication ≤ 2 times weekly, no night symptoms or activity limitations due to asthma [12].

4.1 Primary Objective

The primary objective of this research project was to determine whether the consumption of two fatty fish meals per week (≥ 150g cooked fillet/meal) in the context of the Greek Mediterranean diet improves pulmonary function and asthma symptoms in children with clinically diagnosed asthma.

Primary Outcome 1: Pulmonary function as assessed by spirometry (FEV_1, FVC, FEV_1/FVC, PEF, $FEF_{25-75\%}$)

Hypothesis 1: The primary hypothesis challenged was that increased fatty fish consumption in the context of a Mediterranean diet (the intervention) for 6 months improves pulmonary function as indicated by an increase in spirometry measurements (FEV_1, FVC, FEV_1/FVC, PEF, $FEF_{25-75\%}$) compared to a control condition.

Primary Outcome 2: Bronchial inflammation measured by Fractional exhaled Nitric Oxide analysis (FeNO).

Hypothesis 2. There will be a decrease in FeNO in the intervention group as compared to the control at 6 months.

4.2 Secondary Objectives

Secondary Objective 1: to investigate whether the consumption of a Mediterranean diet enriched with fatty fish improves asthma control in children.

Hypothesis 1: There will be a decrease in asthma control scores for the intervention group at 6 months compared to the control.

Secondary Outcome 1: Asthma control as measured by the Asthma Control Questionnaire score.

Secondary Objective 2: To examine whether consumption of a Mediterranean diet enriched with fatty fish improves quality of life in asthmatic children.

Hypothesis 2: There will be greater increase in paediatric asthma quality of life scores for the intervention group compared to the control at 6 months.

Secondary Outcome 2: Quality of life in asthmatic children assessed using the mini Paediatric Asthma Quality of Life Questionnaire score.

Secondary Objective 3: To examine whether adherence to the dietary intervention improves compliance to the Mediterranean dietary pattern and diet quality in the intervention group at 6 months as compared to the control.

Hypothesis 3: There will be better adherence to the Mediterranean diet and improvement in diet quality for the intervention group as compared to the control.

Secondary Outcome 3: Diet quality and adherence to the Mediterraean diet as measured by the KIDMED score.

Secondary Objective 4: To examine the relationship between plasma vitamin D levels and lung function in asthmatic children following a dietary intervention.

Hypothesis 4: There is a positive association between plasma Vitamin D and lung function tests in the study population.

Secondary Outcome 4: Vitamin D levels reflected by plasma 25 (OH)D and lung function/bronchial inflammation using spirometry and FeNO.

Secondary Objective 5: To investigate the association between weight status and pulmonary function in the total sample of asthmatic children at baseline.

Hypothesis 5: There is an association between high BMI and pulmonary function in asthmatic children as compared to normal weight.

Secondary Outcome 5: Weight status as measured by BMI and lung function via spirometry parameters (FEV_1, FVC, FEV_1/FVC, PEF, $FEF_{25-75\%}$).

Secondary Objective 6: To investigate the relationship between overweight/obesity on bronchial inflammation compared to normal weight in asthmatic children.

Hypothesis 6: There is a positive relationship between overweight/obesity and bronchial inflammation in asthmatic children at baseline as compared to normal weight.

Secondary Outcome 6: BMI was categorized as normal weight, overweight or obese according to the Hellenic Paediatric Growth Charts which is comparable to the age-sex cut-offs proposed by the International Obesity Task Force (IOTF) for children and adolescents 2- 18 years old. These cut points are based on age and sex specific percentile curves that correspond to the adult cut points of 25 and 30 kg/m^2 for overweight and obesity. Bronchial inflammation was measured by FeNO.

SYNOPSIS

The first three chapters of this thesis formed the background and rationale for this PhD study. Three main points were highlighted in the literature search. Firstly, evidence from the ISSAC study (2009) showed that, asthma is 20% less prevalent in Mediterranean countries in Greece, Italy, Spain, Portugal and Algeria, as compared to English-speaking countries, thus suggesting that the Mediterranean lifestyle including a diet rich in seasonal fruits, vegetables, wild greens and fish may be a major factor that contributes to prevalence and severity of this disease. In Chapter 2 the systematic literature review conducted and published by the candidate concluded that adherence to the Mediterranean dietary pattern may reduce asthma burden and severity in children, recommending future well-designed intervention studies to confirm the promising findings. In Chapter 3, the systematic review and meta-analysis of observational data on the role of fish intake in childhood asthma, completed and published by the candidate, suggested that regular consumption of all fish (lean and fatty), at least once per week reduced asthma incidence and wheeze in infancy (< 5 years old). Regarding older children (8-14 years old), fatty fish as compared to no fish intake reduced asthma incidence. Nevertheless, no clinical trials have yet been conducted in children to clarify the benefit of fish intake in paediatric asthma. Based on this promising data and given the limitations of cross-sectional studies in determining a cause-effect relationship, the objective of this PhD study was to assess whether increased fatty fish intake as part of the Mediterranean dietary pattern improves pulmonary function and reduces symptoms in asthmatic children using clinical trial methodology. To the candidate's knowledge this is the first paediatric intervention study examining the impact of fatty fish intake in combination with a dietary pattern, specifically the Mediterranean diet. If shown to be efficacious such a dietary intervention could be the basis for a low-cost, safe public health intervention to reduce the prevalence and burden of asthma in children with obvious long-term benefits for the individual.

CHAPTER 5 Methods & Materials

The purpose of Chapter 5 is to give a detailed description of the methods used in this PhD clinical study to test the hypotheses raised earlier in Chapter 4. Study design, participants, procedures and assessment tools are described in section 5.1 by the following publication authored by the candidate *Papamichael et al, 2018. A clinical trial of Mediterranean diet enriched with fatty fish in pediatric asthma: Study protocol. J Pharmacy Pharmacol 6: 225-239 doi: 10.17265/2328-2150/2018.03.004.* Features of the study design and rationale not included in this paper are summarized in Appendix 2B. Nutritional tools and statistical methods are explained below.

J Pharmacy Pharmacology 6 (2018) 225-239 doi: 10.17265/2328-2150/2018.03.004

5.1 A Clinical Trial of Mediterranean Diet Enriched with Fatty Fish in Pediatric Asthma: Study Protocol

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A Clinical Trial of Mediterranean Diet Enriched with Fatty Fish in Pediatric Asthma: Study Protocol

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Abstract: Background: Considerable interest exists in the therapeutic potential of dietary omega 3 fatty acids in asthma, due to anti-inflammatory and immune-modulating effects. The aim of this Randomized Controlled Trial is to investigate the efficacy of fatty fish consumption in the context of the Mediterranean diet in ameliorating symptoms in pediatric asthma. Methods: Children aged 5-12 years with physician-diagnosed "mild asthma" will be recruited from a pediatric asthma clinic in Athens and randomized to two groups. The intervention group will consume two meals of fatty fish (≥ 150g cooked fish) per week over a period of 6 months as part of the Greek Mediterranean diet. The control group consumes their usual diet. Questionnaires will be used to collect data on socio-demographics, medical history, dietary habits, adherence to the Mediterranean dietary pattern, asthma control and quality of life. Pulmonary function will be assessed using spirometry and exhaled nitric oxide analysis. In addition, blood tests will be undertaken to assess metabolic profile, biomarkers of nutritional status and dietary intake. Discussion: This clinical dietary intervention will evaluate the therapeutic potential of a Mediterranean diet enriched with fatty fish on pediatric asthma and assist in devising dietary guidelines for the management of pediatric asthma.

Key words: Asthma, children, fish, Mediterranean diet, Mediterranean dietary pattern, omega 3 fatty acids

1. Introduction

Childhood asthma has become a major public health concern [1, 2] causing a physical and economic burden for the family and society due to hospitalization, medication expenses, missed school and work days [3]. It has been estimated that the mean cost per patient per year is $1,900 USD in Europe and $3,100 USD in the USA [3]. Therefore, substantial cost of asthma could be saved by better management of asthma symptoms.

Accumulating scientific evidence suggests that diet is interwoven in the pathogenesis of asthma [4]. The Western diet consists of foods that are highly processed, stored and transported over great distances thus affecting nutrient density (or quality). It has been postulated that reduced consumption of foods rich in antioxidants (fruits, vegetables), increased n-6 PUFA intake (from margarine, vegetable oils) and low n-3 PUFA intake (from oily fish) may have contributed to the rise in allergic diseases including asthma by impacting immunity [5, 6]. Previous studies have reported that adherence to healthy dietary patterns that include fresh fruits, vegetables and fish to be associated with a lower prevalence of asthma among children [7-13]. In contrast, a Western-type diet [14] that is high in processed foods and high dietary ratio of n-6: n-3 PUFA intake from increased intake of margarines, vegetable oils and low intake of fish was associated with increased risk of asthma [15, 16] and wheeze among children as compared to low n-6 PUFA intake [17]. In a recent systematic review examining the impact of the Mediterranean dietary pattern in childhood asthma it identified fifteen observational

studies and no clinical trials [18]. The majority of studies (12/15) reported that adherence to the Mediterranean dietary pattern reduced risk and prevalence of asthma in children, two studies (2/15) no effect and one study (1/15) an increase in symptoms. This suggests that healthy dietary patterns like the Mediterranean diet might have a role in the prevention and management of asthma.

The Mediterranean diet has been acknowledged by UNESCO not only as an intangible heritage but as a sustainable dietary pattern and lifestyle [19]. The Mediterranean-style diet is not a specific diet, but rather a collection of eating habits traditionally shared by people in the different countries bordering the Mediterranean Sea. Unlike the Western-type diet, this traditional diet is produced and marketed locally and many foods are eaten shortly after harvesting [20, 21]. The traditional Greek Mediterranean diet, is characterized by a high intake of vegetables, legumes, fruits, and wholegrain cereals including bread; a low intake of saturated fats but high intake of monounsaturated fats from olive oil; a low to moderate intake of dairy products, mostly cheese and yogurt; and a low intake of meat and a moderate to high intake of fish, a rich source of omega 3 fatty acids, EPA and DHA [21].

The significance of EPA and DHA is that they play a crucial role in inflammation. Increased consumption of fish, in particular fatty fish results in enhanced incorporation of EPA and DHA in immune cell membranes at the expense of n-6 PUFA arachidonic acid, a pro-inflammatory mediator [22]. EPA is a precursor for less potent eicosanoids than arachidonic acid derived eicosanoids. Apart from EPA inhibiting the metabolism of arachidonic acid to pro-inflammatory eicosanoids, EPA and DHA is precursors for the production of resolvins, protectins and maresins which have anti-inflammatory effects [22].

The high content in the Mediterranean diet of vegetables, fresh fruits, cereals, and the abundant use of extra virgin olive oil guarantees an adequate intake of fibre, vitamins C, D, E, β-carotene, minerals, a-linolenic acid, magnesium, selenium, glutathione and beneficial bioactive compounds such as polyphenols and antioxidants that seem to play a key role in the pathogenesis of respiratory disease [4, 23, 24].

Although the evidence from epidemiological studies regarding fish intake and the Mediterranean diet in childhood asthma is promising, there is lack of clinical trials. This highlights the need to obtain high-level evidence before considering any global public health strategies. The objective of this RCT is to test the primary hypothesis of whether a Mediterranean diet enriched with fatty fish improves pulmonary function (FEV_1) and reduces asthma symptoms in children. And secondly, if there will be a decrease in bronchial inflammation (eNO), reduction in medication use and need for medical care, better asthma control, improved quality of life and higher adherence to the Mediterranean dietary pattern. To better understand the mechanisms that underlie how dietary changes may modify the risk of asthma, we will use metabolomics to evaluate metabolic profile including plasma fatty acid composition (EPA, DHA), organic acids, Vitamin D, and inflammatory markers.

2. Materials and Methods

2.1 Experimental Design

Preparation and reporting of this RCT has been conducted according to the SPIRIT (Standard Protocol Items: Recommendations for Interventional Trials) guidelines [25] (Appendix).

This is a parallel randomized controlled dietary intervention trial of six months duration in children with mild asthma. Details of this clinical trial are available at the Australian and New Zealand Clinical Trials Registry (www.ANZCTR.org.au/ACTRN12616000492459p) which will be updated as required. This study was conducted according to the principles of the declaration of Helsinki (1989). Protocol approval was granted from La Trobe University, Human Ethics Committee Australia (Approval no: HEC 16-035).

Pulmonary function and asthma symptoms will be measured using spirometry (FEV_1, FVC, FEV_1/FVC, PEF, FEF 25-75%) [25, 26], exhaled Nitric Oxide analysis (eNO) [27] and Asthma Control questionnaire (ACQ) [27, 28]. The primary outcome will be assessed using Forced Expiratory Volume in 1 sec (FEV_1) [25, 26].

A pilot study will be undertaken in a small representative sample of children with asthma in order to assess feasibility, detect any ambiguity or language problems in the content of the questionnaires and to estimate the time required for a full-scale RCT to respond to these questions. Due to children's age range (5-12 y.o), questionnaires will be completed jointly by children and parent(s)/or caregiver(s). It is estimated that approximately 15-20 minutes will be required for completion.

2.2 Materials

Children will be recruited from a pediatric asthma clinic in the greater city of Athens, Greece. During routine medical consultation, children will be screened and enrolled by a pediatric respiratory physician. Recruitment will continue until the target population is achieved (at least 32 children per group). Children will be included if they are: 5-12 years of age with physician-diagnosed "mild" asthma as defined by the GINA (Global Initiative for Asthma guidelines) [2] and willing to comply to the dietary intervention including "fatty fish" in the context of a Mediterranean diet for a period of six months. According to GINA, "mild asthma" is "well-controlled" asthma. A patient that has day symptoms of not more than twice a week, no night-waking symptoms, need for reliever medication less than twice a week and no activity limitations due to asthma is considered to have "mild asthma" [2]. Children will be excluded if they have severe or chronic asthma [29], are taking oral glucocorticoid medication, food allergies (especially to fish and seafood), taking high-dose multi-vitamins or fish oil supplements, suffering with gastroesophageal reflux disease, cystic fibrosis or congenital respiratory disease [2]. Also excluded will be children that are vegetarians and not willing to modify their diet.

Children will be randomized into two groups. The intervention group will be instructed to consume two fatty fish meals per week (at least 150 g cooked fish) [10] as opposed to "lean fish" as part of the Greek Mediterranean diet over a period of 6 months [20, 30]. The control group will consume their usual diet.

During routine medical consultations parents and children will be informed verbally by the physician and researchers about the purposes and study requirements. As an incentive for participation, pulmonary and biochemical tests will be provided free of charge.

Proxy consent will be obtained from parents/or carers since children are under 12 years old. Potential participants will be officially enrolled by the physician when parents read the information sheet and provide written, informed consent. It will be emphasized to children and parents that participation in this study is voluntary. And at any time that children should refuse to participate or should his/her health deteriorate, participation will be terminated immediately and withdrawn from the study. In the case of adverse effects parents will be instructed to seek medical attention immediately and to inform the pediatric-pneumologist. Under such circumstances, parents will be required to complete a withdrawal notification form as evidence of termination from participation in the study trial and withdrawal of consent for data use in the project. Parents will be assured that all data collected will be confidential, anonymous, coded and used exclusively for the purpose of this study. And that the research findings will be disseminated in a PhD thesis, presented at conferences and published in journals and that neither names nor will any other identifying information be used. As the parents and children will be Greek-speaking all written materials (information sheets, consent/withdrawal forms, questionnaires) have been translated into the Greek language and back-translated by a professional linguistic teacher.

To ensure that the intervention assignment is unbiased, all eligible participants that give consent for participation will be randomized into 2 groups (control versus intervention) with a 1:1 allocation ratio using an internet platform (http://www.randomization.com). This validated system automates the random assignment of patient number to randomization number which is linked to the different intervention arms. Allocation concealment will be ensured until the patient has been recruited into the trial, which will occur after spirometry, eNO, anthropometry measurements and questionnaires have been completed at baseline. A unique identification code based on randomization number/date of birth/gender and intervention group will be assigned to all participants. Due to the nature of this dietary intervention trial, the dietary intervention will not be blinded from the participants, their families or from the dietician.

However, the statistician who will be analysing the data will be blinded to the group allocation.

After randomization and group allocation, participants will be assessed in the following order: anthropometry, spirometry, eNO and finally completion of questionnaires, medical history and biochemical tests. The same procedure will be repeated at the end of 6 months (Table 1).

Children will be assessed using the following tools: Anthropometry will be conducted according to standard protocol [26, 32]. Children's height will be measured to the nearest 0.1 cm using a SECA stadiometer after shoes had been removed and children are positioned in the standard Frankfort horizontal plane. Body weight will be measured to the nearest 0.1 kg on calibrated electronic scales (SECA, Hanover, MD) without shoes and heavy clothing. Three measurements will be taken and the mean value will be recorded and used for analytical purposes. Body mass index [BMI= weight (kg) divided by height squared (m^2)] will be calculated in order to classify weight either as normal, overweight or obese using the cut-off points for BMI for overweight and obesity by sex, as proposed by the IOTF (International Obesity Task Force) for children aged 2-18 years [33]. Children's growth rate will be assessed using the Hellenic Pediatric Growth Charts [34].

Childhood asthma is often characterised by decreased lung function, airway hyper-responsiveness and elevated exhaled nitric oxide [35]. Poorly controlled asthma as indicated by variable expiratory airflow limitation, is associated with greater variability in lung function than well-controlled asthma [2]. According to the Global Initiative for Asthma [2], the gold standard for the evaluation of pulmonary function in children (at least 5 years old) is spirometry [26, 35]. FEV_1 (Forced expiratory volume in 1 second) from spirometry is more reliable than PEF (peak expiratory flow) as an indicator of pulmonary function [26] and for this reason we will use the value of FEV_1 to evaluate lung function in patients. Normal pulmonary function will be considered values of FEV_1 greater than 80% predicted [26, 35] and variation in FEV_1 of 10-12% to be clinically significant in children [2, 26]. In pediatric asthma, eNO (exhaled Nitric Oxide) analysis is a useful prognostic tool in assessing eosinophilic airway inflammation, asthma control, monitoring treatment response and predicting asthma exacerbations [27, 36]. In children no lung inflammation and good asthma control is indicated by eNO values less than 20 ppb [27, 36].

Pulmonary function will be measured by trained technicians using a portable spirometer (MIR Spirobank II, MIR Inc USA) and eNO analyser (NO Breath, Benfont Inc, UK) according to European Respiratory Society protocol [26, 27, 37, 38] at baseline and 6 months. Patients will be requested to abstain from taking their asthma medication at least four hours before the tests. For the purpose of this study we will use values of FEV_1 and eNO pre-bronchodilator administration.

The dietary intervention is based on the Greek Mediterranean diet [20] as recommended by the Hellenic Ministry of Health and Welfare (1999) [30] and evidence on the beneficial effect of fatty fish intake in childhood asthma [10, 39]. At both time-points a qualified dietician will issue parents an envelope containing contact details, information regarding biochemical examinations, intervention requirements and a self-administered questionnaire. For children younger than 12 years of age, questionnaires will be completed by parents. Participants in the intervention group will be issued a pamphlet explaining the various types of fatty fish available in the marketplace such as salmon, sardines, anchovies, mackerel, trout and amount of fatty fish to be consumed (frozen and cooked) at each meal, as well as lean fish to be excluded from consumption over the study period. It will be emphasized that fatty fish only will be consumed over the six-month period and not lean fish. To reinforce compliance to the intervention, the intervention group will be issued a poster as a reminder to consume two meals of fatty fish per week as part of the Greek Mediterranean diet pyramid. In addition, a table will be given for the recording of the two days per week that fatty fish is consumed by children including the amount (grams) and type of fish that will be e-mailed or faxed to the dietician on a monthly basis. In comparison, the control group will be instructed to consume their usual diet and will be provided with advice on general healthy dietary guidelines according to the Hellenic Ministry of Health and Welfare (1999) [30].

During enrolment week a telephone interview will be conducted by the research dietician to collect information regarding the participant's medical history, medicine use and dietary habits using a 24 hour dietary recall and KIDMED test. In order to ensure program efficacy, dietary requirements for each group will be recapitulated to parents. In addition, parents will be informed that blood and urine tests must be completed within ten days of enrolment into the study and within ten days after the medical consultation at the end of six months.

To assist retention to the intervention two supermarkets are willing to provide economically disadvantaged families with coupons for the purchase of fish. During this six-month study period, participants in both groups will be monitored fortnightly by the research dietician via telephone, e-mails, text and face-to-face consultation. During these sessions, the characteristics and importance of adherence to the Traditional Greek Mediterranean diet due to health benefits will be explained and repeated regularly to parent's in both intervention groups.

A composite questionnaire will be used to collect data on socio-demographics, asthma control, quality of life, dietary habits and medical history. Socio-demographic information will include contact details and information pertaining to dwelling area, postcode, parents' ethnicity, race, marital status, employment, education level, family income, number of siblings, birth rank of participant in the family and type of school attended. Medical history will include details on parent's smoking habits, parental allergy during childhood and in adulthood, pregnancy and breast-feeding duration, participant's birth weight, participant's age of asthma onset and related allergies including food allergy. Medicine and nutritional supplement use will also be collated.

According to GINA guidelines, the ACQ (Asthma Control Questionnaire) is a validated questionnaire to evaluate asthma control in pediatric patients [2, 28].

The ACQ is a short and easy self-administered questionnaire for children aged 6-12 years. For young children parents will be instructed to assist children during completion of the questionnaire. More specifically, the questionnaire consists of 7 items that assess the presence of symptoms, medication use and pulmonary function % FEV1 predicted during the past 7 days. Responses for the first 6 questions are based on a 7-point scale (0 = totally controlled to 6 = extremely poorly controlled). The 7th question, regarding spirometry measurement FEV_1, is completed by the clinic staff. A final score is the mean of the responses to the 7 questions. A score < 0.75, indicates no manifestation of symptoms, no activity limitations, no rescue bronchodilator use and an FEV_1 % > 95% and is considered as having "totally-controlled" asthma. On the other hand, a score of ≥ 1.5 indicates "extremely poorly controlled" [28, 40].

Asthma control is the principal aim of asthma management. Uncontrolled asthma decreases the quality of life, increases risk of exacerbations and mortality as well as increased costs due to need for medication and admissions to hospitalization [41]. Children's quality of life will be measured using the validated mini PAQLQ (Pediatric Asthma Quality of Life Questionnaire) which was developed by Juniper et al. [42] for asthmatic children aged 6-16 years. The mini PAQLQ is a concise version of the original PAQLQ questionnaire and consists of 13 items which measure physical, emotional and social problems experienced by children with asthma [42]. Children are asked to recall their experiences during the past week and to respond to each question on a scale from 1-7. (1 = severe impairment; 7 = no impairment). The overall score is the mean of the 13 responses [28]. The mini PAQLQ is considered to be a reliable responsive measuring tool which is applicable for long-term monitoring in clinical trials [43]. Both the ACQ and the PAQLQ are available in Greek translations.

Children's dietary habits will be assessed using a questionnaire which includes information on the breakfast meal and FFQ (Food Frequency Questionnaire) that is based on the validated semi-quantitative PANACEA-FFQ for Greek children 10-12 years old [31]. Since children are younger than 12 years, parents will be used as surrogates to evaluate children's dietary intake. In addition, multiple 24 hour dietary recalls will be used to validate information recorded in FFQs that will be administered during three telephone interviews conducted on two weekdays and one weekend [44] at baseline, 3 months and 6 months.

Adherence to the Mediterranean dietary pattern will be assessed using the KIDMED Index which is a 16-item test that has been developed specifically for Spanish children and adolescents [45]. This index is based on the principles of the Mediterranean dietary pattern as well as factors which are contrary and detrimental to health. The KIDMED score ranges from 0-12. Questions describing dietary habits which are contrary to the Mediterranean dietary pattern are assigned a score of -1, whereas habits favouring this eating pattern are given a value of +1. Individual scores are summed to produce a final score. A score of 0-3 reflects low Mediterranean diet, 4-7 improvement needed and 8-12 optimal Mediterranean diet [45].

Physical activity participation will be evaluated using the question from the ISAAC (International Study of Asthma and Allergies in Childhood) phase 3 environmental questionnaire [46]. Any missing data will be retrieved during telephone interviews.

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Table 1 Schedule of enrolment and assessments at each time-point[2]

		Time-points		
	Description	Baseline	3 Months	6 Months
Enrolment/Screening				
Eligibility Screening questionnaire	Inclusion/exclusion criteria	✓		
Information sheet	Study description and intervention	✓		
Informed consent	Written consent	✓		
Randomization		✓		
Group allocation				
Assessment:				
Anthropometry	Height, weight, BMI, gender	✓		✓
Spirometry	FEV_1, FVC, FEV_1/FVC, PEF, FEF 25-75%	✓		✓
Exhaled Nitric Oxide analysis	eNO (ppb)	✓		✓
Questionnaires:				
Socio-demographics	Parents' history: ethnicity, SES, employment status, education level; number of siblings, family rank	✓		
ACQ	Validated 7-item child Asthma Control Questionnaire	✓		✓
PAQLQ	Validated 13-item mini Pediatric Asthma Quality of Life			
Dietary Habits:	Questionnaire	✓		✓
FFQ	Food Frequency Questionnaire[1]	✓		✓
KIDMED Test	Mediterranean diet adherence tool for children and adolescents			
24-hour Dietary Recall	Dietary intake	✓		✓
Medical History	Parents information (parental atopy, smoking status), child's birth weight, delivery mode, breast-feeding, presence of other allergies other than asthma (rhinitis, eczema), food allergy, medication use, physical activity level	✓	✓	✓
Biochemical testing: Blood/urine samples	Plasma fatty acid composition, nutrient status, inflammatory markers	✓		✓

Key: ✓—Assessments undertaken at each time-point; ACQ—Asthma Control Questionnaire; PAQLQ—Pediatric Asthma Quality of Life questionnaire; eNO—Exhaled Nitric Oxide; FFQ—Food Frequency Questionnaire. FEV_1—Forced Expiratory Volume in 1 second; FVC—Forced Vital Capacity; FEV_1/FVC—ratio of Forced Expiratory Volume and Forced Vital Capacity, PEF—Peak Expiratory Flow, FEF 25-75%—Mid Expiratory Flow; SES—socioeconomic status.

[1]Food Frequency questionnaire is based on the validated (PANACEA) FFQ for Greek children, 10-12 years [31].

[2]This figure has been prepared according to SPIRIT guidelines [25].

Metabolomics is a rapidly growing field in medicine which is based on the concept that an individual's metabolic state provides a close representation of the individual's overall health status [47, 48]. The purpose of using metabolomics in our clinical trial is primary to measure nutrient status [49], compliance to the dietary intervention and to determine metabolic profiles specific to children suffering with mild asthma. Secondly, to discover imbalances and deficiencies of metabolites in the disease process that is influenced by dietary factors and can be resolved using personalized nutritional therapy. Participants will undergo biochemical testing at baseline and at the end of six months at a private metabolomics laboratory in the city of Athens, Greece. All specimens will be prepared, analysed and stored at this clinic. Blood and urine samples will be collected and assessed to validate dietary information from FFQs, 24-hour dietary recall, KIDMED test and to assess compliance to the intervention. More specifically, plasma fatty acid composition (omega 3 fatty acids, omega 6 fatty acids, EPA, DHA) Vitamin D, nutritional status and inflammatory markers will be evaluated at both time-points.

2.3 Statistical Analysis

Sample size and power calculations were estimated based on FEV_1. Using G Power Statistical Analysis (version 3.1) [50] we estimate that for a two-tailed analysis with an alpha of 0.05 and power of 90% we will require a sample size of fifty two (52) participants in order to detect a statistically significant difference in mean FEV_1 between the groups. Allowing for a 20% drop out rate gives a final sample size of sixty four (64) participants, which is thirty two (32) per group. Data analysis will be conducted using SPSS, version 20 (IBM, Chicago, IL). Continuous variables will be assessed for normality using descriptive statistics, graphical methods and Shapiro-Wilks statistical test. Differences between the intervention groups will be compared using T-test for normally distributed variables and Mann-Whitney test in the case of non-normality. Categorical variables will be examined using Chi-squared test. *P*-value will be considered to be statistically significant at the 5% level. In the case of missing values, values will be replaced using data imputation.

All records that contain names and medical data including spirometry, eNO, biochemical assessment and questionnaires will be stored in a safe at the asthma clinic in the care of the chief investigator. Electronic data will be coded and saved in a password secured database by the key researcher at the study site. Data saved in software discs will be kept safe in a password protected file. At a later date, coded data will be transferred and stored at La Trobe University, School of Allied Health Research drive. Only the study investigators will have access to the cleaned data sets. To ensure confidentiality, data dispersed to project investigators will be blinded of any identifying participant information

3. Results and Discussion

The universal abandonment of healthy dietary habits by children including in Mediterranean countries [51, 52] and adoption of a Westernized diet highlights that poor diet is a global problem which might, in part, explain the surge in pediatric asthma [4]. Perhaps the considerable morbidity related to asthma may be ameliorated by addressing modifiable risk factors such as diet [53]. A meta-analysis of eight observational studies was conducted by Garcia-Marcos et al. [54] to assess the influence of Mediterranean diet exposure on asthma prevalence in children. According to the authors, a high Mediterranean diet score was associated with lower occurrence of "current wheeze" (OR: 0.85; 95% CI 0.75-0.98; $p = 0.02$) and "severe wheeze" OR: 0.66; 95% CI 0.48-0.90; $p = 0.008$) mainly in Mediterranean regions and "asthma ever" (0.86, 0.78-0.95, $p = 0.004$) in both Mediterranean and non-Mediterranean countries.

Fish a component of the Mediterranean diet, is a rich source of n-3 PUFA, EPA and DHA fatty acids that are able to inhibit the metabolism of n-6 PUFA, arachidonic acid and interrupt the inflammatory process [39, 55]. EPA and DHA are substrates for less potent prostaglandins, leukotrienes and thromboxanes than arachidonic acid, thereby potentially acting to reduce airway inflammation that is a characteristic of asthma [39, 55]. Therefore, sufficient omega 3 fatty acids in the diet is critical for proper prostaglandin metabolism and deficiencies in omega 3 fatty acids would suggest higher levels of inflammation in the lungs would be present. By these anti-inflammatory mechanisms omega 3 fatty acids may improve lung function and achieve better asthma control. Based on the promising data documented above, the intention of this present clinical trial is to provide first-level evidence on the potential of Mediterranean diet and dietary n-3 fatty acids as an adjunct therapy in the management of childhood asthma. . Dietary guidelines can be safely issued when consistency is found between observational and experimental studies.

Relating dietary patterns to disease outcome within a population is a new approach in nutritional epidemiology as compared to isolated nutrients or food. Since a child's dietary pattern consists of a variety of food groups and nutrients in each meal, it is deemed appropriate that this intervention trial should focus on the assessment of a dietary source of omega 3 fatty acids (fish) within a specific dietary pattern [56]. The Mediterranean diet consists of an array of food groups providing a constellation of antioxidants, vitamins, minerals, mono-unsaturated fatty acids, fibre, n-3 PUFA as well as optimal n-6: n-3 PUFA ratio that might act synergistically promoting beneficial effects on respiratory function [21]. The results of this study are important in establishing a cause-effect relationship between Mediterranean diet enriched with fatty fish in pediatric asthma. This study will fill in the gap in long-term studies on asthma symptom management using dietary approaches.

This study is novel for a number of reasons. It is the first RCT to investigate the effectiveness of an overall dietary pattern (Mediterranean diet) and fatty fish intake in children with pre-existing asthma. Another strength of this study is the randomized design, the objective assessment of asthma symptoms using spirometry and eNO analysis as well as metabolomics for measuring intervention compliance, nutrient status and in determining a metabolic profile for asthmatic children. In addition, the validated questionnaires are used to measure asthma control, quality of life and dietary habits. The advantage of using fish as opposed to fish oil supplementation is that it is part of the traditional eating habits, palatable, readily available in supermarkets, easily incorporated into the family diet, requires little preparation time and cooking skills and has no side-effects. In contrast, fish oil tablets may cause stomach discomfort, burping, and nausea and unlike fish do not generally improve diet quality [57]. Another strength of the present trial is the close monitoring and continual support provided to participating families by a qualified dietician.

A possible weakness of this study might be study duration, as a longer time period covering all four seasons throughout the year might be required to improve pulmonary function. Also, difficulty in changing long-established dietary habits of families exists such as increasing the consumption of fruit and vegetables. Bearing in mind the economic crisis in Greece, for some families the purchasing of fish may have a financial impact. Nevertheless, any benefits shown from the trial will outweigh the costs to both the individual, family and the health budget. Regarding future implications, clinical trials are warranted in non-Mediterranean settings to investigate whether a Mediterranean-type diet enriched with fatty fish translates to lower asthma symptoms in children.

4. Conclusions

Diet is a modifiable risk factor that can be targeted in the prevention and management of asthma. Due to the lack of evidence-based clinical intervention data, currently there are no dietary recommendations for the management of pediatric asthma. Findings from observational studies suggest that adherence to a Mediterranean-type diet and consumption of fish may reduce risk and symptoms of asthma in children but no clinical trials have been conducted. This clinical trial is important as it will be the first to establish whether a dietary source of omega 3 fatty acids in combination with the Mediterranean dietary pattern can be used as an adjunct therapy in the management of childhood asthma. This has significant future implications for public health, because such a dietary intervention is practical, non-invasive, cost-effective, promises long-term health benefits and could contribute to a decrease in asthma burden.

Conflicts of interest

The authors declare no financial interests or potential conflicts of interest.

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Contributions

MP has devised the protocol, study design and is the principal author of the paper. CI is the principal investigator and CK, DT and MP co-investigators. CI and BE co-authored the study and BE is responsible for the statistical analysis. All co-authors declare that we have seen and approved the final version of the manuscript being submitted. The authors confirm that the article is the authors' original work, has not received prior publication and is not under consideration for publication elsewhere.

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Appendix

SPIRIT 2013 Checklist: Recommended items to address in a clinical trial protocol and related documents*

Section/item	Item No	Description	Addressed on page number
Administrative information			
Title	1	Descriptive title identifying the study design, population, interventions, and, if applicable, trial acronym	1
Trial registration	2a	Trial identifier and registry name. If not yet registered, name of intended registry	2
	2b	All items from the World Health Organization Trial Registration Data Set	2
Protocol version	3	Date and version identifier	2
Funding	4	Sources and types of financial, material, and other support	17-18
Roles and responsibilities	5a	Names, affiliations, and roles of protocol contributors	1, 17
	5b	Name and contact information for the trial sponsor	N/A
	5c	Role of study sponsor and funders, if any, in study design; collection, management, analysis, and interpretation of data; writing of the report; and the decision to submit the report for publication, including whether they will have ultimate authority over any of these activities	N/A
	5d	Composition, roles, and responsibilities of the coordinating centre, steering committee, endpoint adjudication committee, data management team, and other individuals or groups overseeing the trial, if applicable (see Item 21a for data monitoring committee)	14
Introduction			
Background and rationale	6a	Description of research question and justification for undertaking the trial, including summary of relevant studies (published and unpublished) examining benefits and harms for each intervention	2-5
	6b	Explanation for choice of comparators	6
Objectives	7	Specific objectives or hypotheses	4
Trial design	8	Description of trial design including type of trial (eg, parallel group, crossover, factorial, single group), allocation ratio, and framework (eg, superiority, equivalence, non-inferiority, exploratory)	4,7
Methods: Participants, interventions, and outcomes			
Study setting	9	Description of study settings (eg, community clinic, academic hospital) and list of countries where data will be collected. Reference to where list of study sites can be obtained	5
Eligibility criteria	10	Inclusion and exclusion criteria for participants. If applicable, eligibility criteria for study centres and individuals who will perform the interventions (eg, surgeons, psychotherapists)	5-6
Interventions	11a	Interventions for each group with sufficient detail to allow replication, including how and when they will be administered	6-14
	11b	Criteria for discontinuing or modifying allocated interventions for a given trial participant (eg, drug dose change in response to harms, participant request, or improving/worsening disease)	6
	11c	Strategies to improve adherence to intervention protocols, and any procedures for monitoring adherence (eg, drug tablet return, laboratory tests)	10,13
	11d	Relevant concomitant care and interventions that are permitted or prohibited during the trial	-

Outcomes	12	Primary, secondary, and other outcomes, including the specific measurement variable (eg, systolic blood pressure), analysis metric (eg, change from baseline, final value, time to event), method of aggregation (eg, median, proportion), and time point for each outcome. Explanation of the clinical relevance of chosen efficacy and harm outcomes is strongly recommended	4-6
Participant timeline	13	Time schedule of enrolment, interventions (including any run-ins and washouts), assessments, and visits for participants. A schematic diagram is highly recommended (see Figure)	6-9
Sample size	14	Estimated number of participants needed to achieve study objectives and how it was determined, including clinical and statistical assumptions supporting any sample size calculations	13
Recruitment	15	Strategies for achieving adequate participant enrolment to reach target sample size	6,10
Methods: Assignment of interventions (for controlled trials)			
Allocation:			
Sequence generation	16a	Method of generating the allocation sequence (eg, computer-generated random numbers), and list of any factors for stratification. To reduce predictability of a random sequence, details of any planned restriction (eg, blocking) should be provided in a separate document that is unavailable to those who enrol participants or assign interventions	7-8
Allocation concealment mechanism	16b	Mechanism of implementing the allocation sequence (eg, central telephone; sequentially numbered, opaque, sealed envelopes), describing any steps to conceal the sequence until interventions are assigned	7
Implementation	16c	Who will generate the allocation sequence, who will enrol participants, and who will assign participants to interventions	5, 7-8
Blinding (masking)	17a	Who will be blinded after assignment to interventions (eg, trial participants, care providers, outcome assessors, data analysts), and how	7
	17b	If blinded, circumstances under which unblinding is permissible, and procedure for revealing a participant's allocated intervention during the trial	7
Methods: Data collection, management, and analysis			
Data collection methods	18a	Plans for assessment and collection of outcome, baseline, and other trial data, including any related processes to promote data quality (eg, duplicate measurements, training of assessors) and a description of study instruments (eg, questionnaires, laboratory tests) along with their reliability and validity, if known. Reference to where data collection forms can be found, if not in the protocol	7-13
	18b	Plans to promote participant retention and complete follow-up, including list of any outcome data to be collected for participants who discontinue or deviate from intervention protocols	10,13
Data management	19	Plans for data entry, coding, security, and storage, including any related processes to promote data quality (eg, double data entry; range checks for data values). Reference to where details of data management procedures can be found, if not in the protocol	6-7; 13-14
Statistical methods	20a	Statistical methods for analysing primary and secondary outcomes. Reference to where other details of the statistical analysis plan can be found, if not in the protocol	13
	20b	Methods for any additional analyses (eg, subgroup and adjusted analyses)	-
	20c	Definition of analysis population relating to protocol non-adherence (eg, as randomised analysis), and any statistical methods to handle missing data (eg, multiple imputation)	12,13
Methods: Monitoring			
Data monitoring	21a	Composition of data monitoring committee (DMC); summary of its role and reporting structure; statement of whether it is independent from the sponsor and competing interests; and reference to where further details about its charter can be found, if not in the protocol. Alternatively, an explanation of why a DMC is not needed	-
	21b	Description of any interim analyses and stopping guidelines, including who will have access to these interim results and make the final decision to terminate the trial	-
Harms	22	Plans for collecting, assessing, reporting, and managing solicited and spontaneously reported adverse events and other unintended effects of trial	6

		interventions or trial conduct	
Auditing	23	Frequency and procedures for auditing trial conduct, if any, and whether the process will be independent from investigators and the sponsor	-
Ethics and dissemination			
Research ethics approval	24	Plans for seeking research ethics committee/institutional review board (REC/IRB) approval	5
Protocol amendments	25	Plans for communicating important protocol modifications (eg, changes to eligibility criteria, outcomes, analyses) to relevant parties (eg, investigators, REC/IRBs, trial participants, trial registries, journals, regulators)	5
Consent or assent	26a	Who will obtain informed consent or assent from potential trial participants or authorised surrogates, and how (see Item 32)	6
	26b	Additional consent provisions for collection and use of participant data and biological specimens in ancillary studies, if applicable	-
Confidentiality	27	How personal information about potential and enrolled participants will be collected, shared, and maintained in order to protect confidentiality before, during, and after the trial	6-7; 14
Declaration of interests	28	Financial and other competing interests for principal investigators for the overall trial and each study site	17
Access to data	29	Statement of who will have access to the final trial dataset, and disclosure of contractual agreements that limit such access for investigators	14
Ancillary and post-trial care	30	Provisions, if any, for ancillary and post-trial care, and for compensation to those who suffer harm from trial participation	6
Dissemination policy	31a	Plans for investigators and sponsor to communicate trial results to participants, healthcare professionals, the public, and other relevant groups (eg, via publication, reporting in results databases, or other data sharing arrangements), including any publication restrictions	6
	31b	Authorship eligibility guidelines and any intended use of professional writers	-
	31c	Plans, if any, for granting public access to the full protocol, participant-level dataset, and statistical code	-
Appendices			
Informed consent materials	32	Model consent form and other related documentation given to participants and authorised surrogates	32
Biological specimens	33	Plans for collection, laboratory evaluation, and storage of biological specimens for genetic or molecular analysis in the current trial and for future use in ancillary studies, if applicable	14

Unpublished details of study methodology

Letters of ethics approval for all procedures in this clinical trial from La Trobe University as well as the Australian and New Zealand Clinical Trials Registry (ANZCTR) can be found in Appendix 1. All printed materials issued to participants that include recruitment forms, questionnaires, pamphlets, posters, records, 24-hr food recall, summary of main findings, end-of-study educational materials and letters to collaborators and supermarkets were designed by the candidate and are available in Appendix 2A. A detailed outline of the rationale behind the study design and materials used are available in Appendix 2B. An example of completed questionnaires, output of spirometry and biochemical testing are shown in Appendix 2C.

5.2 Study Procedure

The progress through the phases of the RCT at baseline is outlined in Figure 17.

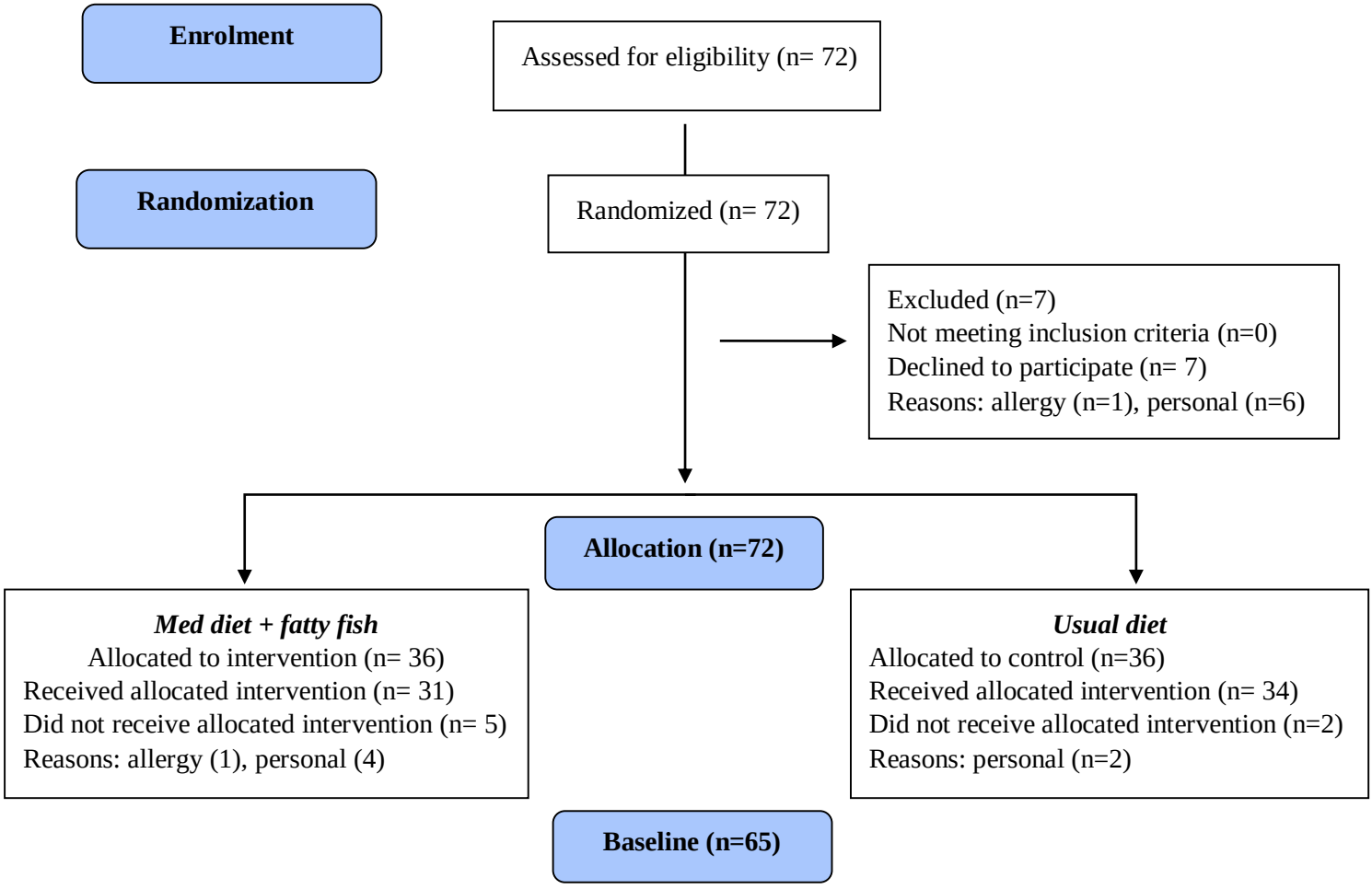

Figure 17 Consort flow diagram of study design

Clinical Assessment and Time-points

Assessments were undertaken at two time-points, baseline and 6 months, except for 24-hour dietary recalls that were administered at three time-points (baseline, 3 months and 6 months) in order to assess adherence to the dietary intervention as well as to capture 'true' dietary intake of participants. It must be noted that all children were assessed during the same season throughout the year (November 2016 to August 2017) to minimize bias. An overview of all the outcome measurements and corresponding time-points can be found in Table 1 of the published protocol paper above in Section 5.1.

Summary of measurements

Table 9 summarizes measurements conducted in this study and acknowledgement of the contributions made by the investigators and PhD candidate.

Table 9 Summary of measurements and contribution by investigators

Measurement	Investigator	Location/mode of administration
Recruitment/screening	Dr. Katsardis	Paediatric asthma clinic
Questionnaires (ACQ, PAQLQ, socio-demographics, dietary habits, physical activity, participant evaluation)	Candidate	Paediatric asthma clinic
Medical history	Candidate	Telephone interview
KIDMED test	Candidate	Telephone interview
24-hr Dietary Recall	Candidate	Telephone interview
Mediterranean diet nutrition education	Candidate	Telephone/ consultation
Anthropometry	Candidate	Paediatric asthma clinic
Spirometry	Dr. Katsardis	Paediatric asthma clinic
FeNO	Dr. Katsardis	Paediatric asthma clinic
Biochemical tests	Dr. Tsoukalas	Metabolomics clinic
Collection of biochemical/ spirometry results	Candidate	Metabolomics clinic; Asthma clinic
Design of SPSS databases	Candidate	
Data entry, statistical compilations*	Candidate	

*All statistical analyses were conducted by the candidate under the supervision of Professor Bircan Erbas.

5.3 Assessment Tools

All written materials required for this project (questionnaires, nutrition education material) were designed in English by the candidate according to the guidelines provided on-line by La Trobe University Ethics Committee. Materials were checked and granted permission by the principle supervisor and by the institution after which they were translated to the Greek language by the candidate who is a native speaker. All documents were checked by a linguistics expert and back-translated, afterwhich hard copies were printed by the candidate. One month prior to the study commencement all written material were seen by the collaborating physician and approved to be used for the study. Two weeks before recruitment of participants into this study, the candidate delivered all questionnaires to the medical clinic. The enrolment procedure as well as questionnaires were explained by the candidate to the physician and staff, then distributed to eligible participants.

5.4 Anthropometry

Hellenic Paediatric Growth Charts

Anthropometric variations exist between populations due to genetics and environmental factors, hence population-specific growth charts might provide a more accurate estimation of children's growth status [310]. With respect to infant feeding practices, in Greece, with more mothers in the work-force, formula-feeding as opposed to breast-feeding is a more popular option [293]. For this reason it was deemed by the Institute of Child Health and the Hellenic Ministry of Health, that national growth charts are more appropriate for use in this population rather than WHO growth charts that are based on healthy breast-fed infants [311]. Application of the Hellenic Pediatric Growth Charts using real data for a participant in this RCT is shown in Appendix 2B, Section 5.6 (Figure 5).

5.5 Pulmonary function tests and Pulse Oximetry

A detailed description on performing spirometry, FeNO and pulse oximetry in children is available in Appendix 2B, Sections 5.7- 5.9.

5.6 Questionnaires

The rationale that formed the basis for the design of the questionnaires including a complete description is available in Appendix 2B, Sections 5.10-5.14.

5.7 Nutritional Biomarkers

Biochemical testing, nutritional biomarkers and laboratory procedures are explained in Appendix 2B, Sections 5.15-5.17.

5.8 Measuring dietary intake in children

5.8.1 Portion size determination for fish intake

Rationale

Up to now, there did not exist a consensus for frequency of consumption and portion size of food groups for children in Greece. Recommendations for child nutrition were based on the Hellenic dietary guidelines for adults as stated by the Hellenic Ministry of Health and Welfare in 1999 [(294)]. Recently, new dietary guidelines on healthy eating for adults, children and adolescents have been proposed by the Institute of Preventive Medicine, Environmental and Occupational Health (PROLEPSIS, 2014) [(293)]. Apart from promoting the Traditional Greek Mediterranean Diet, nutritional recommendations include frequency of consumption of food groups as well as portion size according to age groups. Given the nutritional value and health benefits of fish consumption, the new guidelines suggest that children and adolescents should consume a variety of fish and seafood 2-3 times weekly including fatty fish at least once a week [(293)]. Portion size per meal according to age group is presented in Table 10.

Table 10 Recommended fish intake for children based on the Hellenic dietary guidelines for children and adolescents

Recommended fish intake for children according to the Hellenic dietary guidelines for children and adolescents (2014)					
Age	1-2 years	2-3 years	4-8 years	9-13 years	14-18 years
Meal frequency	2 serves/week	2 serves/week	2-3 serves/week	2 -3 serves/week	2 -3 serves/week
Portion size*	60 g	60-90 g	90-120 g	120-150 g	150 g

**Portion size refers to cooked weight without bones*

Reference: PROLEPSIS, 2014. National dietary guidelines for infants, children and adolescents. Available from http://www.diatrofikoiodigoi.gr/?Page=diatrofikoi-odigoi-paidia

Methods

The amount and frequency of fatty fish consumed by the intervention group, at least 150 grams cooked filleted fish per meal twice weekly for a period of six-months was consistent with the Hellenic dietary guidelines for children and adolescents 5-12 years old [(293)]. Two months before the commencement of this clinical trial the candidate purchased various types of fresh fatty fish commonly sold in Greek fish markets and supermarkets (Basilopoulos and Sklavenitis) such as sardines, trout, anchovies, gilthead sea-bream, chubb mackerel, and salmon. Before cooking, each type of raw fish was weighed, then grilled or boiled. After they were deboned and reweighed in order to determine the amount of fresh fish (g) equivalent to 150 g cooked fish. Due to the economic crisis only affordable reasonably priced fish either fresh or frozen were suggested to families in the intervention group. In general, the cost of small fish (sardines, anchovies, chubb mackerel) ranged from 3-6 € per kilo, trout at 6 €/ kilo, gilthead sea bream at 8-9 €/kilo and salmon at 15 €/ kilo. It was estimated that, for each participant approximately 700 g of raw fatty fish was required on a weekly basis, costing about 4-5 € per week for the small fish and approximately 10 € for salmon. In order to educate the intervention group about the types of fish classified as 'fatty fish' and 'lean fish' not to be consumed over the study period, a pamphlet was designed illustrating the types of fatty fish available in the Greek market and the amount of raw fish equivalent to 150 g of cooked filleted fish as well as the types of lean fish not to be consumed during the six-month study period (Appendix 2A, 11.0 a/b). For example, 10-12 sardines and 350 g raw trout, were equivalent to approximately 150 g cooked and deboned.

Canned tuna was not recommended for consumption by children under 16 years due to low omega-3 fatty acid content, added oil, salt and preservatives that could cause an allergic reaction [(312)].

Enhancing intervention adherence- Fatty fish poster

As a reminder for parents to incorporate fish meals into the family menu, a poster was designed by the candidate illustrating the Greek Mediterranean Diet Pyramid [4] and a cartoon image of a physician dictating that participants should consume two fatty fish meals per week (Appendix 2A, 12a/b). It was culturally relevant to include the physician in the poster to reinforce to participants the importance of adhering to the dietary instructions (Figure 18). Also indicated in the poster was that 350 g serve of fresh fish is equivalent to 150 g cooked fillet. This poster was deliberately illustrated in bright colours so that it would be noticeable and plastic coated for durability. It was suggested that it should be placed in a visible place in the family kitchen such as adhered to the refrigerator.

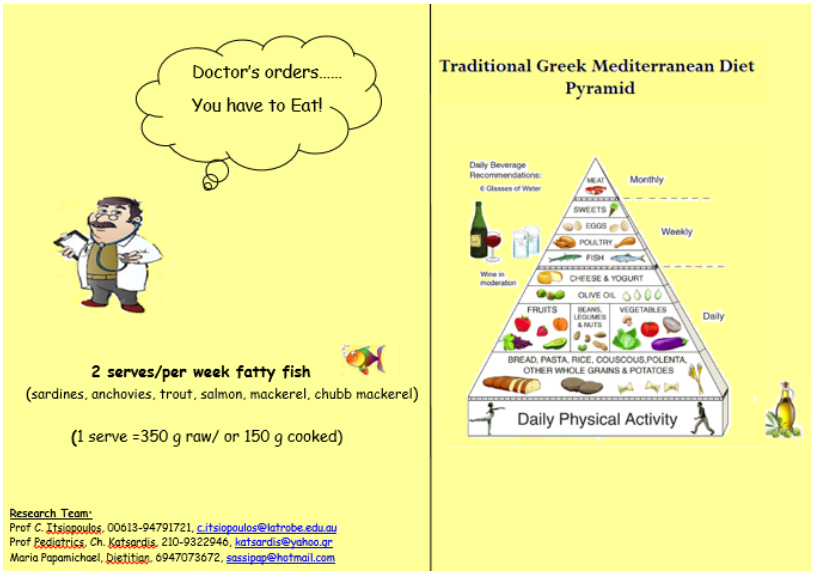

Figure 18 Fatty fish poster

This poster depicts a physician dictating to participants to consume two serves of fatty fish weekly (150 g cooked fish) as part of the Traditional Greek Mediterranean Diet.

With regards to meal planning, the candidate advised parents to allocate two days per week for the consumption of fish in the family menu, for example every Wednesday and Saturday. To enhance and monitor compliance, parents were issued a table to record on a weekly basis the two days that participants consumed fish, the type of fatty fish and amount per meal (Appendix 2A, 13 a/b). This table was scanned and forwarded to the

candidate via e-mail, fax or mobile on a monthly basis and collected at the end of the six month study period.

To summarize, at the end of the baseline medical appointment parents/guardians in the intervention group were issued a white envelope that contained:

1. Fatty fish pamphlet
2. A table to record weekly fatty fish intake
3. Fatty fish poster
4. Instructions for blood tests and candidate's contact details
5. Advice on the Traditional Greek Mediterranean dietary guidelines

In comparison, the control group received in a yellow envelope:

1. Instructions for blood tests and candidate's contact details
2. Advice on the the Hellenic dietary recommendations for children/adolescents which coincide with the Traditional Greek Mediterranean Diet pyramid.

5.8.2 Dietary Habits Questionnaire

During usual medical appointments, each participant was allocated a time limit of 30 minutes for all assessments. Bearing in mind the time restriction and after discussion with the research team, it was decided that a concise targeted FFQ should be designed for the purpose of this study. Short targeted FFQs have been used in other intervention [291; 313; 314] and epidemiological studies including ISAAC and the NHANES (2009-2010) study [315; 316] to gather information regarding eating habits in children and adolescents. Hence, a concise 31-item dietary habits questionnaire was developed which retrieved information on the breakfast meal, fast-food consumption, type of oil used in cooking and included a short FFQ. Details regarding breakfast meal habits, fast food intake and use of olive oil in cooking were evaluated by the following four questions of the 31-item dietary habits questionnaire (Appendix 2A, 14a/b)

Q1. *Does your child consume more than once a week fast food or eat out at fast food restaurants (e.g Goody's)?*

YES □ *NO* □

Q2. How many times during the week does your child consume breakfast?

0 □ 1 □ 2 □ 3 □ 4 □ 5 □ 6 □ 7 □

Q3. If YES your child does consume breakfast, does he/she consume any of the following foods and how many times per week?

Breakfast cereals or bread or rusks or toast *YES* □ *NO* □ *times per week …….*

Milk products (milk, yogurt, cheese) *YES* □ *NO* □ *times per week …….*

Pies/ croissants/biscuits/cookies or cake *YES* □ *NO* □ *times per week …….*

If NO, what does your child usually eat for breakfast and how many times per week?

……

Q4. Do you use olive oil in cooking, for frying and or add to rice or pasta?

YES □ *NO* □

If NO, what do you usually use? ……..

The PANACEA FFQ

The 27-item FFQ was designed based on the validated child questionnaire from the Physical Activity, Nutrition and Allergies in Children Examined in Athens (PANACEA) study [317]. The PANACEA study was a large epidemiological study designed to evaluate possible childhood asthma associations with several environmental factors including diet. The PANACEA FFQ was a semi-quantitative FFQ which gathered information on daily or weekly intake of 63 foods and beverages usually consumed by children and adolescents in Greece and are part of the Traditional Greek Mediterranean diet, fast-food and snacks consumed outside of the home including school-canteens, the cooking method usually used, type of oils and fats along with meal behaviour patterns. More specifically, the FFQ included frequency of consumption (per week and per day) of a variety of foods that included fish, poultry, dairy products, breakfast cereals, red meat, meat products, eggs, white bread, whole grain bread, potatoes, rice, fruits, fruit juice, legumes, vegetables, soft drinks, low-calorie soft drinks, beverages (tea, chamomile) and traditional Greek cooked meals, salty snacks, sweets and fats used in cooking. Weekly intake of the breakfast meal was assessed (in times per week) and by a closed-type question with eight possible responses: milk, yogurt, cereals, fruit juice, honey/marmalade, bread/toast, butter/margarine and cake. Typical serving sizes of the aforementioned food and beverages were standard units of measurements. Information regarding frequency of intake was never, daily, weekly and monthly basis [317].

The Modified 27-item semi-quantitative FFQ

The 63 food items in the original PANACEA questionnaire were grouped to form 27 questions relating to individual foods and food groups (Appendix 2A, 14a/b). The consumption patterns for the following 27 food items/groups assessed were: milk, chocolate milk, yogurt, cheese (white and yellow), cereals (bread, breakfast cereal, rusks), fruit, fruit juice, boiled vegetables (broccoli, cauliflower, marrows, spinach, wild greens, antiva, silver beet, beetroot), raw salads (lettuce, rocket, cabbage, carrots, tomato, cucumber), ladera (stewed vegetables in tomato sauce), legumes, pasta, rice, white meat, red meat, lean fish (2% fat), fatty fish, seafood (calamari, prawns, oysters, mussels, octopus), margarine, nuts, olive oil, fast food (hamburger, souvlaki, pizza, hot

dog), salty snacks (chips, popcorn), pies (cheese pie, spinach pie), sweets (cream cakes, cookies, biscuits, cake, Danish pastries, ice-cream, milkshake, chocolate), soft drinks including energy drinks along with composite meals common in the Greek Mediterranean diet (mousaka, pastitsio, cabbage rolls, spaghetti bolognaise). Frequency of consumption of foods was based on daily, weekly or monthly. Possible responses were: never or rarely; 1-3 times per month, once a week; 2-3 times per week, 4-6 times per week, once a day, 2-3 times per day, ≥ 4 times per day. Quantity of food consumed was assessed by usual portion sizes that were clearly indicated for every food/drink item in the FFQ and represented by household measuring tools (1 cup, ½ cup, 1 tablespoon, 1 teaspoon) that were made available at the asthma clinic. For example, one serve was considered to be one cup (250 ml) of milk, 150 g portion of cooked meat/ fish or chicken and one souvlaki by 1 medium-sized pitta with 1 skewer of meat.

Food intake calculations

Daily consumption of 27 food items as grams per day was assessed from FFQs at both time points. Based on participants' responses, the reported frequency of consumption of the FFQ items were converted to times per day as follows:

Frequency	Conversion factor (times per day)
Once per week	0.14
2-3 times per week	0.36
4-6 times per week	0.71
1-3 times per month	0.07

Then daily intakes of all food items in grams per day were calculated by multiplying the standard portion size by the value corresponding to each consumption frequency as times per day. For example, if participant (ID 1) consumed cheese 2-3 times per week and the standard portion size was 30 g, then multiplying 0.36 by 30 g would give 10.8 grams of cheese per day. The same procedure was undertaken for each participant and food item listed in the FFQ. In addition, foods with similar nutritional properties were classified into food groups. Twelve main food groups (dairy products, fruit, vegetables, legumes, starch, meat, seafood, fish, sweets, fast food, savoury snacks and fats) were formed reflecting a dietary pattern usually followed by the population. For example the savoury group was calculated by combining data (in grams per day) for salty snacks and

pies. Similarly, the fish group consisted of fatty and lean fish. Finally, the mean and standard deviation for each food item and food group per intervention group were estimated at both time-points. Daily intakes of energy and nutrient analysis of all food items and groups were derived using nutrient data from McCance and Widdowson's (2010) and Trichopoulou (2004) [318; 319]. A total of 39 nutrients (water, carbohydrates, protein, fat, energy, fibre, saturated fat, monounsaturated fat, polyunsaturated fat, EPA, DHA, trans fat, cholesterol, sodium, potassium, calcium, magnesium, phosphorus, iron, copper, zinc, chloride, manganese, selenium, iodine, retinol, carotene, vitamin D, vitamin E, thiamine, riboflavin, niacin, tryptophan, vitamin B6, vitamin B12, folate, pantothenate, biotin and vitamin C) were examined.

5.8.3 Assessment of Mediterranean diet quality in children

As yet, no tool has been designed to assess the degree of compliance to the Mediterranean diet for Greek children or adolescents. In most paediatric studies, the Mediterranean diet score developed by Trichopoulou [297] and Psaltopoulou [320] [321; 322; 323; 324] for adults as well as the KIDMED score have been used [325; 326].

The KIDMED Index is a Mediterranean diet quality index constructed exclusively to evaluate the food habits and degree of adherence to the Mediterranean dietary pattern of a population of 3850 Spanish children and adolescents aged between 2–24 years in the Enkid study [242; 313]. The KIDMED questionnaire is a concise, simple 16-item test that is based on the principles that sustain the Mediterranean dietary pattern as well as factors which hinder its adherence (Appendix 2A, 15a/b). Questions describing dietary habits that are detrimental and contrary to the Mediterranean dietary pattern are assigned a score of -1, whereas habits coinciding, are given a value of +1. Individual scores are then added together to give a final total score. The theoretical score ranges from 0-12 points. A score of 0-3 reflects poor adherence to the Mediterranean dietary pattern; 4-7 improvement needed and 8-12 optimal adherence [242].

More specifically, the KIDMED questionnaire presumes a daily consumption of at least 1 serving of fruit and vegetables. The recommended servings of dairy products are at least 3 servings daily: one dairy product for breakfast and at least two servings of yogurt and /or cheese during the rest of the day. Consumption of grains and cereals is recommended daily for breakfast, while pasta and rice should be consumed at least 5

times per week. A weekly consumption of at least 2-3 servings of nuts and fish, and two servings of legumes are suggested. Dietary behaviours considered to be detrimental and not part of the principles of the Mediterranean diet include the frequent intake of sweets and lollies (more than twice daily), commercially baked goods and pastries (for breakfast), fast-foods and breakfast skipping (Table 11).

Table 11 Assessment of Mediterranean diet quality in children

KIDMED Questionnaire	
Score	Question
+1	Takes a fruit or fruit juice every day
+1	Has a second fruit every day
+1	Has fresh or cooked vegetables regularly once a day
+1	Has fresh or cooked vegetables (more than once a day)
+1	Consumes fish regularly (at least 2–3 times per week)
-1	Goes more than once a week to a fast-food (hamburger) restaurant
+1	Likes pulses and eats them more than once a week
+1	Consumes pasta or rice almost every day (5 or more times per week)
+1	Has cereals or grains (bread, etc.) for breakfast
+1	Consumes nuts regularly (at least 2–3 times per week)
+1	Uses olive oil at home
-1	Skips breakfast
+1	Has a dairy product for breakfast (yoghurt, milk, etc.)
-1	Has commercially baked goods or pastries for breakfast
+1	Takes two yoghurts and/or some cheese (40 g) daily
-1	Takes sweets and candy several times every day

KIDMED categories:

Poor adherence ≤ 3; Need improvement 4-7; Optimal Mediterranean diet adherence ≥ 8.

Reference: Serra-Majem L, Ribas L, Ngo J et al. (2004) Food, youth and the Mediterranean diet in Spain. Development of KIDMED, Mediterranean Diet Quality Index in children and adolescents. Public Health Nutr ***7****, 931-935. doi: 10.1079/PHN2004556.*

5.8.4 Multiple 24-hr dietary recalls

Multiple 24-hr recalls were used to evaluate participants' dietary intake, validate information collected in the dietary habits questionnaire and to assess dietary change as the result of the intervention [315]. A total of three 24-hr dietary recalls were administered (baseline, 3 months and 6 months) on two week-days and one weekend in order to capture day-to-day and seasonal variation (winter and summer) (Appendix 2A, 16 a/b). Dietary recalls consisted of an open-ended response structure to prompt parents/care-takers in providing a comprehensive and detailed report of all foods and beverages consumed by the participant over the past 24 hours as well as to describe cooking preparation methods, type of foods consumed (e.g white or wholemeal bread) and portion size. In order to capture a more accurate picture, parents and siblings eating habits were also queried. An extensive list of prompt questions used during these telephone interviews is available in (Appendix 2A, 16c/d). Data from 24 hr food recalls at baseline and six months were analysed for micro and macronutrient content using Food Works 9 (Xyris Software, Australia).

Methods

At both assessment time-points the dietary habits questionnaire was completed by parents/carers according to detailed guidelines that accompanied the FFQ. At all times, trained staff and the candidate were available to assist parents/carers with queries. Parents required 10-20 minutes to complete questionnaires which were placed in envelopes, stored in a safe-cabinet at the medical centre and collected by the candidate at the end of each week. During the same week of the medical consultation, a telephone-interview was conducted by the candidate to collect details on the participant's medical history as well as to conduct the KIDMED questionnaire and 24-hour food recalls. Additional information regarding the breakfast meal, lunch, dinner as well as mid-morning and afternoon snacks were also retrieved. In particular, details regarding children's food preferences, fish intake and daily intake of bread, vegetables, salads, fruit, milk, chocolate milk, sweets, fast food, lollies and soft drinks were questioned with possible reasons given. Portion size was estimated using kitchen measuring tools and typical restaurant serving sizes. Missing data in questionnaires was discussed and completed during the telephone interview. The time required to complete the interview was approximately 30 minutes.

5.9 Motivational materials

Christmas cards

Continual support to participants was provided over the holidays. In order to maintain dietary compliance during Christmas and Easter vacation, greeting cards were designed, printed and posted to participants in both groups. Two sets of greeting cards were designed for Christmas, different for each group. With respect to the intervention group, apart from season greetings, the card contained a reminder for participants to continue to consume two fatty fish meals/week during the two week Christmas holidays (Figure 19).

Figure 19 Christmas greeting card designed for the intervention group

Figure 19 illustrates the inscription printed in the Christmas cards for the intervention group

Left-hand side: Inscribed "We would like to remind you to continue consuming fatty fish during the Christmas holidays. Right-hand side: "Merry Christmas & Happy New Year". Best wishes from the Research Team.

In contrast, for the control group only season's greetings were inscribed in Christmas cards (Figure 20).

Figure 20 Christmas greeting card for the control group

Figure 20 shows that "Merry Christmas & a Happy New Year!" was inscribed in Christmas cards designed for the control group, signed best wishes from the research team.

Over the Christmas break both groups were reminded by telephone calls, e-mails and text messages that the diagnostic clinic would be open during Christmas for biochemical testing.

Easter cards

Given that the follow-up assessment was scheduled shortly after Easter, the purpose of Easter cards was to remind parents about the six month appointment and assessments to be performed. Nevertheless, the candidate contacted all parents by telephone one week prior to appointments in order to confirm and inform parents about clinical testing and questionnaires to be completed. One set of Easter cards was designed for both groups (Figure 21).

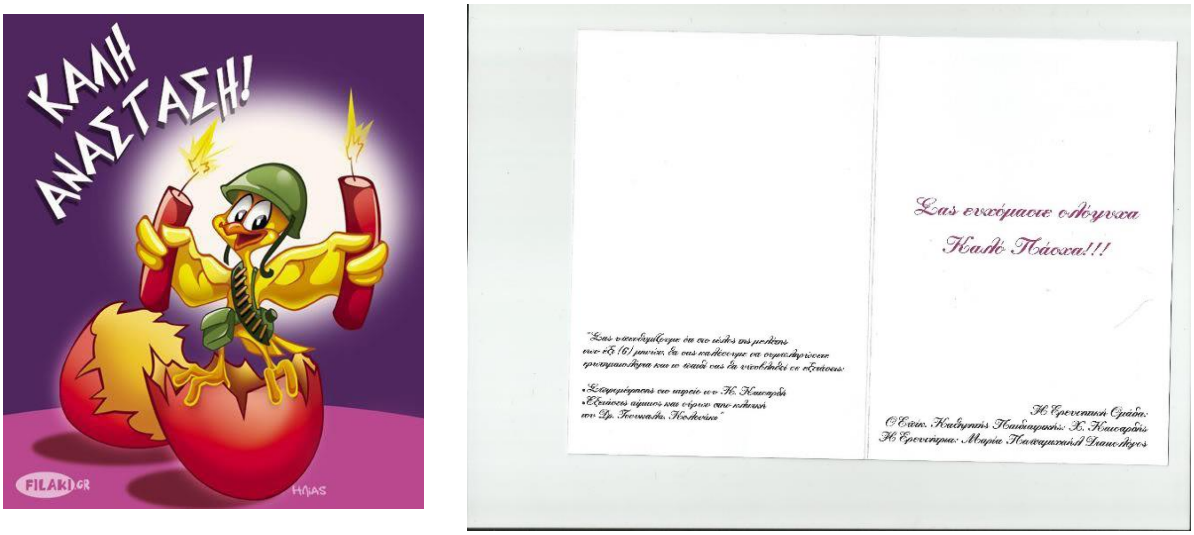

Figure 21 Easter card

Figure 21 shows that "Happy Easter Sunday" is illustrated on the cover of this card.

Right-hand side inscribed "On behalf of the research team, we wish you whole-heartedly Happy Easter." Left-hand side 'We would like to remind you that at the six-month follow-up a questionnaire will be completed and participants will undergo spirometry, FeNO and biochemical tests".

5.10 End of study nutrition education materials

At the end of participants' follow-up medical visit, after questionnaires and clinical tests had been completed, the candidate provided both groups with a fact-sheet summarizing the Hellenic dietary guidelines for children and adolescents which is centred on promoting the Traditional Greek Mediterranean diet prototype [293] (Figure 22). (Appendix 2A. 17a/b).

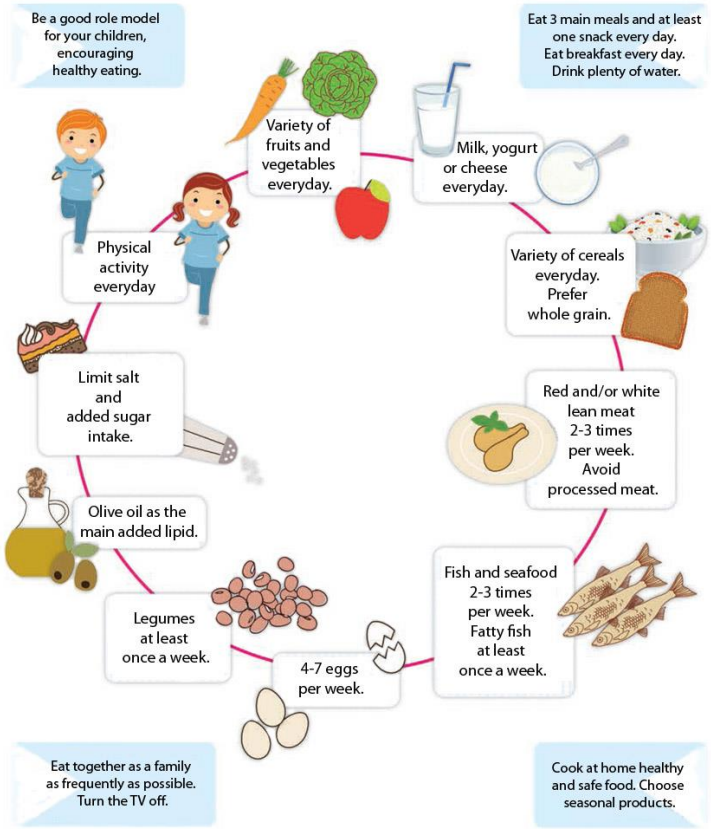

Figure 22 Ten steps to health eating for children and adolescents

Reference: PROLEPSIS (2014) National dietary guidelines for infants, children and adolescents. http://www.diatrofikoiodigoi.gr/?Page=diatrofikoi-odigoi-paidia

5.11 Participant evaluation of intervention protocol: Acceptability

At follow-up parents were provided with a short questionnaire that consisted of closed-ended questions addressing parent's experiences regarding acceptability of the intervention, any concerns, problems and suggestions to improve the trial (Appendix 2A, 18a-18d). Two questionnaires were developed, one for each group. The questionnaires were similar in content and consisted of two sections. Section A evaluated ease of implementation, comprehensibility of questionnaires, support provided by the research team, participant's health status and quality of life during the six month intervention as well as participant's attitude, barriers or difficulties encountered and future intentions. Section B comprised of two questions that referred to participation in future studies as well as suggestions for themes.

5.12 Funding

No funding was received for this project. The candidate declares no conflict of interest.

5.13 Study Sponsorship/Donations

Taking into account the economic crisis in Greece, high rate of unemployment and to motivate participants to participate in this study, financial assistance was provided for biochemical tests and spirometry testing. At both time-points, biochemical tests were provided free-of-charge to all participants in this study by co-investigator, Dr Tsoukalas at the Metabolomic Clinic. The cost of biochemical tests was 350 € per participant. In addition, participants were exempted from spirometry costs (40 € per test) which were paid by the PhD candidate's scholarship allowance.

Regarding unemployed parents and families of low-economic status, the cost of fish was an issue. In August 2016, three months prior to commencement of this project, two supermarket chains, *Basilopoulos and Sklavenitis* were approached by the candidate and kindly invited to participate in this study by donating fresh fatty fish to participants (Appendix 2A, Letter 1a/b). In exchange for their generosity, it was agreed that the supermarket logo would be advertised and acknowledged in all publications, conferences, and on questionnaires. Unfortunately, due to the economic crisis and lack of funds, a small donation was offered by the two supermarket. *Basilopoulos Supermarkets* were able to donate 1000 € for the purchase of fatty fish which was given in the form of food vouchers valued at 6 € each (Figure 23). This amount of money was equivalent to 167 vouchers. In order to be able to purchase approximately 1 kilogram of fatty fish per week at a price of 6 €/ kg over a period of 6 months, each participant required 24 coupons. Given that the intervention group comprised of 32 participants, a total of 768 coupons would be required. Since only 167 coupons were available these were not enough for all of the 32 participants in the intervention group. After consultation with the study's respiratory physician, Dr. Katsardis, it was decided that the coupons should be issued to two families whose parents were unemployed. The left over vouchers were distributed to the remaining 30 participants in the intervention group as a Christmas bonus which was mailed together with Christmas cards.

Figure 23 Coupons (6 €) donated by Basilopoulos Supermarkets

Figure 23 illustrates the 6 € food voucher issued to participants in the intervention group.

Sklavenitis Supermarkets donated 27 kilos of farmed Gilthead Sea bream (tsipoura) which was worth 215 €. Greek farmed gilthead sea bream is categorized as a 'fatty fish' variety [(293)]. The candidate arranged with the supermarket that each fish weighed 350 grams raw (which is equivalent to at least 150g when cooked and deboned). By doing this one fish would be needed per meal. This simplified the problem of estimating 150g portion size/meal by parents. On the day that the 27 kilos of fresh fish (n=56) was delivered to the supermarket, they were collected by the candidate and distributed into 28 plastic freezer bags. Each bag contained 2 fish meals, so one bag was required per week. Fish were maintained frozen in the refrigerator at the candidate's residence. During the enrolment period, the candidate contacted economically disadvantaged families and appointments were arranged to deliver the frozen fish in person.

5.14 Professionalism and Team work

On January 9th, 2017, at the end of recruitment a letter was sent to the financial supporters Basilopoulos, Sklavenitis and Metabolomic clinic expressing appreciation for their involvement in the project (Appendix 2A, 20a-20f). Also attached to the e-mail was a copy of the first two pages of the socio-demographic questionnaire portraying the supermarkets' and clinic's logo as agreed.

During the four years of this PhD project, funders were updated regularly regarding abstracts accepted for presentation at conferences and manuscripts published in international scientific journals.

In December 2018, one year after the end of the study, participants were provided with a summary of the main findings of the clinical trial (Appendix 2A, 19a/b).

5.15 Statistical Methods

5.15.1 Data management

Identification Code

All data used was anonymous, coded and unidentifiable. Each participant was allocated an identification (ID) number in order to maintain anonymity. A unique ID number was devised for each participant based on the National Health Service (NHS) Community Health Index Number [(327)]. This code comprised of 4 variables: enrolment number, date of birth, gender, and allocated group. Participants satisfying the eligibility criteria and after signed consent were assigned a participant number (ranging from 1 to 72) based on entry into the RCT. Date of birth was recorded as day followed by month and last 2 digits of the year (dd/mm/yy). Gender was coded as M for male, F female. Based on the randomization sequence, the participant was allocated to intervention (I) or Control (C). More specifically, an ID of 28/120908/F/I represents the characteristics of a patient with a participant number of 28, born on the 12th of September 2008, belonging to the female gender (F) and allocated to the intervention group.

5.16 Randomisation and group allocation

Randomization has been extensively used in clinical trials and other biological experiments [(328)]. It eliminates selection bias, produces comparable groups with respect to known and unknown confounding variables and eliminates the source of bias in treatment assignments. Additionally, it permits the use of probability theory in analysing data and to express the likelihood of chance as a source for the difference of end outcome. In general, a randomized experiment is an essential tool for testing the efficacy of the treatment [(323)].

Randomization was conducted by the candidate supervised by the statistician using an internet platform http:///www.randomization.com. This validated system automates the random assignment of participant number to randomization number which is linked to the different intervention arms. The first original generator was used. Treatment labels were assigned ‘intervention group’ and ‘control group’. Given that G Power analysis produced a sample size of 64 participants, then 64 participants were randomized into 2 blocks. After consulting with the physician and taking into account the attrition rate, an additional 8 subjects were recruited, i.e a total of 72 subjects were enrolled at baseline.

The generated randomization plan was as follows:

1. Intervention group_____
2. Control group_____
3. Control group_____
4. Control group_____
5. Control group_____
6. Control group_____
7. Intervention group_____
8. Intervention group_____
9. Control group_____
10. Control group_____
11. Intervention group_____
12. Intervention group_____
13. Control group_____
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61. Intervention group_____
62. Intervention group_____
63. Control group_____
64. Control group_____
65. Intervention
66. Control
67. Control
68. Intervention
69. Intervention
70. Intervention
71. Control
72. Control

**Randomization plan created on 18/04/2016, 22:48:24

Reference: http:///www.randomization.com (seed no: 24503)

The randomization sequence for 72 participants was provided to the pneumologist three weeks prior to commencement of the study (on 20.10.16). Enrolment of the first participant by the physician commenced on 11/11/2016 according to the randomization protocol until the final participant was recruited on 23/12/2016. The first (1st) participant

in this study was enrolled to the intervention group and the 72nd participant, the control group. Blinding of the intervention from the participants, parents, physician and candidate was not possible due to the nature of the dietary intervention.

5.17 Statistical Analysis

A complete explanation of statistical tests used for all analyses in this thesis has been accounted for in the published papers. Briefly, pulmonary function parameters ($FEV_{1,}$ FVC, FEV_1/FVC , PEF , $FEF_{25-75\%}$), FeNO and scores were analysed as continuous variables. These variables were assessed for normality using graphical methods, descriptive statistics and Shapiro-Wilks test. Differences between intervention groups were examined using T-test for normally distributed variables and non-parametric tests Mann-Whitney and Kruskal-Wallis otherwise. In the case of categorical variables, Chi Square test was applied. Within group differences were assessed using Paired t-test and Wilcoxon sign rank test. In tables, continuous variables are presented as means ± standard deviations (S.D) and categorical data as absolute frequencies (with proportions). The effect of the intervention on spirometry, FeNO, asthma control and quality of life were examined using Multiple Linear Regression model adjusted for confounders of age, sex and possible confounding variables such as regular physical activity and BMI. Regular physical activity was considered to be exercising at least three times per week. A sub-group analysis was performed by stratifying data to sufficient/insufficient plasma vitamin D levels at baseline with sufficient vitamin D defined as 25(OH)D $\geq$ 25 ng/mL. The effect of the intervention in children with sufficient serum vitamin D levels at baseline was investigated using linear regression and interaction analyses. An additional analysis was conducted to further examine the effect of serum 25(OH)D on pulmonary function tests by subdividing data according to categories based on bone health [deficient $\leq$ 10ng/mL, insufficient 10-20 ng/mL, inadequate 20-30 ng/mL and desirable $\geq$ 30 ng/mL]. Data are presented as estimated effect size (β) from the regression, 95% CI and p-value significant at the 5% level. All compilations were conducted using Statistical Package for the Social Sciences (SPSS Version 20.0) (IBM Incorp. Armonk, NY, USA) and interactions by STATA release 14.1 (College Station, TX, USA) software.

SYNOPSIS

Chapter 5 described in detail the rationale behind the methods and materials employed in this PhD study. The study protocol was outlined in a publication written by the candidate. In brief, this study was a 2-arm parallel randomized controlled trial (RCT) that included 72 Greek children with doctor-diagnosed 'mild asthma', aged 5-12 years old. The primary hypothesis to be challenged was that increased fatty fish intake as part of the Mediterranean diet will improve pulmonary function and decrease asthma symptoms; secondary hypotheses, there will be better asthma control, quality of life and adherence to the Mediterranean dietary pattern as assessed by the KIDMED score. The intervention diet was a Mediterranean diet enriched with two meals of fatty fish weekly ($\geq$ 150 g cooked filleted fish/meal) for a period of six months. The control was the usual diet of the participant's family. Outcome measures assessed at baseline and six-months were: anthropometry, spirometry, FeNO, metabolomic profile (fatty acid composition, vitamin D, organic acids), asthma control, quality of life, dietary habits and adherence to the Mediterranean dietary pattern. Included in this chapter were statistical methods used to determine sample size, randomization sequence as well as statistical tests applied in data analysis.

CHAPTER 6 Results

Chapter 6 presents the results of data analysis. The purpose of this chapter is to answer the primary and secondary hypotheses posed earlier in Chapter 4. The main findings have been summarised in four manuscripts of which three papers have been recently published, and one is currently under-review. The hypothesis and corresponding publication is shown in tabulated form as follows:

PRIMARY HYPOTHESIS	**PUBLICATION**	**Section**
Hypothesis 1: The primary hypothesis challenged was that increased fatty fish consumption in the context of a Mediterranean diet (the intervention) for 6 months improves pulmonary function as indicated by an increase in spirometry measurements (FEV_1, FVC, FEV_1/FVC, PEF, $FEF_{25-75\%}$) compared to a control condition.	***Efficacy of a Mediterranean diet supplemented with fatty fish in ameliorating inflammation in paediatric asthma: a randomized controlled trial.*** *J Hum Nutr Diet. 2019 Apr; 32(2): 185-197.*	6.5
Hypothesis 2: There will be a decrease in FeNO in the intervention group as compared to the control at 6 months	***Efficacy of a Mediterranean diet supplemented with fatty fish in ameliorating inflammation in paediatric asthma: a randomized controlled trial.*** *J Hum Nutr Diet. 2019 Apr; 32(2): 185-197*	6.5
SECONDARY HYPOTHESES		
Hypothesis 1: There will be a decrease in asthma control scores for the intervention group as assessed by the Asthma Control Questionnaire compared to the control at six months.	***Efficacy of a Mediterranean diet supplemented with fatty fish in ameliorating inflammation in paediatric asthma: a randomized controlled trial.*** *J Hum Nutr Diet. 2019 Apr; 32(2): 185-197*	6.5
Hypothesis 2: There will be greater increase in paediatric asthma quality of life scores for the intervention group	***Efficacy of a Mediterranean diet supplemented with fatty fish in ameliorating inflammation in paediatric asthma: a randomized controlled trial.*** *J Hum Nutr Diet. 2019 Apr; 32(2): 185-197*	6.5

compared to the control at six months.		
Hypothesis 3: There will be better adherence to the Mediterranean dietary pattern and an improvement in diet quality in the intervention group compared to the control as assessed by the KIDMED questionnaire.	***Efficacy of a Mediterranean diet supplemented with fatty fish in ameliorating inflammation in paediatric asthma: a randomized controlled trial.*** *J Hum Nutr Diet. 2019 Apr; 32(2): 185-197*	6.5
Hypothesis 4: There is a positive association between plasma 25(OH)D and lung function in the study population as measured by pulmonary function tests (spirometry and FeNO).	***The impact of vitamin D status on lung function in asthmatic children adhering to Mediterranean diet enriched with fatty fish*** *(under-review in Nutr Res Manuscript ID: NR_2019_902)*	6.6.3
Hypothesis 5: There is an association between high BMI and pulmonary function in asthmatic children as compared to normal weight as reflected by spirometry (FEV_1, FVC, FEV_1/FVC, PEF, $FEF_{25-75\%}$).	***Weight status and respiratory health in asthmatic children.*** *Lung. 2019* Dec;197(6):777-782. *doi: 10.1007/s00408-019-00273-w. [Epub 2019 Sep 14]*	6.3
Hypothesis 6: There is a positive association between overweight/obesity and FeNO in asthmatic children as compared to normal weight.	***Weight status and respiratory health in asthmatic children.*** *Lung. 2019* Dec;197(6):777-782. *doi: 10.1007/s00408-019-00273-w. [Epub 2019 Sep 14]*	6.3
Novel to this PhD project		
Hypothesis: There is a unique urinary organic acid profile in asthmatic children	***Urinary organic acids as biomarkers in the assessment of pulmonary function in children with asthma"*** *Nutr Res. 2019 Jan; 61: 31 – 40.*	6.7

In addition, results that are yet to be published are presented in this chapter. Firstly in section 6.1 baseline socio-demographic characteristics as well as risk factors for asthma exacerbations are presented followed by anthropometry in section 6.2 and dietary habits

of Greek asthmatic children in section 6.4. The answers to the hypotheses raised in this PhD study is discussed in the publications authored by the candidate in sections 6.3, 6.5 and 6.6.3. Innovative to this thesis is the application of metabolomics in the investigation of a relationship between urinary organic acids and pulmonary function in asthmatic children The results of this study are shown by the published manuscript in section 6.7. And finally included in section 6.8 of this chapter, are the findings of the participant evaluation questionnaire.

6.1 Socio-demographic characteristics of total sample and per intervention group at baseline

Socio-demographic characteristics of the total sample and for intervention/control group at baseline are shown in (Table 12).

Table 12 Baseline socio-demographic characteristics, anthropometry and associated allergies for total sample and per intervention and control group.

Variable	**Group**			
	Total (n = 72)	**Control (n = 36)**	**Intervention (n = 36)**	***P*[c]**
Age (years)	7.97 ± 2.2	8 ± 2.0	8 ± 2.0	0.23[b]
Sex				
Male	54.2% (39)	31.9% (23)	22.2% (16)	0.09
Female	45.8% (33)	18.1% (13)	27.8% (20)	
Height (cm)	133.4 ± 13.3	133.5 ± 13.78	133.4 ± 13.0	0.95[b]
Height category				
Normal	63.9% (46)	41.7% (30)	22.2 (16)	**0.00**
Tall	36.1% (26)	8.3% (6)	27.8% (20)	
Weight (kg)	33.7 ± 12.5	33.4 ± 12.7	34.1 ± 12.5	0.85[b]
Body Mass Index (kg/m^2)	18.3 ± 3.7	18.1 ± 3.7	18.6 ± 3.8	0.73[b]
BMI categories *				0.14
Severely Underweight (BMI < 16 kg/m^2)	1.4% (1)	1.4%(1)	0.0%	
Slightly Underweight (BMI 17-18.5 kg/m^2)	2.8% (2)	2.8% (2)	0.0%	
Underweight (BMI 16-17.5 kg/m^2)	0.0%	0.0%	0.0%	
Normal weight (BMI 18.5-24.99 kg/m^2)	56.9% (41)	30.6% (22)	24.6% (19)	
Overweight (BM1 25-29.99 kg/m2)	27.8% (20)	8.3% (6)	19.4% (14)	
Obese (BMI> 30 kg/m2)	11.1% (8)	6.9% (5)	4.2% (3)	
Parent's details				
Race				
Caucasian	97.8% (69)			
Marital Status				
Married	91.5% (65)			
Parents' education level				
Mother				
Primary school	2.8%(2/71)	2.8% (1/35)	2.8% (1/35)	0.58
Junior High	5.7% (4)	5.7%(2)	5.7% (2)	
Senior High	42.8% (30)	37.1% (13)	48.6% (17)	
College	19.7% (14)	22.9% (8)	17.1%(6)	
Technical College	8.5% (6)	14.3% (5)	2.9% (1)	
University	16.9% (12)	17.1% (6)	17.1% (6)	
Master	2.8% (2)	0.0%	5.7% (2)	
PhD	0.0%	0.0%	0.0%	
Father				
Primary school	7.0% (5)	14.3%(5)	0.0%	0.74
Junior High	11.2% (8)	14.3%(5)	8.5% (3/35)	

Senior High	45.1% (32)	45.7%(16)	45.7% (16)	
College	12.7% (9)	17.1% (6)	8.5% (3)	
Technical College	5.6% (4)	0.0%	11.4%(4)	
University	15.5%(11)	17.1%(6)	14.3% (5)	
Master	7.0% (5)	2.8%(1)	11.4% (4)	
PhD	2.8% (2)	2.8%(1)	2.8% (1)	
Parent's Employment Status				
Mother				
Unemployed	21.0% (15)			
Part-time	7.3% (5)			
Full-time	15.5% (11)			
Public sector	11.3% (8)			
Private sector	26.8%(19)			
Self-employed	9.9% (7)			
House-wife/child-care	8.5% (6)			
Dismissed from work	0.0%			
Father				
Unemployed	8.5%(6)			
Part-time	7.0% (4)			
Full-time	23.9%(17)			
Public sector	19.7%(14)			
Private sector	28.2%(20)			
Self-employed	12.7%(9)			
House-wife/child-care	0.0%			
Dismissed from work	1.4%(1)			
Average monthly family income (mean)	1587.29 €			
Type of school participant attends				
Public	93.0% (66)			
Private	7.0% (5)			
Smoking habits				
Mother smoked during pregnancy				
Yes	12.7 (9)	13.9% (5)	11.1% (4)	0.75
Mother smoked during 1st year of child's life				
Yes	28.2% (20)	30.6% (11)	25% (9)	0.65
Mother smokes today				
Yes	49.3% (35)	61.1% (22)	36.1% (13)	**0.04**
Father smoked during first year of child's life:				
Yes	52.9% (36)	58.3% (21)	41.7%(15)	0.14
Father smokes today				
Yes	49.3% (35)	60.0% (21)	38.9%(14)	0.15
e-cigarettes	-	-	5.6%(2)	
Parental Allergy				
Maternal allergy during childhood:				
Yes	33.8% (24)	33.3% (12)	33.3% (12)	0.93
Rhinitis	23.9% (17)	(12/36)	19.4% (7)	0.44
Asthma	12.7% (9)	27.8% (10)	19.4% (7)	0.07
Eczema	2.8% (2)	5.6% (2)	5.6% (2)	0.15

Maternal allergy during adulthood:				
Yes	47.9% (34)	55.6% (20)	38.9% (14)	0.19
Rhinitis	40.8% (29)	47.2% (17)	33.3% (12)	0.27
Asthma	14.1% (10)	11.1% (4)	16.7% (6)	0.46
Eczema	0.0%	0.0%	0.0%	-
Paternal allergy during childhood:				
Yes	13.6%(9)	17.1% (6)	8.3% (3)	0.20
Rhinitis	4.5% (3)	5.7% (2)	2.9% (1)	0.48
Asthma	9.1% (6)	11.4% (4)	5.6% (2)	0.31
Eczema	0.0%	0.0%	0.0%	-
Paternal allergy during adulthood				
Yes	36.6% (26)	51.4% (18)	22.2% (8)	**0.02**
Rhinitis	32.4% (23)	45.7% (16)	19.4% (7)	**0.03**
Asthma	5.6% (4)	8.6% (3)	2.8% (1)	0.32
Eczema	0.0%	0.0%	0.0%	-
Number of children in family				
1	35.2% (25)			
2	59.1% (42)			
3	4.2%(3)			
4	1.4% (1)			
Participant's birth order				
1	71.8%(51)			
2	23.9%(17)			
3	2.8%(2)			
4	1.4%(1)			
Pregnancy details				
Gestation (weeks)				
32-37 weeks	11.3% (8)	11.1(4)	11.4% (4)	0.97
37-40 weeks	88.7% (63)	88.9% (32)	86.1% (31)	
>40 weeks	0.0%	0.0%	0.0%	
Delivery:				
Vaginal delivery	49.3% (35)	28.2% (20)	21.1% (15)	0.28
Caesarean-section	50.7% (36)	22.5% (16)	28.2% (20)	
Participant's birth weight				
<2500g	8.5% (6)	8.3% (3)	8.6% (3)	0.97
2500-4000g	91.5% (65)	91.7% (33)	91.4% (32)	
>4000g	0.0%	0.0%	0.0%	
Breastfeeding:				
Yes	85.9% (61)	88.9% (32)	80.5% (29)	0.46
No	14.1% (10)	11.1% (4)	17.1% (6)	
<3 months	49.1% (30/61)	53.1% (17/32)	44.8% (13/29)	0.70
3-6 months	29.5% (18)	31.3% (10)	27.6% (8)	
6-12 months	18.0% (11)	12.5% (4)	24.1% (7)	
>12 months	3.3% (2)	3.1% (1)	3.4% (1)	
Asthma & related allergy details				
Child's age of asthma onset (months)	34.4 mo ± 26.8	36 mo ± 21.7	36 mo ± 31.4	0.19[b]
Does your child suffer from other allergies?				
Yes	28.2% (20)	30.6% (11)	25.0% (9)	0.65
No	71.8% (51)	69.4% (25)	72.2% (26)	
Rhinitis	26.8% (19)	30.6% (11)	22.2% (8)	0.46

Conjunctivitis	5.6% (4)	8.3% (3)	2.8% (1)	0.32
Eczema	1.4% (1)	0.0%	2.8% (1)	0.31
Food Allergy	11.3% (8)	11.1% (4)	11.1% (4)	0.97

In bold statistical significant p-values

Total sample $N = 72$

* BMI categories according to Hellenic Paediatric Growth Charts [311].

P^b -P-value from Mann-Whitney test; P^c -P-value from Chi- Square test

P-value was considered statistically significant at the 5% level.

6.2 Anthropometry

Latest findings of the WHO European Childhood Obesity Surveillance Initiative (COSI) (2015-2017), documented that Greece has one of the highest rates of overweight in children in Europe, sharing second rank together with Italy and Spain where 42% of boys are overweight [329]. Specifically, 4 in 10 Greek school children are overweight, with 42% of boys and 38% of girls being overweight, while 20% of boys and 14% of girls were obese [329]. In addition, there is common belief that long-term use of oral corticosteroids in asthmatic children stunts growth and causes weight gain [270].

Distribution of children's height at baseline and six-months is presented in Figure 24 and BMI in Figure 25.

Figure 24 Distribution of height for total sample at baseline and six-months

Figure 24 illustrates that by the end of the six month study there was an increment in the frequency of children with normal height from 64% to 75% . According to the US Centre for

Disease Control (CDC), normal stature is considered to be height ranging from the 5th percentile to less than 95th percentile and stunting less than 5th percentile [330].

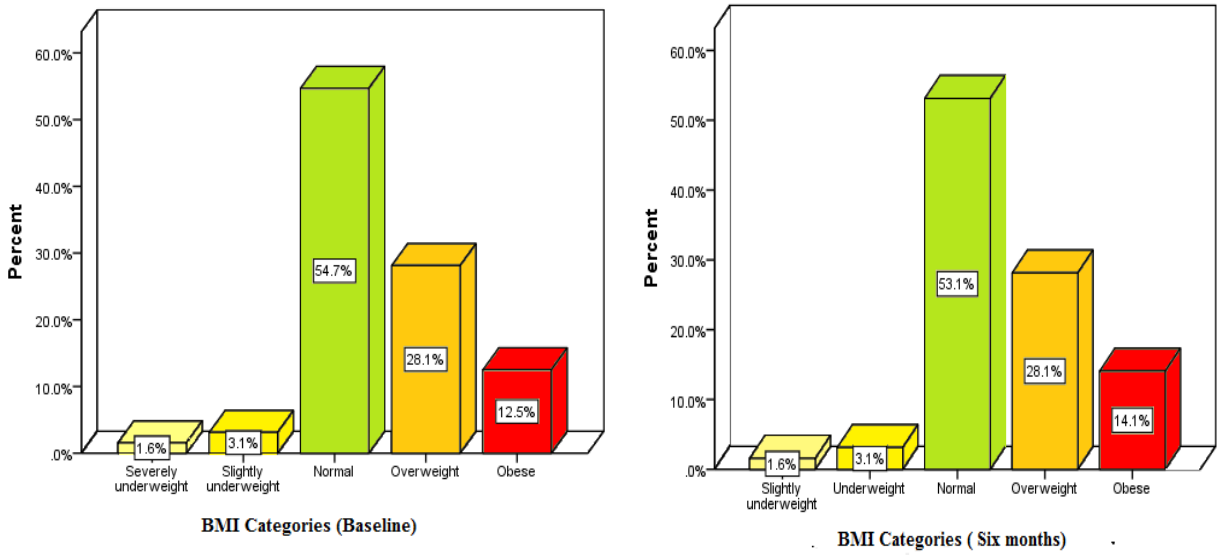

Figure 25 BMI categories for total sample at baseline and six months

With respect to weight status, Figure 25 shows that at baseline, 1.6% (1/64) of children was 'severely underweight', 4.7% (3/64) 'slightly underweight', 54.7% (35/64) 'normal weight', 28.13% (18/64) 'overweight' and 10.9% (7/64) 'obese' according to the Hellenic paediatric growth charts. At six months, 1.6% (1/64) of children was 'slightly underweight', 3.1% (2/64) 'underweight', 53.1% (34/64) 'normal weight', 28.1% (18/64) 'overweight' and 14.1% (9/64) 'obese'. Investigating differences in BMI levels per sex, at baseline showed that girls were more overweight than boys [38.7% (12/31) vs 18.2% (6/33) respectively], but boys (5-8 years) more obese than girls [18.2% (6/33) vs 6.5% (2/31) respectively], although non-significant [X^2 (4, N = 64) = 6.97, p = 0.14)]. The same trend was observed for obesity at six months (obesity: boys 15.2% (5/33) vs girls 12.9% (4/31) [X^2 (4, N = 64) = 1.05 p = 0.90)]. No differences between girls and boys were observed for BMI as the continuous variable at both time-points [(baseline: (U = 508.5, p = 0.97); six months (U = 488.5, p = 0.76) Mann-Whitney test]. These findings confirm the magnitude of the child obesity problem in Greece and is consistent with the literature.

Investigation of the effect of high BMI on pulmonary function in asthmatic children is assessed in the following short communication *Papamichael et al, Weight status and respiratory health in asthmatic children,* recently published in *Lung J. 2019* Dec;197(6):777-782. *doi: 10.1007/s00408-019-00273-w [Epub 2019 Sep 14].*

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6.3 Weight status and respiratory health in asthmatic children: A short communication

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ASTHMA

Weight Status and Respiratory Health in Asthmatic Children

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Abstract

In this study,we explored the effect of adiposity as measured by BMI on lung function in 72 asthmatic school children (5–12 years) using baseline data from the Mediterranean diet enriched with fatty fish intervention study. Bronchial function was assessed using spirometry and fractional exhaled nitric oxide (FeNO). BMI categories were classified as normal and overweight/obese based on International Obesity Task Force cut-offs. Weak correlations were observed between BMI and FVC (p = 0.013) and FEV_1 (p = 0.026). Median FeNO was lower in the overweight/obese as compared to normal weight group (p= 0.027). Linear regression showed an increment in $FEF_{25–75\%}$ in the overweight/obese group as compared to normal weight after controlling for confounders namely age, height, sex, regular physical activity, medication and KIDMED score (p= 0.043; β =11.65 units, 95% CI 0.36–22.94), although with no effect on FeNO. In conclusion, the findings of this study suggest that excess body weight could impact pulmonary dynamics in childhood asthma.

Keywords Asthma · Body mass index · Children · Lung function · Spirometry · Overweight

Abbreviations

BMI Body mass index
FEV1 Forced expiratory volume in 1 s
FVC Forced vital capacity
FEV1/FVC Ratio of forced expiratory volume in 1 s and forced vital capacity
PEF Peak expiratory flow
$FEF_{25–75\%}$ Mid expiratory flow 25–75% vital capacity
FeNO Fractional exhaled nitric oxide

Introduction

The universal rise in paediatric asthma and obesity are two chronic conditions that warrant urgent attention in public health [1]. Both conditions are associated with adverse effects on lung function and contribute to increased morbidity and disability during childhood[1].There is compelling epidemiological evidence supporting an association between obesity and asthma prevalence/severity in children suggesting a common pathophysiology between these two conditions [1]. Nevertheless, there is limited understanding of the effect of adiposity on pulmonary function in paediatric patients with mild asthma. Previous research has shown that 70% of asthmatic patients suffer from 'mild asthma'[2]. Mild asthma is more frequent, more symptomatic, and less well controlled in children than in adults.

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[2]. Severe exacerbations in mild asthma represent 30–40% of asthma exacerbations requiring emergency consultation [2]. Our recent work on the Mediterranean diet and asthma revealed that increased fatty fish intake reduced bronchial inflammation in asthmatic children [3]. Using baseline data from this dietary intervention study, we opted to explore the effect of adiposity as measured by Body Mass Index (weight/height2) on lung function in Greek asthmatic school children.

Methods

The present study was a cross-sectional analysis of the Mediterranean diet enriched with fatty fish and paediatric asthma intervention study. The sampling procedure took place from November 1st to December 31st, 2016. The design and study protocol have been described in detail in Online Resource1. In brief, after signed informed consent, 72 children aged 5–12 years, with physician-diagnosed mild asthma [4]were recruited from a private paediatric asthma clinic in the greater city of Athens, Greece. The primary outcomes were pulmonary function indices (FEV_1, FVC, FEV_1/FVC, PEF, $FEF_{25-75\%}$), reported as percent predicted values of pre- bronchodilator administration and bronchial inflammation biomarker, fractional exhaled nitric oxide (FeNO). In this manuscript the primary exposure of interest is BMI [5]. Pulmonary function tests were performed by trained personnel following standard protocol [6] using a portable spirometer MIR Spirobank II (MIR Inc. USA) and eosinophilic airway inflammation by a portable FeNO analyser (NO breath, Benfont Inc.UK) [7]. Normal pulmonary function in children was defined as FEV1, FVC, FEV1/FVC, PEF ≥ 80% predicted, and FEF25–75% > 60–65% [8]. A threshold of 20 ppb was used as cut-off to define eosinophilic inflammation in children [9]. Body weight and height were assessed using calibrated electronic scales and stadiometer (SECA, Hanover, MD) followed by BMI calculation. BMI categories were classified either as normal, overweight or obese as stated by the International Obesity Task Force (IOTF) cut-offs [5]. All procedures performed in this study were in accordance with the ethical standards of the institution (La Trobe University Human Ethics Committee, HEC 16-035) and with the 1964 Helsinki declaration.

Statistical Analysis

Continuous variables were assessed for normality using graphical methods and Shapiro–Wilks test. Given that only seven subjects were obese, data for overweight and obese were combined and BMI was stratified to two groups, normal weight versus overweight/obese. Differences between groups were compared using independent *t* test, Mann–Whitney test for skewed variables and Chi Square Test for frequencies. Spearman's correlation and linear regression were fitted to estimate the association between BMI and lung function parameters and FeNO. Potential confounders namely age, sex, height, regular physical activity (≥ 3 times/week), KIDMED score and medicine were identified by review of the literature. The effect size was estimated by unstandardized β coefficient and 95% confidence interval. Statistical significance was set a priori at $p < 0.05$.

Results

At baseline, of the 72 children recruited, 54.2% (39) were male, 45.8% (33) were female, with a mean age of 7.97 ± 2.21 years, and 39.7% (27/68) were overweight/obese (BM1 ≥ 25 kg/m2) with 48.6% (34) exercising at least three times per week and 83% (59) taking asthma medication daily. Girls were slightly more overweight/obese than boys [40.6% (13/32) vs. 38.9% (14/36) respectively;$p = 0.88$]. With respect to pulmonary function, in general, children had well-controlled asthma. Spirometry showed normal lung function (FVC, FEV_1, FEV_1/FVC, PEF ≥ 80% predicted, FEF 25–75% > 60–65%) and absence of bronchial inflammation (FeNO < 20 ppb). Baseline data is summarised according to BMI status (normal weight vs. overweight/ obese) in Table 1.

There were weak positive correlations between BMI and FVC ($p = 0.013$, $\rho = 0.29$), FEV_1 ($p = 0.026$, $\rho = 0.26$) and$FEF_{25-75\%}$ ($p = 0.345$, $\rho = 0.11$). In contrast, a negative trend was observed between BMI and FEV_1/FVC, PEF and FeNO although non-significant. Stratification by sex showed a positive correlation between BMI and FVC (p= 0.004; ρ= 0.49) and FEV_1 ($p = 0.027$; $\rho = 0.38$) in girls; and a negative correlation between BMI and PEF in boys (p=0.049, ρ= − 0.32).

Univariate analysis showed no difference in spirometry parameters between normal weight and overweight/obesegroups ($p > 0.05$). Contrastingly, there was a statistically significant difference in FeNO between the overweight/obese and the normal weight groups (p = 0.027). Median FeNO was slightly lower in the overweight/obese group as compared to the normal weight group (median 5 vs. 11 ppb respectively; p =0.027) (Fig. 1).

No effect on BMI group was observed for FEV_1, FVC, FEV_1/FVC, PEF, FeNO in the unadjusted and adjusted models ($p > 0.05$). As for $FEF_{25-75\%}$, in the unadjusted model no difference was noted between BMI groups ($p = 0.40$, β = 4.363, 95% CI − 6.00–14.73). However, after controlling for confounders namely age, height, sex, regular physical activity, medication and KIDMED score, a positive effect of overweight/obese on $FEF_{25-75\%}$ was observed (Table 2). $FEF_{25-75\%}$ increased by 11.65 units for children in

Table 1 Baseline subject characteristics and lung function for total sample and summarised by BMI group

Variable	Total	BMI Group				*p* value
		Normal		Overweight/obese[1]		
		Mean (*n*)	SD	Mean (*n*)	SD	
Male	54.2% (39)	53.7% (22)		50.00% (14)		0.88[c]
Age	7.97 ± 2.21	7.83	2.38	8.33	1.98	0.23[b]
BMI	18.34 ± 3.74	16.49	1.74	21.93	3.23	**0.00**[b]
FVC (L)*	95.11 ± 9.87	93.29	9.17	97.63	9.92	0.07[a]
FEV_1 (L)	97.67 ± 10.16	95.95	9.98	100.41	10.70	0.08[a]
FEV_1/FVC	102.01 ± 6.40	102.17	6.51	102.04	5.61	0.93[a]
PEF (L/s)	94.65 ± 19.11	93.95	19.09	93.59	14.58	0.93[a]
$FEF_{25-75\%}$	102.06 ± 20.88	100.41	21.49	104.78	20.08	0.40[a]
FeNO (ppb)	13.59 ± 13.36	15.35	12.33	11.44	15.33	**0.03**[b]
FeNO < 20 ppb	76.1% (54)	70.00% (28)		85.71% (23)		0.15[c]
Regular exercise ≥ 3 times/week	48.6% (34)	46.15% (18)		50.00% (14)		0.65[c]
Asthma medication over past month yes	83.1% (59)	82.93% (34)		81.48% (22)		0.86[c]

Bold character represents significant *p*-values. BMI group: normal weight ($n = 41$), overweight/obese ($n = 27$)

BMI body mass index, *FEV_1* forced expiratory volume in 1 s, *FVC* forced vital capacity, *FEV_1/FVC* ratio of forced expiratory volume in 1 s and forced vital capacity, *PEF* peak expiratory flow, *$FEF_{25-75\%}$* mid expiratory flow 25–75% vital capacity, *FeNO* fractional exhaled nitric oxide

*% Predicted pre-bronchodilator; *p*-value significant at the 5% level

[1]Overweight/obese refers to combined data for overweight and obese subjects

[a]*p* value calculated using independent *t* test

[b]Mann–Whitney test

[c]Chi Square test

the overweight/obese group as compared to normal weight ($p = 0.043$; $\beta = 11.65$ units, 95% CI 0.36–22.94).

Discussion

In this report, we demonstrated a positive association of overweight/obesity on dynamic lung volume represented by $FEF_{25-75\%}$, an indicator of small airway calibre, which is consistent with the literature [10]. This is clinically significant because small airway function has been associated with asthma symptoms (dyspnoea), impaired control and health-related quality of life [11]. No significant association of weight status was detected for FeNO in the adjusted regression model controlling for age, height, sex, regular physical activity, medication and KIDMED score. Most studies have examined the effect of BMI on FEV_1, FVC and FEV_1/FVC [1]. Our finding is novel and adds to the limited evidence on the impact of body weight on lung function in small airways in asthmatic children. To our knowledge, this is the second study since that undertaken by Spathopoulos in 2009 to investigate the relationship between overweight, lung function and FeNO in Greek asthmatic school children [12]. The finding of a positive association of excess weight on lung volume and airway flow as reflected by increments in FEV_1, FVC and $FEF_{25-75\%}$ in overweight/obese children as compared to the normal weight has been documented in prior paediatric studies [10, 13–16]. The sex effect might be because of the earlier growth spurt and onset of puberty in girls as compared to boys [16]. As for biomarkers of airway limitation, FEV_1/FVC and PEF, a negative correlation was observed although it is non-significant, most likely due to small sample size. Further studies with larger samples are warranted to clarify this association.

Recently, Ekstrom et al. demonstrated that overweight/obesity was associated with increased lung volume (FVC, FEV_1) and airway obstruction (reduced FEV_1/FVC) in children up to 16 years [13]. Jones et al. found an increase in FVC and FEV_1 by 13.6% and 7.6% respectively as well as a 3.5% reduction in FEV_1/FVC in overweight asthmatic children [15]. Comparable results were described by Yao et al. in the PATCH study [10]. BMI disproportionately increased FVC, FEV_1, PEF and $FEF_{25-75\%}$ but decreased FEV_1/FVC and FeNO in Asian children. However, in contradiction to

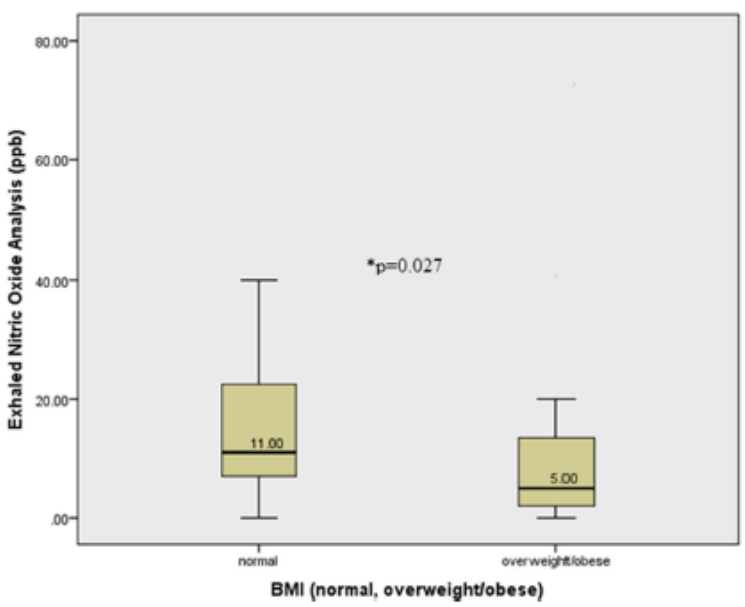

Fig. 1 Box-plot of FeNO versus BMI group. It is apparent that median FeNO was lower in the overweight/obese group than in the normal weight group (5 vs .11 ppb respectively, $p=0.027$). *Mann–Whitney test

Table 2 Adjusted multiple linear regression model

Spirometry parameter	Co-variate	β	95% CI	p value
$FEF_{25-75\%}$	BMI group	11.65	0.36, 22.94	**0.043**
	Age	3.59	− 2.10, 9.27	0.21
	Height	−0.76	− 1.76, 0.23	0.13
	Sex	−4.42	− 14.65, 5.79	0.39
	Regular physical activity	−3.67	− 14.21, 6.87	0.49
	Medication	3.74	− 10.52, 18.00	0.60
	KIDMED score	−1.64	− 4.19, 0.90	0.20

Bold character represents marginally significant p-values. $FEF_{25-75\%}$ mid expiratory flow 25–75% vital capacity. Spirometry variables presented as percent predicted pre-bronchodilator administration

β Unstandardized coefficient, 95% *CI* 95% confidence interval

*BMI as the dichotomous variable (overweight/obese vs. normal weight)

[a]p value evaluated applying multiple linear regression model controlling for confounders namely age, height, sex, regular physical activity, KIDMED score and medication

our findings, Spathopoulos et al. documented that high BMI reduced spirometry parameters, FVC, FEV_1,$FEF_{25–75\%}$ and FEV_1/FVC, in overweight/or obese Greek school children [12]. Discrepancies among studies could be due to variability in sample size, age, population diversity, ethnicity,asthma definition and tools used to assess adiposity in children (BMI, waist to hip ratio, % body fat).

With respect to the absence of airway limitation as reflected by FEV_1/FVC, a feasible explanation could be that abnormal lung growth caused by surplus weight could be a consequence of high BMI and not indicative of airway dysfunction. Then again, there were only seven obese children in our sample. Therefore, any expected decline in spirometric parameters due to large increases in fat mass would have been underpowered to detect any significant dif-ferences. Also, the apparent discrepancy between changes in forced volumes and their ratio in obese children could be attributed to the variable effect of weight according to the degree of overweight. It has been suggested that obesity may affect spirometry measurements when BMI ≥ 40 kg/m^2 [17].
The highest value for BMI in our children was 30.61 kg/m^2.

One potential mechanism by which overweight could impact lung function in asthmatic children is that excess bodyweight promotes disproportionate or abnormal lung growth represented by a mismatch between growth of airways and that of lung parenchyma that is manifested as high or supranormal FVC, FEV_1 and $FEF_{25-75\%}$ but decreased FEV_1/FVC (indicative of airway obstruction) [10]. Airway dysanaptic growth or large lung size is also seen in swimmers, which does not have any association with lung disease [18]. The above-mentioned findings have important implications in the interpretation of lung function tests because spirometry may have reduced specificity and sensitivity for asthma in overweight paediatric patients. In addition, most of the studies linking obesity and lung function in children are based on spirometry which mainly reflects large airway function [19]. Impulse oscillometry may be more appropriate to measure lung resistance and reactance ('true' obstruction) in peripheral and central airways as well as to confirm the presence of dysanapsis in patients with obese-asthma phenotype, as was done in the study by Ekstrom et al.(2018) [13, 19]. Application of impulse oscillometry to analyse the 'true obstructive disorder' as compared to spirometry deserves further investigation.

FeNO is established as a robust non-invasive biomarker of eosinophilic airway inflammation [9]. With respect to low FeNO measurements in overweight/obese asthmatic children as compared to normal weight or no associations, these results might primarily originate from differences in airway calibre or lung volumes between the two groups. Our findings might be related to anatomical and mechanical obstructive effect on airways that favour a ventilation perfusion imbalance rather than a result of inflammation [14].
This paradox/anomaly of low or no relationship between FeNO and BMI has been documented by prior cross-sectional studies in children [10, 13, 20, 21]. Collectively, these revelations suggest that increased adiposity might lead to neutrophilic rather than eosinophilic systemic inflammation [14]. Nevertheless, we did not measure blood neutrophil or eosinophil cell count in the present study. Additionally, reduced FeNO might be the result of limited production of NO in the airways as well as increased NO metabolism [22]. Excessive fat leads to an increase in arginase expression relative to L-arginine concentration and subsequently to lower FeNO levels [22].

A limitation of this study is small sample size. Our findings are based on cross-sectional data, and a causal relationship cannot be inferred. Although BMI is not the gold stand-ard to assess body composition, most studies investigating the obesity-asthma link have used BMI as a proxy for total body fat and to monitor changes in body composition. Lung volume decline is related to 'fat distribution' which comprises of central and peripheral fat as well as tissue composition [17].
Most of the fat deposits in central obesity are in the abdominal area and excessive accumulation of fat mass can alter the pressure–volume characteristics of the thorax and restrict the descent of the diaphragm leading to limited lung expansion [17]. Abdominal adiposity as measured by waist circumference and waist/hip ratio might be better predictors of pulmonary function than weight or BMI [17]. Despite adjusting main confounders in the multivariate analyses, we cannot exclude that pulmonary test measurements could be affected by residual or unmeasured factors that have not been controlled (such as social economic status, maternal smoking or education level). In conclusion, the findings of this study suggest that excess bodyweight could impact pulmonary dynamics in childhood asthma. Future studies are recommended to establish the underlying mechanism behind the obesity and asthma link.

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Author Contributions MMP conceptualized, designed and drafted the manuscript, collected data and performed the statistical analyses. CI was in charge and co-ordinated the study. BE supervised the statistical analyses and interpretation of data. All co-authors critically revised, edited the manuscript and approved the final version as submitted. The authors confirm that the article is the authors' original work, has not received prior publication, and is not under consideration for publication elsewhere.

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Compliance with Ethical Standards

Conflicts of interest The authors declare that they have no conflict of interest.

Ethical Approval All procedures performed in this study involving human participants were in accordance with the ethical standards of the institution (La Trobe University Human Ethics Committee, HEC 16-035) and with the 1964 Helsinki declaration and its later amendments.

Informed Consent Informed consent was obtained from all individual participants included in this study.

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6.3.1 Sex differences between BMI and pulmonary function tests

At baseline, when investigating for sex differences in ventilatory function and FeNO per BMI group, no differences were seen (Table 13). However, at six months a significant difference in PEF between boys and girls was observed in the normal weight group [t (32) = 2.11, p = 0.043]. No sex differences were observed for FeNO in either weight group at six months (Table 14).

Table 13 Gender differences in spirometry and FeNO per BMI group at baseline

Baseline						
	BMI Group					
	Normal			Overweight/obese		
	Gender			Gender		
	Male (n=18)	Female (n=17)		Male (n=12)	Female (n=13)	
Variable	**Mean ± SD**	**Mean ± SD**	***P*[a]**	**Mean ± SD**	**Mean ± SD**	***P*[a]**
FVC* (L)	96.22 ± 10.49	91.41 ± 8.26	0.14	95.50 ± 11.02	99.38 ± 8.09	0.32
FEV_1 (L)	99.06 ± 10.30	93.76 ± 9.62	0.13	99.75 ± 8.95	101.77 ± 9.92	0.59
FEV_1/FVC	102.61 ± 6.55	101.47 ± 7.17	0.62	103.67 ± 4.75	101.77 ± 4.83	0.33
PEF (L/s)	94.89 ± 16.45	90.82 ± 21.42	0.53	94.50 ± 12.15	91.31 ± 15.18	0.56
$FEF_{25-75\%}$	101.61 ± 17.01	97.18 ± 24.06	0.53	108.58 ± 18.24	104.08 ± 18.23	0.54
FeNO (ppb)	14.11 ± 11.27	17.47 ± 14.27	0.46[b]	11.58 ± 19.20	12.23 ± 12.62	0.68[b]

*%predicted pre-bronchodilator administration;

[a] P-value calculated using t-test; [b] Mann-Whitney test

Table 14 Gender differences in spirometry and FeNO per BMI group at six-months

Six-months						
	BMI Group					
	Normal			Overweight/obese		
	Gender			Gender		
	Male (n=17)	Female (n=17)		Male (n=14)	Female (n=13)	
Variable	**Mean ± S.D**	**Mean ± S.D**	***P*[a]**	**Mean ± S.D**	**Mean± S.D**	***P*[a]**
FVC* (L)	95.53± 10.37	93.71±9.01	0.58	96.43± 8.54	100.00 ±8.02	0.27
FEV_1 (L)	100.29±10.76	95.71± 9.25	0.19	100.36±6.66	103.46 ±8.44	0.29
FEV_1/FVC	104.53±4.52	101.76±5.12	0.10	103.36±4.89	102.77±2.55	0.70
PEF (L/s)	109.76±29.33	93.53±12.01	**0.043**	99.07± 22.65	99.08± 14.44	0.99
$FEF_{25-75\%}$	102.29±14.45	99.12± 22.05	0.62	106.07 ±17.05	105.77±12.77	0.95
FeNO (ppb)	12.94± 13.25	25.47± 38.80	0.22[b]	15.50± 19.44	11.23± 7.18	0.90[b]

*%predicted pre-bronchodilator administration

P[a –] P-value calculated using t-test; [b] Mann-Whitney test

6.4 Dietary Habits

6.4.1 Breakfast meal consumption

The FFQ revealed that at baseline 76.6% (49/64) of children consumed breakfast seven days/week with 73.4% (47/64) consuming breakfast cereals/or bread, 87.5% (56/64) milk products and 54.7% (35/64) cheese pies/croissant/or cake. Regarding fast food intake, 15.6% (10/64) of parents replied that fast food was consumed by children more than once a week. With respect to oils used in cooking, frying and added to salads/vegetables, pasta or rice, 96.9% (62/64) of parents replied 'yes' that olive oil was used as the main source of fat.

A similar trend was observed at follow-up. About 75% (48/64) of children consumed breakfast daily, 89.1% (57/64) ate breakfast cereals/or bread, 90.6% (58/64) milk products and 55.6% (35/64) cheese pie/croissant/or cake for the breakfast meal. As for use of olive oil, 98.4% (63/64) of parents responded affirmatively that they used olive oil in cooking and added to all dishes. As for fast food intake, 12.5% (8/64) of children consumed fast food more than once weekly. Breakfast meal, fast food and olive oil intake per group is shown in Table 15. No differences in consumption of breakfast, fast food or olive oil were observed between the groups at both time-points.

Table 15 Olive oil, breakfast and fast food intake for the total sample and per group at baseline and six months

DIETARY HABITS QUESTIONNAIRE									
		BASELINE				SIX MONTHS			
			GROUP				GROUP		
QUESTION	Response	Total sample %(n)	I %(n)	C %(n)	*P**	Total sample %(n)	I %(n)	C %(n)	*P**
Q1. How many times per week does your child eat breakfast? 7 days per week	Yes	76.6(49)	80.6(25)	72.7 (24)	0.16	75.0(48)	77.4(24)	72.7(24)	0.67
Q2. If your child eats breakfast, does he/she eat:									
a) Breakfast cereal or bread or rusks or toast?	Yes	73.4(47)	74.2(23)	72.7(24)	0.89	89.1(57)	90.3(28)	87.9(29)	0.75
b) Milk products (milk, yogurt, cheese) ?	Yes	87.5(56)	80.6 (25)	93.9(31)	0.11	90.6(58)	83.9(26)	97.0(32)	0.07
c) Cheese-pies or croissant or cookies or cake?	Yes	54.7(35)	48.4(15)	60.6(20)	0.33	55.6(35)	63.3(19)	48.5(16)	0.24
Q3. Do you use olive oil for cooking, frying, or add to pasta/ rice?	Yes	96.9(62)	96.8(30)	97.0(32)	0.96	98.4(63)	100.0(31)	97(32)	0.33
Q4. Does your child eat fast food more than once/week?	Yes	15.6(10)	16.1(5)	15.2(5)	0.91	12.5(8)	12.9(4)	12.1(4)	0.92

Total sample N=64; n(intervention) = 31; n(control) = 33

*Chi Square Test

Key: I = Intervention; C = Control

6.4.2. Dietary intake vs Hellenic dietary guidelines 2014

In general, when comparing dietary intake according to the Hellenic dietary recommendations for children and adolescents [(293)] which is based on the Greek Traditional Mediterranean diet prototype, children in the intervention and control groups at both time-points were low in consumption of dairy products, fruit (< 3 serves/day), vegetables (< 400 g/day), starch (as represented by cereals, rice, bread, pasta < 5-6 serves/day), nuts (< 3-4 serves/day) and olive oil. Fish intake was low in both groups at baseline (< 2-3 serves/week) and remained low in the control at six months. As for legume consumption, it was low in the control group (< 3 serves/week) as compared to the intervention at six months.

Table 16 displays the recommended frequency of consumption and serving size for each main food group per age category as suggested in the Hellenic dietary guidelines for children and adolescents[(293)].

Table 16 Dietary intake of main food groups and olive oil according to the Hellenic dietary guidelines for children and adolescents

Food group/ Age	**4-8 years**	**9-13 years**	**Standard portion size for children**
Dairy products (serves/day)	2-3	3-4	250 ml milk, 30 g cheese, 200g yogurt
Vegetables (serves/day)	1-2	2-3	1C or 240 mls raw or cooked vegetables
Fruit (serves/day)	1-2	2-3	1 medium fruit (220g), ½ C fruit juice
Starch (serves/day)	4-5	5-6	30g breakfast cereal, 30g bread, 90 g cooked rice/pasta, 120g potato
Legumes (serves/week)	3	>3	90- 120g (4-8 y.o), 120-150g (9-13 y.o)
Fish (serves/week)	2-3	2-3	90-120g (4-8 y.o), 120-150g (9-13 y.o)
Meat (serves/week)	2-3	2-3	60-90 g (4-8 y.o), 90-120 g (9-13 y.o)
Olive oil (serves/day)	2-3	3-4	15 g, 10-12 olives
Nuts (serves/day)	2-3	3-4	1 handful = 18 almonds, 6 walnuts, 1.5 tbsps. tahini
Water (cups/day)	4-5	6-8	1 Cup = 250 ml

6.4.3 Food consumption patterns estimated from FFQs

Children's food consumption patterns was assessed from FFQs by the question "How many times in the last month did your child consume……..? Frequency of consumption of food at baseline and six months are presented in Tables 1 and 2 of Appendix 2D. Percentage of children responding that they consumed food items on a weekly and daily basis calculated from FFQs is shown graphically in Figures 26 and 27 at baseline and for six months in Figures 28 and 29.

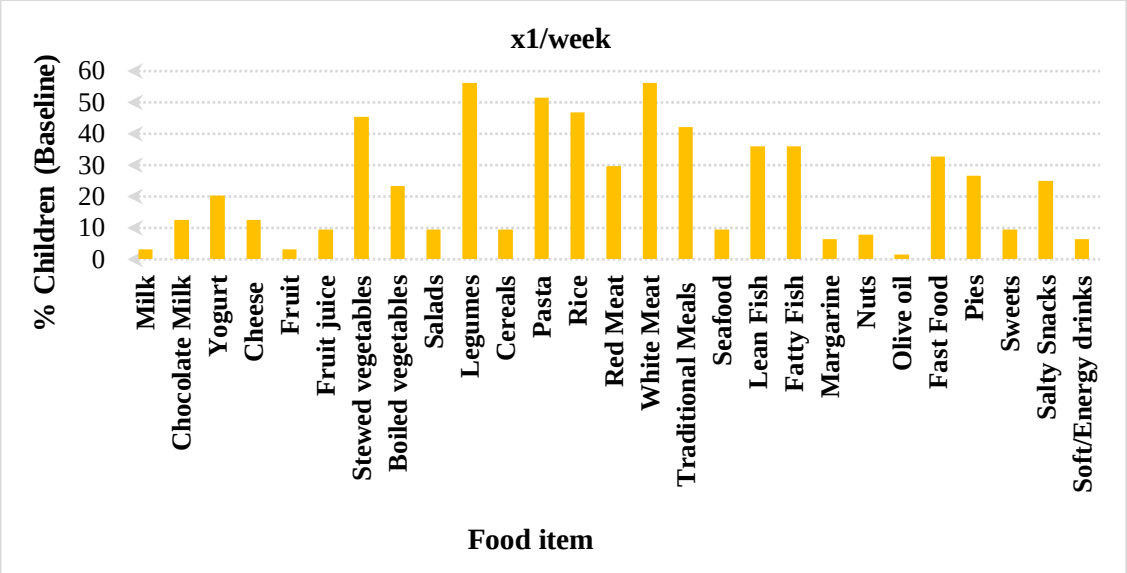

Figure 26 Frequency of children's food intake (%) per week for total sample at baseline

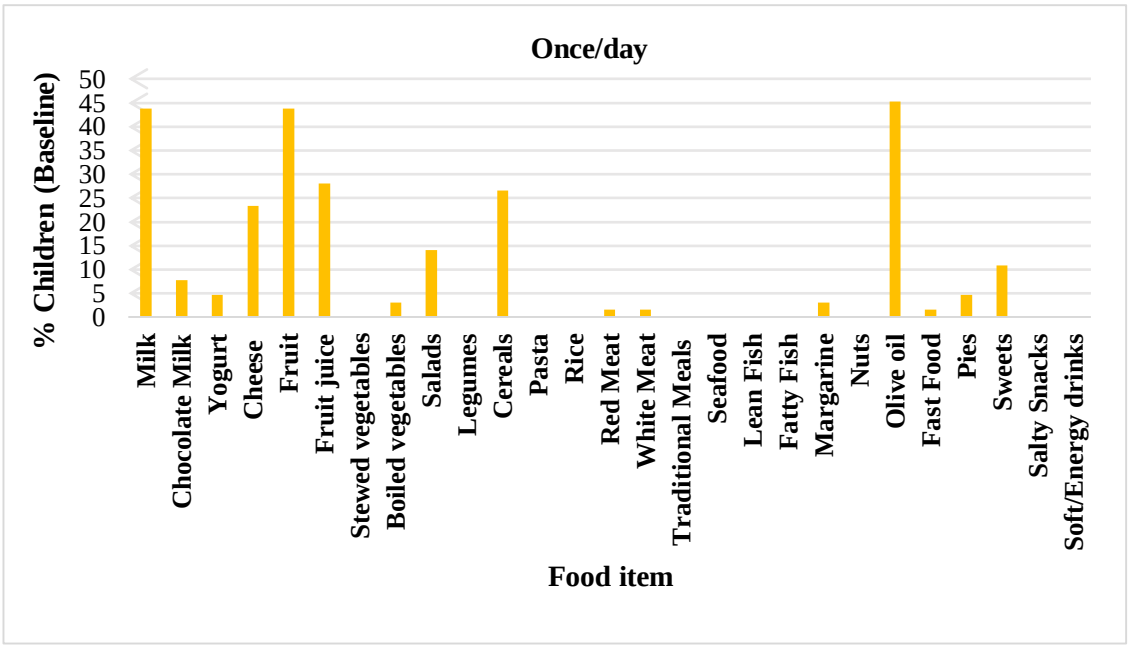

Figure 27 Frequency of children's food intake (%) per day for total sample at baseline

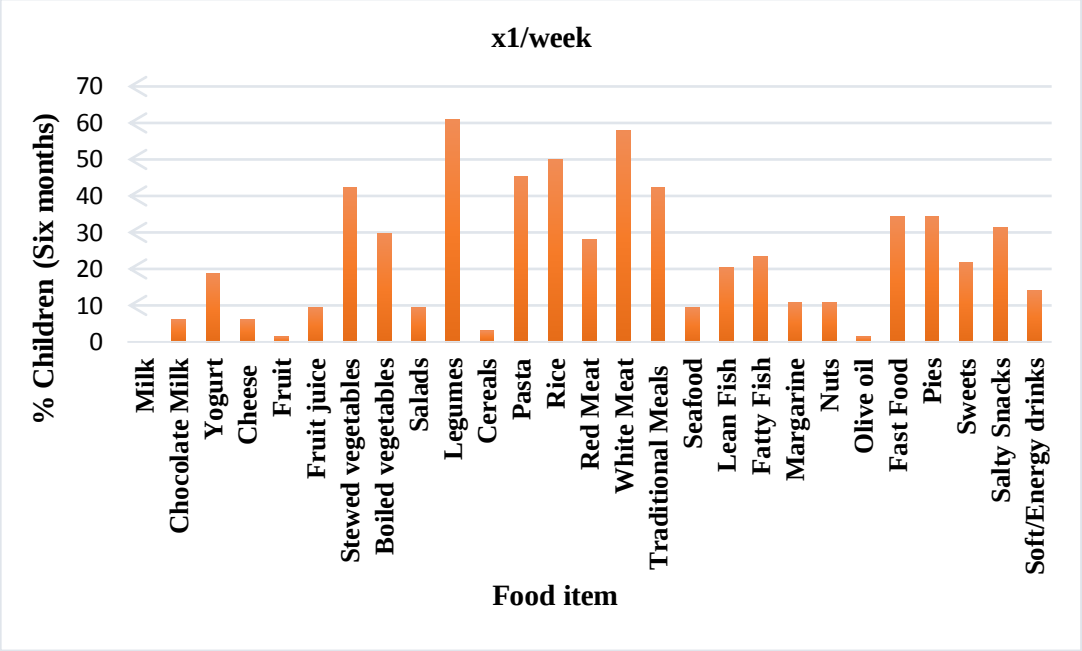

Figure 28 Frequency of children's food intake (%) per week for total sample at six months

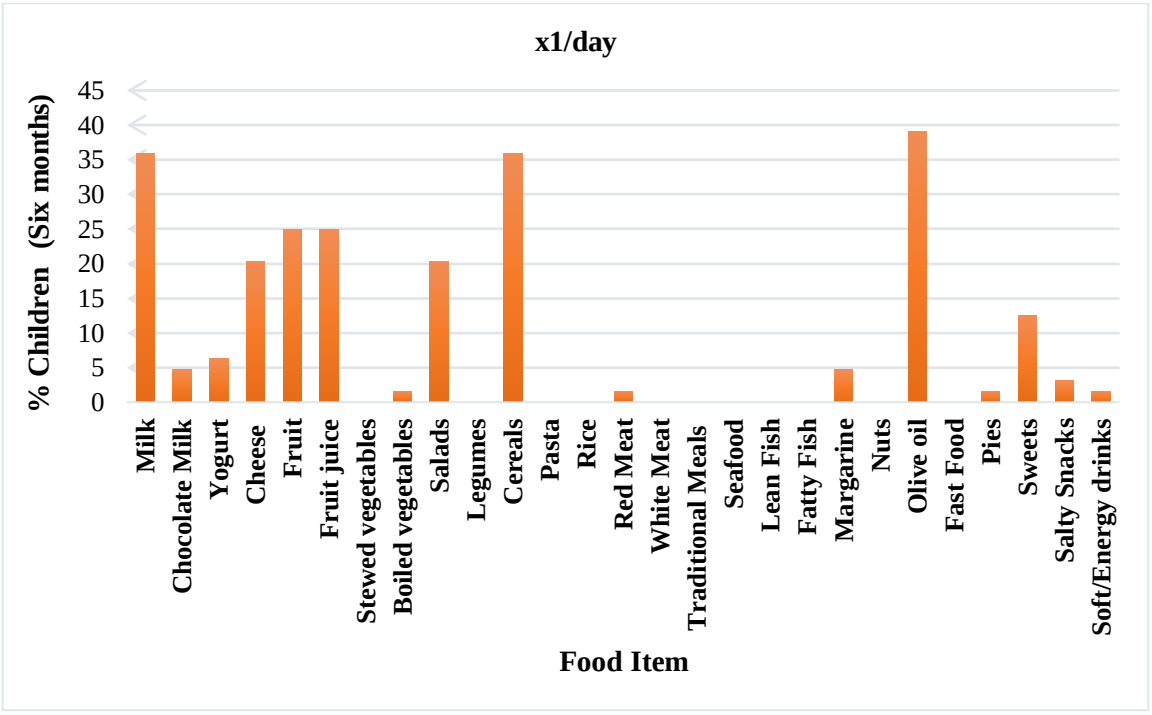

Figure 29 Frequency of children's food intake (%) per day for total sample at six months

Figures 26 and 27 depict that at baseline, children's frequency of intake for milk, yogurt, cheese, fruit, vegetables, salads, cereals, nuts and olive oil were below the Hellenic recommended dietary intake for children and adolescents [293]. Only 43.8% of children consumed milk daily, 4.7% yogurt, 23.4% cheese (yellow/white), 43.8% fruit, 23.4% fruit

juice, 3.1% boiled vegetables, 14.1% salads, 26.6% cereals, and 45.3% olive oil. Regarding weekly intake, 45.3% consumed stewed vegetables in sauce, 23.4% boiled vegetables, 9.4% salads, 56.3% legumes, 51.6% pasta, 46.9% rice, 56.3% white meat, 42.2% traditional meals (pastitisio, mousaka etc), 35.9% lean fish and fatty fish, 32.8%, fast food, 7.8% nuts and 26.6%. pies. The majority of children (42.2%) consumed sweets 2-3 times per week, 59.4% red meat and 37.5% salads. As for fast food, salty snacks and soft drinks, parents responded that these foods were consumed 1-3 times/months at a frequency of 40.6%, 28.1% and 20.3% respectively.

Similar results were apparent from the six months analysis in Figures 28 and 29 with less than 50% of children conforming to the Hellenic dietary recommendations for children/adolescents.. Compared to baseline, at six months daily milk intake decreased to 35.9% of children consuming milk, 25% fruit and 39.1% for olive oil, while cereal intake increased slightly to 35.9% and pies to 34.4%. No change was observed for daily intake of yogurt (6.3%), cheese (20.3%), fruit juice (25%), boiled vegetables (1.6%) and salads (20.3%). The same trend was observed for weekly consumption of white meat (57.8%), traditional meals (42.2%), rice (50%), pasta (45.3%), stewed vegetables (42%), legumes (60.9%), boiled veges (29.7%) and lean fish (20.3%). No change was observed for intake of fast food, salty snacks or soft drinks. As at the commencement of this study, 37.5% of children consumed salads 2-3 times per week, red meat 54.7% and sweets (43.8%). By the end of six months frequency of fatty fish intake increased to 2-3 times per week in 34.4% of all children as compared to only once weekly in 35.9% at baseline.

Frequency of children's food intake by intervention and control groups at both time points is illustrated in Tables 3 and 4 in Appendix 2D. A pictorial illustration of children's weekly and daily dietary pattern per group at baseline is given in Figures 30 and 31. At baseline, a higher percentage of children in the intervention group consumed weekly fatty fish [% children intervention vs control: 45.2% vs 27.3%; $X^2(3, N = 64) = 9.83$, $p = 0.02$], lean fish (% intervention vs control: 48.4% vs 24.2%; $X^2(3, N = 64) = 9.26$, $p = 0.03$) and traditional Greek dishes [% intervention vs control: 51.6% vs 30.3%; $X^2(4, N = 64) = 11.43$, $p = 0.02$)] compared to the control group. Daily fruit juice and fruit intake (% intervention vs control: 35.5% vs 21.2%, $p = 0.24$; 48.4% vs 39.4%, $p = 0.95$ respectively) and weekly stewed vegetables in sauce (% intervention vs control: 51.6% vs 39.4%; $p = 0.42$) was consumed by more children in the intervention group than in the control, although non- significant. In contrast, the control group consumed more soft

drinks 2-3 times per week than the intervention group (% control vs intervention: 18.2% vs 0.0%; $X^2(3, N = 64)=8.25$, $p = 0.04$). Similarly, weekly pie intake was higher in the control than in the intervention group (% control vs intervention: 33.3% vs 19.4%; $p = 0.26$) and daily sweets (% control vs intervention: 12.1% vs 9.7%; $p = 0.99$) as well as daily milk consumption (% control vs intervention: 48.5% vs 38.7%; $p = 0.71$), however not significant.

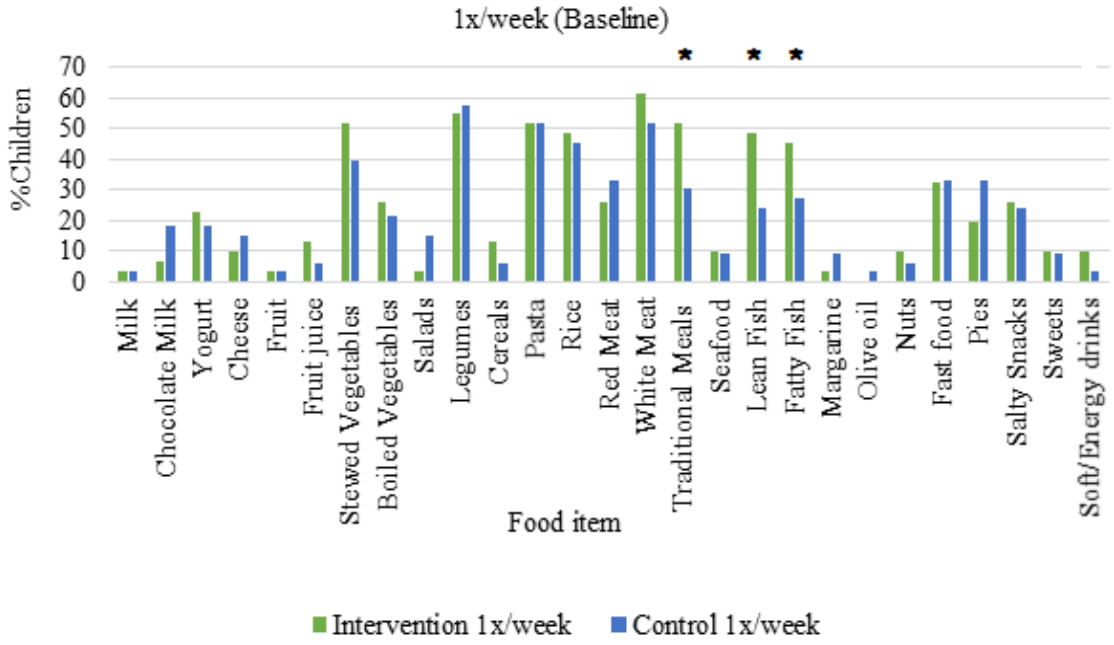

Figure 30 Weekly frequency of food intake per intervention and control group at baseline
*$p < 0.05$ (Chi Square test)

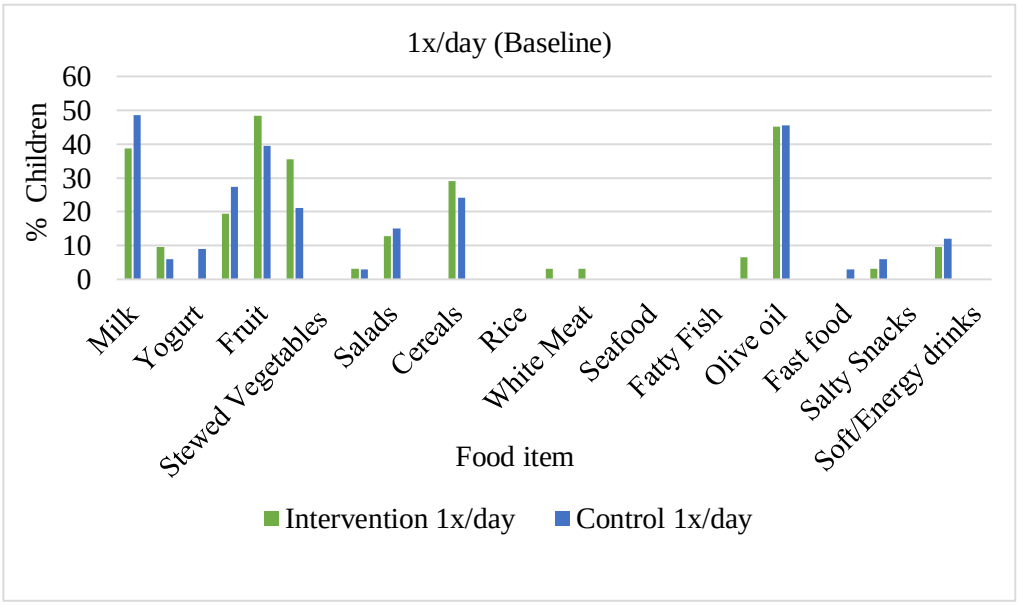

Figure 31 Daily frequency of food intake per intervention and control group at baseline.

Frequency of main food group intake in times per day by intervention and control groups at baseline is shown in Figure 32 and in tabulated form in Table 5 in Appendix 2D.

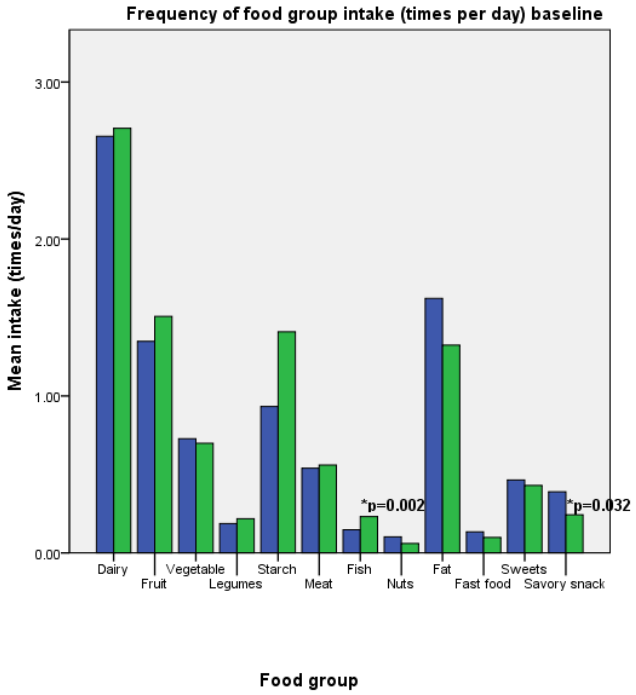

Figure 32 Frequency of main food group intake (times/day) per intervention and control group at baseline

Figure 32 shows that at baseline there was a significant difference in frequency of total fish intake (lean and fatty) in times/day in the intervention group as compared to the control [intervention vs control (mean):0.23 vs 0.15 times/day; $U = 287.00$, $p = 0.002$ (Mann-Whitney test)]. However, there was a higher frequency of savoury snacks intake in the control versus the intervention [control vs intervention (mean): 0.39 vs 0.24 times/day; $U = 352.5$, $p = 0.032$]. The same trend was observed for fat intake, though non-significant (control vs intervention: 1.62 vs 1.32 times/day; $p = 0.21$)

Percentage of children's weekly and daily consumption of food items from FFQs per intervention and control group at six months is shown in Figures 33 and 34. At six months, a higher percentage of children in the intervention group consumed fruit daily [% intervention vs control: 38.7% vs 12%; $X^2(6, N = 64) = 13.09$, $p = 0.042$], legumes weekly (%intervention vs control: 67.7% vs 54.4%; $X^2 (3, N = 64) = 8.11$, $p = 0.044$)and fatty fish 2-3 times/week (% intervention vs control: 67.7% vs 3.0%; $X^2 (4, N = 64) = 41.49$, $p < 0.001$) than the control, due to the nature of the intervention. Although, the control

consumed more lean fish weekly as expected (% intervention vs control: 6.5% vs 32.3%; X^2 (4, N = 64) = 17.31, p = 0.002). Daily consumption of olive oil was higher in the intervention group than in the control, but not significant (% intervention vs control: 48.4% vs 30.3%; p = 0.15). Contrastingly, more children in the control consumed sweets daily than in the intervention and pies weekly (% control vs intervention: 18.2% vs 6.5%; p = 0.23; 39.4% vs 29.0%; p = 0.18 respectively).

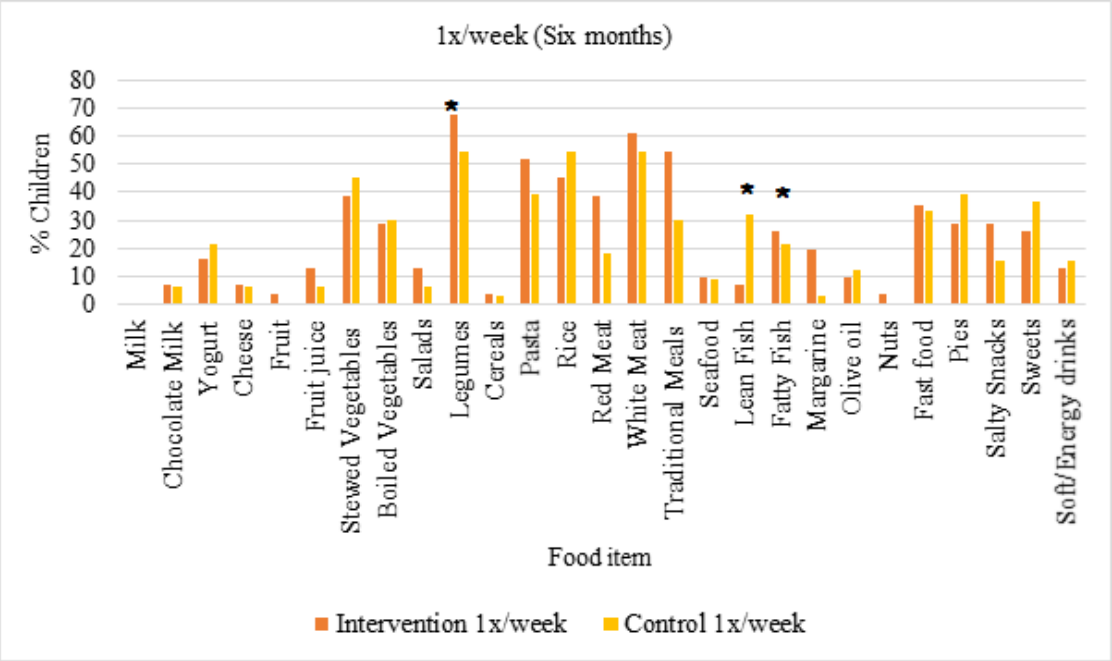

Figure 33 Weekly frequency of consumption of food per intervention and control group at six months

* P-value for weekly intake of legumes (p = 0.044), fatty fish (p < 0.001) and lean fish (p = 0.002)

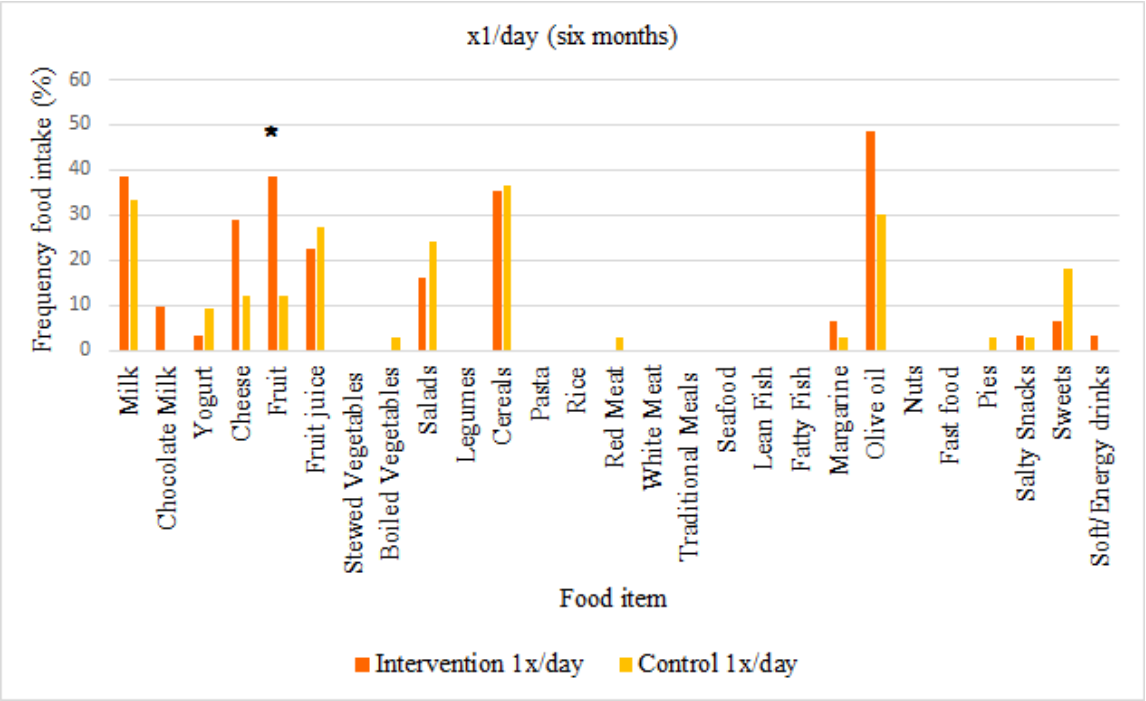

Figure 34 Daily frequency of consumption of food per intervention and control group at six months

Frequency of food group intakes in times per day estimated from FFQs at six months is shown in Appendix 2D in Table 6 and graphically in Figure 35.

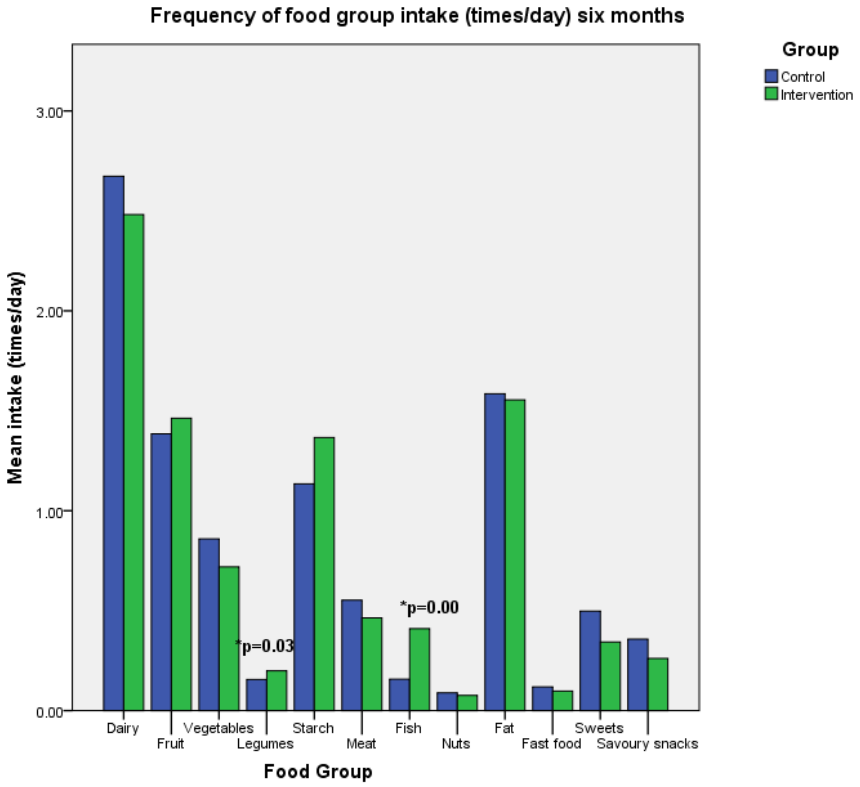

Figure 35 Frequency of food group intake by intervention and control group at six months

In Figure 35 it is apparent that at six months there was a significant increase in frequency of legumes and fish intake in the intervention group as compared to the control [intervention vs control (mean): 0.20 vs 0.16 times/day; U = 374.00, p = 0.034]; [intervention vs control (mean): 0.41 vs 0.16 times/day; U = 102.50, p < 0.001] respectively. Contrastingly, a slightly higher intake of dairy [control vs intervention (mean) 2.67 vs 2.48 times/day; p = 0.90] ,vegetables [control vs intervention (mean) 0.86 vs 0.72 time/day; p = 0.49], sweets [control vs intervention (mean): 0.48 vs 0.34 times/day; p = 0.22] and savoury snacks [0.36 vs 0.26 times/day; p = 0.08] was observed in the control group than in the intervention, nevertheless insignificant.

Comparison of frequency of food group intake (times per day) from FFQs according to group from baseline to six months is shown in Appendix 2D, Table 7.

A comparison of data per intervention and control groups at both time-points is clearly manifested in Figures 36 and 37. Assessment of within group differences in frequency of food group intake (in times per day) between the two time-points showed that there was a significant difference in frequency of fish intake in the intervention group only [baseline vs six months (mean); 0.23 vs 0.40 times/day; Z = -3.59, p < 0.001 (Wilcoxon rank test)]. In addition, at six months there was an increase in legume and fat intake in the intervention group, although not significant (baseline vs six months (mean): 0.22 vs 1.37 times/day ; p = 0.59; 1.32 vs 1.55 times/day; p = 0.14 respectively).

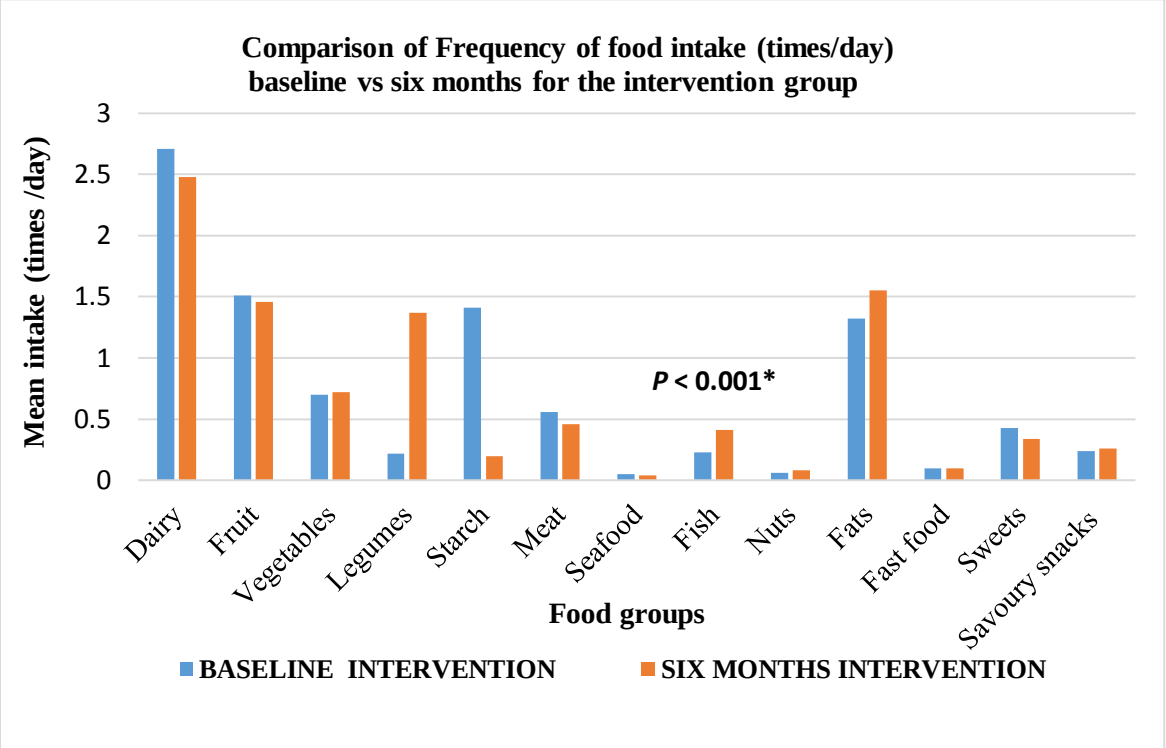

Figure 36 Comparison of frequency of food group intake (in times/day) between baseline and six months for the intervention group**.**

*Wilcoxon rank sign test

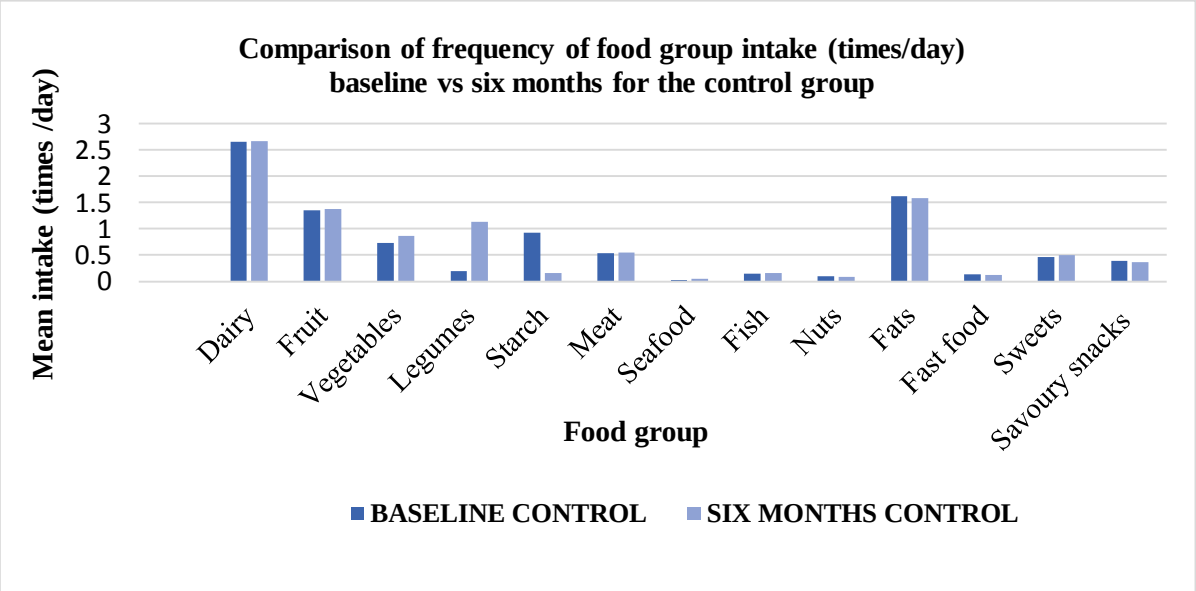

Figure 37 Comparison in food group intake (times/day) from FFQs between baseline and six months for the control group

In contrast, with respect to the control group, from Figure 37, at six months there was a non-significant increase in legume intake (baseline vs six months: 0.19 ± 0.11 vs 1.13 ± 0.53; p = 0.19) and decrease in starch (baseline vs six months: 0.93 ± 0.48 vs 0.16 ± 0.11; p = 0.08).

No significant difference in any other food group between baseline and six months was evident for the control group.

Dietary intake from Food Frequency questionnaires

Dietary intake (in grams per day) of participants at baseline and six months as calculated from FFQs is displayed in Table 17.

Table 17 Dietary intake of participants (grams per day) from FFQs at baseline and six-months

DIETARY INTAKE										
	Baseline					Six Months				
	Group					Group				
	Intervention		Control			Intervention		Control		
Food Item (grams/day)	Mean	SD	Mean	SD	*P*[b]	Mean	SD	Mean	SD	*P*[b]
Milk	343.3	235.8	340.6	191.8	0.79	331.3	229.7	363.0	213.8	0.48
Chocolate milk	51.9	124.8	86.5	177.9	0.38	47.3	124.7	74.5	175.7	0.69
Yogurt	68.5	106.8	68.4	70.7	0.77	54.1	59.5	61.9	75.2	0.82
Cheese	23.2	22.5	17.7	15.2	0.57	20.4	14.0	17.8	17.4	0.15
Fruit	132.4	95.1	119.4	82.4	0.56	142.6	113.1	117.4	94.8	0.32
Fruit juice	149.9	86.2	132.5	147.3	0.11	135.6	117.9	137.1	117.9	0.97
Stewed vegetables	46.2	29.9	39.7	31.3	0.27	41.2	31.4	37.7	30.9	0.73
Boiled vegetables	41.9	54.1	40.4	54.3	0.75	32.4	32.4	27.7	45.8	0.26
Salads	79.6	78.4	94.5	80.0	0.37	99.0	80.6	140.8	116.3	0.13
Legumes	65.5	35.3	56.1	33.7	0.22	59.8	32.2	46.8	31.7	**0.03**
Breakfast cereals	24.1	22.5	17.9	14.5	0.21	27.5	25.9	23.3	16.6	0.84
Pasta	50.5	61.8	32.0	20.4	0.16	36.3	22.8	32.0	17.3	0.49
Rice	38.8	69.4	17.1	13.3	**0.01**	30.3	28.8	20.4	14.0	0.24
Red meat	47.2	24.8	45.1	25.9	0.60	40.1	21.4	50.3	31.3	0.23
White meat	36.9	26.3	35.9	21.2	0.98	29.5	16.3	32.5	24.7	0.94
Seafood	7.0	11.1	4.6	6.9	0.45	5.6	6.9	7.1	6.6	0.30
Lean fish	17.5	11.7	11.7	13.6	**0.01**	15.8	25.7	13.2	10.5	0.09
Fatty fish	17.2	11.8	10.4	11.6	**0.01**	45.8	19.4	10.3	11.0	**0.00**
Traditional meals	22.9	14.6	13.7	9.8	**0.00**	19.1	10.6	16.7	13.5	0.09
Margarine	0.8	1.8	1.4	1.8	0.09	2.1	3.6	1.5	3.4	0.10
Nuts	3.0	6.4	5.1	6.5	0.10	3.8	6.9	4.4	5.6	0.38
Olive oil	16.4	12.5	19.3	14.8	0.31	18.1	14.9	18.9	14.0	0.99
Fast food	19.7	17.0	26.8	36.5	0.64	19.6	12.8	23.7	20.4	0.65
Pies	19.1	29.7	31.3	36.0	**0.03**	19.9	21.1	26.7	28.5	0.18
Sweets	25.8	17.5	27.9	17.7	0.63	20.6	14.9	29.9	29.0	0.22
Salty snacks	8.1	8.4	12.7	11.8	0.13	8.9	12.7	12.5	15.7	0.22
Soft drinks	8.5	16.0	30.1	47.5	0.07	36.1	72.3	36.0	46.4	0.28

Shown in bold are the statistically significant p-values.

Intervention group (n=31), Control group (n=33)

[b]*P*- *P*-value calculated using Mann-Whitney Test

Table17 shows that at baseline the intervention group consumed more rice [(mean): 38.8 vs 17.1 g/day; U = 342.50, p = 0.015], lean fish (17.5 vs 11.7; U = 332.00, p = 0.011) and traditional Greek Mediterranean meals (22.9 vs 13.7 g/day; U = 298.50 ,p = 0.002) than the control group. On the other hand, the control group consumed more pies than the intervention (mean: 31.3 vs 19.1 g/day; U = 355.00, p = 0.030). With regards to the six-month follow-up, a higher intake of legumes (mean: 59.8 vs 46.8 g/day; U = 374.00, p = 0.034) and fatty fish (mean: 45.76 vs 10.35 g/day; U = 61.50, p < 0.001) were consumed by the intervention group than the control, thus confirming good compliance to the dietary intervention.

Differences in dietary intake at baseline by group presented in Table 17 is clearly illustrated in Figure 38.

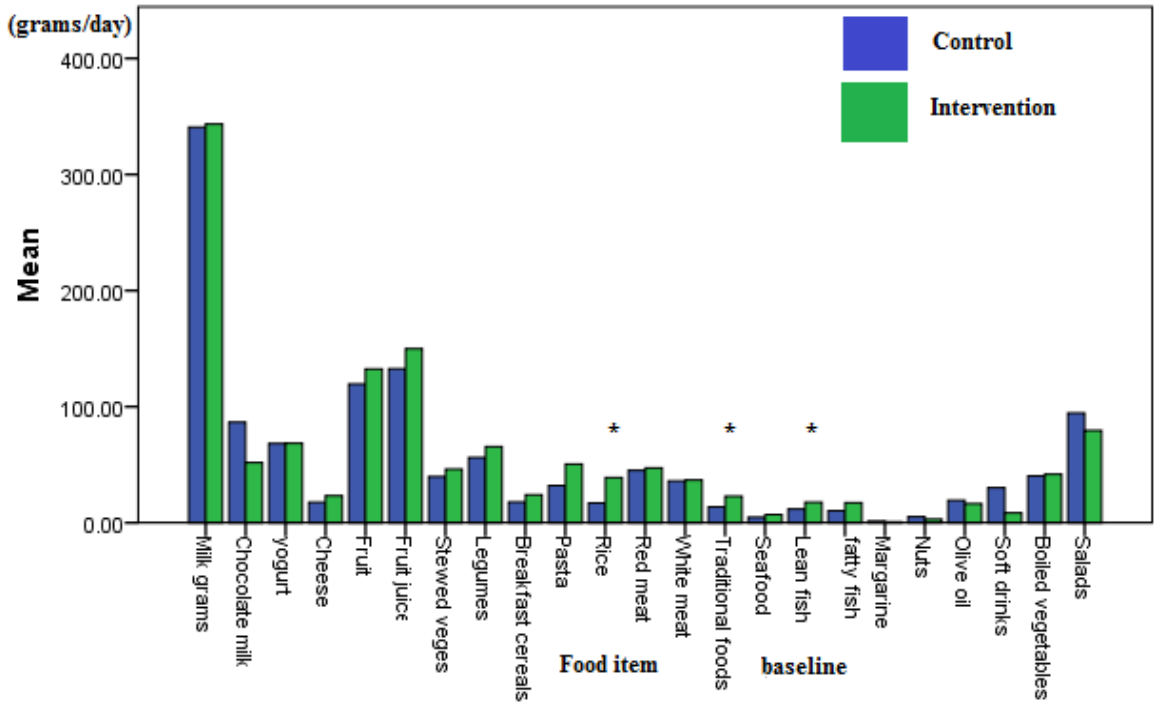

Figure 38 Dietary intake at baseline in grams per day per intervention and control group

*Significant p-values calculated by Mann-Whitney test

As for food groups, Figure 39 shows that at baseline the intervention group consumed more starch (intervention vs control (mean): 113.4 vs 67.0 g/day; U = 351.50, p = 0.031) and 'any' fish (intervention vs control (mean): 34.7 vs 22.1 g/day, U = 287.00 , p = 0.002) than the control group. The reverse was observed for savoury snacks [control vs intervention: (mean) 43.9 vs 27.2 g/day, U = 351.00, p = 0.031].

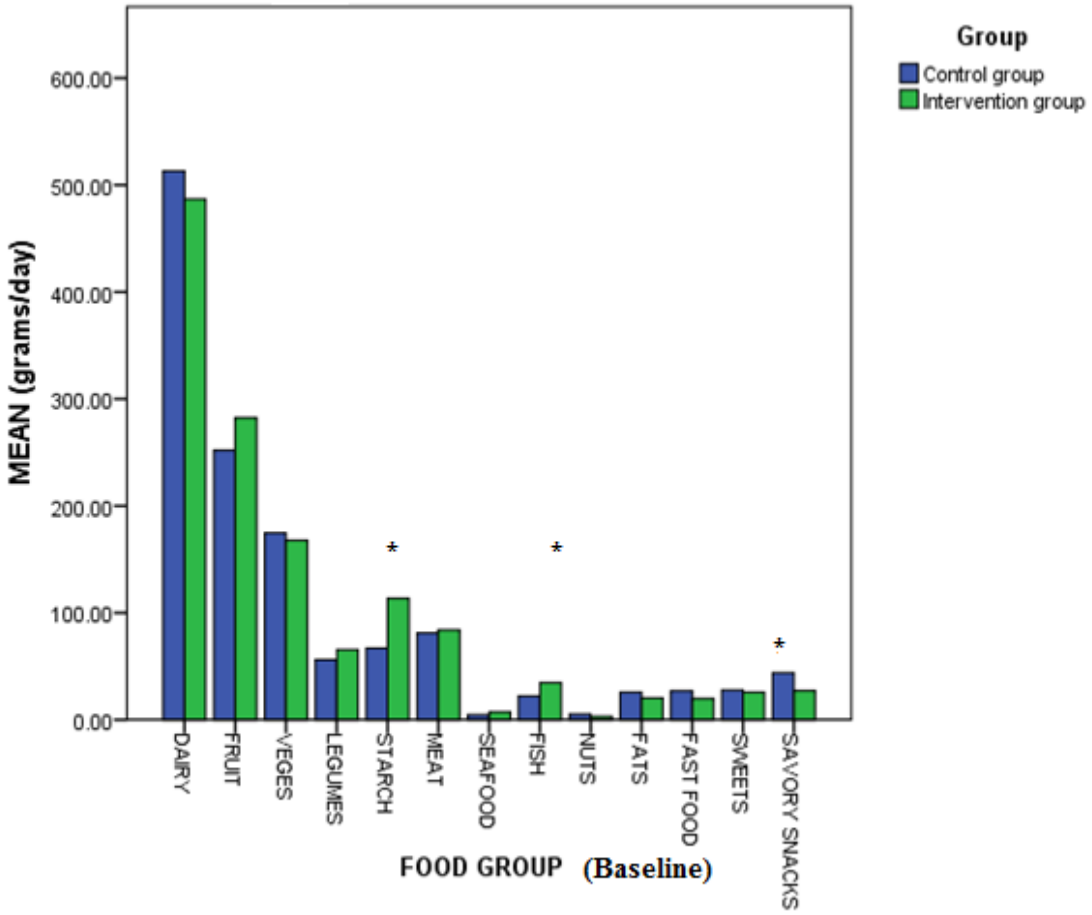

Figure 39 Main food group intake (in grams per day) at baseline per intervention and control group

*Significant *p*-values for starch ($p = 0.03$), fish ($p < 0.001$) and savoury snacks ($p = 0.03$) calculated using Mann-Whitney Test.

In comparison, dietary intake of food items listed in FFQs at six months per intervention and control group is shown in Figure 40 and by main food groups in Figure 41.

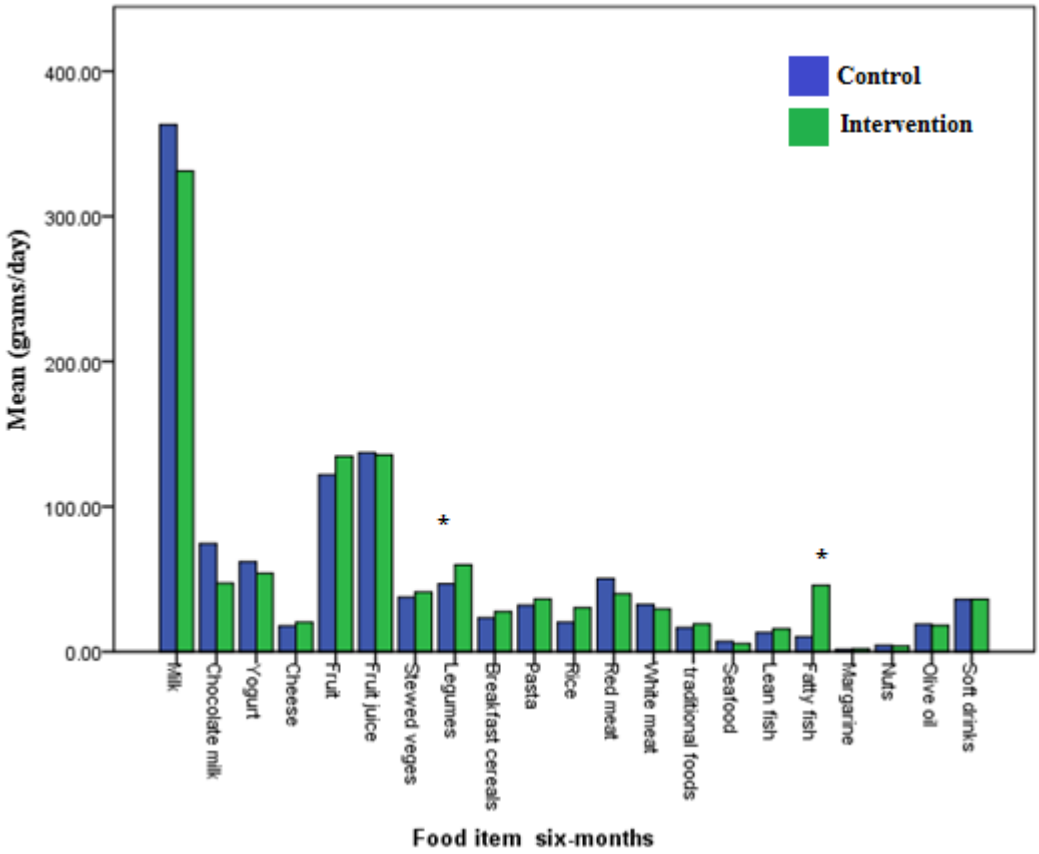

Figure 40 Dietary intake at six months in grams per day by intervention and control group

* $p < 0.05$ (Mann-Whitney test)

Figure 40 illustrates that at six months the intervention group had higher intake of fatty fish than the control ($p < 0.001$) as well as legumes ($p = 0.03$).

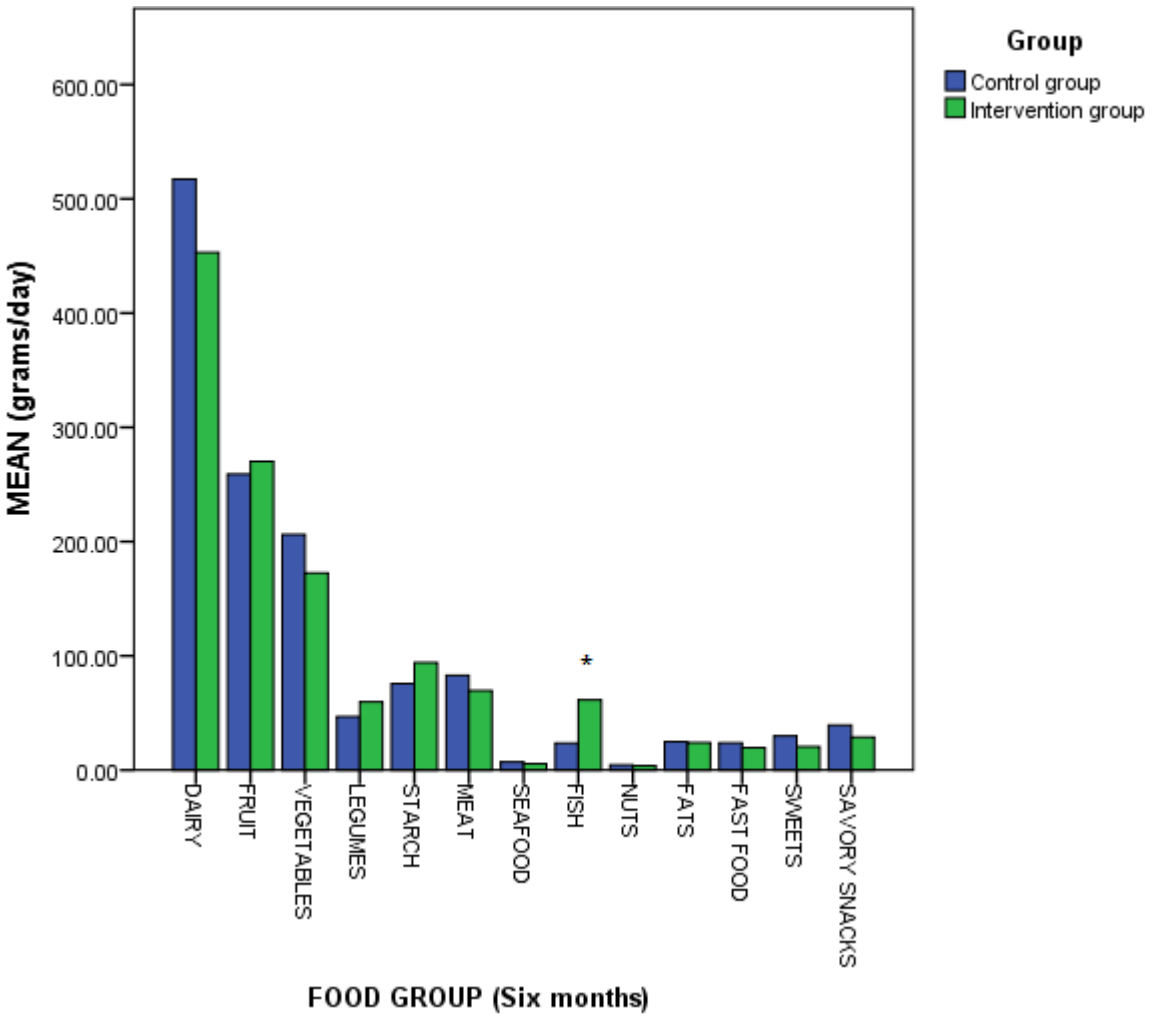

Figure 41 Main food group intake (in grams per day) at six months by intervention and control group *$P < 0.05$ (Mann-Whitney test)

Throughout the six month duration high fish intake was maintained by the intervention group as compared to the control (61.5 vs 26.5 g/day, $p < 0.001$).

When assessing for within group differences in food group intake (in grams/day) between baseline and six months, Wilcoxon sign rank test showed no differences for the control group. In contrast, a significant difference in fish intake at six months was observed in the intervention group compared to baseline ($Z = - 3.59$, $p < 0.001$); which is consistent with the aforementioned data.

6.4.4 Nutrient analysis of FFQs

Nutrient analysis of dietary intake measured using FFQs at both time-points was calculated using *McCance and Widdowson's "The Composition of Foods" (UK)* and Trichopoulou's *"Composition tables of food and Greek dishes"* [318; 319]. To the candidate's knowledge, there is no general consensus on the threshold for macro and micronutrient intake for Greek children. Therefore, dietary reference values for food, energy and nutrients for children in the United Kingdom [331] was used as a guide in this thesis which is summarized in Table 18.

Table 18 Energy and nutrition requirements for children in the UK.

Energy & Nutrition Requirements for children in the UK	
Energy 5-12 years	Estimated Average Requirements for children
Male	1720-2220 kcal/day
Female	1550-1845 kcal/day
Macronutrients: Dietary Reference Values (DRVs) as % daily energy intake (EI)	
Total Carbohydrate (CHO)	50%
Total Protein (P)	15%-20%
Protein:	
Male (5-12 years)	19.7- 42.1 g/day
Female (5-12 years)	19.7-41.2 g/day
Total Fat (F)	30-35%
Saturated fat	11%
Trans fat	2%
Monounsaturated fat (MUFA)	13%
Polyunsaturated fat (PUFA)	6.5%
Eicosapentanoic acid (EPA)/ Dodecahexanoic acid (DHA)(mg/day)	0.45[c]
Cholesterol	300 mg/day [d]
Dietary fibre (5-12 years)	20-25 g/day
Water intake	1ml/kcal/day
Micronutrients Recommended Nutrient Intakes for children 4-14 years	
Thiamin (mg/day)	0.7-0.9
Riboflavin (mg/day)	0.8-1.2
Niacin (mg/day)	11-15
Pantothenate (mg/day)	0.2[a]
Vitamin B6 (mg/day)	0.9-1.2

Vitamin B12 (µg/day)	0.8-1.2
Folate (µg/day)	100-200
Vitamin C (mg/day)	30-35
Vitamin A (µg/day)	400-600
Vitamin D (µg/day)	10
Vitamin E (mg/day)	0.4 mg/g PUFA
Calcium (mg/day)	450-800
Phosphorus (mg/day)	350-775
Magnesium (mg/day)	120-280
Sodium (mg/day)	700-1600 *
Potassium (mg/day)	1100-3100
Chloride (mg/day)	1100-2500
Iron (mg/day)	6.1-14.8
Zinc (mg/day)	6.5-9.0
Copper (mg/day)	0.6-0.8
Selenium (µg/day)	20-45
Iodine (µg/day)	100-130
Biotin (µg/day)	10-200[b]
Manganese (µg/day)	16
Molybdenum (µg/day)	0.5-1.5 µg/kg/day
Chromium (µg/day)	0.1-1.0 µg/kg/day
Fluoride (mg/day)	0.5mg/kg/day
Retinol & β-carotene as Vitamin A	µg/day
Tryptophan (mg)	12 mg/kg bodyweight

*WHO recommendation for Sodium <2g Na/day or 5g salt/day for children (332)
a (333) ; b (334); c (312); d (335)

Reference: Department of Health UK, 2016. Dietary reference values for food, energy and nutrients for the United Kingdom. TSO London, UK.

Nutrient composition of energy, macro and micronutrients as well as fatty acid composition for all food items in FFQs and food groups per intervention and control group at both time points are shown in Appendix 2D in Tables 9-24. Total macro and micronutrient intake of children's dietary intake per group at both time-points is summarized in Table 19.

Table 19 Total macronutrient and micronutrient intake per intervention and control group at baseline and six months calculated from FFQs.

Nutrient	Baseline		Six months	
	Group			
Macronutrients	**Intervention**	**Control**	**Intervention**	**Control**
H_20 (g)	1700.1	1605.7	2880.9	3114.1
Carbohydrate (g)	331.5	279.2	373.7	379.5
Protein (g)	125.1	113.4	346.8	371.4
Fat (g)	147.5	162.4	380.9	426.9
Energy(Kcal)	3006.6	2902.0	6134.6	6671.9
Fibre (g)	29.4	26.1	27.7	28.6
Fatty acid composition				
Saturated fats (g)	157.0	218.1	196.2	224.2
Monounsaturated fats (g)	274.0	290.8	274.3	308.6
Polyunsaturated fats (g)	20.6	21.1	37.9	23.3
EPA (g)	0.2	0.1	0.4	0.1
DHA (g)	0.4	0.3	0.7	0.3
Trans (g)	9.4	10.3	9.1	10.2
Cholesterol (mg)	393.9	400.4	1108.6	1209.4
Micronutrients				
Sodium (mg)	1844.3	1801.0	2869.8	3028.6
Potassium (mg)	7856.9	8136.6	6226.9	6336.0
Calcium (mg)	5990.6	6254.3	5638.9	6335.9
Magnesium (mg)	886.3	864.7	837.0	888.4
Phosphorus (mg)	6924.1	7016.6	6550.1	7115.8
Iron (mg)	78.9	53.8	68.0	59.2
Copper (mg)	2.8	2.4	2.5	2.5
Zinc (mg)	33.9	38.6	36.1	39.8
Chlorine (mg)	2350.6	2436.6	4480.6	4608.5
Manganese (mg)	5.3	4.6	4.9	4.9
Selenium (µg)	167.3	148.7	172.6	152.5
Iodine (µg)	707.6	678.1	729.5	697.7
Retinol (µg)	2603.5	2731.2	2464.1	2792.8
β-carotene (µg)	10029.0	10620.4	10208.6	12110.2
Vitamin D (µg)	9.8	9.1	11.6	9.3
Vitamin E (mg)	20.0	15.9	21.0	22.7
Vitamin C (mg)	495.1	463.3	480.8	512.6
Thiamine (mg)	2.8	2.6	2.7	2.7
Riboflavin (mg)	7.8	7.8	2.7	2.6
Niacin (mg)	32.5	25.7	32.3	28.5
Tryptophan (mg)	78.1	79.2	74.7	80.5
Vitamin B_6 (mg)	6.9	5.3	5.2	5.3
Vitamin B_{12} (µg)	31.1	30.1	31.4	30.8
Folate (µg)	1076.6	1014.2	1017.4	1076.4
Pantothenate (mg)	17.0	16.9	16.1	17.2
Biotin (µg)	82.7	81.1	79.0	82.9

When investigating for differences between the groups at both time-points, no differences were observed. The same outcome was obtained for within group differences.

The percentage of dietary energy intake derived from each of the energy providing nutrients was calculated as follows:
% Carbohydrate (CHO) energy= [(g CHO x 4 kcal)/ total kcal/day] X 100
% Protein (P) energy= [(g P x 4 kcal)/ total kcal/day] X 100
% Fat (F) energy= [(g F x 9 kcal)/ total kcal/day] X 100
The same method was used to estimate % saturated fatty acids, %polyunsaturated fatty acids (PUFA) and % monounsaturated fatty acids of total daily energy intake.

Using this formula the % average macronutrient contribution of total daily energy intake per group at baseline was:

Macronutrient composition baseline (% of daily energy intake) per group:
Intervention group: CHO: 39.2%, Protein: 16.6%, Fat: 44.2%
Control group: CHO: 38.5%, Protein: 15.6%, Fat: 50.4%
% Saturated fatty acids of EI: Intervention group: 47.03%; Control group: 67.63%
% MUFA of EI: Intervention: 55.47%; Control: 90.2%
% PUFA of EI: Intervention: 6.1%; Control: 6.5%
% Trans fatty acid of EI; Intervention: 2.82%; Control: 3.18%

Macronutrient composition at six months (% of daily energy intake) per group:
Intervention group:CHO 24.4%; Protein 22.6%; Fat 55.9%
Control: CHO: 22.8%, Protein: 22.3%; Fat: 57.6%
% Saturated fat of EI: Intervention group: 28.8%; Control group : 30.24%
%MUFA of EI: Intervention: 40.23%; Control: 41.62%
% PUFA of EI: Intervention: 5.56; Control: 3.14
% Trans fatty acid of EI: Intervention: 1.34%; Control: 1.38%

Table 19 shows that at baseline the daily energy intake for both groups exceeded the recommended estimated average requirements for children aged 5-12 years old [1550

kcal-1845 kcal (females); 1720 kcal-2220 kcals (males)] according to the Hellenic Dietary guidelines for children and adolescents [293]. From the composition of macronutrient intake for both groups, it is apparent that children's eating patterns are not consistent with the guidelines (50% CHO, 20% P, 30% fat) [294]. Children's diets were low in cereals, pasta, rice, fruits, legumes, vegetables (< 400 g/day), average in protein (< 20%) but high in fat (> 30%) [293] which is consistent with the low KIDMED score obtained from both groups. In other words, it seems that Greek asthmatic children have low adherence to the Mediterranean diet and are adopting a Western–type of eating pattern. On the other hand, fibre intake was within the recommended range of 20-25 g/day [331]. Nevertheless the macronutrient profile was close to the Traditional Mediterranean diet of 40% carbohydrates, 20% protein and 40% fat [294]. As for water intake, hydration was low in the intervention group (< 2 litres/d) [293] and above 2 litres in the control group, probably due to the high intake of chocolate milk (as evident from FFQs) which would contribute to the water content of the diet.

With respect to children's daily intake of fatty acids, in both groups percentage of saturated fats, MUFA and trans fat exceeded the recommended dietary reference values of 11% , 13% and 2% respectively. Thus explaining the reason for the high percentage of overweight/obese (~40% collectively) in this sample. However, PUFA intake was borderline, about 6.5% of daily total energy intake as recommended [331]. As for the intake of omega 3 fatty acids (EPA/DHA), children in the intervention group consumed 0.6 g/day of EPA and DHA combined, whereas the control group only 0.40 g/day which does not meet current guidelines of 0.45 g/day [312]. The Hellenic dietary guidelines for children and adolescents recommend at least two fish meals per week (150 g) [293] with one meal containing fatty fish. According to the Scientific Advisory Committee on Nutrition (SACN), two fish meals per week provide 450 mg of omega-3 fatty acids EPA/DHA per day (0.45 g/day) [312]. SACN emphasized that this recommendation represents a minimal and achievable average population goal and does not correspond to the level of fish consumption required for maximum nutritional benefit. In other words, with higher intakes more health benefits would be expected. As for dietary cholesterol, both groups were above the recommended intake of 300 mg/day [335]

Micronutrient composition of children's diet revealed that at baseline all vitamins and minerals exceeded the daily RNI values, except for sodium which was below 2 g/day

(equivalent to one teaspoon of table salt per day) as recommended by WHO guidelines [332]. WHO recommends a reduction in sodium intake (< 2g Na/day) in an effort to control blood pressure in children [332]. Dietary vitamin D was also below the RNI of 10 μg/day which in combination with low sun exposure contributes to hypovitaminosis D [226] and is in accordance with low vitamin D concentrations determined from children's biochemical tests.

In contrast, at six months, Table 19 indicates that energy intake in both groups had doubled from baseline and exceeded the recommended EAR of 1550-2220 kcal/d [293]. Carbohydrate intake in both groups decreased to below 40% (< 50% as suggested by the Hellenic dietary guidelines [293]) and fat intake increased to over 40%. In fact, compared to baseline, fat intake increased from 44% to 56% at six months in the intervention group and from 50 % to 58% in the control, which is contradictory to the Hellenic dietary recommendations of 30-35% [293]. However, PUFA and trans fatty acids were less than the DRVs of 6.5% and 2%. Protein intake increased in both groups from approximately 16% at baseline to 22% by the end of the six month study which corresponds to the recommended protein intake according to the Traditional Mediterranean diet of 20% of total daily EI. Fibre intake was within the suggested range of 20-25g/day [331]. As for cholesterol, mineral and vitamin intake the same trend was observed in both groups, as in baseline. Although sodium intake increased by approximately1000 mg in both groups, especially in the control indicating high intake of sodium rich foods and addition of table salt to meals.

6.4.5 Nutrient analysis of 24 hr food recalls

FoodWorks 9 (Xyris, Australia 2017) was used to estimate daily energy intake, macro and micronutrient content of 24 hr food recalls at baseline and six month assessments. Nutrient analysis of baseline 24 hr food recalls are displayed in Table 20.

Table 20 Nutrient analysis of 24 hr food recalls per intervention and control group at baseline

	Nutrient Analysis Baseline				
	Group				
Nutrient	**Intervention**		**Control**		
Macronutrients	Mean	SD	Mean	SD	P[a]
Energy (Cals)	2,127.0	464.0	2,248.8	402.6	0.26
Carbohydrate (g)	228.5	74.8	229.5	62.4	0.95
Protein (g)	87.4	20.3	93.5	19.9	0.23
Total fat (g)	90.8	34.3	102.5	24.7	0.12
Saturated fat (g)	36.15	11.8	38.8	10.6	0.33
Trans fatty acids (g)	-	-	-	-	
Polyunsaturated fat (g)	11.9	5.9	13.2	5.8	0.39
Monounsaturated fat (g)	36.1	19.4	43.8	13.4	0.07
Cholesterol (mg)	235.0	211.3	308.5	241.7	0.13[b]
Water (g)	1,057.4	240.0	1,077.0	238.9	0.74
Dietary fibre (g)	23.4	8.7	19.9	5.6	0.06
Micronutrients					
Thiamin (mg)	1.3	0.5	1.7	1.1	0.06
Riboflavin (mg)	2.2	0.9	2.8	1.4	**0.04**
Niacin (mg)	14.8	5.8	16.3	5.8	0.29
Vitamin C (mg)	116.5	71.8	122.2	77.1	0.76
Vitamin D (µg)					
Vitamin E (mg)	11.2	7.7	13.2	9.3	0.31 [b]
Tocopherol α (mg)	9.9	6.5	10.3	5.0	0.74
Tocopherol β (mg)	2.4	2.7	2.7	2.3	0.39 [b]
Tocopherol γ (mg)	2.9	2.2	14.8	72.2	0.28 [b]
Tocopherol δ (mg)	0.1	0.0	0.1	.1	0.94 [b]
Vitamin B6 (mg)	1.0	0.4	0.9	.4	0.76
Vitamin B12 (µg)	3.3	2.3	3.49	1.85	0.65
Pantothenic Acid (mg)	3.6	2.1	3.8	1.6	0.17 [b]
Biotin (µg)	34.1	11.9	33.8	11.7	0.90
Total folate (µg)	412.0	177.1	491.7	204.3	0.09
Folic acid (µg)	196.2	124.1	241.3	143.9	0.15 [b]
Folate food (µg)	222.2	81.6	256.9	119.9	0.18
Total Vitamin A (µg)	838.7	638.0	843.4	418.1	0.97
Retinol (µg)	468.7	196.4	510.0	250.4	0.46

Beta carotene equiv (µg)	2,210.5	3,814.7	1,998.9	2,209.1	0.55 [b]
Beta carotene (µg)	1,842.7	2,928.2	1,820.0	1,959.8	0.55 [b]
Alpha carotene (µg)	653.9	1,896.8	209.1	684.9	0.65 [b]
Cryptoxanthine beta (µg)	192.5	145.7	198.5	196.9	0.89
Lycopene (µg)	811.1	437.2	844.6	812.6	0.92
Lutein (µg)	59.7	147.2	98.9	159.7	0.75 [b]
Sodium (mg)	2,655.8	1,018.5	2,630.1	798.7	0.91
Potassium (mg)	2,454.6	590.7	2,793.4	803.0	0.06
Magnesium (mg)	242.1	56.5	242.8	44.9	0.95
Calcium (mg)	1,087.0	366.2	1,297.4	473.8	**0.05**
Phosphorus (mg)	1,516.7	285.2	1,529.2	339.7	0.87
Iron (mg)	10.6	4.7	14.7	14.7	0.08
Zinc (mg)	10.4	3.1	11.9	2.9	**0.05**
Manganese (µg)	3,327.3	1,488.6	3,268.2	1,176.7	0.85
Sulphur (mg)	273.7	149.2	288.4	169.9	0.71
Copper (mg)	1.2	0.5	1.2	0.4	0.58
Selenium (µg)	40.3	19.4	50.1	20.1	**0.05**
Iodine (µg)	159.2	51.1	182.2	55.9	0.09
Chromium (µg)	96.2	44.6	115.6	65.9	0.17
Molybdenum (µg)	45.7	21.2	54.7	28.0	0.08 [b]
Fluoride (mg)	0.6	0.4	0.5	0.4	0.25
Kj from protein %	16.9	3.4	16.9	2.9	0.97
Kj from fat %	37.4	9.5	40.2	6.6	0.17
Kj from saturated fat %	15.0	3.7	15.3	3.4	0.77
Kj from trans fat %	-	-	-	-	
Kj from carbohydrate %	42.5	10.0	40.4	7.5	0.34
Kj from fibre %	2.1	0.8	1.7	0.4	**0.01**
Kj from others %	1.0	0.7	0.7	0.4	**0.02**
Fat as mono %	39.4	9.3	43.7	8.9	0.26
Fat as poly %	12.7	5.1	13.3	4.2	0.72
Fat as saturated %	47.9	11.5	42.9	8.0	0.22
Fatty acid composition					
Omega 3 (g)	1.9	1.9	1.4	0.8	0.72 [b]
Omega 6 (g)	10.2	5.3	11.7	5.2	0.28
MUFA Oleic (g)	33.4	18.7	41.2	12.9	**0.05**
Linoleic (g)	10.2	5.3	11.5	5.2	0.29
α-Linolenic ALA (g)	1.2	0.6	1.3	0.7	0.48 [b]
Arachidonic (g)	0.1	0.1	0.1	0.1	0.18
Eicosapentaenoic (EPA) (g)	0.3	0.4	0.0	0.0	0.22 [b]
Docosahexaenoic (DHA) (g)	0.6	0.7	0.1	0.0	0.13 [b]

[a] P-value calculated using t-test; [b] Mann-Whitney; - Negligible;

Total sample N = 65; Intervention group n = 31; Control group n = 34.

*Vitamin D intake was negligible and not estimated.

Sex differences in mean daily energy intake per group at baseline:
Intervention group (male vs female):
2,203.2 ± 427.4 kcal vs 2,078.9 ± 490.8; [t(29) = 0.72, p= 0.48 (t-test)]
Control group (male vs female):
2,283.2 ± 361.9 kcal vs 2,193.3 ± 471.2; [t(32) = 0.63, p = 0.53]
The percentage of dietary energy derived from each of the energy providing nutrients were:
Intervention group: % CHO: 42.9%, % P: 16.4%, %F: 38.4%,
% Saturated fatty acids 15.3%; % MUFA 15.3%, %PUFA 5.0%
Control group:% CHO: 40.8%, P: 16.6%, %F: 41.0%, %
Saturated fatty acids 15.2%, % MUFA 17.5%, % PUFA 5.3%.

In Table 20, examination of energy intake, macro and micronutrient analysis of 24 hr food recalls shows that children in both groups did not comply with the Hellenic dietary recommendations and is consistent with data obtained from the analysis of FFQs.

It is evident that, in general for children in both groups, the daily energy intake surpassed the recommended upper limit for children (5-12 years) of 1845 kcal in females and 2220 kcal for males [293], although marginally, due to high intake of energy dense foods.

In comparison to international paediatric guidelines, carbohydrate intake in both groups was below the recommended 45-60% as anticipated from the low intake of fruit, vegetables, dairy, cereals, pasta, rice and legumes observed in FFQs [293]. Nonetheless, dietary fibre intake was within the lower range of 20-25 g/day [331]. Protein intake was close to the recommended nutrient intake of 15% [331]. As for fat intake, saturated fats, MUFA and cholesterol were high, above the recommended 30-35%, 11%, 13% of daily energy intake and 300 mg/day respectively [335]; [331]; [293]. Nevertheless, PUFA intake did not exceed the recommended 6.5% [331] [293]. Regarding fish intake, EPA/DHA intake was above the daily allowance of 0.45 g/day for the intervention group (0.9 g/day) but below for the control group (0.1 g/day). Indeed, baseline FFQs revealed that more children in the intervention group consumed fatty fish once per week than the control (intervention vs control: 26% vs 21%; $p < 0.001$), whereas more children in the control consumed lean fish once per week (control vs intervention: 32.3% vs 6.5% respectively, $p = 0.002$). Fatty fish is a rich source of omega 3 fatty acids EPA/DHA as compared to

lean fish [312]. Vitamin D was minute in value and not computable, thus explaining the reason for low vitamin D status in the participants of this clinical trial. Low intake of vitamin D rich foods such as fatty fish, together with lack of skin exposure to sunlight are determinants of hypovitaminosis D in children [226].

When examining for significant differences in nutrient intake between the intervention and control group discrepancies were observed for riboflavin (p=0.04), energy from fibre (p = 0.01), % MUFA (p = 0.03). Intake of riboflavin and % MUFA was slightly higher in the control group (control vs intervention group (mean): 2.84 vs 2.22 mg; 42% vs 38% respectively). However, fibre intake was higher in the intervention group as compared to the control (mean: 2.13 vs 1.72 mg). Most likely from a modestly higher intake of legumes in the intervention group (p = 0.03) as evident from FFQs.

On the other hand, the Cretan Mediterranean diet is moderate in protein (20%) and carbohydrate (40%) but high in fat (40%) primarily from the abundancy of olive oil intake [294; 336]. Olive oil is rich in monounsaturated fatty acid oleic acid [320]. Therefore, children's carbohydrate and fat intake is consistent with the Mediterranean dietary pattern. Furthermore, daily energy and macronutrient intake including MUFA and cholesterol are in line with other paediatric studies undertaken in Greece [293].

With respect to micronutrient intakes including sodium, analogous results were observed as those found in the nutrient analysis of FFQs. Sodium intake exceeded the recommended allowance of 2 g/day or 1 teaspoon of table salt (NaCl) as set by the WHO guidelines [332]. With respect to hydration, water intake in both groups was below 2 litres/day [293].

Mean daily energy, macro and micro nutrient intake including fatty acid composition by group at baseline estimated from 24 hr food recalls are exhibited schematically in Figures 42-44.

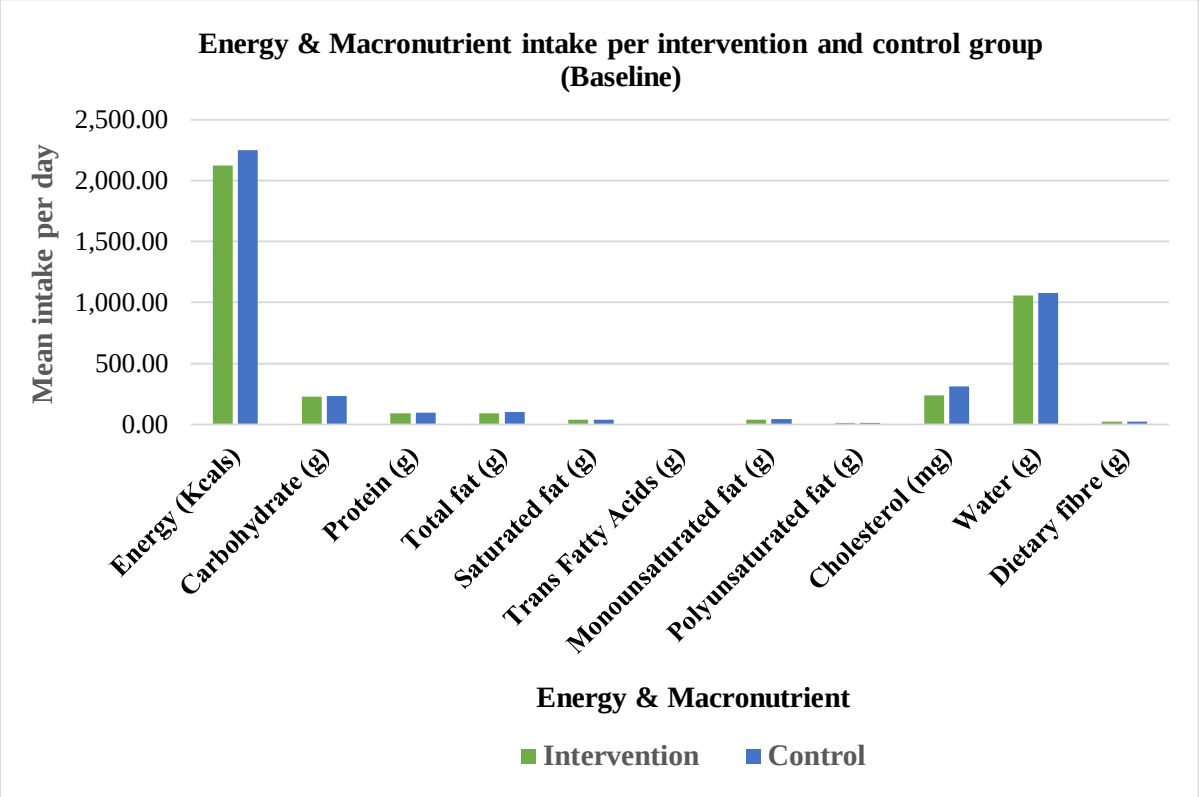

Figure 42 Energy and macronutrient intake estimated from 24 hr food recalls per intervention and control group at baseline

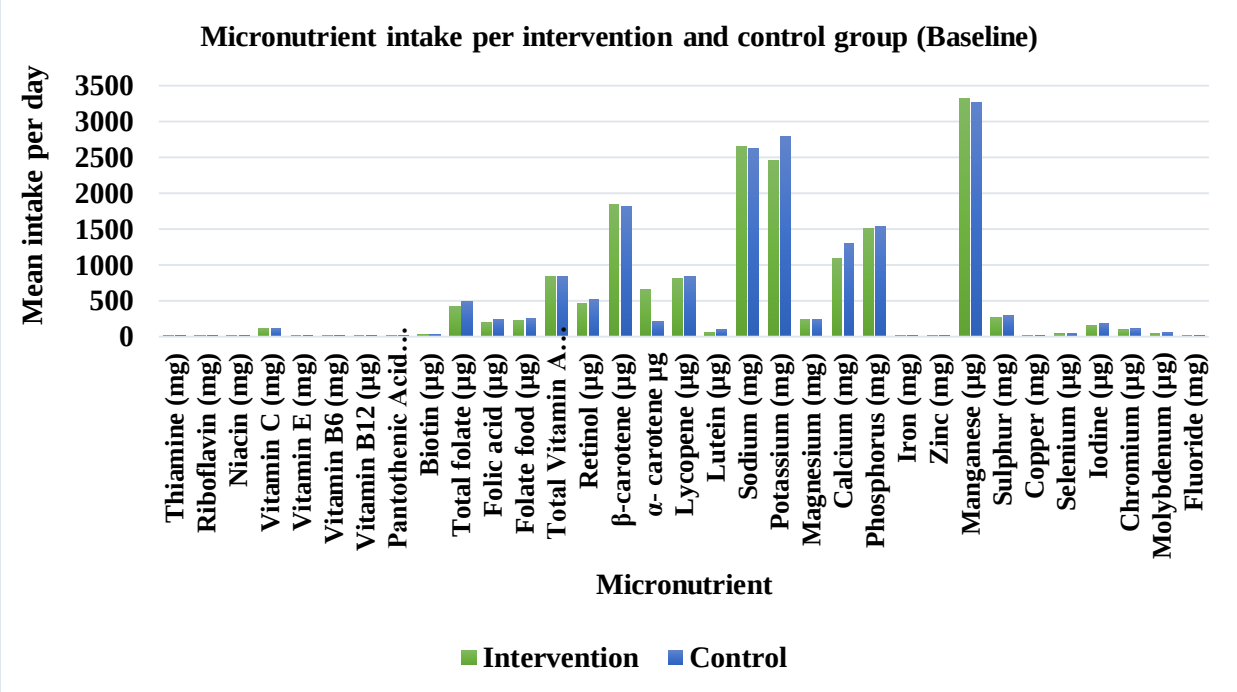

Figure 43 Micronutrient intake estimated from 24 hr food recalls per intervention and control group at baseline

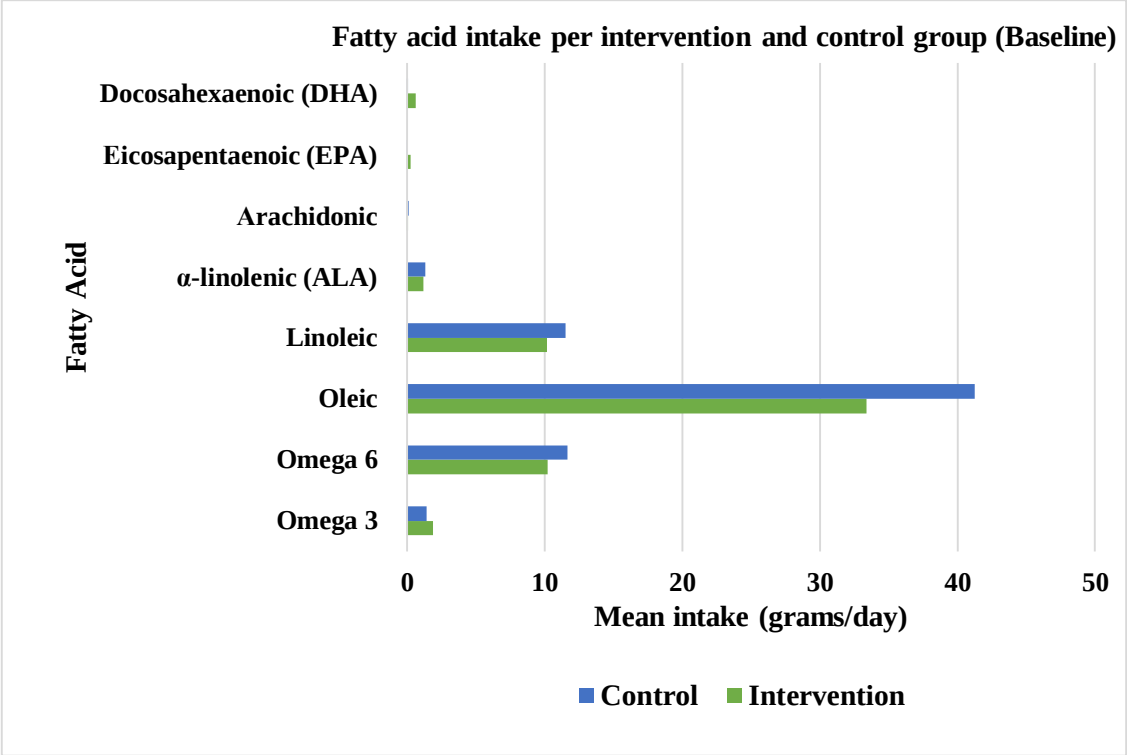

Figure 44 Fatty acid intake estimated from 24 hr food recalls per intervention and control group at baseline.

Nutrient analysis of 24 hr food recalls per intervention and control group at six months is shown in Table 21 and is consistent with data mentioned above.

Table 21 Nutrient analysis of 24 hr food recalls per intervention and control group at six months

Nutrient Analysis Six months					
	Group				
	Intervention		**Control**		**P[a]**
Nutrient	Mean	SD	Mean	SD	
Macronutrients					
Energy (Kcal)	1,759.9	392.1	1,752.4	460.0	0.94
Carbohydrate (g)	161.3	48.5	168.6	61.5	0.60
Protein (g)	85.8	23.7	82.4	37.2	0.66
Total fat (g)	82.8	22.6	79.8	29.0	0.65
Saturated fat (g)	33.1	9.2	32.8	11.3	0.90
Trans fatty acids (g)	1.6	0.6	1.6	0.8	0.67[b]
Monounsaturated fat (g)	35.3	12.3	33.1	15.7	0.54
Polyunsaturated fat (g)	7.8	2.5	7.8	4.2	0.95
Cholesterol (mg)	322.8	200.5	244.7	125.4	0.07
Water (g)	890.0	225.9	1,009.8	294.9	0.08
Dietary fibre (g)	13.7	6.3	15.6	8.1	0.29
Micronutrients					
Thiamine (mg)	1.0	0.6	1.2	0.7	0.28[b]
Riboflavin (mg)	1.8	0.6	1.8	0.6	0.61[b]
Niacin (mg)	13.9	5.6	13.9	8.0	0.99
Vitamin C (mg)	94.9	107.8	142.9	159.9	0.12[b]
Vitamin D (µg)	-	-	-	-	-
Vitamin E (mg)	12.0	6.1	10.1	4.8	0.18
Tocopherol α (mg)	9.9	3.7	8.9	3.9	0.28
Vitamin B6 (mg)	1.4	0.7	1.1	0.4	0.19 [b]
Vitamin B12 (µg)	5.6	2.1	5.3	2.9	0.16 [b]
Total folate (µg)	352.5	126.4	377.5	197.9	0.56
Folic acid (µg)	130.5	106.4	122.4	120.8	0.78
Folic acid food (µg)	222.0	83.1	255.2	113.8	0.20
Total vitamin A (µg)	608.2	242.5	564.1	230.4	0.46
Retinol (µg)	423.0	155.7	362.3	135.0	0.10
β-carotene equiv (µg)	1,132.0	1,052.1	1,233.2	900.7	0.17[b]
β- carotene (µg)	962.3	653.4	1,061.6	562.6	0.52
Lycopene (µg)	-	-			-
Sodium (mg)	1,714.0	551.7	1,870.4	1,158.7	0.50
Potassium (mg)	2,220.6	631.6	2,443.1	877.6	0.26
Magnesium (mg)	195.0	43.2	211.4	69.3	0.27
Calcium (mg)	952.7	285.4	932.4	339.4	0.79
Phosphorus (mg)	1,347.9	290.0	1,308.2	489.9	0.70
Iron (mg)	7.6	2.2	8.4	3.33	0.27
Zinc (mg)	9.6	3.7	10.5	5.6	0.87

Selenium (µg)	65.3	28.2	58.9	41.7	0.48
Iodine (µg)	164.53	48.08	157.61	58.51	0.61
Kj from protein %	20.00	4.14	18.76	5.75	0.33
Kj from fat %	41.40	6.44	39.88	8.87	0.43
Kj from saturated %	16.60	3.00	16.73	4.75	0.90
Kj from trans %	0.87	0.35	0.91	0.46	0.72 [b]
Kj from carbohydrate %	36.40	7.48	38.55	11.62	0.42 [b]
Kj from fibre %	1.50	0.63	1.73	0.76	0.23 [b]
Kj from others %	0.83	0.65	1.03	0.73	0.32 [b]
Fat as mono %	45.73	6.77	43.61	7.19	0.23
Fat as poly %	10.17	1.90	10.61	4.05	0.58 [b]
Fat as saturated %	44.13	7.86	45.76	9.40	0.46
Fatty acid composition					
Omega 3 (g)	1.20	0.61	1.15	0.71	0.80 [b]
Omega 6 (g)					-
Linoleic (g)	6.50	2.27	6.61	3.61	0.89
α-Linolenic ALA (g)	0.90	0.40	1.06	0.66	0.32 [b]
Eicosapentaenoic EPA (g)	0.03	0.18	0.03	0.17	0.95 [b]
Docosahexaenoic DHA (g)	0.07	0.37	0.03	0.17	0.93 [b]

[a] t-test [b] Mann Whitney test

Total sample N = 63; Intervention group n = 30; Control group n = 33

-Negligible

*Vitamin D intake was neglible and not estimated.

Sex differences in mean daily energy intake per group at six months:

Intervention group (male vs female):

1,828.8 ± 416.5 kcal vs 1,720.1 ± 383.13 kcal; $p = 0.47$

Control group (male vs female):

1,787.71 ± 450.81 kcal vs 1, 690.50 ± 489.43 kcal; $p = 0.57$

The % average macronutrient contribution of total daily energy intake per intervention group at six months was:

Intervention group: % CHO: 36.7%; % P: 19.5%; % F: 42.3%

% Saturated fat: 17.0%; % MUFA: 18.1% % PUFA: 4.0%

Control group : % CHO: 38.5%; % P: 18.8%; % F: 41.0%

% Saturated fat: 16.7%; % MUFA: 16.9%; %PUFA : 4.0%

Table 21 shows that six month results were comparable to baseline. No significant differences in daily energy and nutrient intake were observed between the groups

Overall, at six months there was a reduction in daily energy intake between the groups and sexes from approximately 2100 kcal at baseline to 1800 kcal at six months. A probable explanation is an improvement in dietary habits as a result of nutrition education on healthy eating and the basics of the Mediterranean diet given by the candidate on a fortnight basis via text, SMS, e-mail, telephone and face-to-face consultations. Behavioural change in dietary habits was confirmed by the modest increase in KIDMED score for the intervention group at the end of six months. Daily energy intake for both groups and sexes is within the RNI for children aged 5-12 years of 1550-1845 kcal for females and 1720-2220 kcals for males [(293)]. In comparison to baseline, there was a further reduction in the percentage of carbohydrate intake (< 40% in both groups at baseline), which is in accordance with data from FFQs at six months. More specifically, FFQs showed that the frequency of daily intake of dairy, cereals, rice, pasta, fruit, vegetables and legumes was less than 50%. This would explain the decrease in fibre intake observed in both groups (< 20-25 g/day) [(293)]. There was no change in protein intake and total fat intake surpassed the recommended RNI of 30% [(293)]. Likewise for saturated fats and MUFA (above 11% and 13% of total energy intake respectively) [(293);(331)]. However, PUFA intake was below 6.5% [(293);(331)]. Cholesterol intake increased slightly in the intervention group compared to baseline (mean: 235 vs 322 mg/day) but decreased in the control (mean: 308 vs 244 mg/day), although non-significant. Overconsumption of energy-dense foods high in fat have been implicated for the rise in overweight in children [(337)]. No change in overweight status was observed at six months. Overall micronutrient intake exceeded the recommended nutrient intake for children (4-14 year old), except for sodium intake which decreased (< 2000 mg/day). In both groups, omega 3 fatty acid intake (EPA/DHA) was below the RNI of 0.45 g/day, possibly because the day of the 24 hr food recall interview did not include a day that fish was consumed. Nevertheless, FFQs indicated that at six months 84% of the intervention group consumed fish 2-3 times per week from 13% at baseline which is in accordance with the Hellenic dietary guidelines for children [(293)]. Contrastingly, ~50% in the control consumed fish once per week at both time-points. The increase in fatty fish intake was confirmed by the 120% increase in plasma DHA levels from baseline in the intervention group. Studies have shown that biomarkers provide more accurate measures of dietary intake than using conventional dietary assessment tools [(274)]. As for water intake, in both groups daily intake was well below 2 litres [(293)]. Compared to

baseline, there was a reduction in hydration, since children tend to drink less fluids during winter months than in summer.

A schematic presentation of nutrient data from 24 hr food recalls at six months per intervention and control group is shown in Figures 45-47.

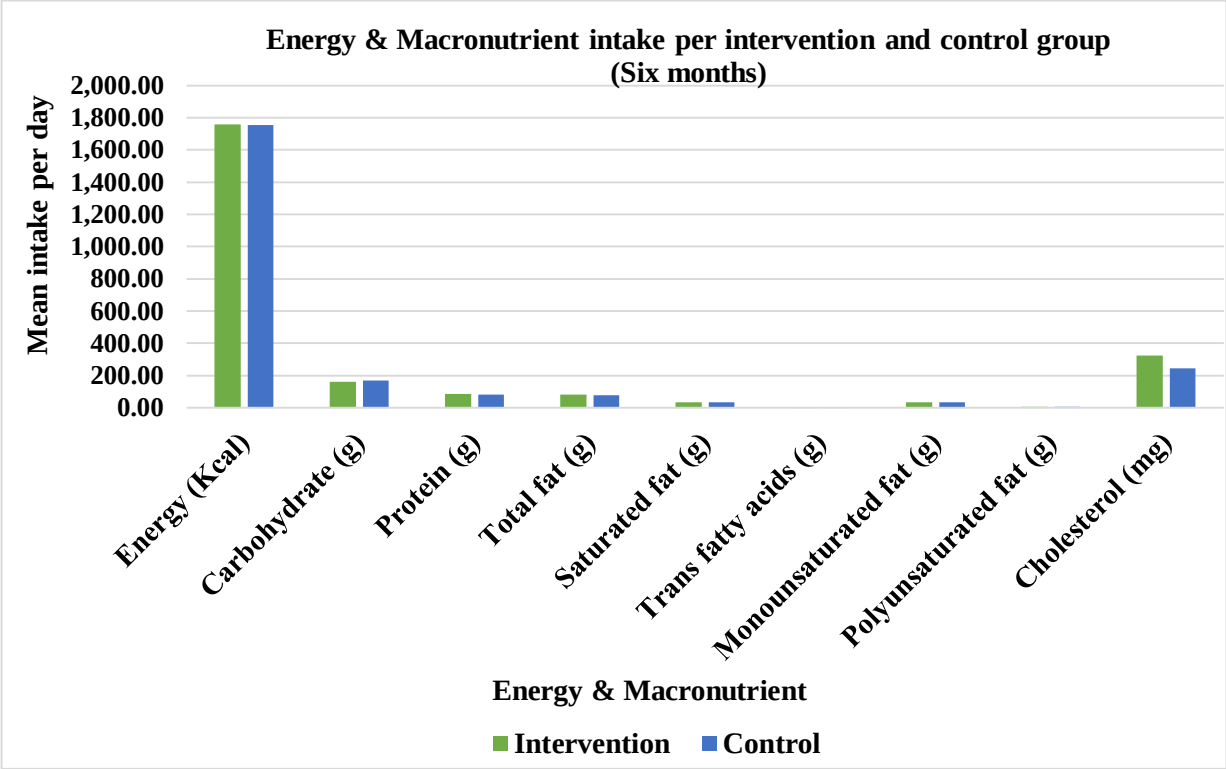

Figure 45 Energy and macronutrient intake estimated from 24 hr food recalls at six months by intervention and control group.

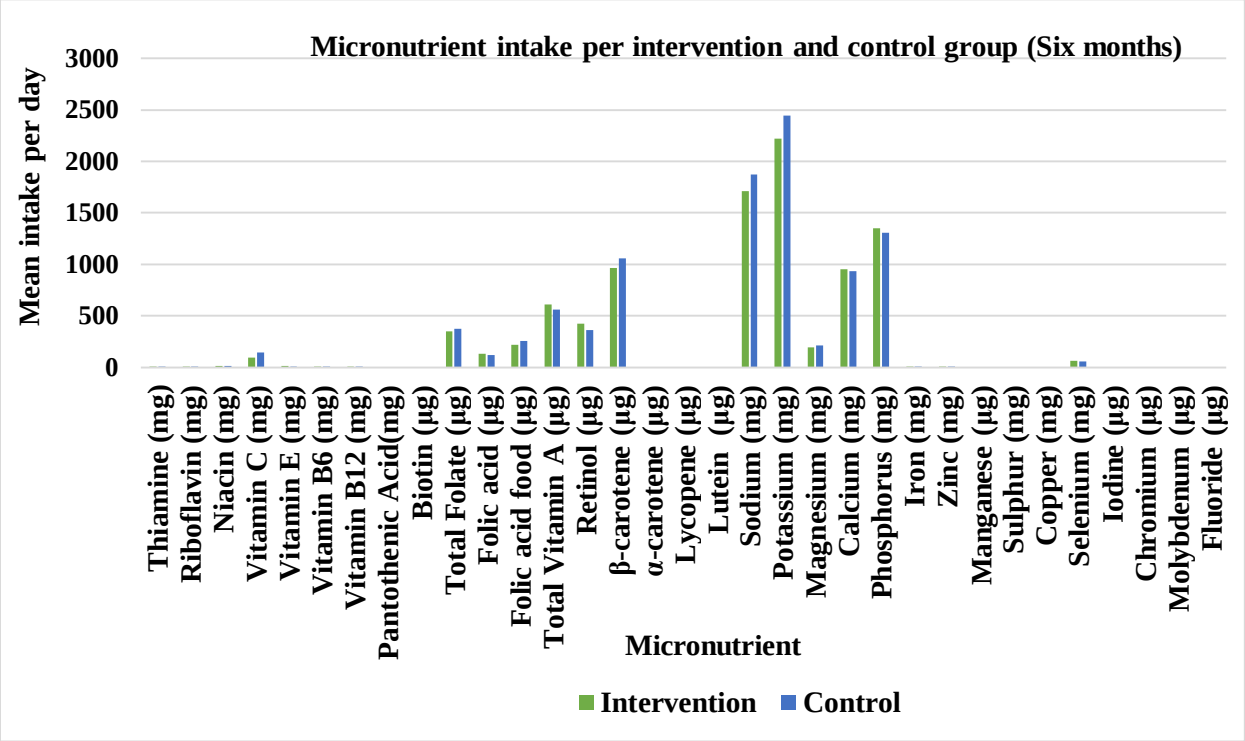

Figure 46 Micronutrient intake estimated from 24 hr food recalls at six months by intervention and control group

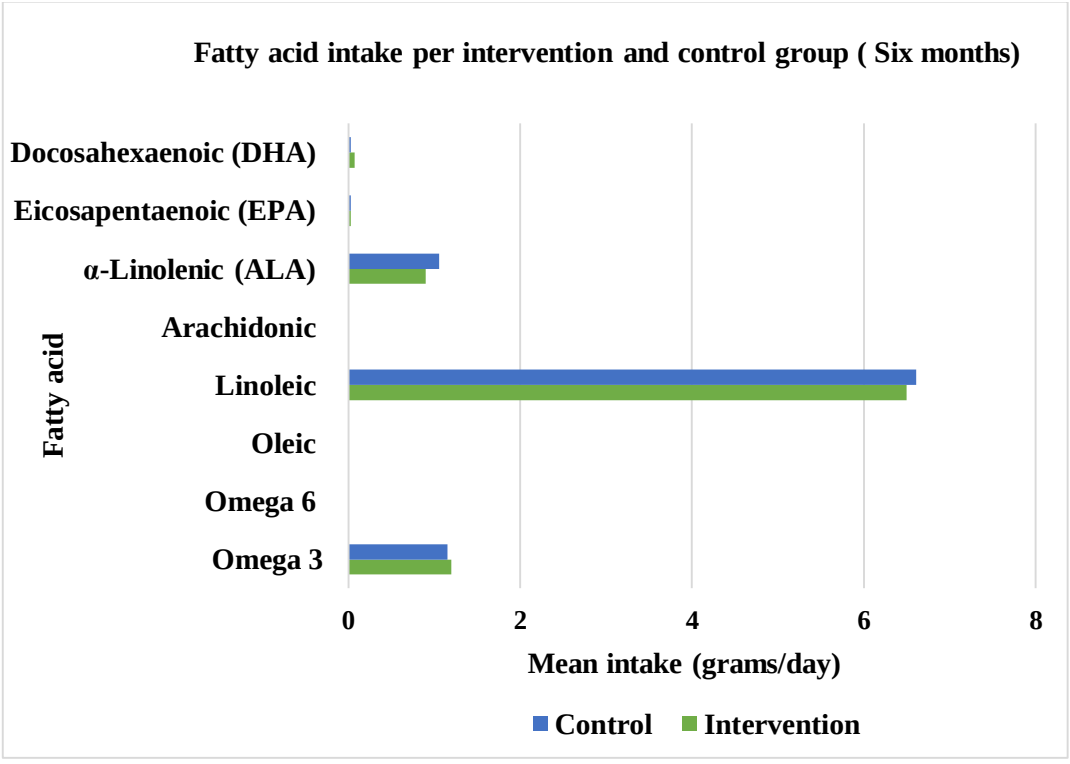

Figure 47 Fatty acid intake estimated from 24 hr food recalls at six months by intervention and control group

Comparison of energy and nutrient intake estimated from FFQs and 24 hr dietary recalls in both groups and time-points showed an overestimation of energy intake, macro and micronutrient intake by FFQs. One disadvantage of the FFQ is that it lacks the detail and specificity of 24 hr dietary recalls and may not provide an accurate estimation of nutrient intake due to recall bias and under or over-reporting of foods [403], as it may seem the case in this study. The tendency of overestimation of dietary intake by FFQs in the paediatric population has been reported in another study [403]. Energy intake computated from 24 hr dietary recalls approached the EAR for children 5-12 years old, although micronutrient intake was above reference values by a small difference and credible.

6.4.6 Mediterranean diet adherence

Mediterranean diet adherence at baseline and six months per KIDMED category are displayed in Figure 48. At both time-points adherence to this eating pattern was low-moderate where KIDMED score ranging from 4-7 indicates improvement needed [mean KIDMED score baseline vs six-months; 5.28 ± 1.99 vs 5.59 ± 1.82, (Z = -1.61, p = 0.107 (Wilcoxon sign rank test)]

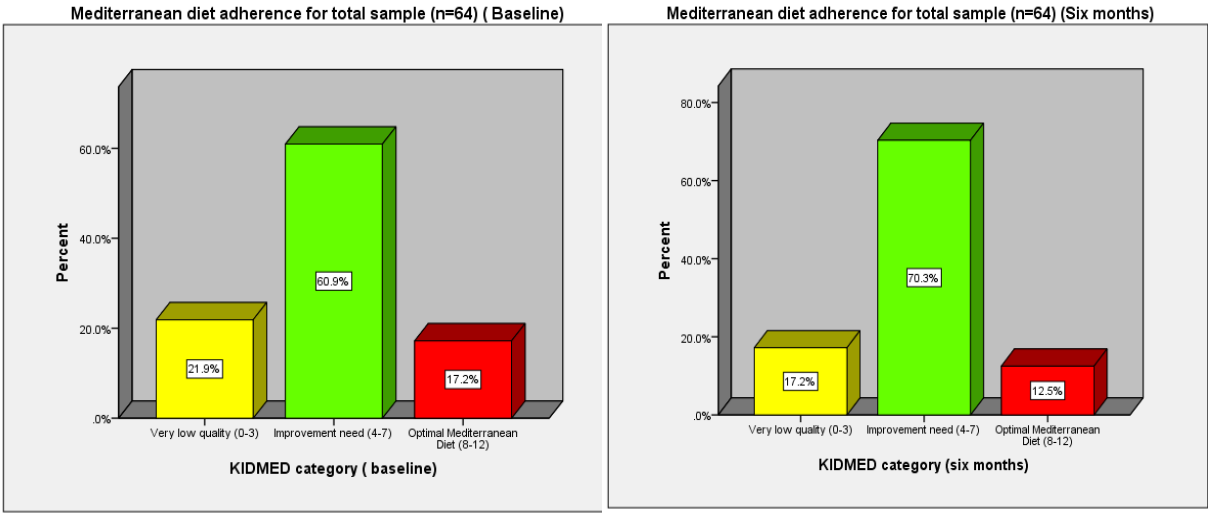

Figure 48 Adherence to the Mediterranean dietary pattern per KIDMED category for total sample at baseline and six months

Figure 48 shows that at baseline only 17.2% of children had ‘optimal adherence’ to the Traditional Greek Mediterranean dietary pattern which declined to 12.5% at the six-month follow-up. The majority of children (60.9% at baseline and 70.3% at six-months) have a ‘need for improvement’.

The same trend was exhibited when investigating Mediterranean diet adherence per intervention and control group at baseline (Figure 49) and six months (Figure 50).

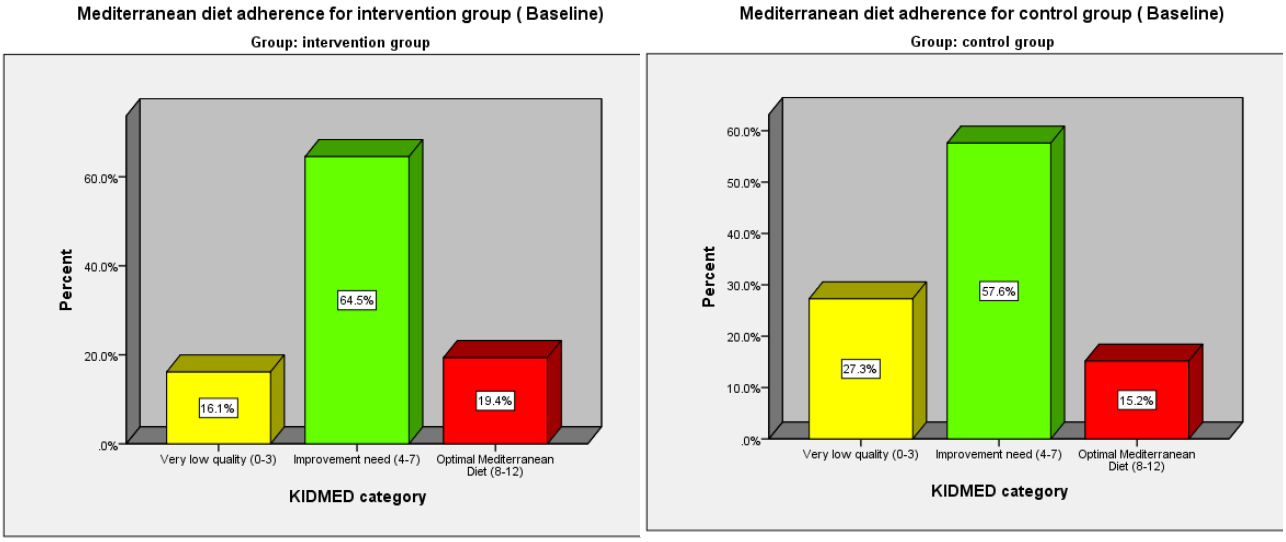

Figure 49 Mediterranean diet adherence as measured by the KIDMED tool per intervention and control group at baseline

Figure 49 shows that at baseline in both groups optimal Mediterranean diet adherence was low and that the majority of children have 'need for improvement'. KIDMED score [(baseline) intervention vs control: 5.3 ± 2.0 vs 5.2 ± 2.0; X^2 test, p = 0.55)]

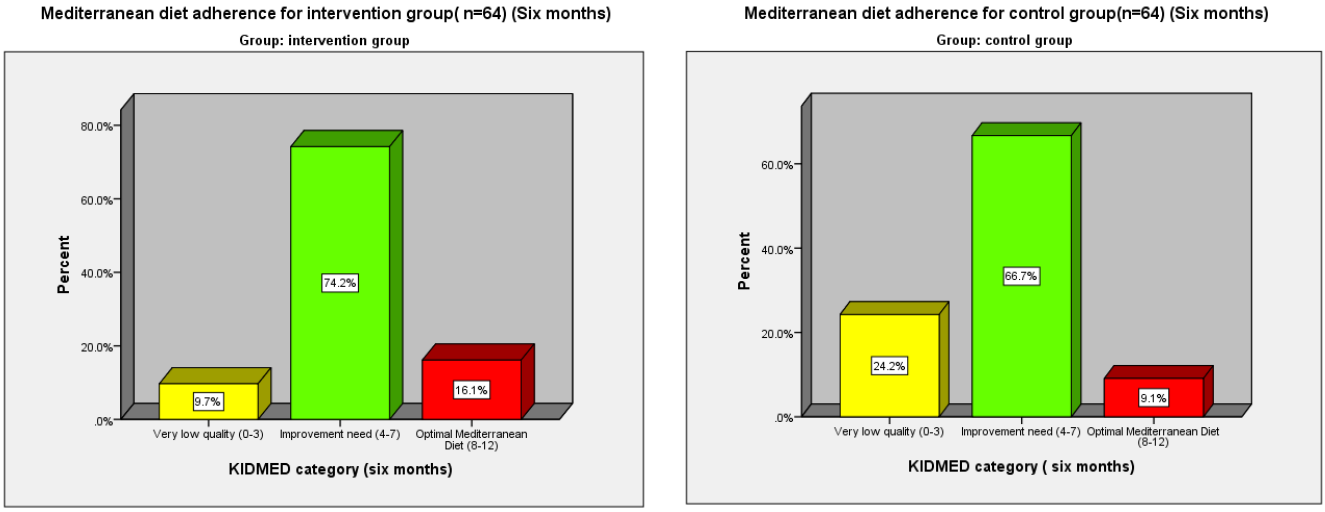

Figure 50 Mediterranean diet adherence as measured by the KIDMED tool per intervention and control group at six months.

From Figure 50 at six months in both the intervention and control groups Mediterraean diet adherence continued to be low (KIDMED score (six months) intervention vs control: 6.1 ± 1.5 vs 5.1 ± 2.0; X^2 test: $p = 0.26$). Although, there was a modest increase in the KIDMED score for the intervention group by the end of six months due to the increase in fatty fish intake [mean KIDMED score intervention group (baseline vs six months): 5.3± 2.0 vs 6.1 ± 1.5; $Z = -2.48$, $p = 0.013$ (Wilcoxon test)]. No change in KIDMED score from baseline to six months was observed for the control group [$p = 0.85$ (Wilcoxon test)]. Contribution of different food groups to total KIDMED score at baseline is depicted in the KIDMED questionnaire shown in Table 22.

Table 22 KIDMED questionnaire at baseline for total sample

KIDMED QUESTIONNAIRE BASELINE				
QUESTION	**Response (Yes/No)**	**(%)**	**n**	***P****
Q1. Does your child take a fruit or fruit juice every day?	Yes	81.2%	52	**0.00**
	No	18.8%	12	
Q2. Eat two fruits every day?	Yes	60.9%	39	0.10
	No	39.0%	25	
Q3. Eat fresh salad or cooked vegetables regularly once a day?	Yes	56.2%	36	0.38
	No	43.7%	28	
Q4. Eat fresh salad or cooked vegetables more than once a day	Yes	7.8%	5	**0.00**
	No	92.2%	59	
Q5. Eat fish regularly (at least 2-3 times per week)?	Yes	9.4%	6	**0.00**
	No	90.6%	58	
Q6. Go to a fast-food restaurant (hamburger) more than once a week?	Yes	4.7%	3	**0.00**
	No	95.3%	61	
Q7. Eats legumes more than once/week	Yes	34.4%	22	**0.02**
	No	65.6%	42	
Q8. Eats pasta or rice almost every day (≥5/week)?	Yes	9.4%	6	**0.00**
	No	90.6%	58	
Q9. Eats cereals or grains for breakfast	Yes	42.2%	27	0.26
	No	57.8%	37	
Q10. Eat dairy products for breakfast	Yes	84.4%	54	**0.00**
	No	15.6%	10	
Q11. Eat baked goods or pastries for breakfast?	Yes	3.1%	2	**0.00**
	No	96.9%	62	
Q12. Skips breakfast?	Yes	12.5%	8	**0.00**
	No	87.5%	56	
Q13. Eat nuts regularly (≥ 2-3 times per week)?	Yes	10.9%	7	**0.00**
	No	89.1%	57	
Q14. Eat 2 yogurts and/or some cheese (40g) daily?	Yes	81.2%	52	**0.00**
	No	18.7%	12	
Q15.Eat sweets and candy several times every day?	Yes	29.7%	19	**0.00**
	No	70.3%	45	
Q16.Eat olive oil with meals?	Yes	100%	64	**0.00**
	No	0	64	

Statistically significant *p*-values shown in bold.

*- *P*-value calculated by Binomial test; P considered to be significant at 5% level. *N* =64.

From Table 22 it is evident that at baseline, in general, children have low intake of vegetables, fish, cereals, pasta/rice, legumes and nuts with the majority of children consuming one fruit or fruit juice which is consistent with data retrieved from FFQs.

Frequency of consumption of foods pertaining to the Mediterranean dietary pattern (including fish intake) at both time-points per group are presented in Table 3 of the published manuscript in Section 6.5.

When investigating for correlations between KIDMED score and spirometry, FeNO and pulse oximetry, no correlations were observed at both time-points. Regarding the association between family income (as the continuous variable) and KIDMED score, no correlation was found (p = 0.94). However, applying one-way ANOVA showed a positive relationship between mother's education level and KIDMED score ($F(5, 36)$ = 2.94, p = 0.025) but not for father's education level (p = 0.97), which is consistent with the literature [338]. No associations were observed between maternal/or paternal education levels (primary, secondary and tertiary) and KIDMED categories in the categorical analysis (X^2 test: p = 0.19, 0.92 respectively).

6.4.7 Assessment of children's dietary intake during telephone conversations

As mentioned in methods of Chapter 5, 24 hr food recalls were undertaken at three time-points during telephone interviews (baseline, 3 months and 6 months) in an effort to record children's eating habits, meal frequency, food preferences and diet quality as well as parents' and siblings' consumption patterns. Also included were details on meal preparation, cooking mode and serving size. Open-end questions were used so that parents would describe in details children's eating habits and their own.

An extensive list of the questions used in retrieval of dietary information is available in (Appendix 2A, 16c/d). The outcome of telephone interviews is summarized below and is in accordance with data obtained from FFQs and the KIDMED questionnaire.

Regular telephone conversations with parents throughout the study period revealed, that the majority of families did not comply with the Hellenic dietary recommendations for healthy eating and the Traditional Greek Mediterranean diet pyramid, in particular for consumption of fruit, vegetables, salads, wholegrain cereals, nuts, fish and milk products. Barriers mentioned by parents were children's likes/dislikes, time required for preparation and cooking of meals, parents' and siblings' food preferences, hectic work and school programs. With respect to the breakfast meal, some parents admitted that children skipped the breakfast meal because children did not 'wake up early enough' or 'didn't feel like eating'. In these cases, a snack (such as toast with ham/ cheese, cheese-pie/croissant) was consumed by children during the morning recess at school. Regarding fruit, on average, one fruit or pre-packed juice (250 g) was consumed per day. As for vegetables and salads, the majority of children had an aversion and avoided consuming these vegetables when offered during meals. Many parents were uncertain about the amount of vegetables and fruit children should consume per day and asked how they could make them more appetizing in order to enhance consumption. On the other hand, few parents did admit that they skipped breakfast, did not consume fruit, vegetables or salads with meals.

In families comprising of more than one child, children tended to mimic dietary patterns of siblings. On the topic of fish consumption, some mothers (mainly in the control group) admitted that due to heavy work schedules, busy school programs, limited time to prepare and cook, fish was rarely consumed or only on weekends. Another factor was

that children preferred to consume meats more than fish due to the taste and small bones. During the intervention the cost of fish was not an issue since it was emphasized by the candidate that cheaper small fish (such as anchovies, sardines, chubb mackerel and trout) were rich in omega-3 fatty acids and that both frozen, farmed and fresh fish were suitable for the purpose of the study. Also, supermarket vouchers and frozen fish were given to families experiencing economic difficulties.

Summary of dietary assessment

To summarize, at baseline, the daily energy intake exceeded the recommended estimated average requirements for children aged 5-12 years old of 1550-2220 kcals [(293)], which is consistent with the high percentage of overweight children in this sample. Dietary analysis showed that, in general, children had poor dietary habits that did not comply to the Hellenic dietary recommendations for children and adolescents. More specifically, children had low intake of milk, vegetables, fruit, cereals, nuts, legumes and fish; and a high intake of red meat and sweets. Poor eating habits were confirmed by the low KIDMED score of 5 out of a maximum of 16. At baseline, children's diet was low in carbohydrates (~ 40%), protein (~ 16%) and high in fat (~ 48%). Intake of saturated fats and cholesterol exceeded the recommended daily intake of 11% total energy intake and 300 mg respectively [(293)]. In comparison, PUFA was below 6.5% of the total daily energy intake [(293)]. Although, fibre intake was within the recommended range of 20-25 g/day [(293)]. The same pattern was observed at the six month assessment. Comparison of food intake patterns between the groups revealed that at baseline more rice, lean/fatty fish and traditional Greek meals were consumed in the intervention group than in the control; while at six months fatty fish intake remained high as well as legumes in the intervention group. However, apart from fatty fish, intake of these food items remained below the national dietary recomendations for children. As for the control group, at baseline more pies were consumed than in the intervention group. At six months there was a higher intake of sweets and salty snacks although non-significant.

As stated by the Hellenic dietary guidelines for healthy eating in children that is based on international guidelines, carbohydrate intake should range from 45-60% of daily

energy intake, protein 10-15% and fat 30-35% [293]. Nevertheless, the macronutrient profile of children's diets was close to the Traditional Greek Mediterranean diet prototype of 40% carbohydrates, 20% protein and 40% fat [336]. Then again, total fat intake of the sample (48%) did exceed 40%, thus explaining the excessive caloric intake at both time-points.

Throughout the study duration both groups had good diet quality as demonstrated by sufficiency in minerals and vitamins. However, sodium intake was above the recommended intake of 5 g/day indicating over-consumption of sodium-rich foods (for example red meat) and addition of table salt to meals. As for hydration, the intervention group consumed less than 2 litres per day (baseline) which increased to above 2 litres by the end of the study. In contrast, the control group was well-hydrated during the six-month term, probably due to higher intake of chocolate milk as compared to the intervention, which would add to the water content of the diet.

So with respect to the overarching research question in this thesis, 'Does a Mediterranean dietary intervention enriched with fatty fish improve pulmonary function in asthmatic children?' is addressed in Section 6.5 by the following publication *Papamichael et al, 2019. Efficacy of a Mediterranean diet supplemented with fatty fish in ameliorating inflammation in paediatric asthma: a randomised controlled trial. J Hum Nutr Diet. 2019 32(2): 185-197.*

J Hum Nutr Diet.2019, 32:185– 197. http://doi.org/10.1111/jhn.12609.

6.5 Efficacy of a Mediterranean diet supplemented with fatty fish in ameliorating inflammation in paediatric asthma: a randomised controlled trial

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The final version of the manuscript is available in J Hum Nutr Diet (Wiley publications)

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ALLERGY AND ATOPY

Efficacy of a Mediterranean diet supplemented with fatty fish in ameliorating inflammation in paediatric asthma: a randomised controlled trial

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Keywords
asthma, bronchial inflammation, children, fatty fish, omega-3 fatty acids.

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Abstract

Background: Childhood asthma is the most common respiratory disorder worldwide, being associated with increased morbidity and a decreased quality of life. Omega-3 fatty acids have anti-inflammatory and immunomodulating properties; however, their efficacy in asthma is controversial. The present study aimed to examine the efficacy of a Mediterranean diet supplemented with a high omega-3 'fatty' fish intake in Greek asthmatic children.
Methods: A single-centred, 6-month, parallel randomised controlled trial compared the consumption of a Mediterranean diet supplemented with two meals of 150 g of cooked fatty fish weekly (intervention) with the usual diet (control) with respect to pulmonary function in children (aged 5-12 years) with mild asthma. Pulmonary function was assessed using spirometry and bronchial inflammation by fractional exhaled nitric oxide analysis.
Results: Sixty-four children (52% male, 48% female) successfully completed the trial. Fatty fish intake increased in the intervention group from 17 g day^{-1} at baseline to 46 g day^{-1} at 6 months ($P < 0.001$). In the unadjusted analysis, the effect of the intervention was of borderline significance ($P = 0.06$, $\beta = -11.93$; 95% confidence interval = -24.32 to 0.46). However, after adjusting for age, sex, body mass index and regular physical activity, a significant effect was observed ($P = 0.04$, $\beta = -14.15$ ppb; 95% confidence interval = -27.39 to -0.91). No difference was observed for spirometry, asthma control and quality of life scores.
Conclusions: A Mediterranean diet supplemented with two fatty fish meals per week might be a potential strategy for reducing airway inflammation in childhood asthma. Future robust clinical trials are warranted to replicate and corroborate these findings.

Introduction

Childhood asthma has become the most common respiratory disorder worldwide [(1)], as well as in Greece [(2)], being associated with increased morbidity and a poor quality of life. Asthma causes substantial physical, mental and economic burden as a result of increased rates of hospitalisation, emergency visits for medical care, school absence and parent's time off work (3). According to the Global Initiative for Asthma (GINA), it has been estimated that, by 2025, there will an additional 100 million people suffering with asthma(4). Therefore, identifying potential asthma therapies is of great public health significance.

Asthma is a heterogeneous disease caused by genetic and environmental factors. A multitude of environmental factors have been associated with asthma risk, namely respiratory infections, smoking, pollution, pet hair, house dust mites, mould and diet [5] . There is evidence that diet can influence the development and progression of asthma in children. In general, a diet that is high in fat, processed foods, sugar and salt has been shown to increase the prevalence and risk of asthma in children and adolescents [6–10]. By contrast, the International Study of Allergies and Asthma in Childhood (ISAAC) showed that a regular intake of fruit, vegetables and fish, as well as adherence to the Mediterranean diet, has a prophylactic effect on asthma in children and adolescents [6,11–14]. In particular, a lower prevalence of asthma was found in Mediterranean centres of Western Europe that share a common dietary pattern [15]. The term 'Mediterranean diet' refers to dietary patterns found in olive-growing areas of the Mediterranean region. The key features of the traditional Mediterranean diet are a high intake of vegetables, wild edible greens, fruits, unrefined cereals, bread, legumes and olives that are fresh, seasonal, locally grown and minimally-processed, as well as an abundance of olive oil; a low to moderate intake of dairy, poultry, fish (depending on the proximity of the sea), nuts and seeds; and a low intake of meat and sweets, including a regular intake of wine with meals [16]. This diet is rich in monounsaturated fatty acids, a balanced ratio of n-6 : n-3 essential fatty acids and high amounts of fibre and antioxidants, such as vitamins E and C, resveratrol, polyphenols, selenium, glutathione, which interact synergistically to promote good health [16].

Research studies have confirmed that excessive amounts of omega-6 fatty acids leading to a high omega-6 : omega-3 fatty acid ratio can promote the pathogenesis of chronic diseases including asthma [17]. Specifically, a ratio of 5 : 1 (as in Mediterranean diets) had a beneficial effect on patients with asthma, whereas a ratio of greater than 10 : 1 (as is common in Western diets) had adverse consequences [18]. Thus, an optimal ratio of these two fatty acids and a high intake of omega-3 fatty acids might have prophylactic potential with respect to asthma symptoms. Fatty fish (salmon, mackerel, herring, sardines and trout) is a rich source of long-chain omega-3 fatty acids, eicosapentanoic acid (EPA) and docosahexanoic acid (DHA). One fish meal can provide between 1.5–3.0 g of EPA/DHA and 1 g of fish oil capsule per day can provide approximately 300 mg [19]. Most of the epidemiological evidence giving rise to the hypothesis that marine omega-3 fatty acids might have a prophylactic effect was generated from observational studies reporting that an early introduction in life and regular consumption of fish in children had a protective effect on asthma in children aged up to 14 years old [20]. Thus, fish, consisting of an array of bioactive nutrients, including EPA, DHA and antioxidants, might have a different health impact compared to fish oil supplementation [21,22]. However, to date, there are no universal dietary guidelines for asthma and the efficacy of marine omega-3 fatty acid therapy in asthma has not been well established. Further research is required to validate this therapy. Exploring the potential of nonpharmacological treatments is important because of the comparatively low risk associated with their use. A dietary modification could reduce asthma burden and improve the quality of life in children suffering with asthma. The present study aimed to investigate the efficacy of a Mediterranean diet supplemented with high omega-3 'fatty' fish intake in Greek asthmatic children.

Materials and methods

Study design

The present study was a 6-month parallel randomised controlled trial (RCT) investigating the effect of the Greek Mediterranean diet supplemented with high omega-3 'fatty' fish intake on asthma in children. The study design has been described in detail elsewhere using CONSORT recommendations [23] and only key features are presented here (24). This RCT was conducted in accordance with the ethical standards in the Declaration of Helsinki and all procedures involving human subjects were approved by the institutional review board of La Trobe University Human Ethics Committee. The study protocol was registered with the Australian and New Zealand Clinical Trial Registry(www.ANZCTR.org.au/ACTRN12616000492459p).

Participants

Seventy-two children [54.60% boys; 46.40% girls; mean (SD) age 7.98 (2.24) years] with asthma were recruited from a paediatric asthma clinic in the greater city of Athens, Greece from 1 November to 31 December, 2016. An internet platform (http://www.randomization.com) was used to automate the random assignment of a patient number to a randomisation number, which was linked to the intervention arms. Eligible participants were randomised equally to intervention or control groups with a 1 : 1 allocation ratio by the physician after written informed consent was obtained from parents. Inclusion criteria included children aged between 5–12 years and having physician-diagnosed 'mild' asthma as defined by the GINA guidelines [1]. According to GINA, 'mild asthma' is 'well-controlled' asthma. A patient that has day-time symptoms and the need for relief medication

less than twice a week, as well as no night-waking symptoms or limitations in daily activities as a result of asthma, is considered to have 'mild asthma' [1]. Exclusion criteria were children with severe or chronic asthma [25], gastroesophageal reflux disease, cystic fibrosis,congenital respiratory disease [1], food allergies, taking multiple glucocorticoid medication, high-dose multivitamins or fish oil supplements, as well as being vegetarian and not willing to modify their diet.

Intervention

The intervention group was instructed to consume two fatty fish meals per week (at least 150 g of cooked fish) [26] as part of the Greek Mediterranean diet over a per-iod of 6 months [27]. In comparison, the control group consumed their usual diet. The intervention group was provided with a detailed dietary education delivered by a dietitian on the key principles of a Mediterranean diet, including a pamphlet indicating the types of fatty fish to be consumed (such as sardines, trout, salmon, mackerel and anchovies), amounts of fresh fish equivalent to 150 g of cooked fillet fish and a list of lean fish not to be consumed over the study period. To monitor and facilitate intervention compliance, parents were issued a table to record the type and amount of fatty fish consumed per meal, as well as the 2 days per week that fatty fish was consumed during the 6-month period. This record was returned to the dietitian on a monthly basis and collected at the end of the 6-month study. In comparison, the control group was instructed to consume their usual diet and was provided with advice on general healthy dietary guidelines in accordance with the Hellenic Ministry of Health and Welfare (1999) [27].

Measurements

Children were assessed at two time-points: baseline and at 6 months during usual medical consultations. Because children were younger than 12 years of age, parents were used as a proxy to complete questionnaires [24]. During the same week of medical consultations, a telephone interview was conducted by the dietitian to collect information regarding the participant's medical history, medicine use and adherence to the Mediterranean diet. Dietary intake was measured using a food frequency questionnaire (FFQ) based on the validated semi-quantitative PANACEA-FFQ for Greek children aged 10–12 years [28] and adherence to the Mediterranean diet using the KIDMED tool [29]. Throughout the study, all participants were monitored fortnightly by the dietitian via telephone, e-mails, text and face-to-face consultation. Any missing data were retrieved during telephone interviews.

Food intake calculations

Daily consumption of each food item (g day^{-1}) was assessed from FFQs. The reported frequency of consumption of FFQ items was converted to frequency of consumption per day.

Then g day^{-1} was calculated by multiplying portion size by the value corresponding to each consumption frequency [28]. Based on the participant's responses, the information was then aggregated into food groups. Eleven main food groups (dairy products, fruit, vegetables, legumes, starch, meat, sweets, fast food, savoury snacks, fats and soft drinks) were formed, reflecting a dietary pattern followed by the population.

Anthropometry

Children's height was measured to the nearest 0.1 cm using a stadiometer (Seca Corp., Hanover, MD, USA) after their shoes had been removed and children were positioned in the standard Frankfort horizontal plane [30]. Body weight was measured to the nearest 0.1 kg on calibrated electronic scales (Seca Corp.) without shoes and heavy clothing. Body mass index (BMI) was calculated (kg m^{-2}) and study participants were classified normal weight, overweight and obese using the Hellenic paediatric growth charts [31].

Pulmonary function and bronchial inflammation

Pulmonary function was measured by trained technicians according to European Respiratory Society (ERS) protocol [32] using a portable spirometer (MIR Spirobank II; MIR Inc., New Orleans, LA, USA) providing age, sex, weight and height. Spirometry was undertaken in the standing position with a nose-clip. The mouth-piece was placed into the participant's mouth with lips sealed firmly around the mouth-piece. The participant was instructed to inhale to total lung capacity and to exhale as hard and as fast as possible without a pause and then a deep breathe was inhaled to total lung capacity. The best of three technically acceptable tests was selected. Normal pulmonary function was considered values of forced expiratory volume in 1 s (FEV_1) greater than 80% predicted and variation in FEV_1 of 10–12% to be clinically significant in children [33].

Levels of fractional exhaled nitric oxide (FeNO) were measured using a FeNO analyser (NO Breath, Benfont Inc., UK) in accordance with American Thoracic Society (ATS)/ERS guidelines [34]. Absence of lung inflammation

and good asthma control was indicated by FeNO values less than 20 ppb [35,36].

Questionnaires

Asthma control

Asthma control was evaluated using the Greek translation of the Asthma Control Questionnaire (ACQ), which is a validated questionnaire assessing asthma control in paediatric patients aged 6–12 years [37]. A score < 0.75 is considered as having 'well-controlled' asthma and a score of≥ 1.5 indicates 'extremely poorly controlled' [37].

Quality of life

Children's quality of life was measured using the Greek translation of the validated mini Paediatric Asthma Quality of Life Questionnaire (PAQLQ) for asthmatic children aged 6–16 years [38,39]. Parents assisted children in completing questionnaires. Children were asked to recall their experiences during the past week and to respond to each question on a scale from 1 to 7, where 1 indicates severe impairment and 7 indicates no impairment [39].

Adherence to Mediterranean dietary pattern

Adherence to the Mediterranean dietary pattern was measured using the KIDMED Index which is a 16-item test that has been developed specifically for Spanish children and adolescents [29] and has been applied previously to assess Mediterranean diet compliance in Greek children and adolescents [40,41]. The KIDMED score ranges from 0 to 12. A score of 0–3 reflects low Mediterranean diet adherence, 4–7 indicates improvement is needed and 8–12 reflects an optimal Mediterranean diet [29].

Physical activity status

Physical activity status was estimated using the ISAAC Phase 3 Environmental Questionnaire [42]. Regular physical activity was considered to be more than or equal to three times per week.

Biochemical tests

Patients were requested to abstain from fluid and food consumption at least 2 h after the last meal before testing. Venous samples (4 mL) were collected from children following a 2-h fast. The samples were centrifuged and plasma decanted from the supernatant and were stored at -20°C until analysis within 24 h to avoid degradation. In the case of haemolysis, blood collection was repeated. The internal standard mixture (200 lL of methyl nonadecanoate in hexane containing butylated hydroxytoluene) was added to 100 mL of plasma. Fatty acid hydrolysis and derivatisation into methyl esters was performed by adding 5% v/v methanolic HCl. Transmethylation was performed at 90°C for 60 min. The samples were then brought to room temperature and extraction of fatty acid methyl esters was performed using hexane. These were then transferred to gas chromotography injection vials with a crimp cap. Mass spectrometry allows direct detection and identification of fatty acids in plasma without affecting quantity or quality; thus, lipid extraction before methylation was not included [43] (see Supporting information, Appendix S1).

Sample size

Sample size was based on spirometry measure, FEV_1 and was determined using G*POWER analysis [44]. Assuming a modest effect size of 0.4 [45] to show a significant difference in FEV_1, we estimated that a sample of at least 64 patients was adequate to provide a power of 90%, to evaluate two-sided hypotheses regarding statistically differences in FEV_1 between groups at a probability level less than 0.05 and allowed for a 20% dropout rate.

Statistical analysis

Data were analysed using SPSS, version 20.0 (IBM Corp., Armonk, NY, USA) software. Continuous variables were assessed for normality using the Shapiro–Wilks test and are presented as the mean (SD). And categorical variables are shown as frequencies. Differences between the intervention groups were compared using t-test for normally-distributed variables and a Mann–Whitney test or chi-squared test otherwise. The effect of the intervention on pulmonary function, asthma control and quality of life was assessed using multiple linear regression models controlling for potential confounding factors, including age, sex, physical activity and BMI. The results from the regression model are presented as unstandardised b coefficients and corresponding 95% confidence interval (CI). $P < 0.05$ was considered statistically significant. According to the ATS guidelines, a reduction in FeNO by at least 10 units for values lower than 50 ppb should be used as the cut-off point to indicate a significant response to anti-inflammatory therapy [36].

Results

At baseline, 72 children were recruited and randomly allocated into two groups (Fig. 1). Sixty-four children (51.6% male and 48.4% female), of whom 31 children were in the intervention group and 33 in the control, completed the trial and baseline and follow-up assessments. The overall participation rate was 88.9% (64/72). Eight children dropped out: one as a result of an allergy (not related to the intervention) and seven for personal reasons.

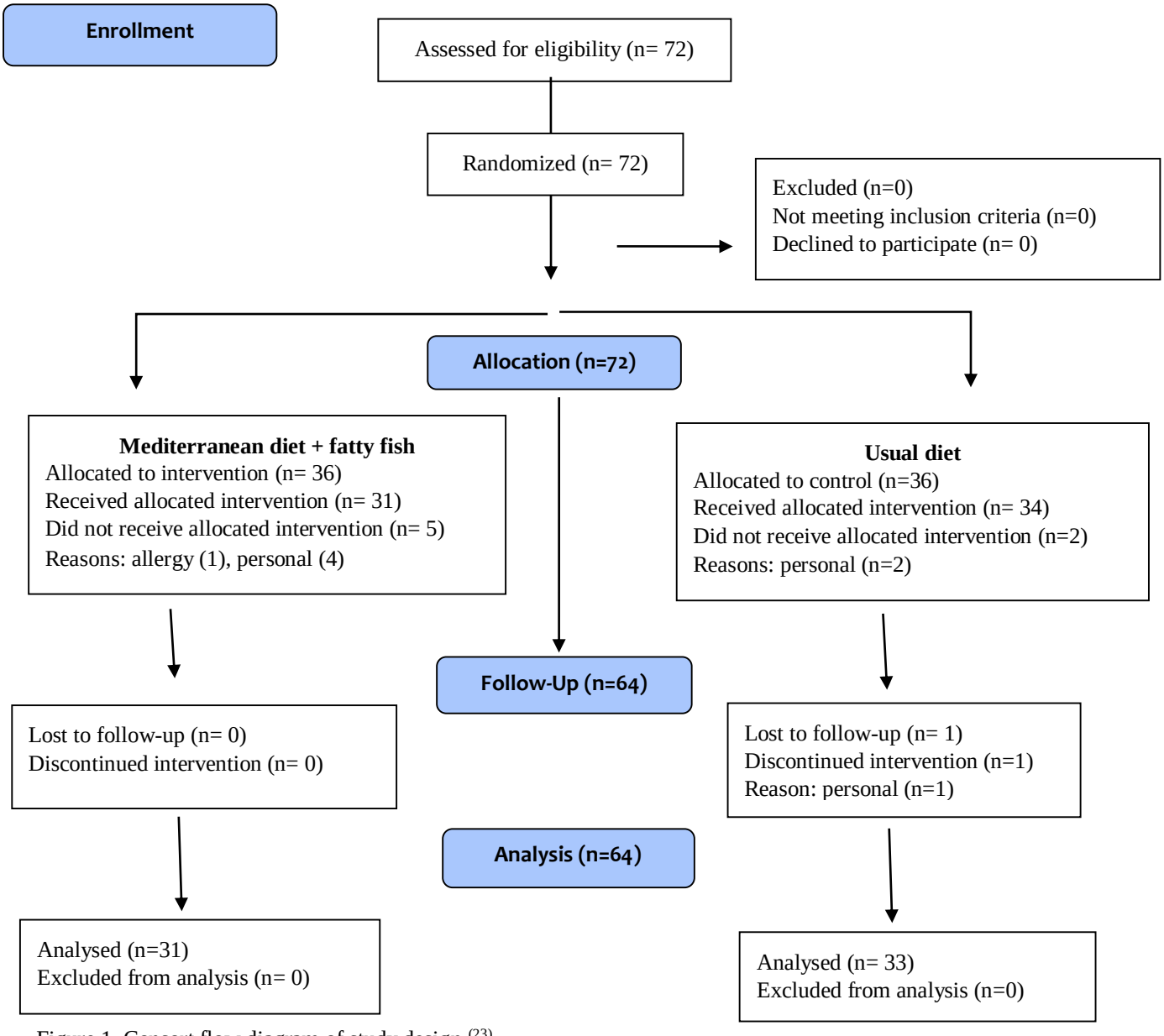

Figure 1. Consort flow diagram of study design [23]

At baseline, homogeneity between the groups was observed for demographic and clinical characteristics, as well as for adherence to the Mediterranean dietary pattern (Table 1).

The same trend was observed in the 6-month follow-up, except for the KIDMED score. There was a modest improvement in adherence to the Mediterranean dietary pattern from baseline to follow-up for the intervention group compared to the control group [KIDMED score: 5.32–6.10 (intervention group) versus 5.24–5.12 (control group); P = 0.02], most likely as a result of increased fish intake, although scores indicate that an improvement in Mediterranean diet adherence is needed.

Dietary intake of the main food groups, fatty/lean fish, nuts, olive oil, fats, sweets, fast food, savoury snacks and soft drinks is presented in Table 2, whereas adherence to the Mediterranean diet is shown in Table 3.

Table 2 shows a statistically significant increase in fatty fish intake for the intervention group (P < 0.001) from approximately 17 g day^{-1} at baseline to 46 g day^{-1} at 6 months compared to the control group (10 g day^{-1} at baseline and 6 months), hence validating good compliance to the dietary intervention by the intervention group. The same trend is apparent from the KIDMED questionnaire in Table 3. A statistically significant increase (P < 0.001) in the frequency of consumption of fish (at least two or three times per week) from approximately 13% at baseline to 84% at 6 months was observed in the intervention group compared to 6.1% at both time-points in the control group.

Table 1 Demographics, clinical tests and Mediterranean diet adherence at baseline and 6 months

Demographic and clinical characteristics per intervention group										
	Baseline group					Six months group				
	Intervention		Control			Intervention		Control		
Variable	Mean	SD	Mean	SD	P[a]	Mean	SD	Mean	SD	P[a]
Age (years)	7.78	2.25	8.18	2.26	0.47	8.19	2.18	8.76	2.35	0.30[b]
Male n (%)	12 (38.7%)		21 (63.6%)		0.05					
Height (cm)	133.70	13.48	133.30	13.62	0.91	136.40	13.50	135.8	13.67	0.98[b]
BMI (kg/m^2)	18.62	3.95	18.20	3.81	0.72	18.70	3.90	18.73	3.84	0.98[b]
Overweight/obese*	15(48.4%)		11 (33.4%)		0.22	13(41.9%)		14 (42.4%)		0.86
Regular physical activity (%)(≥ 3x/wk)	13 (41.9%)		19 (57.6%)		0.46	13 (41.9%)		18 (54.5%)		0.59
Medication n (%)										
Yes	26(83.9%)		27(81.8%)		0.83	14 (45.1%)		21(63.6%)		0.18
Anti-leukotriene therapy n (%)	24 (77%)		27(81.8%)		0.16	12 (38.7%)		19(57.5%)		0.21
Pulmonary function										
FEV_1 (% predicted)	97.23	8.80	99.09	10.55	0.45	100.19	9.44	100.09	8.76	0.96
FVC (% predicted)	94.61	8.68	96.30	11.11	0.50	96.94	9.20	96.79	9.14	0.95
FEV_1/FVC(%predicted)	101.97	4.40	102.39	7.78	0.79	102.87	3.66	102.88	5.60	0.99
PEF (% predicted)	94.32	19.28	93.48	18.79	0.86	100.58	21.02	101.21	21.68	0.91
FEF_{25-75} (% predicted)	100.29	17.80	103.94	21.47	0.46	103.45	14.09	102.73	19.78	0.87
FeNO (ppb)	17.94	17.61	10.15	7.16	0.16[b]	14.61	15.07	18.09	29.41	0.81[b]
ACQ score	0.35	0.34	0.36	0.39	0.96	0.23	0.49	0.20	0.28	0.87[b]
PAQLQ score	6.77	0.32	6.70	0.44	0.47	6.83	0.56	6.90	0.18	0.91[b]
KIDMED Score	5.32	2.01	5.24	2.02	0.87	6.10	1.49	5.12	1.98	**0.02[b]**

Bold characters represent statistical significant p-values.

ACQ- Asthma Control Questionnaire; PAQLQ- Paediatric Asthma Quality of Life Questionnaire; FEV_1- Forced Expiratory Volume in 1 second; FVC- Forced Vital Capacity; FEV_1/FVC- ratio of Forced Expiratory Volume and Forced Vital Capacity; PEF- Peak Expiratory Flow; $FEF_{25-75\%}$-Mid Expiratory Flow 25-75% vital capacity; FeNO- Fractional exhaled Nitric Oxide analysis.

P[a] - P-values shown were calculated using t-test or Chi-square test;

P[b] -P-value estimated using non-parametric Mann-Whitney test.

*Hellenic paediatric growth charts {Hellenic Institute of Child Health [38]

Regarding biochemical tests, at baseline, no significant difference in plasma fatty acid composition was observed between the groups ($P > 0.05$). In comparison, at 6 months, significant differences in DHA ($P < 0.001$), total plasma omega-3 fatty acids levels ($P < 0.001$) and the omega-6 : omega-3 fatty acid ratio ($P < 0.001$) were observed between the groups (Table 4). The percentage change in DHA was 119.80% in the intervention group and 43.39% in the control (crude analysis). Regarding EPA levels, differences between the groups were not significant. At 6 months, total plasma omega-3 fatty acid levels were higher in the intervention group compared to the control group (mean value: 168.20 versus 115.82 μmol L^{-1}, respectively) and the omega- 6: omega-3 fatty acid ratio was lower in the intervention group than in the control (14.5 : 1 versus 19.4 : 1, respectively). Elevated levels of total plasma omega-3 fatty acids (or lower omega-6 to omega-3 fatty acid ratio) in the intervention group are a marker of compliance to the dietary intervention, thus confirming that biomarkers are a good surrogate for dietary intake [46].

Regarding asthma control and quality of life, the mean change in scores from baseline to follow-up was not significantly different between the two groups in the Univariate and multivariate analysis ($P > 0.05$) (Table 5). The same trend was noted for spirometry. No significant change in spirometry from baseline was observed between groups in the univariate and multivariate analysis (Table 5).

Regarding bronchial inflammation, crude analysis showed that FeNO increased by 78.23% in the control group but decreased by 18.56% for the intervention group (see Supporting information, Fig. S1).

When applying the multiple linear regression model, in the unadjusted analysis, the effect of the intervention was of borderline significance ($P = 0.06$, b = -11.93; 95% CI = - 24.32 to 0.46). However, after adjusting for age, sex, BMI and regular physical activity, a significant effect

Table 2 Consumption of main food groups and items (grams per day) baseline and 6 months

	Consumption of food groups and fish									
	Baseline Group					Six months Group				
	Intervention		Control			Intervention		Control		
Food group/Item (g day^{-1})	Mean	S.D	Mean	S.D	P[b]	Mean	SD	Mean	SD	P[b]
Dairy products	486.91	301.96	513.21	305.61	0.71	453.09	283.90	517.24	303.22	0.38
Fruit	282.33	139.18	251.94	172.36	0.23	270.25	187.53	259.08	187.70	0.65
Vegetables	167.74	129.24	174.68	133.47	0.85	172.58	103.97	206.23	155.48	0.49
Legumes	65.49	35.32	56.07	33.70	0.22	59.81	32.22	46.81	31.75	**0.03**
Starch	113.47	131.38	67.02	25.56	0.03	94.18	59.38	75.78	24.63	0.42
Meat	84.03	47.03	81.04	38.43	0.77	69.60	30.80	82.88	45.93	0.27
Seafood	7.02	11.14	4.65	6.89	0.45	5.60	6.98	7.09	6.57	0.29
Lean fish	17.54	11.73	11.71	13.63	0.01	15.77	25.69	13.20	10.50	0.09
Fatty fish	17.18	11.79	10.41	11.62	**0.01**	45.76	19.40	10.35	11.01	**0.00**
Nuts	3.01	6.45	5.12	6.46	0.09	3.81	6.90	4.45	5.58	0.38
Olive oil	16.43	12.49	19.34	14.76	0.31	18.08	14.96	18.98	14.02	0.98
Fats	20.28	14.38	25.87	19.48	0.32	24.01	19.43	24.88	15.92	0.49
Fast food	19.73	16.99	26.81	36.49	0.64	19.61	12.84	23.75	20.36	0.65
Sweets	25.82	17.50	27.89	17.71	0.63	20.58	14.98	29.89	29.03	0.22
Savoury snacks	27.22	31.78	43.97	41.89	0.03	28.93	24.55	39.26	35.64	0.16
Soft drinks	8.52	16.02	30.08	47.50	0.07	36.14	72.29	36.02	46.38	0.28

Bold characters represent statistical significant P-values.
[b] P-value calculated with Mann–Whitney test.

was observed (P = 0.04, β = -14.15 ppb; 95% CI = -27.39 to -0.91). Specifically, two meals of fatty fish (at least 150 g of cooked filleted fish/meal) in the context of a Mediterranean diet resulted in a decrease in bronchial inflammation as measured by FeNO by 14.15 ppb (95% CI = -27.39 to -0.91; β = -14.15; P = 0.04) (Table 6).

Medication use

Regarding asthma medication use, at baseline, 53 out of 64 children were taking medication as part of their daily asthma therapy, of whom 51 children were taking anti-leukotriene agonists [intervention (24) versus control (27)]. At 6 months, 35 out of 64 children were taking medication [intervention (14) versus control (21)], with 31 children taking anti-leukotriene therapy (intervention (12) versus control (19). In the crude analysis, a greater reduction in anti-leukotriene use from baseline to 6 months was seen in the intervention group compared to the control group (24 versus 12 and 27 versus 19, respectively), although the result was not significant (X^2 test: P = 0.21).

Discussion

The present clinical trial explored the hypothesis that a Mediterranean diet supplemented with high omega-3 fatty acid intake as 'fatty' fish improves pulmonary function and decreases symptoms in asthmatic children. The KIDMED score revealed that, in both the intervention and control groups and across all time-points, children did not have optimal adherence to the Traditional Mediterranean diet. Previous studies undertaken in Mediterranean regions have reported an abandonment of the Mediterranean dietary pattern in children and adolescents [47]. Nevertheless, the most significant finding of the present study is that fatty fish intake, specifically eating two meals of fatty fish (at least 150 g of cooked fillet fish per meal) weekly in the context of a Mediterranean diet, resulted in a decrease in bronchial inflammation (FeNO) by 14 units in the intervention group. According to the ATS guidelines, a reduction in FeNO by at least 10 units for values lower than 50 ppb as the cut-off point indicates a significant response to anti-inflammatory therapy [36]. No statistical significant differences in spirometry, asthma control or quality of life were observed between the groups. A possible explanation as to why we did not observe any change in spirometry with fatty fish intake could be because children had normal lung function and well-controlled asthma. On the other hand, our recent meta-analysis investigating the role of fish intake in childhood asthma documented that early introduction and regular intake of fish (at least once a week) was beneficial on 'current wheeze' [odds ratio (OR) = 0.62; 95% CI = 0.48–0.80] and 'current asthma' (OR = 0.75; 95%

Table 3. KIDMED Questionnaire baseline and six month follow-up

KIDMED Questionnaire							
		Baseline Group			Six-months Group		
Question	Response	Intervention % (n)	Control % (n)	P[a]	Intervention % (n)	Control % (n)	P[a]
Q1. Does your child take a fruit or fruit juice every day?	Yes	80.6% (25)	81.8% (27)	0.90	87.1% (27)	84.8% (28)	0.79
Q2. Eat two fruits every day?	Yes	61.3% (19)	60.6 % (20)	0.95	48.4 % (15)	57.6% (19)	0.46
Q3. Eat fresh salad or cooked vegetables regularly once a day?	Yes	58.1% (18)	54.5% (18)	0.78	54.8% (17)	45.5% (15)	0.45
Q4. Eat fresh salad or cooked vegetables more than once a day	Yes	6.5% (2)	9.1% (3)	0.69	6.5% (2)	12.1% (4)	0.44
Q5. Eat fish regularly (at least 2-3 times per week)?	Yes	12.9% (4)	6.1% (2)	0.35	83.9% (26)	6.1% (2)	0.00
Q6. Go to a fast-food restaurant (hamburger) more than once a week?	Yes	3.2% (1)	6.1% (2)	0.59	0	9.1% (3)	0.09
Q7. Eats legumes more than once a week	Yes	32.3% (10)	36.4% (12)	0.73	19.4%(6)	15.2% (5)	0.66
Q8. Eats pasta or rice almost every day (5 or more times per week)?	Yes	12.9% (4)	6.1% (2)	0.35	25.8% (8)	6.1% (2)	0.03
Q9. Eats cereals or grains (bread etc.) for breakfast?	Yes	35.5% (11)	48.5% (16)	0.29	35.5% (11)	45.5% (15)	0.42
Q10. Eat dairy products for breakfast?	Yes	80.6% (25)	87.9% (29)	0.43	80.6% (25)	90.9% (30)	0.24
Q11. Eat baked goods or pastries for breakfast?	Yes	3.2% (1)	3.0% (1)	0.96	0.0% (0)	3% (1)	0.33
Q12. Skips breakfast?	Yes	12.9% (4)	12.1% (4)	0.92	12.9% (4)	9.1% (3)	0.62
Q13. Eat nuts regularly (at least 2-3 times per week)?	Yes	6.5% (2)	15.2% (5)	0.26	12.9% (4)	12.1% (4)	0.92
Q14. Eat 2 yogurts and/or some cheese (40g) daily?	Yes	83.9% (26)	78.8% (26)	0.60	80.6% (25)	78.8% (26)	0.85
Q15. Eat sweets and candy several times every day?	Yes	19.4% (6)	39.4% (13)	0.08	12.9% (4)	21.2% (7)	0.38
Q16. Eat olive oil with meals?	Yes	100% (31)	100% (33)	k	100% (31)	100% (33)	k

Bold characters represent statistical significant P-values.
P[a] - P-value calculated using Chi Square Test; k- constant

Table 4. Plasma fatty acid composition of children at baseline and six months per group

Plasma fatty acids composition										
	Baseline Group					Six-months Group				
	Intervention		Control			Intervention		Control		
Plasma fatty acid (mcmol/L)	Mean	S. D	Mean	S.D	P[b]	Mean	S.D	Mean	S.D	P[b]
Omega-3 fatty acids										
alpha-Linolenic acid	10.81	5.0	12.33	4.94	0.38	14.72	5.23	14.40	4.20	0.91
EPA	30.22	13.53	33.01	21.57	0.54	29.99	15.78	25.08	8.10	0.33
DHA	54.54	30.94	50.07	33.67	0.33	119.88	41.43	71.80	26.36	**0.00**
Omega-6 fatty acids										
Gamma- Linolenic acid	10.15	6.92	11.67	6.35	0.25	25.80	16.01	28.37	19.86	0.79
Linoleic acid	1,124.29	443.25	1.147.32	428.68	0.87	1,724.34	546.64	1,602.04	433.88	0.34
Arachidonic acid	446.55	643.37	362.12	85.64	0.24	381.54	84.42	411.59	122.76	0.33
Total plasma fatty acids	4,939.01	1,084.76	5,118.12	1,175.32	0.64	5,973.66	1,391.99	5,678.64	1,229.67	0.62
Total plasma Ω3 fatty acids	99.17	46.61	99.45	57.31	0.35	168.20	56.40	115.82	34.61	**0.00**
Total plasma Ω6 fatty acids	1,557.90	443.45	1,618.86	479.28	0.79	2,254.36	607.29	2,167.93	547.56	0.62
Ratio Ω6 : Ω3	24.24	26.48	23.21	25.81	0.13	14.48	4.92	19.44	4.64	**0.00**

Bold characters represent statistical significant P-values.
P[b] - P-values estimated using Mann-Whitney test;
PUFA-polyunsaturated fatty acids; Ω3- omega-3 fatty acids; Ω6- omega-6 fatty acids

Table 5.Univariate and Multivariate analysis showing mean change in asthma control, quality of life and pulmonary function parameter estimates from baseline to follow-up in the intervention and control groups.

Mean change	Group		Difference			
	Intervention	Control	Mean	95% CI	P^a	P^c
ACQ score	-0.13	-0.16	0.03	-0.19; 0.24	0.80	0.66
PAQLQ score	0.05	0.19	-0.14	-0.34; 0.05	0.15	0.23
FVC (% predicted)	2.45	-0.12	2.57	-1.81; 6.96	0.24	0.33
FEV_1 (% predicted)	2.84	0.61	2.23	-1.87; 6.34	0.28	0.38
FEV_1/FVC (%predicted)	0.58	0.79	-0.21	-2.54; 2.12	0.86	0.86
PEF % (%predicted)	6.06	7.06	-0.99	-11.18; 9.18	0.85	0.71
FEF_{25-75} (% predicted)	2.35	-1.24	3.60	-3.09; 10.28	0.29	0.44
FeNO (ppb)	-3.84	8.09	-11.93	-24.32; 0.46	0.06	**0.04**

Bold characters represent statistical significant P-values.

P^a- P-value calculated using t-test;

P^c- P-value from multiple linear regression analysis adjusted for confounders age, sex, BMI and regular physical activity.

ACQ- Asthma Control Questionnaire score; PAQLQ- Paediatric Asthma Quality of Life Questionnaire score. FEV_1- Forced Expiratory Volume in 1 second; FVC- Forced Vital Capacity; FEV_1/FVC- ratio of Forced Expiratory Volume and Forced Vital Capacity; PEF- Peak Expiratory Flow; $FEF_{25-75\%}$- Mid Expiratory Flow 25-75% vital capacity; FeNO- Fractional exhaled Nitric Oxide analysis.

Table 6. Multiple linear regression analysis showing mean change in bronchial inflammation (FeNO) for intervention and control groups from baseline to follow-up

Mean score change	Variable	Difference		
		β	95% CI	P^c
FeNO(ppb)				
	Group	-14.15	-27.39; -0.91	**0.04**
	Age	-1.69	-4.97; 1.59	0.31
	Sex	6.89	-6.23; 20.10	0.30
	Regular physical activity	1.69	-11.51; 14.89	0.80
	BMI	0.22	-1.64; 2.09	0.81

Bold characters represent statistical significant P-values

β-unstandardized beta; BMI-body mass index; CI-confidence interval; FeNO- fractional exhaled nitric oxide

[c] P-value evaluated applying multiple linear regression model adjusted for confounders age, sex, BMI and regular physical activity.

CI = 0.60–0.95) in children up to 4.5 years old, whereas fatty fish intake was protective for 'current asthma' in children aged 8–14 years (OR = 0.35; 95% CI = 0.18– 0.67) [20]. However, in the majority of these studies, asthma outcome was assessed using a questionnaire and not by spirometry.

Another finding warranting further exploration was that there was a higher reduction in medication use for children in the intervention group compared to the control group. Dotterud et al., (2013) reported that fatty fish intake reduced asthma medication use in the last 12 months among girls at age 2 years [48]. Hence, it was proposed that medication use could be decreased in some patients with asthma with an increased dietary omega-3 fatty acid intake from fatty fish if both the drug and omega-3 fatty acids exert their therapeutic effects through the same molecular actions. Thus, the possibility exists for a synergistic effect of drug–diet interactions that confer greater anti-inflammatory benefits when combined than either intervention alone or similar effects with less side-effects.

Regarding the slight increase in plasma DHA concentration (43%) observed in the control group, a plausible explanation for this increase is that DHA was derived from other foods. It has been reported that DHA status can be improved by the long-term intake of vegetable oils. However, the increase in tissue DHA may not be immediate and not as effective as the direct consumption of DHA from fish or fish oil supplements [17]. The Mediterranean dietary pattern is a varied diet that consists of a high intake of vegetables and wild edible greens, as well as a low to moderate intake of free-range animal products such as meat, poultry and eggs [16] that are terrestrial sources of DHA, although not as much as is present in fatty fish. For example, Atlantic cod contains 277 mg of DHA per 180 g fillet, 29 mg in 180 g of chicken breast, 12 mg in an egg, 2 mg in a pork chop and 1 mg in 90 g of beef steak compared to 2477 mg in 180 g of farmed Atlantic salmon [49]. By contrast, for the intervention group, increased fatty fish intake a rich source of DHA/EPA resulted in approximately 120% increase in plasma DHA along with a decrease in bronchial inflammation biomarker FeNO.

Several mechanisms have been proposed by which marine omega-3 fatty acids decrease bronchial inflammation in asthma. In vitro experiments have demonstrated that the consumption of fish leads to a shift in omega-3/omega-6 fatty acid balance resulting in a reduced production of inflammatory mediators involved in disease development [17]. Fatty fish is rich in EPA and DHA, which can inhibit cyclooxygenase and lipo-oxygenase enzyme activity, and also decrease pro-inflammatory mediators

from n-6 fatty acid arachidonic acid such as 2-series prostaglandins and 4-series leukotrienes (leukotriene E4, leukotriene B4), eosinophils and tumor necrosis factor-a, which promote airway oedema, mucus secretion, bronchial inflammation, bronchospasm and onset of asthma symptoms [19]. The 2-series PG have an immunomodulatory function, which modifies the activity of macrophages and lymphocytes and suppresses the production of T-helper (Th)1-related cytokines, promoting the expression of the Th2 phenotype [19]. Th2 responses include the production of interleukin (IL)-4, IL-5, IL-9 and IL-13, and are associated with increased levels of immunoglobulin E via B lymphocytes and eosinophil production leading to severe bronchial inflammation and the onset of asthma symptoms [50,51]. Furthermore, EPA gives rise to eicosanoids with a lower biological potency than those generated from arachidonic acid [19] and thus they are weaker inducers of inflammation. Unique to EPA and DHA, both of these fatty acids are precursors to resolvins, and DHA is also a precursor to protectins and maresins, which are mediators with anti-inflammatory resolving properties. For example, the DHA derived molecule, resolvin D1, facilitates the phagocytic engulfment and clearance of apoptotic neutrophils, which is essential in the resolution of inflammation [52], along with reduced eosinophil activation and infiltration into the lung, decreased concentration of IL-5, Th2 cytokines, Th17, airway mucus metaplasia as well as airway hyperactivity and promoted inactivity of pro-inflammatory transcription activator nuclear factor kappa B. In addition, DHA has an important role in oxidative stress associated with inflammation [53,54]. DHA exerts anti-oxidant effects by reducing the intracellular accumulation of reactive oxygen species and reactive nitrogen species, as well as maintaining optimal levels of glutathione and anti-oxidant enzymes [52]. During inflammation, excessive production of nitric oxide (NO) in the lungs causes tissue damage. DHA is able to inhibit the expression of inducible NO synthase, an enzyme responsible for NO production [52].

Strengths/limitations

To our knowledge, this is the first clinical trial investigating the effect of high omega-3 'fatty' fish intake added to a Mediterranean diet in children with 'mild' asthma. Few studies have examined the effect of fatty fish in asthma [26,48,55,56] and the present study adds to the existing evidence. A strength of our study is that we used fish as opposed to fish oil in the dietary intervention. It has been reported that fish consumption can significantly increase serum levels of DHA and EPA in humans compared to fish oil supplementation [52]. Fish is a source of high-quality protein and trace minerals, especially selenium and iodine, which are not provided in fish oil supplements that may have other beneficial effects. By contrast, fish oil supplements might not provide sufficient anti-inflammatory activity because of impaired enzymatic activity in asthma patients [57]. Also, fish oil is known to have an unpleasant taste, odour and adverse effects such as gastrointestinal disturbances [58,59] and therefore is less palatable and not sustainable. Another strength of the present study is that pulmonary function and bronchial inflammation were assessed quantitatively compared to a parent's report of symptoms or the use of a questionnaire. Treatment and monitoring of asthma are guided by symptom scores or lung function parameters, which are not always accurate markers of disease severity [35]. Exhaled nitric oxide is an important biomarker for bronchial inflammation in asthma because a patient can be asymptomatic and spirometry may not always reflect the underlying inflammation [35]. Also, it is valuable for identifying eosinophilic inflammation, adherence and effectiveness of medication therapy and also for predicting the risk of future exacerbations [60]. In the present study, patient adherence was assessed by independent dietary biomarkers rather than using dietary data (46). Furthermore, plasma fatty acid composition is a reliable indicator of dietary fat intake in children [61].

The primary weakness of the present study is the short duration period and possible limited power to adjust for multiple confounders and conduct analysis of effect modification. Changes in inflammatory cytokines were not measured, which could have added to the extent of inflammation reduction. It would have been interesting to examine whether fish oil in fatty fish could suppress the production of cytokines to levels similar to those attained with appropriate asthma therapy and is associated with clinical improvement. Another drawback is that questionnaires were self-administered by parents, which might have led to misinformation and recall bias. In addition, at baseline, FeNO was higher in the intervention group and lower in the control group and it is possible that the difference in FeNO was driven by the increase in the control group and regression to the mean. Nevertheless, at baseline, there was no bronchial inflammation in both groups because FeNO was less than 20 ppb. Moreover, we may have not taken all potential confounding variables into consideration such as environmental tobacco exposure, maternal education, residence area, parental atopic disease, social economic status and number of siblings. However, the multivariate analysis decreases the probability of confounding and an effort was made to correct for age, sex, regular physical activity and BMI. A potential issue for some families may have been the time required for the preparation and cooking of fish meals. Nevertheless, the health benefits would outweigh the burden.

In conclusion, our findings suggest that a Mediterranean diet supplemented with fatty fish might be a potential non pharmacological strategy to combat airway inflammation. This has important public health implications because dietary interventions are easily applied in 'real-life' situations, are of low cost, have multiple health benefits, and might assist in reducing asthma burden in children. Given that there are no adverse effects of regular fish consumption, a healthy diet incorporating two fatty fish meals per week provides overall health benefits and well-being. Future robust clinical trials are warranted to replicate and corroborate the promising findings documented.

Transparency statement

The lead author affirms that this manuscript is an honest, accurate and transparent account of the study being reported. The reporting of this work is compliant with CONSORT guidelines. The lead author affirms that no important aspects of the study have been omitted and that any discrepancies from the study as planned have been explained.

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Conflicts of interest, sources of funding and authorship

The authors declare that they have no conflicts of interest.

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MP is the principal author of the paper. CI is the principal investigator and DT, CK, MK, BE and MP are co-investigators. BE and KL assisted with the statistical analysis and supported the interpretation and revisions of the manuscript. All co-authors declare that they have seen and approved the final version of the manuscript submitted for publication.

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Supporting information

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Appendix S1. Biochemical tests.

Figure S1. Fractional exhaled nitric oxide (FeNO) values from baseline to 6 months for participants in the intervention group.

Additional results not discussed in the above manuscript are described below.

6.5.1 Plasma fatty acid composition and lung function

Regarding biochemical tests, at baseline, no significant difference in plasma fatty acid composition was observed between the groups except for lauric acid [4.74 (intervention) vs 6.14 mcmol/L (control); $p = 0.04$]. In comparison, at six months significant differences in DHA ($p < 0.001$), eicosatrienoic acid ($p = 0.033$), nervonic acid ($p = 0.01$), total plasma omega-3 fatty acids levels ($p < 0.001$) and omega-6: omega-3 fatty acid ratio ($p < 0.001$) were observed between the groups (Table 23). As for eicosatrienoic acid, plasma concentration of this fatty acid was lower in the intervention group at six months than in the control group (intervention vs control: 4.60 vs 5.54 mcmol/L) respectively. The reverse was observed for nervonic acid levels (intervention vs control: 77.64 vs 65.49 mcmol/L) respectively. Eicosatrienoic and nervonic acids (Ω9) are intermediates in the breakdown of omega-3 fatty acids and MUFA from dietary sources such as olive oil [339]. The arachidonic and EPA ratio is a sensitive index for determining changes in total blood fatty acid composition. At both time-points the ratio of arachidonic acid and EPA was >3 signifying a pro-inflammatory state due to the high intake of omega-6 fatty acids and low omega-3 fatty acids in the diet, as was evident by the high Ω6: Ω3 ratio (approximately 20:1) [339].

Table 23 Plasma fatty acid composition of children at baseline and six months

PLASMA FATTY ACID COMPOSITION OF CHILDREN BASELINE AND SIX MONTHS

VARIABLES (mcmol/L)	Baseline Group					Six-Month Group				
	Intervention		Control			Intervention		Control		
Fatty acids	**Mean**	**SD**	**Mean**	**SD**	***P*[b]**	**Mean**	**SD**	**Mean**	**SD**	***P*[b]**
Omega 3 fatty acids										
a-Linolenic	10.81	5.00	12.33	4.94	0.38	14.72	5.23	14.40	4.20	0.91
Eicosatrienoic	4.60	2.71	5.02	2.94	0.79	4.60	1.78	5.54	2.62	**0.03**
EPA	30.22	13.53	33.01	21.57	0.54	29.99	15.78	25.08	8.10	0.33
DHA	54.54	30.94	50.07	33.67	0.33	119.88	41.43	71.80	26.36	**0.00**
Omega 6 fatty acids										
gamma- Linolenic	10.15	6.92	11.67	6.35	0.25	25.80	16.01	28.37	19.86	0.79
Linoleic	1.124.29	443.25	1.147.32	428.68	0.87	1,724.34	546.64	1,602.04	433.88	0.34
Arachidonic	446.55	643.37	362.12	85.64	0.24	381.54	84.42	411.59	122.76	0.33
Homo-gamma-linolenic	92.08	25.76	98.76	31.08	0.52	123.68	34.36	126.92	39.80	0.54
MUFA										
Myristoleic	5.67	6.56	3.49	3.78	0.23	3.45	2.66	2.70	2.05	0.34
Cis-10 pentadecenoic	33.20	23.95	32.01	19.92	0.98	34.05	23.06	27.82	14.30	0.39
Palmitoleic	87.68	38.16	94.68	46.46	0.65	83.80	38.46	80.77	45.40	0.56
Oleic	702.75	328.23	693.87	243.85	0.79	955.11	342.99	935.32	319.11	0.89
Elaidic	.	.	65.31	.	-	-	-	1.33	-	-
Cis-11 Eicosenoic	4.01	3.42	4.41	3.40	0.62	7.83	2.88	7.15	2.35	0.52
Erucic	2.68	5.28	1.95	3.16	1.00	1.15	0.37	1.27	0.70	0.76
Nervonic	54.97	12.34	56.38	13.13	0.99	77.64	18.01	65.49	16.11	**0.01**

Saturated fatty acids										
Lauric	4.74	1.29	6.14	4.14	**0.04**	15.33	20.01	12.74	16.83	0.67
Myristic	67.58	46.28	62.96	38.98	0.76	73.03	50.31	59.79	30.54	0.39
Pentadecanoic	11.92	4.44	12.41	4.10	0.50	13.45	5.03	11.38	3.54	0.23
Palmitic	1,780.53	376.3	1,816.97	374.22	0.85	1,654.62	350.09	1,587.83	313.49	0.62
Heptadecanoic	12.93	3.27	13.53	3.27	0.34	14.99	3.43	13.35	3.30	0.10
Stearic	523.37	113.92	542.55	135.99	0.63	530.20	112.08	509.11	102.95	0.56
Arachidic	11.41	3.39	13.46	3.34	0.08	15.97	3.54	15.35	3.50	0.40
Behenic	31.6	9.86	33.89	9.77	0.27	43.24	9.85	40.46	8.81	0.31
Lignoceric	27.12	8.61	29.25	7.71	0.28	40.32	9.57	37.32	8.51	0.25
Total plasma	4,939.01	1,084.76	5,118.12	1,175.32	0.64	5,973.66	1,391.99	5,678.64	1,229.67	062
Total plasma saturated	2,410.86	599.02	2,527.15	552.06	0.61	2,397.15	509.09	2,283.32	442.40	0.52
Total plasma MUFA	873.08	342.25	874.66	273.95	0.78	1,155.94	383.93	1,113.60	363.62	0.64
Total plasma PUFA	1,655.06	427.95	1.716,29	497.15	0.83	2,420.55	622.47	2,281.72	569.21	0.44
Total plasma Ω 3	99.17	46.61	99.45	57.31	0.35	168.20	56.40	115.82	34.61	0.00
Total plasma Ω 6	1,557.90	443.45	1,618.86	479.28	0.79	2,254.36	607.29	2,167.93	547.56	0.62
Ratio Ω6 : Ω3	24.24	26.48	23.21	25.81	0.13	14.48	4.92	19.44	4.64	**0.00**
Ratio Arachidonic/EPA	16.82	20.82	13.61	6.08	0.48	15.44	6.78	17.10	4.59	0.08

Total sample N = 64, Intervention n = 31, Control n = 33

Shown in bold are the statistically significant *p*-values. [b]*P*-values estimated using Mann-Whitney test

Key: fa- fatty acids; MUFA- Mono unsaturated fatty acids, PUFA- Polyunsaturated fatty acids; Ω3- omega-3 fatty acids; Ω6- omega-6 fatty acids - Negligible values < 0.00. Values for saturated fatty acids octanoic, decanoic and undecanoic were negligible.

With respect to asthma therapy, by the end of the six month study there was a significant downwards trend (reduction) in medication use for the intervention group (Intervention group: baseline 83.9% (26/31) vs six months 45.1% (14/31) $p = 0.002$) as compared to the control (Control group: baseline: 81.8% (27/33) vs six months 63.6% (21/33); $p = 0.07$ (McNemar test)].

6.6 Vitamin D and respiratory health

Regarding the secondary hypothesis which examines the role of vitamin D status in respiratory health the outcome of this study is discussed in Section 6.6

6.6.1 Correlation between vitamin D and clinical tests

When investigating for associations between plasma vitamin D levels and clinical tests, no correlations were observed between plasma vitamin D and FEV_1 ($p = 0.67$), FVC ($p = 0.66$), FEV_1/FVC ($p = 0.86$), PEF ($p = 0.54$) , $FEF_{25-75\%}$ ($p = 0.53$), and blood oxygen saturation ($p = 0.52$). There was a weak association between vitamin D and bronchial inflammation biomarker, FeNO, although borderline significant, probably due to small sample size and low power [Spearman's rho: $r(62) = 0.24$, $p = 0.058$)].

6.6.2 Correlation between BMI and vitamin D baseline

Another interesting finding was that BMI was inversely correlated to vitamin D status at baseline. Spearman's correlation showed a negative association between BMI and plasma 25 (OH) D, although weak ($r(62) = - 0.27$, $p = 0.031$).

Investigation of vitamin D status per weight group showed that vitamin D was slightly lower in the overweight/obese group compared to normal weight, but non-significant [Mean(overweight/obese vs normal weight): 22.22 ± 5.84 ng/mL vs 24.50 ± 7.81 ng/mL, $p = 0.12$ (Mann-Whitney test)]. When stratifying data to 25(OH)D < 25 ng/ml (insufficient) and 25(OH)D ≥ 25ng/ml (sufficient), at baseline 80.8% of overweight/obese children had 25(OH)D < 25ng/ml compared to normal weight (52.6%) ($X^2(1, 64)= 5.31$, $p = 0.021$;) with more girls insufficient than boys in both BMI groups, although modestly significant ($X^2(1, 64) = 3.98$, $p = 0.046$). No difference

in vitamin D status was observed between BMI categories (p = 0.67) at six months or between the sexes (p = 0.51).

The association between 25(OH)D and pulmonary function tests was further investigated via two analyses. Firstly, using the 50th percentile cut-off for plasma 25 (OH)D as a surrogate for 'insufficient' (25(OH)D < 25 ng/ml) and 'sufficient' (25(OH)D ≥ 25 ng/mL). Secondly, data was stratified based on the international cut-offs for bone health [deficient ≤ 10ng/mL (≤ 25 nmol/L), insufficient 10-20 ng/mL (25-50 nmol/L), inadequate 20-30 ng/mL (50-75 nmol/L) and desirable ≥ 30 ng/mL (> 75 nmol/L]. The findings are presented in Section 6.6.3 in the manuscript (as submitted to *Nutrition Research, Elsevier*) with title *Papamichael et al, 2019 Impact of plasma vitamin D status on lung function in asthmatic children adhering to Mediterranean diet enriched with fatty fish* which is currently under-review *(Manuscript ID: NR_2019_902).* Proof of manuscript submission to *Nutrition Research* is available in Appendix 3 (page 634).

6.6.3 Impact of plasma vitamin D status on lung function in asthmatic children adhering to a Mediterranean diet enriched with fatty fish.

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Title: The impact of vitamin D status on lung function in asthmatic children adhering to a Mediterranean diet enriched with fatty fish.

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Abstract

Asthma in children is the most prevalent allergic disease worldwide that has become a major public health priority. This study examined whether baseline serum vitamin D levels modify the beneficial response of fatty fish intake on pulmonary function in asthmatic children. We studied the relationship between serum vitamin D status at baseline, spirometry and Fractional exhaled Nitric Oxide levels (FeNO) in 64 Greek children with 'mild asthma' aged 5-12 years (51.6% male) in a dietary intervention study. The intervention group consumed two fatty fish meals/week (≥ 150 g cooked filleted fish/meal) as part of the Mediterranean diet for six months, and the control group, their usual diet. Baseline serum 25(OH)D was determined using Enzyme-Linked Immuno Assay and defined as sufficient levels of 25(OH)D ≥ 25 ng/mL. Only 36% of children were graded as sufficient in 25(OH)D levels on entry into the study with a higher proportion of girls insufficient than boys (61% vs 39% respectively). Participants with sufficient levels of serum 25(OH)D at baseline, consuming the intervention diet increased FEV_1/FVC by 4.89 units (β= 4.89; 95%CI: 1.19- 8.61; p=0.013) and $FEF_{25-75\%}$ by 12.83 units (β= 12.83; 95%CI: 4.27-21.40; p= 0.006) compared to controls. No significant differences in pulmonary function or

FeNO were observed for those with insufficient levels of 25(OH) D in either the intervention or control groups.

In conclusion, sufficient Vitamin D status appears to be important in facilitating improvements in pulmonary function in response to dietary intervention in children with asthma.

Keywords:

Asthma; children; Mediterranean diet, pulmonary function; spirometry, vitamin D

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1.Introduction

There is general consensus that asthma is the most prevalent allergic disease affecting 6.3 million children worldwide [340]. It is a chronic inflammatory disorder of the airways associated with increased morbidity, patient burden and poor quality of life as well as substantial health care and societal costs, rapidly becoming a public health concern/priority [341]. Studies show that asthma control is poor in children with under 50% of children adhering to prescribed asthma therapy [342].

Asthma is unequivocally caused by a complex combination of genetic and environmental factors [341]. Poor nutrition has been implicated as one of these factors contributing to increasing asthma prevalence along with vitamin D deficiency in children [343; 344]. It is hypothesised that the increasing prevalence of vitamin D deficiency may be linked to the asthma epidemic which has stimulated research interest in the role of vitamin D in asthma pathophysiology. Recent studies have demonstrated that airway epithelia, lung fibroblasts, airway smooth muscle cells, immune cells (T and B cells, macrophages, monocytes, dendritic cells) contain vitamin D receptors and high levels of the enzyme, 1a-hydroxylase [345]. Vitamin D receptors regulate the transcription of various genes implicated in inflammation and immunomodulation of respiratory epithelium [340]. Vitamin D suppresses pro-inflammatory cytokines Interleukin-17 (IL-17), IL-13 and promotes anti-inflammatory cytokines such as IL-10 [340]. Thus, vitamin D has a number of biological and immunomodulatory effects that might be important in regulating key mechanisms in asthma.

Low vitamin D status has been reported to be common among children with asthma in a variety of settings [218; 340; 346]. According to a recent systematic review and meta-analysis undertaken by Jat et al (2017), emerging evidence from observational studies suggest a link between vitamin D deficiency and asthma in children [205]. Data analysis of 23 relevant studies including 13,160 children under 18 years revealed that vitamin D deficiency and insufficiency was prevalent in 28.5% and 26.7% of asthmatic children respectively. Asthmatic children had lower 25(OH)D levels as compared to non-asthmatic children. Although mixed results were reported for correlations between 25(OH)D and asthma incidence, asthma control and lung function. Plausible

explanations for diversity amongst studies included heterogeneity in study designs, techniques used to measure serum vitamin D, sample size, age, population variability, geographic location, asthma diagnosis at variable ages and partial controlling for confounders. Similar findings have been published in another systematic review [347], reporting a 36% decreased risk of asthma exacerbation in children. In addition, a beneficial effect of vitamin D supplementation on asthma exacerbations in children has been documented in systematic reviews and meta-analyses [348] .

In our latest randomized controlled trial designed to examine the efficacy of a Mediterranean diet supplemented with fatty fish, we found that increased fatty fish intake reduced bronchial inflammation in asthmatic children [349]. Using data from this trial, we conducted a sub-analysis testing the hypothesis whether baseline serum vitamin D levels modify the beneficial response of fatty fish intake on pulmonary function in asthmatic children and extends further to our work on diet and lung function in childhood asthma.

2.Methods and materials

2.1 Study design

The current study used baseline and six-month follow-up data from a single-centre randomised controlled parallel intervention study that tested the efficacy of fatty fish intake in the context of the Greek Mediterranean diet on asthma in children that took place in Athens, Greece from November 2016 to September, 2017 [349]. The trial design and methodology have been published in detail elsewhere [350] and only key features are described here. The study protocol is registered with the Australian and New Zealand Clinical Trial Registry (www.ANZCTR.org.au/ACTRN12616000492459p). The present study was conducted according to the ethical principles of the Declaration of Helsinki (1989), and all procedures involving patients were approved by the Human Ethics Committee of La Trobe University, Australia (HEC 16-035). All parents gave written informed consent prior recruitment of children into the study.

The primary outcomes of this study are pulmonary function as assessed by spirometry (FEV_1, FVC, FEV_1/FVC, PEF, $FEF_{25-75\%}$) and bronchial inflammation biomarker Fractional exhaled Nitric Oxide (FeNO).

2.1.1 Subjects/Intervention

Children were recruited from a pediatric asthma clinic in the greater city of Athens, Greece. Children were 5-12 years of age with physician-diagnosed "mild" asthma as defined by GINA (Global Initiative for Asthma guidelines) [351] and willing to comply to the dietary intervention including "fatty fish" in the context of a Mediterranean diet for a period of six months. Following recruitment children were randomised to the dietary intervention or control group with a 1:1 allocation ratio using an internet platform (http://www.randomization.com). The intervention group was instructed to consume two fatty fish meals per week (at least 150g filleted fish/meal), as part of the Greek Mediterranean diet for a period of six months and the control group their usual diet. Fatty fish included salmon, sardines, trout, mackerel, and anchovies, fresh and frozen. The following assessments were made at baseline and at the completion of the 6 months trial: health and lifestyle questionnaire, physical activity, medical history, dietary intake using a validated food frequency questionnaire and KIDMED questionnaire, anthropometry, biochemical tests and lung function using spirometry and FeNO.

2.2 Assessments

2.2.1 Anthropometry

We measured standing height to the nearest 0.1 cm using a SECA stadiometer after shoes and heavy clothing had been removed and children were positioned in the standard Frankfort horizontal plane [352]. Body weight was measured to the nearest 0.1 kg on calibrated electronic scales (SECA, Hanover, MD). Children's body mass index (BMI) was calculated as weight divided by height squared (kg/m^2) and classified as normal weight, overweight or obese based on the Hellenic pediatric growth charts [353].

2.2.2 Spirometry

Pulmonary function was measured in the standing position with a nose-clip using a portable spirometer (MIR Spirobank II, MIR Inc. USA) in accordance to European Respiratory Society protocol [354]. The highest spirometry value ('best test') of three efforts was used for the analyses. Normal pulmonary function in healthy children was considered spirometry parameter values ($FEV_{1,}$ FVC, PEF)> 80% predicted, $FEF_{25-75\%}$ > 60-65% and FEV_1: FVC ratio above 80% [355; 356]. A variation in FEV_1 of 10-12% is considered to be clinically significant in children [355; 356].

2.2.3 Fractional exhaled Nitric Oxide Analysis

Fractional exhaled Nitric Oxide was used as a marker for bronchial inflammation [357]. FeNO was measured using NO Breath (Benfont Inc., UK) by use of a single-breath method according to European Respiratory Society guidelines [357]. No lung inflammation and good asthma control was represented by FeNO values less than 20 ppb [358] .

2.2.4 Physical activity level

Physical activity status was estimated using the International Study of Asthma and Allergies in Childhood (ISAAC) Phase 3 Environmental Questionnaire [359]. Regular physical activity was considered to be exercise more than or equal to three times per week (Supplemental S1).

2.2.5 Dietary assessment

A Food Frequency Questionnaire (FFQ) based on the validated semi-quantitative PANACEA-FFQ for Greek children aged 10-12 years [360]was administered to all children who participated in the study and was completed by their parents during medical consultations at baseline and six-months. Daily consumption of each food item (in grams per day) was assessed from FFQs.

2.2.6 Adherence to the Mediterranean diet

Adherence to the Mediterranean diet intervention was assessed utilizing the KIDMED questionnaire which was created to estimate adherence to the Mediterraean dietary pattern in children and young adults. The KIDMED index is based on the principles sustaining the Mediterranean dietary pattern and those that undermine it [361]. The KIDMED questionnaire comprises of 16 yes or no questions. Twelve questions denote a positive connotation with respect to the Mediterranean diet and are assigned a value of +1; 4 questions denote a negative connotation and are assigned a value of -1. If the sum of the values of the questionnaire range from 0-3, adherence to the Mediterranean diet is categorized as 'low', 4-7 'moderate' and 8-12 as ' high' [361] .

In order to maintain motivation and adherence to the dietary intervention, all participants were reviewed fortnightly by the dietician via face-to-face consultation, e-mails, text and telephone. During baseline and six-month interviews adherence to the Mediterranean dietary pattern and fish intake was assessed using the KIDMED questionnaire which was also used to validate dietary details from FFQs. In order to optimize health benefits, the importance of complying with dietary instructions was emphasized regularly to parents.

2.2.7 Serum 25-Hydroxyvitamin D levels

At baseline and six-months, venous samples (4ml) were collected from children according to WHO guidelines following a 2 hour fast [362]. The samples were centrifuged and serum decanted from the supernatant and were stored at -20°C until analysis, within 24 hours to avoid degradation. In case of hemolysis blood collection was repeated. Vitamin D status was measured using serum 25(OH)D as it has a half-life of 2-3 weeks and it is the best indicator of vitamin D exposure from diet and dermal synthesis [363]. Determination of 25(OH)D from blood serum was undertaken using Enzyme-Linked Immuno Assay (ELISA) (IDS, Tyne & Wear, UK). The intra- and inter- assay variability was less than 1.9% and 4.6% respectively. The performance of the assay for the quantification of 25(OH)D was assessed in co-operation with Vitamin D External Quality Assessment Scheme Advisory (DEQAS), UK. The blood level of 25(OH)D that is defined as vitamin D deficiency remains somewhat

controversial. Vitamin D deficient has been variably defined as serum 25 (OH)D concentration < 10 to < 30 ng/mL (< 25 to < 75 nmol/L) [364], insufficient 21-29 ng/mL (52-72 nmol/L) and sufficient ≥ 30 ng/mL (≥ 75 nmol/L) which is based on bone health [226]. There is no general consensus on the threshold of vitamin D for respiratory health. For the purpose of this study we used the 50th percentile cut-off as a surrogate for 'insufficient' (25(OH)D < 25 ng/ml) and 'sufficient' vitamin D levels (25(OH)D ≥ 25 ng/mL) in the present population which is in accordance with various studies that have defined vitamin D insufficiency values from 10 ng/ml-35ng/ml (25 nmol/l- 80 nmol/l respectively) [218; 365; 366].

2.3 Statistical Analyses

Data was analysed using the Statistical Package for the Social Sciences (IBM SPSS 20.0, IBM Corp, Armonk NY) and interactions tested for using STATA release 14.1 (College Station, TX, USA) software. Differences between groups were compared using t-tests and Mann-Whitney test when assumptions of normality were not satisfied. Categorical variables were analysed using Chi square test and are presented as frequencies. Vitamin D [25(OH)D] concentrations were used as the continuous variable and 'categorized as 'insufficient' or 'sufficient' (≥ 25 ng/mL). After stratification of data according to 25(OH) D categories at baseline, multiple linear regression model was applied to compare mean change in pulmonary function between the groups from baseline to six months. Based on previous research, factors affecting spirometry, FeNO and vitamin D status namely age, sex, BMI and regular physical activity [356; 358; 367] were used as adjustments and retained in the regression model if they changed the exposure effect size by 10% or were statistically significant. The results from the regression model are presented as unstandardized β coefficients. The level of statistical significance was defined at *p*-values< 0.05. Differences in the effect of the intervention on lung function in children stratified by baseline serum vitamin D status were investigated using an interaction analysis and *p*-values< 0.1 were considered significant in order to capture important interaction effects. To further explore the association of serum 25(OH)D with spirometry and FeNO, data was sub-divided to four strata: deficient ≤ 10ng/mL (≤ 25 nmol/L), insufficient 10-20 ng/mL (25-50 nmol/L), inadequate 20-30 ng/mL (50-75 nmol/L) and desirable ≥ 30 ng/mL (>

75 nmol/L) [368; 369]. Multivariate regression as well as the interaction analysis was also performed taking confounding factors into consideration.

3.Results

Sixty-four (64) children (intervention n=31, control n=33), 51.6% (33) male and 48.4% (31) female, mean age 8.00 ± 2.00 years completed baseline and six-month assessments. At recruitment 40.6% (26) of children were overweight/obese according to the Hellenic pediatric growth charts. Only 36% (23) of children were classified as 'sufficient' (25(OH) D ≥ 25 ng/mL) with more girls 'insufficient' in 25(OH)D than boys (61% (25) vs 39% (16) respectively; p = 0.007). Approximately half of the children exercised regularly (≥ 3 times/week) and 83% were taking daily asthma therapy. Most of children (94%) were not taking dietary supplements. One child was taking folic acid and three a multivitamin containing 5 μg of vitamin D. Adherence to the Mediterranean diet at baseline was 'moderate' in both groups (KIDMED score: intervention 5.32 vs control 5.24, p = 0.72) with 19.4% of children in the intervention group having 'high' adherence versus 15.2% in the control (p= 0.55).

Examination of Vitamin D status revealed that serum 25(OH)D concentrations increased by 20.87% in the intervention group at 6 months and marginally in the control group by 11.53% [(Mean 25(OH)D baseline vs six-months): Intervention group 23.19 vs 28.03 ng/mL (p = 0.002); Control group: 23.95 vs 26.70 ng/mL (p=0.07)] (crude analysis). More specifically, at baseline 56.1% (23) of children in the intervention group were 'insufficient' in 25(OH) D which decreased to 44.4% (12) by six-months (p = 0.007). In comparison, 43.9% (18) in the control group were insufficient in 25(OH)D at baseline and 55.6% (15) at six-months (p>0.05). No differences were observed in KIDMED scores, spirometry or FeNO between intervention or control groups at baseline nor at the end of the trial (p > 0.05).

As the focus of this paper was to evaluate changes in pulmonary function and relevant biomarkers by Vitamin D status, the baseline and 6 months socio-demographic, KIDMED categories, and bio-clinical characteristics are presented in Table 1. Data on food group intake stratified by Vitamin D status (insufficient < 25ng/mL and sufficient

≥25ng/mL) are shown in Table 2. Mediterranean diet adherence based on vitamin D status is shown in Supplemental S2, Table S2.

Table 1. **S**ocio-demographic and clinical characteristics for intervention groups according to insufficient/sufficient serum Vitamin D at baseline

	Vitamin D baseline						**Vitamin D six months**					
	Insufficient 25(OH)D< 25 ng/mL Group			**Sufficient 25(OH)D ≥ 25 ng/mL Group**			**Insufficient 25(OH)D< 25 ng/mL Group**			**Sufficient 25(OH)D ≥ 25 ng/mL Group**		
Variable	**Intervention (n=23)**	**Control (n=18)**		**Intervention (n=8)**	**Control (n=15)**		**Intervention (n=12)**	**Control (n=15)**		**Intervention (n=18)**	**Control (n=16)**	
	Mean ± SD	Mean ± SD	*P*[a]	Mean ± SD	Mean± SD	*P*[a]	Mean ± SD	Mean ± SD	*P*[a]	Mean ±SD	Mean ± SD	*P*[a]
Age (years)	8.00 ±2.00	8.00±2.00	0.76	8.00±2.00	9.00±2.00	0.72	8.08±2.31	8.33±2.55	0.79	8.06±2.01	9.06±2.11	0.16
Male (%)	30.40%	50.0%	0.20[b]	62.5%	80%	0.36[b]	41.7%	66.7%	0.19[b]	33.3%	62.5%	0.09[b]
BMI (kg/m^2)	19.00 ±3.94	19.37±3.69	0.76	17.54±4.04	16.96±3.63	0.73	19.35±3.55	18.07±2.88	0.28[c]	17.98±4.03	19.33±4.70	0.41[c]
Overweight/obese	56.5%	44.4%	0.34[b]	25%	20%	0.60[b]	50.00%	42.9%	0.72[b]	35.3%	46.7%	0.51[b]
Regular Physical Activity	34.80%	50.00%	0.33[b]	62.5%	66.7%	0.84[b]	25.00%	46.7%	1.00[b]	55.6%	62.5%	0.07[b]
Medication Yes	91.30%	77.80%	0.22[b]	62.5%	86.7%	0.18[b]	41.7%	73.3%	0.09[b]	50.0%	50.0%	1.00[b]
Pulmonary function (% pred)												
FVC	94.04 ±9.27	97.28±11.89	0.33	96.25±6.98	95.13±10.37	0.79	99.33±10.14	95.67±11.27	0.39	95.56±8.87	97.13±8.02	0.59
FEV_1	97.26 ±9.74	99.00±11.74	0.61	97.13±5.84	99.20±9.32	0.57	103.75±10.66	100.87±10.51	0.49	97.83±8.10	99.44±7.12	0.55
FEV_1/FVC	102.61±4.46	101.17±8.64	0.49	100.13±3.91	103.87±6.58	0.16	103.92±2.23	104.93±4.83	0.51	101.89±4.13	101.88±6.04	0.99
PEF	93.17±20.81	93.11±15.88	0.99	97.63±14.65	93.93±22.37	0.68	108.92±28.62	106.60±25.44	0.83	95.61±12.51	95.50±18.82	0.98
$FEF_{25-75\%}$	101.96±19.47	101.22±24.43	0.91	95.50±11.48	107.20±17.55	0.10	107.17±8.59	106.93±21.14	0.97	100.39±15.72	100.25±17.83	0.98
FeNO (ppb)	17.87±19.17	7.06±5.12	0.59[c]	18.13±13.20	13.87±7.64	0.88[c]	11.83±12.17	21.27±40.30	0.98[c]	16.06±17.09	16.31±17.42	0.56[c]
Vitamin D (ng/mL)	20.35±3.48	18.62±4.85	0.58[c]	31.64±8.66	29.63±5.00	0.97[c]	19.06±2.89	19.99±4.41	0.30[c]	34.01± 9.43	32.99±6.55	0.98[c]
KIDMED score	5.78±1.70	5.44±2.06	0.53[c]	4.00±2.33	5.00±2.00	0.32[c]	6.58±1.68	4.93±2.25	**0.03[c]**	5.94±1.16	5.31±1.89	0.13[c]
KIDMED category												
Low	8.7%	22.2%	0.46[b]	37.5%	33.3%	0.85[b]	8.3 %	33.3%	0.11[b]	5.6%	18.8%	0.34[b]
Moderate	69.6%	55.6%		50.0%	60.0%		58.3%	60.00%		88.9%	68.8%	
High	21.7%	22.2%		12.5%	6.7%		33.3%	6.7%		5.6%	12.5%	

P-values significant at 5% level in bold.

[a] *P* values shown were calculated using t-test ; [b] Chi-square test; [c] Mann-Whitney Test

Key: %pred- % predicted; FEV_1- Forced Expiratory Volume in 1 second; FVC- Forced Vital Capacity; FEV_1/FVC- ratio of Forced Expiratory Volume and Forced Vital Capacity, PEF- Peak Expiratory Flow, $FEF_{25-75\%}$- Mid Expiratory Flow between 25-75% vital capacity; FeNO- Fractional exhaled Nitric Oxide

Table 2. Intake of food groups (grams/day) at baseline and six-months according to vitamin D status per intervention group.

	Vitamin D Baseline						**Vitamin D Six-months**					
	25 (OH)D < 25 ng/mL			**25(OH)D≥ 25 ng/mL**			**25(OH)D < 25 ng/mL**			**25(OH)D ≥ 25 ng/mL**		
	Group			**Group**			**Group**			**Group**		
Food Group/Item (grams/day)	**Intervention (n=23)**	**Control (n=18)**		**Intervention (n=8)**	**Control (n=15)**		**Intervention (n=12)**	**Control (n=15)**		**Intervention (n=18)**	**Control (n=16)**	
	Mean± SD	Mean± SD	*P*[c]	Mean± SD	Mean± SD	*P*[c]	Mean± SD	Mean± SD	*P*[c]	Mean± SD	Mean± SD	*P*[c]
Dairy	530.25±290.04	500.89±283.83	0.75	362.32±320.10	527.99±339.46	0.09	362.75±229.97	507.53±302.12	0.19	524.56±307.30	471.47±271.23	0.52
Fruit	313.90±135.45	224.25±113.11	**0.03**	191.59±112.53	285.17±224.05	0.46	328.63±252.95	241.07±137.11	0.48	224.69±123.34	251.54±220.69	0.65
Vegetables	182.87±141.65	170.15±119.34	0.86	124.23±74.91	180.13±152.85	0.44	179.96±95.34	254.53±187.31	0.39	169.68±114.32	162.12±116.69	0.86
Legumes	65.05±36.45	65.23±35.76	0.94	66.75±34.16	45.08±28.40	0.09	71.50±39.84	55.34±34.84	0.25	53.00±25.31	40.78±29.27	**0.03**
Starch	120.96±150.77	67.34±25.31	0.12	91.94±42.71	66.63±26.74	0.12	95.99±70.18	73.74±29.07	0.49	95.28±54.14	76.25±21.24	0.51
Meat	85.74±53.50	80.09±45.45	0.68	79.13±21.15	82.17±29.40	0.94	75.00±28.14	91.02±59.90	0.55	63.87±31.93	78.29±30.76	0.19
Seafood	6.80±12.32	3.96±6.25	0.60	7.65±7.34	5.48±7.73	0.41	6.85±8.11	7.43±6.11	0.67	4.52±6.32	7.65±7.09	0.17
Lean fish	18.20±12.86	11.02±13.51	**0.02**	15.64±8.02	12.55±14.20	0.23	8.76±15.78	13.83±12.76	0.06	18.32±29.60	12.96±8.19	0.28
Fatty fish	16.76±10.26	12.18±13.41	0.08	18.39±16.19	8.28±9.02	0.09	45.75±14.92	11.82±14.01	**0.00**	45.31±22.72	8.96±7.51	**0.00**
Fish	34.96±19.95	23.20±24.94	**0.02**	34.03±18.75	20.83±19.79	0.08	54.51±22.44	25.65±25.18	**0.00**	63.62±44.34	21.92±12.74	**0.00**
Nuts	1.93±2.52	3.71±5.71	0.50	6.11±11.99	6.81±7.08	0.25	5.22±9.77	5.16±6.82	0.92	3.08±4.40	3.92±4.69	0.48
Olive oil	18.26±14.07	17.54±13.11	0.98	11.18±2.05	21.50±16.73	0.07	18.03±15.45	21.50±14.84	0.63	18.58±15.37	17.27±14.17	0.44
Fats	20.73±14.52	22.27±16.29	0.97	18.98±14.88	30.19±22.55	0.16	27.27±24.05	28.30±16.49	0.43	22.64±16.45	22.60±16.19	0.94
Fast food	16.84±15.58	34.61±47.78	0.20	28.03±19.18	17.45±9.97	0.17	19.48±7.52	20.47±17.93	0.85	19.22±15.81	24.45±20.26	0.43
Sweets	24.39±18.44	26.19±16.67	0.61	29.90±14.74	29.94±19.27	0.97	17.83±15.03	27.44±25.05	0.54	23.32±14.85	34.04±33.95	0.31
Savoury snacks	24.68±34.66	30.75±22.39	0.10	34.53±21.72	59.84±53.93	0.39	31.53±23.94	37.88±41.35	0.88	27.99±25.98	42.62±32.48	0.07
Soft drinks	5.19±12.49	27.51±46.50	0.05	18.11±21.64	33.16±50.13	0.89	56.20±104.14	26.32±43.06	0.85	22.05±41.23	46.55±50.49	0.06

In bold are shown statistically significant p-values at 5% level

[c] - *P*-value computed using Mann-Whitney Test.

From Table 2 it is apparent that during the six-month period fatty fish intake increased markedly in the intervention group for all vitamin D levels as compared to the control ($p = 0.00$)

Stratification by vitamin D level revealed that in children insufficient in 25(OH)D (< 25 ng/mL) at baseline, there was no effect of the dietary intervention for spirometry parameters and bronchial inflammation indicator (FeNO) in the univariate and multivariate analyses (p >0.05). There was a trend towards significance in the ratio of FEV_1/FVC in the univariate model (p = 0.044) which became borderline in the multivariate model (p = 0.06). However, among children with sufficient 25(OH)D levels (≥ 25 ng/mL), a significant change in spirometry parameters FEV_1/FVC (p = 0.01) and $FEF_{25-75\%}$ (p= 0.00) was observed in the univariate analysis (Table 3).

Table 3. Mean change in pulmonary function parameter estimates from baseline in the intervention and control groups stratified by 25(OH) D levels at baseline

Vitamin D	Mean score change	Group		Difference		
		Intervention	Control	Mean	95% CI	P^a
Sufficient (25(OH)D≥ 25 ng/mL)	FVC*	-3.62	0.27	-3.89	-9.79, 2.00	0.18
	FEV_1	-0.75	-1.00	0.25	-4.43, 4.93	0.91
	FEV_1/FVC	3.62	-1.33	4.96	1.60, 8.31	**0.01**
	PEF	-1.37	5.20	-6.57	-25.04, 11.89	0.47
	$FEF_{25-75\%}$	5.62	-6.87	12.49	4.99, 19.99	**0.00**
	FeNO (ppb)	-2.37	11.40	-13.77	-45.44, 17.88	0.38
Insufficient (25(OH)D < 25ng/mL)	FVC	4.56	-0.44	5.01	-1.00, 11.02	0.10
	FEV_1	4.09	1.94	2.14	-3.82, 8.10	0.47
	FEV_1/FVC	-0.48	2.56	-3.03	-5.98, 0.08	**0.04**
	PEF	8.65	8.61	0.04	-13.00, 13.08	0.99
	FEF_{25-75}	1.22	3.44	-2.23	-11.71, 7.26	0.64
	FeNO (ppb)	-4.35	5.33	-9.68	-21.01, 1.65	0.09

In bold are shown statistically significant p-values at 5% level

*Spirometry parameters as percent predicted

[a] Univariate analysis- t-test

Key: FEV_1- Forced Expiratory Volume in 1 second; FVC- Forced Vital Capacity; FEV_1/FVC- ratio of Forced Expiratory Volume and Forced Vital Capacity; PEF- Peak Expiratory Flow; $FEF_{25-75\%}$- Mid Expiratory Flow between 25-75% vital capacity; FeNO- Fractional exhaled Nitric Oxide.

When applying multiple linear regression model adjusted for age, sex, regular physical activity and BMI, a significant mean change in FEV_1/FVC (β= 4.89, 95% CI: 1.19-8.61; p = 0.013) and $FEF_{25-75\%}$ (β= 12.83, 95% CI: 4.27- 21.40; p = 0.006) was observed in the intervention group with sufficient 25(OH) D levels (Table 4). Comparable results were verified by the interaction analysis, FEV_1/FVC ($p_{interaction}$= 0.002) and $FEF_{25-75\%}$ (**p** $_{interaction}$= 0.033). No significant change was evident for FeNO or other spirometry variables.

Table 4. Multiple linear regression model indicating mean change in FEV_1/FVC and $FEF_{25-75\%}$ for children with sufficient 25(OH) D (≥25ng/mL) at baseline

Baseline 25(OH)D ≥ 25ng/mL		**Difference**		
Mean score change	**Variable**	**β***	**95% CI**	***P*#**
FEV_1/ FVC*	Group	4.89	1.19, 8.61	**0.013**
	Sex	-1.39	-5.53, 2.73	0.48
	Age	-0.46	-1.46, 0.53	0.34
	BMI	0.22	-0.37, 0.81	0.44
	Regular physical activity	-0.35	-4.25, 3.56	0.85
$FEF_{25-75\%}$	Group	12.83	4.27, 21.40	**0.006**
	Sex	-1.26	-10.79, 8.28	0.78
	Age	0.31	-1.98, 2.60	0.77
	BMI	-0.28	-1.64, 1.08	0.67
	Regular physical activity	-3.57	-12.59, 5.45	0.41

* Spirometry parameters as percent predicted

In bold are shown statistically significant *p*-values at 5% level

- *P*-value for group analysis using Multiple linear regression model adjusted for (baseline) age, sex, regular physical activity and BMI; β*-unstandardized beta

Key: FEV_1/FVC - ratio of Forced Expiratory Flow in 1 second on Forced Vital Capacity; $FEF_{25-75\%}$- Mid Expiratory Flow between 25-75% vital capacity

Subdivision of 25(OH)D to four strata [≤ 10ng/mL (≤ 25 nmol/L), 10-20 ng/mL (25-50 nmol/L), 20-30 ng/ml (50-75 nmol/L) and ≥ 30 ng/mL (> 75 nmol/L)] did not alter the results. The adjusted regression analysis showed a positive effect of the intervention on FEV_1/FVC (p = 0.023; β= 23.3; 95%CI: 12.5-34.0) for children with sufficient serum 25(OH) D ≥ 30 ng/mL (≥ 75 nmol/l), although not in the interaction analysis (p = 0.26)(Supplemental S3, Table S3).

Examination of serum 25(OH)D as a continuous variable yielded a significant result for $FEF_{25-75\%}$ (p = 0.04) but not for FEV_1, FVC, FEV_1/FVC, PEF, or FeNO (p > 0.05) (Supplemental S4, Table S4). Results are presented pictorially in Figure S5 and Figure S6 (Supplemental S5).

4.Discussion

In the present study we sought to understand the role of vitamin D status on pulmonary function in children with 'mild asthma' following a Mediterranean diet enriched with fatty fish. Two important findings were highlighted in this study. Firstly, the majority of children (64%) were insufficient in vitamin D predominating in girls [234]. This is consistent with European data documenting the pandemic problem of hypovitaminosis D in children particularly within the age groups of 1-6 and 7-14 years [364]. And further supports the findings of recent studies on the relationship between low vitamin D levels and childhood asthma [218; 370]. The fact that the majority of our patients were insufficient in vitamin D is alarming, although the thresholds used for vitamin D sufficiency was based on bone metabolism, thus proposing that concentrations > 30 ng/ml may be essential for optimal immune functioning and respiratory health.

Secondly, in children with sufficient serum 25(OH)D at baseline, following a Mediterranean diet enriched with fatty fish was associated with enhanced pulmonary function and airway flow in small airways as indicated by an increase in FEV_1/FVC as well as $FEF_{25-75\%}$, an indicator of small airways calibre. No significant effect was observed for bronchial inflammation biomarker, FeNO. This may have clinical significance in managing childhood asthma because FEV_1/FVC has been proposed as

a sensitive measure of airway obstruction and FEF $_{25-75\%}$ a more sensitive marker of obstruction in small airways than FEV_1 or FVC [355; 356]. Also, FEF $_{25-75\%}$ reflects early disease [355; 356]. FEF $_{25-75\%}$ has been found to be reduced in children with uncontrolled asthma even when FEV_1 is normal [371]. However, there is a debate about its usefulness because the above parameter is a highly variable measure that is influenced by expiratory time and change in FVC [372]. Low levels of $FEF_{25-75\%}$ and FEV_1/FVC have been associated with steroid use, hospitalization, asthma exacerbations and asthma severity as compared to children with normal spirometry [371]. Given that the majority of children have normal FEV_1, finding that another spirometry parameter ($FEF_{25-75\%}$) is associated with the presence of airway obstruction, poor asthma outcome and increased risk of future asthma exacerbation has important implications for health professionals performing spirometry in asthmatic children. It is interesting to note that we did not find an association between vitamin D and FEV_1 and FVC both of which are measures of total lung capacity. Although the reason for this discrepancy is yet to be elucidated, one could speculate that the impact of vitamin D on lung function may be mediated through changes in airway obstruction which affects FEV_1/FVC and FEF $_{25-75\%}$ rather than via total lung capacity.

The outcome of this study is comparable to other studies in asthmatic children reporting direct correlations between vitamin D status and lung function test measurements including FEV_1/FVC [218; 365; 370; 373] but not for FeNO [218; 370]. On the other hand, there was no correlation between FVC, FEV_1, FEV_1/FVC, PEF and FEF $_{25-75\%}$ in the studies by Brehm (2012) [209], Chinellato (2011) [218] and Krobtrakulchai (2012) [374] and Yao (2014) [370].Two studies of Vitamin D status in asthma were undertaken in the Mediterranean region [218; 365], one in Iran [373] and Taiwan [370]. Children's age ranged from 4-18 years old. Overall, studies documented a high percentage of vitamin D deficiency in asthmatic children ($\geq$ 40%) and a dose-response relationship between serum vitamin D and spirometry parameters (FEV_1, FVC, FEV_1/FVC) in asthmatic children but not for FeNO [218; 370] which is consistent with our findings and suggests a potentially important role for vitamin D in respiratory health. Regarding FEF $_{25-75\%,}$ only one out of the four studies examined the effect of vitamin D level on FEF $_{25-75\%}$ in asthmatic children, reporting no effect [370]. A major

limitation of these studies is the cross-sectional design which precludes the ability for causal inferences. A feasible explanation for the inconsistency among studies regarding vitamin D and lung function tests is that vitamin deficiency might represent a marker of environmental and lifestyle factors such as dietary, dress habits, time spent indoors and less sun exposure, availability as well as fortification policies. Additionally, heterogeneity among study methodologies such as variability in sample size, methods used for vitamin determination, threshold for vitamin D deficiency/sufficiency, lack of data on dietary and vitamin D supplementation, geographic location (latitude), ethnicity, skin melanin content, population variability in vitamin D metabolism, absorption and cutaneous synthesis of vitamin D, genetic variants in vitamin D receptors [345] and season that blood tests were undertaken, are plausible factors that might have led to spurious results. It has been suggested that levels higher than 40 ng/mL may be necessary for optimal immune functioning and overall health [375].

The results of this study suggest that vitamin D could affect tissue remodelling and might be interrelated with the development of airway limitation. One of the pleiotropic effects of vitamin D is its action on the development and mechanics of lung function. Gupta demonstrated that vitamin D levels were inversely correlated to airway smooth muscle mass and lung function in children with severe asthma [211]. On the other hand, our study showed the benefits of sufficient serum vitamin D status on lung function even in 'mild' asthmatic children. According to a study in mice undertaken by Zosky et al, lung size and volume was significantly smaller in vitamin D deficient mice compared with replete mice which influenced lung mechanics [376]. So, sufficient vitamin D levels in children could have resulted in inhibition of smooth muscle proliferation, improved cell growth and lung structure resulting in less resistance in small airways and airway obstruction, thus improving airflow as evident by the improvement in the ratio of FEV_1/FVC and $FEF_{25-75\%}$. This is significant because long-term treatment of asthma emphasizes the control of airway inflammation and not the prevention of airway remodelling.

Nonetheless, in line with these findings, no effect of vitamin D was observed on FeNO values in our patients. This is not surprising because FeNO detects eosinophilic airway

inflammation in asthma [358] and vitamin D may not be a determinant of eosinophilic inflammation [370]. Although the exact mechanisms underlying the link between vitamin D status and lung function remain unclear, one possibility is that Vitamin D could improve lung function by influencing airway remodelling and affecting smooth muscle cell movement, growth and contractility, collagen synthesis and by inhibiting matrix metalloproteinase and fibroblast proliferation as well as protection from respiratory infections and enhancing asthma therapy [377]. Other plausible mechanisms include the action of vitamin D on regulating airway inflammation such as control of inflammatory innate and adaptive immune response, induction of antimicrobial mechanisms to efficiently resolve infection and promotion of regulatory responses both indirectly by impairment of antigen presenting cell function and directly via effects on T lymphocytes, namely Th1 to Th2 switch and production of anti-inflammatory cytokines (IL10, TGFβ) from Treg cells [230; 340].

Another property of vitamin D worth noting, is its ability to enhance the response to asthma therapy by inducing the production of IL-10, a potent anti-inflammatory cytokine from regulatory T-cells [217; 231; 232]. We observed an additive effect of vitamin D with regards to the intervention on medication therapy, thus suggesting a dual effect. At baseline, 62.5% of children in the intervention group with sufficient vitamin D level were taking asthma medication compared to 91.3% insufficient in vitamin D. By the end of the study period there were fewer children in the intervention group with insufficient vitamin D levels and taking medication (41.7%) as compared to baseline. And as for the intervention group with sufficient vitamin D levels, only 50% of children were on asthma therapy. By the end of the study trial serum vitamin D levels increased in the intervention group by approximately 21% as compared to 11% in the control. Similarly, Searing and Brehm [212; 217] documented an inverse relationship between vitamin D levels in asthmatic children and use of steroids and anti-leukotriene therapy A likely explanation could be that vitamin D interacts synergistically and ameliorates the action of medication as was manifested by the improvement in FEV_1/FVC in the intervention group. In addition, having adequate serum vitamin D levels enhanced the effect of the dietary intervention in asthmatic children. Fatty fish is a rich source of vitamin D as well as a variety of other nutrients, antioxidants and

bioactive molecules, including omega 3 fatty acids, selenium, iodine, potassium, B-vitamins, that when combined these components interact synergistically having positive effects on pulmonary function [378]. Small dietary modifications such as increased fatty fish intake and sun exposure could improve airway conduction and thereby reduce asthma burden in children.

With respect to insufficient vitamin D levels in children, this may be the result of lifestyle factors. Extra-curriculum activities, more time spent indoors watching TV, playing video games and computer-time including both parents in the work-force are some of the barriers to daily physical activity in children [379]. In addition, poor dietary habits and abandonment of the Traditional Mediterranean diet by children [5] may have contributed to hypovitaminosis D in children. In our study, with regards to no association between insufficient Vitamin D (< 25 ng/mL) and lung function tests in children, we found that only 50% of children engaged in regular physical activity and in general, children had moderate adherence to the Mediterranean dietary pattern. Another potential limiting factor is reverse causation where children with poor asthma control are less likely to engage in outdoor activities due to difficulty in breathing [380]. Also, since respiratory infections trigger asthma exacerbations in children [21], parents may confine children indoors especially during winter months and as a result are exposed to less sunlight. Nonetheless, some studies have reported significant positive associations between vitamin D status and physical activity independent of sun-exposure [381]. Then again, hypovitaminosis in asthmatic children could be a consequence of inflammatory disease and not the cause [382].

Strengths/ limitations

There is lack of published data on the relationship between vitamin D status and lung function tests in asthmatic children. To the authors' knowledge, this study is the first clinical intervention trial to address the impact of vitamin D status in asthmatic children consuming a Mediterranean diet enriched with fatty fish. Previous publications evaluating vitamin D status and lung function in children have examined the association of serum 25(OH)D or vitamin D supplementation with FEV_1 and FVC

[218; 348]. A novel element of this study was the investigation of 25(OH)D in relation to FEV_1/FVC and $FEF_{25-75\%}$. Our findings present new evidence suggesting that children with sufficient serum vitamin D have better lung function even in small airways and reduced airway obstruction represented by $FEF_{25-75\%}$ and FEV_1/FVC. Nevertheless, future clinical trials are recommended to corroborate these findings. A potential strength is that pulmonary function and bronchial inflammation were assessed quantitatively using spirometry and exhaled nitric oxide analysis which enforces the accuracy, validity and reliability of our findings [351]. Innovative to asthma research is the use of metabolomics to assess biochemical markers in children. We used ELISA to quantify serum 25(OH)D as an independent biomarker of vitamin D status compared to studies which rely on dietary intake assessment of vitamin D using self-reported diet techniques that are prone to recall bias [383; 384]. In addition, serum concentration of 25 (OH)D is considered to be the most reliable measure of overall vitamin D status and thus can be used to determine whether a patient is vitamin D sufficient [382].

A possible limitation in our study is the cut-off used for vitamin D status, although, we have applied the threshold most commonly used in European pediatric populations [364]. Currently, there is no universal consensus on the concentration of 25(OH)D that defines vitamin D deficiency in children [364] and optimal levels for respiratory conditions such as asthma remain unknown. Another drawback is the small sample size and short-study duration employed for practical reasons such as minimizing study costs and patient burden. The promising findings could be due to chance (small sample size) and this warrants further investigation. A larger sample size would have increased our statistical power to detect a greater effect size. Seasonal differences could explain variance in serum 25 (OH)D between intervention groups and time-points. At baseline, blood tests were undertaken in November–January, encompassing typical winter months and in May-August at follow-up, typical summer months [367; 385]. Although at follow-up 25(OH)D levels increased substantially in the intervention group as compared to the control, most likely to increased fatty fish intake. One more possible determinant could be daily sun exposure which was not included as a potential confounder in the regression model.

In conclusion, vitamin D insufficiency in sun-replete countries including Greece is common in asthmatic children, especially in girls. We found that consumption of a Mediterranean diet enriched with fatty fish markedly enhanced pulmonary function in asthmatic children with sufficient serum 25(OH)D levels. Sufficient Vitamin D status appears to be important in facilitating improvements in pulmonary function in response to dietary intervention in children with asthma. Health professionals should emphasize the importance of a healthy lifestyle for children that includes a diet rich in dietary sources of vitamin D such as fatty fish and fortified foods in combination with outdoor play-time for ample sun exposure. Normalization of vitamin D status could reduce cost and asthma burden in children.

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Contributions:

MP is the principal author of the paper. CI, BE, CK contributed in revising the first draft of the manuscript. BE and KL guided the statistical analysis. All co-authors declare that we have seen and approved the final version of the manuscript being submitted. The authors confirm the article is the authors' original work, hasn't received prior publication and isn't under consideration for publication elsewhere.

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Highlights

- Asthma in children is the most prevalent allergic disease worldwide
- Hypovitaminosis D may have contributed to the rise in childhood asthma
- Sufficient vitamin D levels in children enhanced the effect of the dietary intervention, reduced medication use and improved airway conduction in small airways and decreased airway obstruction as reflected by $FEF_{25-75\%}$ and FEV_1/FVC respectively.

On-line Supplement *Nutrition Research*

Supplemental S1 Assessments

Physical activity level

Physical activity status was estimated using the International Study of Asthma and Allergies in Childhood (ISAAC) Phase 3 Environmental Questionnaire. Physical activity was evaluated by the following question "How many times per week does your child engage in vigorous exercise?" Possible responses were never/rarely, 1-2 times/week and more than 3 times/week. Regular physical activity was considered to be exercise more than or equal to three times per week.

Dietary assessment

A Food Frequency Questionnaire (FFQ) based on the validated semi-quantitative PANACEA-FFQ for Greek children aged 10-12 years was administered to all children who participated in the study and was completed by their parents during medical consultations at baseline and six-months. Daily consumption of each food item (in grams per day) was assessed from FFQs. The reported frequency of consumption of each food item in FFQs was converted to frequency of consumption per day. Grams per day were estimated by multiplying portion size by the value corresponding to each consumption frequency. Based on the participant's responses, the information was then summed into food groups. Thirteen main food groups (dairy, fruit, vegetables, legumes, starch, meat, fish, nuts, fats, sweets, fast food, savoury snacks, and soft drinks) were formed, reflecting a dietary pattern. Types of fish consumed such as fatty and lean as well as olive oil were also included.

Serum 25-Hydroxyvitamin D levels

At baseline and six-months, venous samples (4ml) were collected from children according to WHO guidelines following a 2 hour fast. The samples were centrifuged and plasma decanted from the supernatant and were stored at -20°C until analysis, within 24 h to avoid degradation. In case of hemolysis blood collection was repeated. Vitamin D status was measured using serum (25-OH)D as it has a half-life of 2-3 weeks and it is the best indicator of vitamin D exposure from diet and dermal

synthesis. Determination of 25-OH Vitamin D from blood serum was undertaken using Enzyme-Linked Immuno Assay (ELISA) (IDS, Tyne & Wear, UK). A sample volume of 25µL was added to glass tubes followed by 1mL of 25D-biotin conjugate and the mixture vortexed. Then, 200µL of this mixture was added to the anti-25D antibody coated microtitre plate. The assay mixture was incubated for 2 hours at room temperature and plates were washed to remove unbound 25-D biotin conjugate. Bound 25-D biotin conjugate was identified by adding 200µL Avidin HRP (Horseradish peroxidase) for 30 minutes and then the plate was washed again. Tetramethylbenzidine (TMB) substrate was added followed by incubation for 30 minutes and the reaction terminated with acid. The absorbance recorded at 450 nm was inversely proportional to the concentration of 25-OH vitamin D. Serum 25(OH) D values were estimated for unknown samples directly from the calibration curve. The intra- and inter- assay variability was less than 1.9% and 4.6% respectively. The performance of the assay for the quantification of 25(OH)D was assessed in co-operation with Vitamin D External Quality Assessment Scheme Advisory (DEQAS), UK. The blood level of 25(OH)D that is defined as vitamin D deficiency remains somewhat controversial. Vitamin D deficient has been variably defined as serum 25 (OH)D concentration < 25 to < 75 nmol/L (< 10 to < 30 ng/mL), insufficient 21-29 ng/mL (52-72 nmol/L) and sufficient ≥ 30 ng/mL (≥ 75 nmol/L) which is based on bone health. There is no general consensus on the threshold of vitamin D for respiratory health. For the purpose of this study we used the 50^{th} percentile cut-off as a surrogate for 'insufficient' (25(OH)D < 25 ng/ml) and 'sufficient' vitamin D levels (25(OH)D ≥ 25 ng/mL) in the present population which is in accordance with various studies that have defined vitamin D insufficiency values from 10 ng/ml-35ng/ml (25 nmol/l- 80 nmol/l respectively).

Supplemental Table S2

Table S2. KIDMED questionnaire according to vitamin D status per intervention group at baseline and six-months follow-up

KIDMED Questionnaire	Vitamin D baseline						Vitamin D six months					
	25(OH)D < 25 ng/ml			25 (OH)D≥ 25 ng/L			25(OH)D < 25 ng/ml			25 (OH)D≥ 25 ng/L		
	Group			Group			Group			Group		
	I (n=23)	C (n=18)	P^b	I (n=8)	C (n=15)	P^b	I (n=12)	C (n=15)	P^b	I (n=18)	C (n=16)	P^b
Q1. Does your child take a fruit or fruit juice every day? YES	87.0%	77.8%	0.44	62.5%	86.7%	0.18	83.3%	80.0%	0.82	88.9%	87.5%	0.90
Q2. Eat two fruits every day? YES	65.2%	66.7%	0.92	50.0%	53.3%	0.88	41.7%	60.0%	0.34	50.0%	56.2%	0.72
Q3. Eat fresh salad or cooked vegetables regularly once a day? YES	60.9%	55.6%	0.73	50.0%	53.3%	0.87	50.0%	46.7%	0.86	61.1%	43.8%	0.31
Q4. Eat fresh salad or cooked vegetables more than once a day YES	8.7%	16.7%	0.44	0.0%	0.0%	K	16.7%	13.3%	0.81	0.0%	12.5%	0.122
Q5. Eat fish regularly (≥ 2-3 times per week)? YES	8.7%	5.6%	0.70	25.0%	6.7%	0.21	100.0%	6.7%	**0.00**	72.2%	6.2%	**0.00**
Q6. Go to a fast-food restaurant more than once a week? YES	4.3%	11.1%	0.41	0.0%	0.0%	k	0.0%	0.0%	k	0.0%	12.5%	0.12
Q7. Eats legumes more than once a week YES	30.4%	44.4%	0.35	37.5%	26.7%	0.59	33.3%	13.3%	0.21	11.1%	18.8%	0.53
Q8. Eats pasta or rice almost every day (≥ 5 times per week)? YES	13.0%	5.6%	0.42	12.5%	6.7%	0.64	41.7%	0.0%	**0.01**	16.7%	12.5%	0.73
Q9. Eats cereals or grains for breakfast? YES	43.5%	44.4%	0.95	12.5%	53.3%	0.06	33.3%	46.7%	0.48	38.9%	43.8%	0.77
Q10. Eat dairy products for breakfast? YES	87.0%	83.3%	0.74	62.5%	93.3%	0.06	66.7%	86.7%	0.21	94.4%	93.8%	0.93
Q11. Eat baked goods or pastries for breakfast? YES	0.0%	0.0%	k	12.5%	6.7%	0.64	0.0%	0.0%	k	100.0%	93.8%	0.28
Q12. Skips breakfast?												

Q13. Eat nuts regularly (≥2-3 times per week)?	YES	4.3%	16.7%	0.19	37.5%	6.7%	0.063	16.7%	13.3%	0.81	5.6%	6.2%	0.93
Q14. Eat 2 yogurts and/or some cheese (40g) daily?	YES	4.3%	11.1%	0.41	12.5%	20.0%	0.65	25.0%	13.3%	0.44	5.6%	12.5%	0.48
Q15. Eat sweets and candy several times every day?	YES	87.0%	83.3%	0.74	75.0%	73.3%	0.93	91.7%	66.7%	0.12	77.8%	87.5%	0.46
Q16. Eat olive oil with meals?	YES	8.7%	22.2%	0.22	50.0%	60.0%	0.64	8.3%	26.7%	0.22	16.7%	18.8%	0.87
	YES	100%	100%	k	100.0%	100.0%	k	100.0%	100.0%	k	100.0%	100.0%	k

In bold are shown statistically significant p-values at 5% level

n(25(OH)D < 25 ng/ml) baseline = 41; n(25 (OH)D≥ 25 ng/L) baseline = 23

n(25(OH)D < 25 ng/ml) six-months = 27; n(25 (OH)D≥ 25 ng/L) six-month = 34

[b]*P*- Chi Square Test; k=constant

Key: I- Intervention group; C-Control group

In Table S2 the KIDMED questionnaire indicates that regular fish intake increased in the intervention group during the six-month period as compared to the control group ($p < 0.001$).

Supplemental S3 Table S3

Table S3. Adjusted interaction analysis indicating the effect of the intervention on pulmonary function and FeNO per 25(OH)D strata

Vitamin D Category	Score	Effect of intervention	95% CI	Strata specific *p*-value	Interaction *p*-value
25(OH)D≥ 75 nmol/L (n=7)	FVC	-118.9	-1045, 807	0.35	0.28
	FEV_1	-86.6	-947, 774	0.42	0.85
	FEV_1/FVC	23.3	12.5, 34.0	**0.023**	0.26
	PEF	85.4	-2412, 2583	0.74	0.27
	$FEF_{25-75\%}$	115.0	-1308, 1538	0.49	0.17
	FeNO (ppb)	31.9	-2576, 2639	0.90	0.65
25(OH)D 50-75 nmol/L (n=38)	FVC	6.01	-1.19, 13.21	0.09	0.52
	FEV_1	3.97	-2.78, 10.73	0.24	0.54
	FEV_1/FVC	-1.66	-5.79, 2.47	0.42	0.51
	PEF	1.04	-13.01, 15.09	0.88	0.45
	FEF_{25-75}	4.73	-4.84, 14.29	0.32	0.44
	FeNO (ppb)	-15.44	-37.50, 6.62	0.16	0.67
25(OH)D 25-50 nmol/L (n=19)	FVC	-0.81	-8.01, 6.39	0.81	
	FEV1	0.91	-6.41, 8.23	0.79	
	FEV1/FVC	1.13	-2.21, 4.47	0.48	
	PEF	-11.54	-36.75, 13.68	0.34	
	FEF25-75	-2.23	-19.49, 15.02	0.78	
	FeNO (ppb)	-5.42	-25.39, 14.55	0.57	
25(OH)D ≤ 25 nmol/L (n=0)					

*models adjusted for age, sex, regular physical activity and BMI at baseline

P-value < 0.05 level in bold.

Key: 25 nmol/L = 10 ng/mL; 25-50 nmol/L= 10-20 ng/mL; 50-75 nmol/L= 20-30 ng/mL

Supplemental S4

Table S4. Interaction analysis for serum 25(OH)D and pulmonary function tests.

25(OH)D (ng/mL)*	
Parameter	**Interaction *p*-value**
FVC	0.52
FEV_1	0.94
FEV_1/FVC	0.08
PEF	0.22
$FEF_{25-75\%}$	**0.041**
FeNO (ppb)	0.80

*25(OH)D as a continuous variable

P-values < 0.05 are in bold.

Supplemental S5 Figures

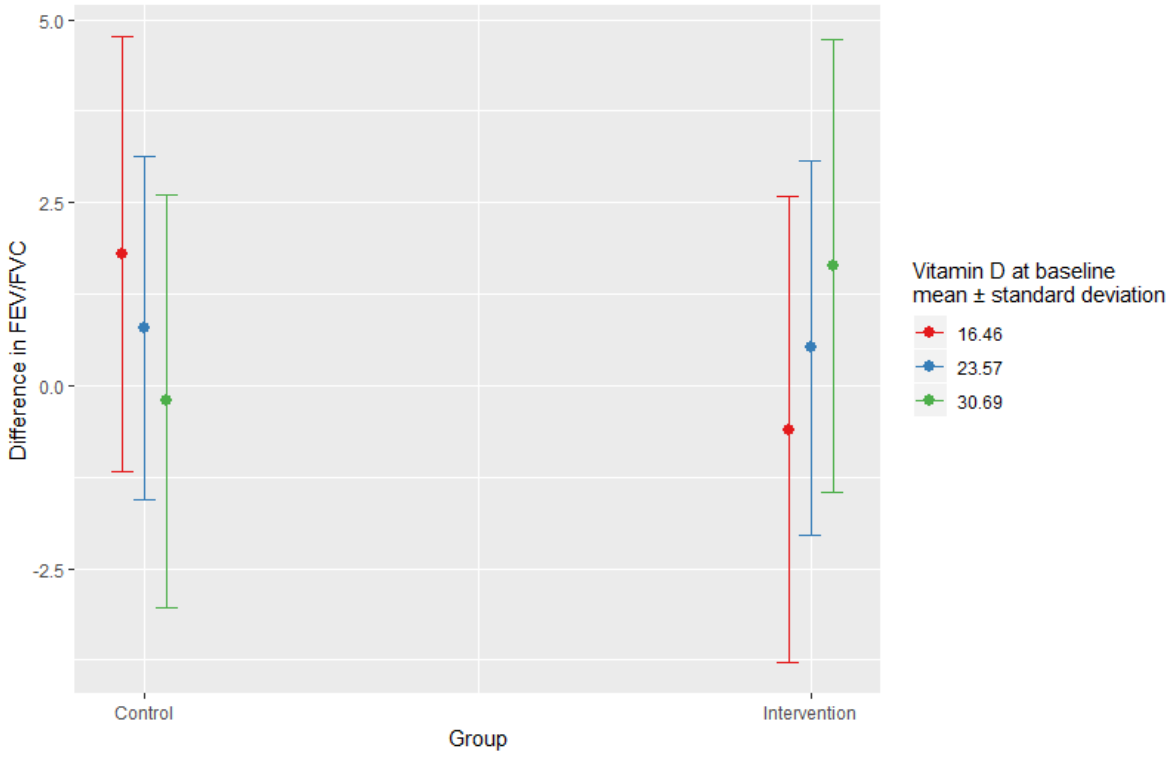

Figure S5. The effect of the dietary intervention on change in FEV_1/FVC by baseline serum 25(OH)D level.

Evidence of an interactive effect was observed between baseline serum 25(OH)D level and the intervention on change in FEV_1/FVC (p = 0.086). Increasing baseline serum 25(OH)D level was associated with an increase in FEV_1/FVC in the intervention group, and decrease in the control group.

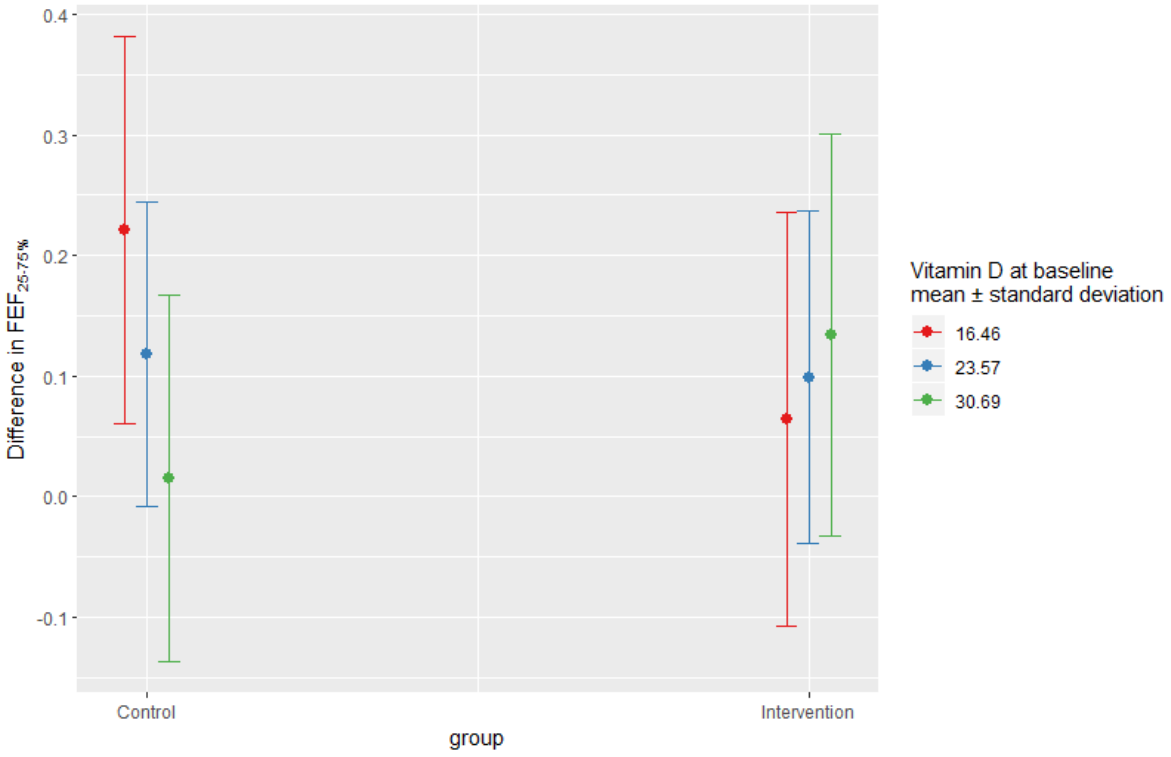

Figure S6. The effect of the dietary intervention on change in FEF $_{25\text{-}75\%}$ by baseline serum 25(OH)D level.

Evidence of an interactive effect was observed between baseline serum 25(OH)D level and the intervention on change in FEF $_{25\text{-}75\%}$ (p = 0.041). Increasing baseline serum 25(OH)D level was associated with a steady increase in FEF $_{25\text{-}75\%}$ in the intervention group, and substantial decrease in the control group.

Additional statistics: Medication use at six months based on plasma 25(OH) D level at baseline

As aforementioned in the unpublished manuscript by the end of the six-month trial vitamin D levels increased by 21% in the intervention group. With respect to medication use, there was a significant decrease in medication use for the intervention group with insufficient vitamin D levels baseline (25(OH)D < 25 ng/ml) from 91% (21/23) at baseline to 42% (5/12) at six months (p = 0.012 McNemar's Test). No change in medication use was observed in the control group for both vitamin D categories at baseline [p = 0.25, 0.38 respectively]. It can be speculated that regular fatty fish intake in combination with the Mediterranean diet improved overall health and lung function as evident from the decrease in need for asthma medication.

6.7 Urinary organic acids as biomarkers in the assessment of pulmonary function in children with asthma

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Unique to asthma research is the application of metabolomics. Exploratory to this thesis was the use of Gas-Chromatrography and Mass-Spectroscopy to determine urinary organic acids profile of asthmatic children. In section 6.7, the outcome of this subanalysis is discussed in the recent publication *Papamichael et al, Urinary organic acids as biomarkers in the assessment of pulmonary function in children with asthma. J Nutrition Research 2019; 61: 31-40. https://doi.org/10.1016/j.nutres.2018.10.004*

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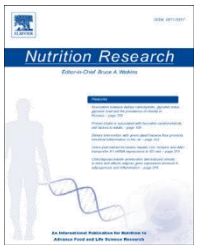

Urinary organic acids as biomarkers in the assessment of pulmonary function in children with asthma

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ABSTRACT

Childhood asthma prevalence continues to rise despite advancements in prevention and medical management strategies. The purpose of this study was to investigate correlations between urinary organic acids and pulmonary diagnostic tests, asthma control in Greek asthmatic children. We hypothesized that urinary organic acids are positively associated with poor pulmonary function in children with asthma. Seventy-two children, 5 to 12 years old with asthma were recruited from a pediatric asthma clinic in Athens, Greece. Pulmonary function was assessed using spirometry and exhaled nitric oxide analysis. Asthma control was measured qualitatively using the Asthma Control Questionnaire. Targeted metabolomic analysis of 34 urinary organic acids in children was conducted by gas chromatography–mass spectrometry. A statistically significant difference between girls and boys was found for asthma control score ($P = .02$), lactic acid ($P = .03$), but not for any other organic acids ($P > .05$). Statistically significant correlations were found between lactic acid and Forced Expiratory Volume in 1 second (FEV_1) ($P = .02$), Forced Vital Capacity (FVC) ($P = .03$); 4- hydroxyphenylacetic acid and FEV_1 ($P = .01$), FVC ($P = .01$); 5-hydroxyindoleacetic acid and FEV_1/FVC ($P = .03$), eNO ($P = .05$); glycolic acid with Peak Expiratory Flow (PEF) ($P = .03$); and malic acid with asthma control ($P = .02$). In conclusion, metabolomics was used to determine correlations between urinary organic acids and conventional pulmonary diagnostic tests in Greek asthmatic children. Metabolomics could be a promising approach for asthma research and in detection of novel biomarkers for asthma monitoring and therapeutic targets for childhood asthma. This study contributes towards a better understanding of the biochemical pathways involved in asthma.

Abbreviations: FEV_1, Forced Expiratory Volume in 1 second; FVC, Forced Vital Capacity ;FEV_1/FVC, ratio of Forced Expiratory Volume in 1 second and Forced Vital Capacity; PEF, Peak Expiratory Flow; $FEF_{25-75\%}$, Forced Expiratory Flow at 25-75% of the pulmonary volume; eNO, exhaled Nitric Oxide analysis.

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1. Introduction

Asthma is the most common chronic respiratory disease in children worldwide and has become a global public health concern [1]. It affects from1–20% of the child population and is highest in English speaking countries [2]. Asthma causes considerable burden for the individual physically, mentally, socially and is associated with increased morbidity and in some cases early mortality [3]. In addition, asthma is an economic burden for the family and society due to increased medical costs for hospitalization, medical care and drug therapy [4,5]. Asthma onset starts early in life and can carry into adulthood [6].

Asthma is a heterogeneous disease characterized by airway inflammation, bronchial hyperresponsiveness and recurrent episodes of reversible airway obstruction that cause symptoms of wheeze, cough, dyspnoea and tightness in the chest [6]. To date, asthma diagnosis is based on patient's history of symptoms, confirmed expiratory airflow limitation (spirometry) and bronchial reactivity of variable severity (hyperresponsiveness) [1]. However, symptoms and pulmonary function measurements may not always reflect the underlying airway inflammation and are insensitive to small variations in inflammatory status that might be of clinical significance [7]. Even though exhaled nitric oxide analysis (eNO) is a non-invasive diagnostic test for monitoring eosinophilic airway inflammation, previous studies in children have reported a weak association among NO levels, asthma control and in predicting exacerbations [8,9]. Secondly, spirometry is not always available in medical clinics, is expensive, time consuming and requires skilled technicians and is not executable for children younger than 5 years old [10]. Thus, the diagnosis of asthma can be difficult to confirm when no clear objective marker of asthma exists in a clinical setting. From a clinical point of view, research focusing on asthma biomarkers would assist in the diagnosis, management and treatment of this disorder.

It is well-established that asthma is a multifactorial disease caused by genetic and environmental factors [11]. Although the advances in knowledge regarding the etiology and pathophysiology of this complex disease, the pathways involved and role of molecular determinants as mediators in asthma development is not yet fully-understood [12]. There is growing interest in the application of metabolomics in research including the study of respiratory disease [13]. Metabolomics takes into account genetic and biochemical variability among individuals regarding disease susceptibility, nutrient requirements and drug responsiveness [14]. It is useful in the study of disorders with an environmental etiology because it is able to capture the cellular response to past exposure. Therefore, metabolic profiles can assist in the understanding of the biological mechanisms involved in asthma and may be useful in detecting imbalances before the onset of disease and manifestation of symptoms [15].

Metabolomics is the systematic analysis of small molecule metabolites, namely carbohydrates, amino acids, organic acids, nucleotides and lipids, in biological specimens including urine, blood, saliva, tissues and breath exhalate for the assessment of nutritional status [16]. This innovative technology involves a simple, non-invasive procedure that has been used to identify new biomarkers and novel biochemical pathways for many complex diseases including cardiovascular disease [17], diabetes [18], obesity [19], inflammatory disease[20], psoriasis[21], chronic obstructive pulmonary disease [22],cystic fibrosis [22], polycystic ovary syndrome [23] and cancer [24]. The examination of organic acids is an accurate test which assesses compounds (the end-products of gene expression) found in urine that are produced during daily metabolism.

Organic acid testing can indicate the functional need for specific nutrients, imbalances, enzymatic insufficiency and dysfunction, oxidative damage, detoxification, methylation factors and microbiome status [25]. Metabolomic profiling is of clinical importance since the results of the organic acid analysis allow greater insight into a patient's biochemical individuality, leading to more targeted therapeutic recommendations [26]. To date a limited number of studies have focused on the generation of a metabolic profile in pediatric asthma [27,28].

The objective of this study is to apply metabolomics to investigate possible relationships between urinary organic acids and pulmonary diagnostic tests, asthma control in a population of Greek asthmatic children that might be useful as a diagnostic tool in the management of childhood asthma. We hypothesized that urinary organic acids are positively associated with poor pulmonary function in children with asthma. Dietary profiles are beyond the scope of this manuscript and will be published at a later date.

2. Methods and materials

2.1. Participants

A total of seventy-two(72) children aged between 5–12 years old [54% (39) boys, 46% (33) girls); mean age 7.97 ± 2.20 years] were recruited from a pediatric asthma clinic in the greater city of Athens, Greece, from1st November to 31st December 2016. The present study was conducted according to the guidelines in the Declaration of Helsinki, and all procedures involving human subjects were approved by the La Trobe University Human Ethics Committee, Australia (approval number ID: HEC 16–035) for conduct in Athens, Greece.

All participants were interviewed and evaluated by trained personnel (physicians, pulmonary function test technicians, nurses, and dietician) during regularly scheduled clinic visits. Parents were informed about the purpose and methods of the study and written informed consent was obtained. Participation of patients was voluntary.Children were recruited into this study by the physician if they were 5–12 years of age and had physician-diagnosed ‘mild’ asthma. Mild asthma was defined according to the GINA criteria [1]. Inclusion criteria included children with mild asthma who had no episodes or need for hospitalization, no day or night symptoms, a need for reliever medication up to two times per week and no restriction in daily activities and had a spirometry measure of FEV_1 greater than 80% predicted [5]. The majority of patients (83%) were taking asthma medication during the past month as part of their usual daily therapy.

2.2. Assessment tools

2.2.1. Anthropometry

Anthropometry was conducted according to standard protocol [29]. Children's height was measured in the standard

Frankfort horizontal plane to the nearest 0.1 cm using a SECA stadiometer after shoes had been removed. Body weight was measured to the nearest 0.1 kg on calibrated electronic scales (SECA, Hanover, MD) without shoes and heavy clothing. Three measurements were taken and the mean value was recorded and used for analytical purposes. Body mass index [BMI = weight (kg) divided by height squared (m2)] was calculated in order to classify weight either as normal, overweight or obese using the cut-off points for BMI for overweight and obesity by sex, as proposed by the International Obesity Task Force, (IOTF) for children aged 2–18 years [30]. Children's growth rate was assessed using the Hellenic Pediatric Growth Charts [31].

2.2.2. Evaluation of pulmonary function

2.2.2.1. *Spirometry.* Pulmonary function was assessed using a portable spirometer MIR Spirobank II (MIR Inc. USA), according to the European Respiratory Society protocol [32]. Patients were requested to abstain from taking their medication within 4 hours of the pulmonary function tests or 8 hours for long-acting bronchodilators. Spirometry was conducted in the standing position. A nose clip was placed on the patient's nose in order to prevent nasal inhalation. A new mouthpiece was given for each patient. The mouthpiece was inserted into the patient's mouth, lips were sealed around the mouth-piece. The patient was instructed to inhale to total lung capacity (TLC) and immediately air was exhaled as hard and as fast as possible without pause, and then a big breathe was inhaled again back to TLC. This process was repeated three times and the highest values of FEV_1, FVC, FEV_1/FVC, PEF, $FEF_{25–75\%}$ were used in the analyses [32]. Normal pulmonary function was indicated by FEV_1, FVC, PEF and $FEF_{25–75\%}$ values greater or equal to 80% predicted, and bronchial obstruction by an FEV_1/FVC ratio less than 90% [1].

2.2.2.2. *Exhaled nitric oxide analysis.* Bronchial inflammation was investigated using a portable exhaled Nitric Oxide analyzer (eNO) (NO breath, Benfont Inc. UK). Exhaled Nitric Oxide analysis was performed in accordance to ATS/ERS recommendations [33]. Patients performed the test in an upright position. The mouthpiece was inserted into the patient's mouth, lips were sealed around the mouth-piece and a big breathe was inhaled over 2 to 3 seconds to total lung capacity and then exhaled immediately as hard as possible into the meter for a period of 10 seconds which allowed a reasonable plateau to be achieved. Three measurements were taken at 30 second intervals that agreed within 10% and the final eNO which is the mean value was recorded. Exhaled nitric oxide values less than 20 ppb were an indication of no lung inflammation, good asthma control, and good compliance to medication therapy [34].

2.2.3. *Asthma control*

The Asthma Control Questionnaire (ACQ) was used to evaluate asthma control in children [35]. For children younger than 12 years old, parents assisted children in completing the questionnaire. This questionnaire consists of 7 items that assess the presence of symptoms, medication use and pulmonary function as % FEV_1 predicted during the past 7 days. Responses for the first 6 questions were based on a 7-point scale (0 = totally controlled to 6 = extremely poorly controlled). The final score was the mean of the responses to the 7 questions. A score <0.75, indicates no manifestation of symptoms, no activity limitations, no rescue bronchodilator use and an FEV_1 > 95%, and is considered as having 'totally-controlled' asthma. On the other hand, a score of ≥1.5 indicates 'extremely poorly controlled' asthma [36].

2.2.4. *Biochemical tests*

All patients were in good health and consumed their usual diet 'ad libitum'. Spot clean-catch urine samples were collected at a metabolomics clinic in Athens, Greece. Urine samples were collected and stored at −80 °C until analysis which was undertaken at the metabolomics clinic in Athens, Greece. Gas chromatography–mass spectroscopy (GC–MS) as previously described by Tanaka et al. [37] was used to identify 34 unique organic acids. Specifically, organic acids were extracted from urine by liquid–liquid extraction after mixing the specimen with an internal standard solution. The oxidation of 2-keto acids with hydroxylamine hydrochloride was performed. Organic acids were converted to their corresponding trimethylsilyl (TMS) ethers with N,O,-bis-(trimethylsilyl) trifluoroacetamide (BSTFA) containing 1% trimethylchlorosilane (TMCS) (both from Supelco, Bellefonte, PA, USA). The derivatization imparts volatility to the organic acids, which is required for GC–MS analysis. The organic acids- TMS ethers were separated in a capillary gas chromatography column containing an immobilized, non-polar stationary phase. Following chromatographic separation, organic acids were routinely detected by electron impact mass spectrometry performed in the scan mode with a mass range between 50 and 550 m/z. Identification was achieved by comparison to published spectra compounds and quantification by comparison to the calibration of pure standard compounds in ratio to an internal standard. To account for hydration status of patients, metabolites were referenced to creatinine.

2.3. Statistical analyses

Sample size was based on spirometry measure, FEV_1 and was determined using G Power Analysis (Version 3.1) [38]. A sample of at least 64 patients was adequate to provide a power of 90%, to evaluate two-sided hypotheses regarding statistically differences in FEV_1 between groups at a probability level less than 0.05 and allowed for a 20% drop out rate. A variation in FEV_1 of 10–12% was considered to be clinically significant in children [1].

SPSS (Version 20)(IBM Inc. USA) was used for all statistical compilations and all statistical analyses were completed under the supervision of co-author (EB) at La Trobe University in Australia. All variables were assessed for normality using Shapiro-Wilks test. Differences in organic acids were analyzed using t-test for normally distributed variables and Mann–Whitney test was applied otherwise. Normally distributed variables are presented as means and standard deviation or as median 25th, 75th percentile in the case of non-normality. Spearman's rho coefficient was applied to estimate bivariate associations between urine organic acids. P value was considered to be significant at the 5% level.

A list of descriptive statistics for tendency (means, median, min and max) and dispersion (standard deviation, standard error of

means, percentiles) were used for describing the levels of organic acids. The physiological values of organic acids were set using 95% confidence intervals of means. To determine reference values for each metabolite, the 5% percentile lower limit and 95% percentile upper limit was defined. Pulmonary function tests and scores were analyzed as continuous.

3. Results

Seventy two children were recruited in this study and completed spirometry and eNO assessments. We had 90% (65/72) participation rate. Seven children dropped out, 6 due to personal reasons and one due to allergy (Fig. 1). Demographic characteristics of children and asthma control scores are displayed in Table 1 and clinical test outcomes in Table 2. In general, pulmonary function tests showed that children had normal pulmonary function (spirometry measurements >80% predicted), no lung inflammation (eNO <20 ppb) and well-controlled asthma (ACQ score <0.75). Although, a statistical significant difference in ACQ scores was noted between boys and girls (P = .02). No statistical significant discrepancies in age, height, BMI, pulmonary function tests were observed between boys and girls (P > .05).

Regarding biochemical tests, Table 3 shows the basic descriptive parameters of 34 unique urinary organic acids for the sample population. Differences in organic acids concentration between boys and girls are displayed in Table 4. No statistically significant differences in mean organic acid levels were exhibited between boys and girls except for lactic acid (P = .03). Girls displayed a higher mean concentration of lactic acid as compared to boys (6.27 vs 5.82) respectively. Urinary organic acid concentrations in children are presented as percentiles in Supplemental Table S1. Gender differences in percentiles of urinary organic acids are displayed in Table 5. Bivariate correlations between organic acids are shown in Supplemental Table S2. Eighty (80) unique statistically significant correlations were detected.

Table 1 - Demographic characteristics of study population

Variable		Sex		
	Total	Male	Female	P[a]
Sex		54% (39)	46% (33)	
Age (years)	7.97 ± 2.20	8.00 ± 2.00	8.00 ± 2.00	.98
Height (cm)	133.43 ± 13.31	132.82 ± 12.54	134.15 ± 14.33	.86
BMI (kg/m^2)	18.34 ± 3.74	18.42 ± 3.89	18.24 ± 3.62	.71
ACQ score	0.37 ± 0.36	0.47 ± 0.41	0.26 ± 0.25	.02

Total sample n=72; Males n=39; Females n=33.
Actual values are shown as means ± standard deviation.
Abbreviations: ACQ- Asthma Control Questionnaire; BMI-Body Mass Index
[a] P-value was calculated using Mann-Whitney Test.

When investigating correlations between pulmonary function tests and organic acids statistically significant correlations were observed between lactic acid and FVC (P = .03, r = -0.27), lactic acid and FEV_1 (P = .02, r = −0.29), 4-hydroxyphenylacetic acid and FVC (P = .01, r = − 0.32), 4-hydroxyphenylacetic acid and FEV_1 (P = .01, r = −0.32), 5-hydroxy-indoleacetic acid and FEV_1/FVC (P = .03, r = 0.27), 5-hydroxyindoleacetic acid and eNO (P = .05, r = − 0.24), glycolic acid and PEF (P = .03, r = − 0.27). As for asthma control a statistically significant association was found between malic acid and ACQ score (P = .02, r = 0.62). No significant correlations were observed for other metabolites and pulmonary function tests or asthma control scores (P > .05).

4. Discussion

In the present study we used GC–MS metabolomics to examine possible associations between urinary organic acid

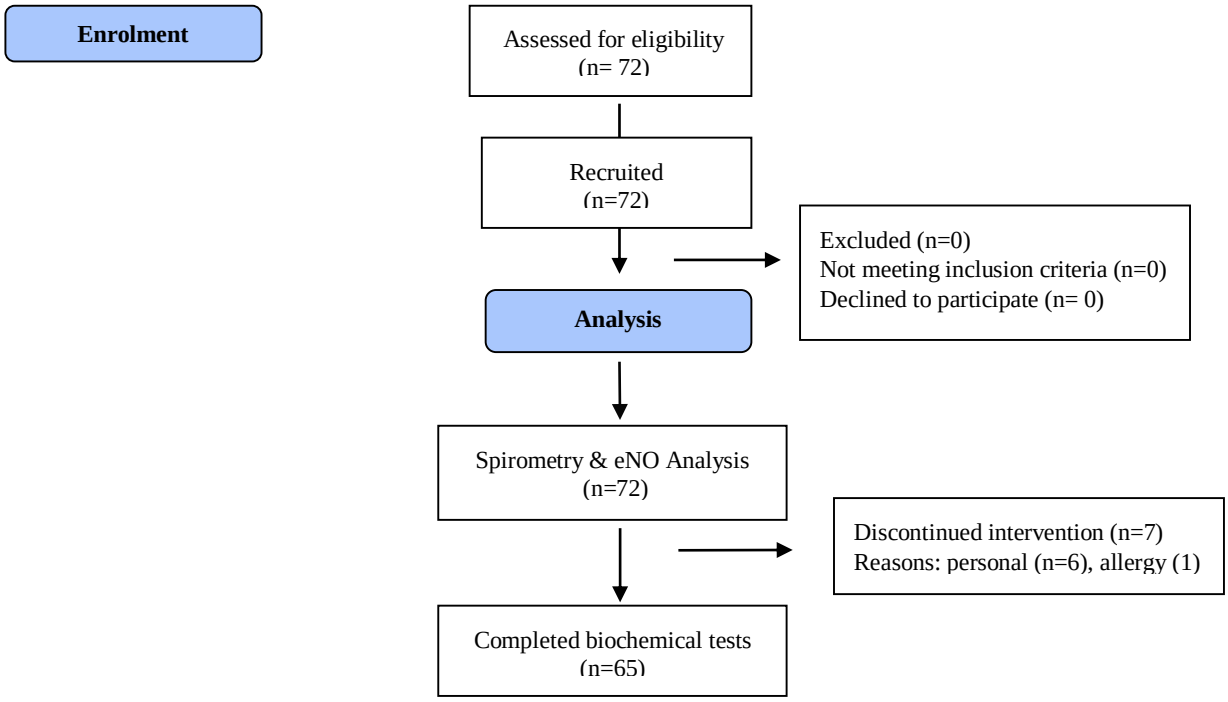

Fig. 1 - Selection of participants in the study

Table 2 - Pulmonary function tests for total sample and per sex

	Total	Sex		P[a]
		Male	Female	
% predicted FVC (L)*	95.11 ± 9.86	95.95 ± 9.92	94.12 ± 9.87	0.44
%predicted FEV_1 (L)	97.67 ± 10.16	98.18 ± 10.21	97.06 ± 10.23	0.64
% predicted FEV_1/FVC	102.01 ± 6.40	101.69 ± 6.69	102.39 ± 6.13	0.65
% predicted PEF (L/s)	94.65 ± 19.11	95.03 ± 19.90	94.21 ± 18.43	0.94
% predicted $FEF_{25-75\%}$ (L/s)	102.06 ± 20.88	103.31 ± 21.25	100.58 ± 20.65	0.58
eNO (ppb)	10; (5,18)[c]	9;(4,18)	10; (5,21)	0.46[b]

Total sample n=72; Males n=39; Females n=33.
Actual values are shown as means ± standard deviation.
Abbreviations: FEV_1- Forced Expiratory Volume in 1 second; FVC- Forced Vital Capacity; FEV_1/FVC- ratio of Forced Expiratory Volume in 1 second and Forced Vital Capacity, PEF- Peak Expiratory Flow, $FEF_{25-75\%}$ -Forced Expiratory Flow at 25-75% of the pulmonary volume, eNO- exhaled Nitric Oxide Analysis
*Spirometry values pre-bronchodilator administration.
[a] p-value calculated using t-test
[b] p-value calculated using Mann-Whitney test
[c] eNO values presented as median, 25th and 75th percentiles.

Table 3 - Description of specific 34 metabolites for total sample of children

Urine OA metabolites (mmol/mol Crea)		Min	Max	Estimated 95%CI
Citric	114.44 ± 75.07[a]	24.10	343.60	95.69-133.19*
Aconitic	44.14 ± 19.31	15.10	143.20	39.31-48.96
Isocitric	7.07 ± 2.22	2.10	12.40	6.52-7.627
2 -Ketoglutaric	25.34 ± 14.11	3.20	96.00	21.81-28.86
Succinic	4.85 ± 6.08	1.00	41.30	3.25-6.45
Fumaric	1.17 ± 0.15	1.00	1.30	0.79-1.55
Malic	1.21 ± 0.43	0.90	2.30	0.96-1.46
3-Hydoxy 3-methylglutaric	5.17 ± 4.88	1.00	30.10	3.96-6.38
Lactic	6.04 ± 5.18	1.80	39.60	4.76-7.32
Pyruvate	7.85 ± 3.05	1.40	15.00	7.09-8.61
3-Hydroxybutyric	30.68 ± 73.70	1.10	274.70	-10.13-71.49
Pyroglutamic	16.20 ± 8.29	4.90	56.50	14.10-18.30
2- Ketoisocaproic	1.30	1.30	1.30	-
2-Keto 3-methylvaleric	1.77 ± 0.49	1.20	2.10	0.54-2.99
3-Hydroxyisovaleric	20.42 ± 14.23	1.40	77.90	16.83-24.00
Methylmalonic	1.50 ± 0.60	1.00	3.80	1.26-1.75
Homovanillic	3.39 ± 1.55	1.00	7.50	2.99-3.78
5-hydroxy-indole-acetic	3.38 ± 2.40	1.00	15.70	2.77-3.98
Vanillilmandelic	2.24 ± 0.75	1.00	4.10	2.05-2.42
4-Hydroxyphenylacetic	14.96 ± 10.23	2.30	71.50	12.40-17.51
Orotic	1.10	1.10	1.10	-
Glutaric	1.23 ± 0.30	0.90	1.60	0.75-1.70
2-Hydroxyglutaric	3.19 ± 1.68	1.00	13.00	2.77-3.62
Glycolic	32.39 ± 15.59	10.30	74.50	28.49-36.28
Oxalic	6.65 ± 5.61	1.40	39.80	5.18-8.13
Glyceric	2.50 ± 1.25	1.10	7.10	2.13-2.87
2-Hydroxyisobutyric	5.67 ± 2.62	1.50	16.00	5.02-6.33
2-Hydroxybutyric	2.65 ± 1.34	1.70	3.60	-9.42-14.72
Ethylmalonic	2.09 ± 1.22	1.00	6.40	1.77-2.40
Methylsuccinic	1.53 ± 0.76	0.90	4.10	1.28-1.78
Adipic	2.04 ± 1.55	1.00	7.00	1.34-2.75
Suberic	1.94 ± 0.96	0.70	3.70	1.43-2.45
Sebasic	1.03 ± 0.54	0.60	1.80	0.16-1.89
4-Hydroxyphenylpyruvic	1.91 ± 1.97	1.00	7.50	0.5-3.23

Total sample n=65; [a] Actual values are shown as means ± standard deviation;
*Estimated 95%CI of means using 1-sample t-test; -: Negligible values <0.00

Table 4 - Description of 34 urinary metabolites according to sex

Urine OA metabolites (mmol/mol Crea)	Sex						
	Male			Female			
		Min	Max		Min	Max	P-value[b]
Citric	97.90 ± 63.91[a]	26.80	304.40	132.05 ± 82.81[a]	24.10	343.60	0.16
Aconitic	40.25 ± 13.27	22.30	72.70	48.28 ± 23.68	15.10	143.20	0.37
Isocitric	6.82 ± 1.91	2.80	12.20	7.35 ± 2.51	2.10	12.40	0.96
2 -Ketoglutaric	25.40 ± 16.59	8.40	96.00	25.27 ± 11.15	3.20	56.30	0.52
Succinic	3.52 ± 2.21	1.10	9.50	6.17 ± 8.17	1.00	41.30	0.76
Fumaric	-	-	-	1.17 ± 0.15	1.00	1.30	0.22
Malic	1.04 ± 0.13	0.90	1.30	1.39 ± 0.56	1.00	2.30	0.63
3-Hydoxy 3-methylglutaric	5.02 ± 4.24	1.00	17.90	5.33 ± 5.52	1.00	30.10	0.46
Lactic	5.82 ± 6.48	1.80	39.60	6.27 ± 3.45	2.40	17.90	0.03
Pyruvate	7.67 ± 2.83	3.30	14.90	8.03 ± 3.30	1.40	15.00	0.26
3-Hydroxybutyric	3.99 ± 35.36	1.10	101.50	49.76 ± 102.07	1.10	274.70	0.81
Pyroglutamic	16.57 ± 9.49	5.20	56.50	15.84 ± 7.02	4.90	32.00	0.30
2- Ketoisocaproic	-	-	-	1.30	1.30	1.30	-
2-Keto 3-methylvaleric	1.60 ± 0.57	1.20	2.00	2.10	2.10	2.10	0.22
3-Hydroxyisovaleric	17.95 ± 11.57	3.10	62.30	23.13 ± 16.45	1.40	77.90	0.80
Methylmalonic	1.30 ± 0.22	1.10	1.70	1.71 ± 0.79	1.00	3.80	0.41
Homovanillic	3.26 ± 1.39	1.30	6.40	3.52 ± 1.73	1.00	7.50	0.74
5-Hydroxyindoleacetic	3.45 ± 2.76	1.10	15.70	3.31 ± 2.03	1.00	7.80	0.08
Vanillilmandelic	2.20 ± 0.61	1.10	3.60	2.28 ± 0.88	1.00	4.10	0.51
4-Hydroxyphenylacetic	14.75 ± 12.26	2.30	71.50	15.17 ± 7.68	3.50	39.40	0.64
Orotic	-	-	-	1.10	1.10	1.10	-
Glutaric	0.90	0.90	0.90	1.33 ± 0.25	1.10	1.60	0.66
2-Hydroxyglutaric	3.35± 2.03	1.00	13.00	3.02 ± 1.20	1.10	5.60	0.96
Glycolic	29.48 ± 12.38	13.30	62.20	35.47 ± 18.11	10.30	74.50	0.54
Oxalic	5.67 ± 2.92	1.50	12.20	7.70 ± 7.42	1.40	39.80	0.56
Glyceric	2.43 ± 1.44	1.20	7.10	2.56 ± 1.09	1.10	4.90	1.00
2-Hydroxyisobutyric	5.89 ± 2.80	2.20	16.00	5.45 ± 2.45	1.50	11.20	0.51
2-Hydroxybutyric	1.70	1.70	1.70	3.60	3.60	3.60	0.52
Ethylmalonic	2.23 ± 1.53	1.00	6.40	1.94 ± 0.80	1.00	3.60	0.82
Methylsuccinic	1.59 ± 0.82	1.00	4.10	1.48 ± 0.71	0.90	4.00	0.98
Adipic	1.70 ± 0.80	1.00	3.40	2.30 ± 1.93	1.00	7.00	0.36
Suberic	1.96 ± 0.53	1.40	2.70	1.94 ± 1.13	0.70	3.70	0.87
Sebasic	0.65 ± 0.07	0.60	0.70	1.40 ± 0.57	1.00	1.80	0.18
4-Hydroxyphenylpyruvic	2.58 ± 2.78	1.00	7.50	1.35 ± 0.86	1.00	3.10	0.16

Males n=39; Females n=33.

[a] Actual values are shown as means ± standard deviation

[b] p-values were calculated using Mann-Whitney test; -: Negligible values <0.00

profile and pulmonary function in a sample of Greek asthmatic children that are not identifiable by conventional pulmonary diagnostic tests and patient symptoms. We hypothesized that urinary organic acids are positively associated with poor pulmonary function in children with asthma [39]. Based on our data we accept that these associations may be plausible. Data analysis revealed that in general, children had normal lung function and well-controlled asthma. However, a significant difference in asthma control was found between girls and boys. Girls showed slightly better asthma control than boys. This is consistent with the published literature that reports a higher prevalence and greater severity of asthma in boys pre-adolescence, after which it is reversed [40]. In our study concentrations of quantified organic acids in Greek children were different compared to recent studies undertaken in other pediatric populations both asthmatic [27, 28,41] and healthy non-asthmatic [42, 43]. The most important finding of this study was that GC-MS analysis identified 34 unique urinary organic acids as well as negative associations specifically between lactic acid, 4-hydroxyphenylacetic acid, 5-hydroxyindoleacetic acid, glycolic acid and spirometry parameters FEV_1, FVC, PEF, eNO, whereas positive associations between FEV_1/FVC, asthma control and 5-hydroxyindoleacetic acid and malic acid respectively. To our knowledge this is the first study to report associations between these metabolites and pulmonary function tests in Greek asthmatic children. A feasible explanation for these observations might be airway dysfunction, increased work of breathing, hypoxemia due to poor oxygenation, oxidative stress and inadequate energy metabolism along with alterations in gut microbiota.

Lactic acid, an indicator of lactic acidosis is produced during cellular respiration under conditions of hypoxia or thiamine (Vitamin B1) deficiency [44]. Therefore, an inverse association between lactic acid and spirometry would indicate an inflam-matory state which is a characteristic of asthma pathogenesis as is expected. With regards to the negative correlation observed between 4-hydroxyphenylacetic acid and FEV_1, research has demonstrated

Table 5- Percentiles of urinary organic acid levels according to sex.

Urine OAs (mmol/mol Crea)	**Sex**									
	Male					**Female**				
	Percentiles									
	05th	**25th**	**50th**	**75th**	**95th**	**5th**	**25th**	**50th**	**75th**	**95th**
Citric	31.20	50.70	75.70	134.50	220.20	36.30	72.30	93.80	189.20	302.80
Aconitic	22.50	29.20	36.20	49.70	68.00	15.40	37.30	42.60	61.30	78.50
Isocitric	4.40	5.50	6.40	8.00	10.00	3.70	5.50	7.50	9.40	12.00
2 -Ketoglutaric	8.90	15.80	22.60	30.40	53.00	8.00	20.00	24.10	32.50	40.00
Succinic	1.10	1.80	3.00	4.50	7.50	1.10	1.60	3.50	6.50	19.30
Fumaric	-	-	-	-	-	1.00	1.00	1.20	1.30	1.30
Malic	.90	1.00	1.00	1.10	1.30	1.00	1.00	1.10	2.10	2.30
3-Hydoxy 3-methylglutaric	1.00	2.30	2.90	6.80	14.30	1.10	2.20	3.75	5.70	15.30
Lactic	1.90	3.10	4.50	5.80	13.60	2.40	3.80	5.35	7.95	13.00
Pyruvate	3.60	5.80	6.90	9.00	13.70	2.00	5.35	8.70	10.50	13.80
3-Hydroxybutyric	1.10	1.20	1.45	2.00	101.50	1.10	1.30	1.70	66.10	274.70
Pyroglutamic	5.70	10.70	15.80	20.50	27.10	4.90	9.40	17.10	21.20	28.20
2- Ketoisocaproic	-	-	-	-	-	1.30	1.30	1.30	1.30	1.30
2-Keto 3-methylvaleric	1.20	1.20	1.60	2.00	2.00	2.10	2.10	2.10	2.10	2.10
3-Hydroxyisovaleric	3.20	10.90	17.70	21.00	36.80	4.40	13.00	17.90	34.10	50.30
Methylmalonic	1.10	1.10	1.20	1.50	1.70	1.00	1.20	1.40	1.80	3.80
Homovanillic	1.30	2.50	3.00	3.90	6.00	1.10	2.20	3.00	5.10	6.60
5-Hydroxyindoleacetic	1.10	1.90	3.00	3.80	8.60	1.00	1.70	3.00	4.20	7.40
Vanillilmandelic	1.20	1.80	2.10	2.60	3.50	1.00	1.60	2.20	2.70	4.00
4-Hydroxyphenylacetic	3.20	9.40	11.60	16.60	34.60	5.60	9.80	15.00	17.80	29.70
Orotic	-	-	-	-	-	1.10	1.10	1.10	1.10	1.10
Glutaric	0.90	0.90	0.90	0.90	0.90	1.10	1.10	1.30	1.60	1.60
2-Hydroxyglutaric	1.60	2.30	3.00	3.80	5.10	1.40	2.10	2.90	3.80	5.30
Glycolic	13.30	21.90	27.20	32.80	52.40	10.60	19.70	33.70	46.40	72.00
Oxalic	1.70	3.60	5.05	7.60	11.80	2.30	3.95	5.30	9.30	19.90
Glyceric	1.30	1.50	1.90	2.60	5.80	1.10	1.70	2.30	3.40	4.30
2-Hydroxyisobutyric	2.80	4.20	5.10	7.10	11.00	1.80	3.50	5.10	7.10	10.10
2-Hydroxybutyric	1.70	1.70	1.70	1.70	1.70	3.60	3.60	3.60	3.60	3.60
Ethylmalonic	1.00	1.10	1.50	2.60	5.60	1.00	1.20	1.95	2.40	3.50
Methylsuccinic	1.00	1.10	1.25	1.70	4.10	.90	1.00	1.30	1.70	4.00
Adipic	1.00	1.00	1.50	2.20	3.40	1.00	1.15	1.65	2.10	7.00
Suberic	1.40	1.50	2.10	2.10	2.70	.70	1.00	1.60	3.20	3.70
Sebasic	0.60	0.60	0.65	0.70	0.70	1.00	1.00	1.40	1.80	1.80
4-Hydroxyphenylpyruvic	1.00	1.00	1.40	2.00	7.50	1.00	1.00	1.00	1.00	3.10

Males n=39; Females n=33; -: Negligible values<0.00

the ability of 4-hydroxyphenylacetic acid to attenuate pulmonary inflammation and edema by suppressing hypoxia in rats [45]. Additionally, increased urinary excretion of 4 hydroxyphenylacetate is a marker of bacterial overgrowth [46]. Anaerobic enteric bacteria that possess L-amino acid decarboxylase hydrolyse amino acids from dietary protein to tyrosine that is degraded in the process to tyramine. Tyramine is subsequently deaminated and oxidized to 4-hydroxyphenylacetic acid which is excreted in urine [46]. Hence dysbiosis could lead to airway inflammation in airways which possibly explains the negative correlation seen between spirometry parameters (FEV_1, FVC) and 4-hydroxyphenylacetate. This is significant because airway inflammation results in poor asthma control.

We also found hydroxyindoleacetate to be positively correlated with spirometry measure FEV_1/FVC and negatively with bronchial inflammatory marker (eNO). Hydroxyindoleacetate is the main end product of serotonin (5-HT) metabolism. Studies have confirmed that serotonin is a mediator involved in allergy development that is generated from tryptophan [47]. Serotonin plays an important role in signaling immune response by modulating chemotaxis, leukocyte activation, proliferation, cytokine secretion, anergy, and apoptosis [47]. According to a recent study undertaken in mice conducted by Nau et al. (2015), activation of serotonin receptors 5-hydroxytryptamine (5-HT) in airways triggered T-cells and innate immune cells in the lungs and blocked the activation and expression of pro-inflammatory mediators including chemokines, nitric oxide synthase, adhesion molecules, cytokines, and translocation of NF-kB, consequently preventing the development of hyperresponsiveness, mucus overproduction, lung eosinophilia, airway inflammation alleviating asthma symptoms [48]. Therefore, the anti-inflammatory properties of serotonin might explain the associations observed between hydroxyindoleacetate and normal spirometry measure-ments for FEV_1/FVC and absence of bronchial inflammation as indicated by eNO.

Regarding the negative correlation found between glycolic acid and spirometry measure (PEF), increased glycolic acid as well as 3,4-dihydroxybutyric acid, glycine, cis-aconitic acid; phenylalanine, tyrosine, p-hydroxyphenylacetic acid, and homovanillic acid are mediators involved in the tyrosine pathway [49]. Tyrosine is a precursor of neurotransmitter catecholamines that are released under conditions of stress (fight or flight response) and are involved in the regulation of the immune system [50]. So in our study, since children were in the healthy state, free from asthma attacks, it would be expected for glycolic acid levels to be inversely related to PEF values which were normal (greater than 80% predicted) [10] as was shown.

As for the positive association observed between malic acid and asthma control scores, this might be explained by the presence of reactive oxygen radicals in airways [51]. Reactive oxidative species are products of normal cellular metabolism and under physiological conditions, participate in maintenance of cellular redox homeostasis. The lung is equipped with enzymatic and non-enzymatic antioxidant systems. Over-production of reactive oxidative species and inadequate antioxidant levels are a hallmark of airway oxidative stress that eventually lead to lung damage and airway inflammation due to damage of lipids, DNA and proteins [52,53]. The glyoxylate and dicarboxylate cycle uses acetate to generate malic acid. Modifications in glyoxylate and dicarboxylate metabolism have been related to mitochondrial dysfunction that would contribute to decreased ability to detoxify reactive oxygen species (ROS) [51].Hence, in our study, the presence of urinary malic acid might be a marker for chronic inflammation and oxidative stress in airways that contribute to asthma exacerbations and consequently poor asthma control [53].

This is the first study to apply metabolomics to investigate organic acids from urine samples of Greek asthmatic children. We showed that metabolomics might be a useful adjunct tool to conventional pulmonary function tests in the management of pediatric asthma. A strength of this study is the application of the gold standard for the evaluation of urinary organic acids using GC–MS which is considered to be a robust metabolomics tool in metabolite identification and quantification due to high sensitivity, specificity as well as good dynamic range [54]. Another strong point is the use of urine samples in children as opposed to blood, nasal lavage fluid, exhaled breath condensates, induced sputum or bronchial lavage fluid [55]. This method is inexpensive, non-invasive, applicable in clinical-setting, easily collected in children of all ages, rapid, abundant for repeated collection and provides a wide spectra of metabolites both exogenous and endogenous [56]. Regarding study design, our population was homogeneous with respect to age, asthma status and medication use which improves consistency of the findings.

Possible limitations in our study might be that we could not compare asthmatic children to healthy non-asthmatic children or explore metabolic changes in various asthma phenotypes due to paucity of recent studies and reference values undertaken in both asthmatic [27,28,41] and non-asthmatic children [42,43]. Another drawback was that we did not assess gut microflora in children. This warrants further investigation. Nevertheless, our study provides preliminary insights into the possible application of metabolomics in studies of asthma in children.

As all procedures metabolomics has its advantages and disadvantages. The metabolic profile of an organism is dynamic, hence metabolites reflect a snapshot of the current cellular activity [55]. Also small variations in metabolites may be undetected [55]. Experimental design, ‘targeted’ as opposed to ‘untargeted’ might be a source of heterogeneity [14] because important metabolites could be missed. On the other hand, the ‘targeted’ approach takes advantage of the established biochemical pathways, known enzymes kinetics and end products in the study of disease [55]. Targeted experiments are appropriate for the study of asthma [27,28] and have been employed in numerous metabolomics studies analyzing human urine that have led to the identification and quantification of hundreds of urine metabolites [56-58].

In conclusion, metabolomics was used to determine correlations between urinary organic acids and conventional pulmonary diagnostic tests in Greek asthmatic children. Possible biological interpretations were given for the observations. Metabolomics could be a promising approach for asthma research and in detection of novel biomarkers for asthma monitoring and therapeutic targets for childhood asthma. This study adds to the existing metabolomics database on urinary organic acids for asthmatic children and contributes towards a better understanding of the biochemical pathways involved in asthma.

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In asthmatic children there is limited data on the correlation between diagnostic tests, quality of life and urinary organic acids. Given that at baseline there is homogeneity among participants, Spearman's correlation rank test was applied to investigate whether there is an association between urinary organic acids and pulmonary diagnostic tests, asthma control and quality of life scores used to evaluate burden in asthmatic children. The additional correlation analysis is summarized in Table 24 and extends to the findings mentioned in the published manuscript above.

Table 24 Baseline correlations between urinary organic acids and pulmonary function tests, pulse oximetry, asthma control and quality of life parameters

Bivariate correlations at baseline		
OA vs Pulmonary function parameters/scores	*P*-value	Spearman's rho (r)
Lactic vs FVC	0.03	-0.27
Lactic vs FEV_1	0.02	-0.28
4-hydroxyphenylacetic vs FVC	0.01	-0.32
4-hydroxyphenylacetic vs FEV_1	0.01	-0.32
5-hydroxyindoleacetic vs FEV_1/ FVC	0.03	0.28
5-hydroxyindoleacetic vs FeNO	0.054	-0.24
Glycolic vs PEF	0.03	-0.27
Glyceric vs oxygen saturation	0.03	0.53
Suberic vs oxygen saturation	0.058	-0.87
Malic vs ACQ score	0.01	0.64
Glutaric vs ACQ score	0.05	-0.95
2-hydroxyisobutyric vs ACQ score	0.02	0.29
4-hydroxyphenylpyruvate vs QOL score	0.04	-0.62
5-hydroxyindoleacetic vs QOL score	0.049	-0.25

Key: ACQ-Asthma Control Questionnaire score; QOL-Paediatric Asthma Quality of Life Questionnaire score; FEV_1- Forced Expiratory Volume in 1 second; FVC- Forced Vital Capacity; FEV_1/FVC- ratio of Forced Expiratory Volume and Forced Vital Capacity; PEF- Peak Expiratory Flow; $FEF_{25-75\%}$- Mid Expiratory Flow between 25-75% vital capacity; FeNO- Fractional exhaled Nitric Oxide

6.8 Participant evaluation of intervention study

At the end of the six month trial participant evaluation of this intervention study was reviewed using a questionnaire that consisted of closed ended questions (Appendix 2A 18a-18d). The purpose of the questionnaire was to detect difficulties, barriers in compliance to the dietary intervention along with health effects (benefits/adverse), unanticipated problems in the study design and assessment tools as well as support provided by the research team and physician. Also included were topics of interest for future study. Parents of all participants completed the questionnaire (n = 64). The results of the questionnaire is summarized in Table 25.

Table 25 Participant evaluation of intervention study

PARTICIPANT EVALUATION QUESTIONNAIRE AT SIX MONTHS	
Questions common for both groups	***Response***
QA1. The questionnaire format was easy to understand.	98% (63) of parents agreed that the format of the questionnaire was easy to understand
QA2. Support was provided by the research team at all times.	96.8% (61) of parents agreed that sufficient support was provided by the research team during the study period.
QA3. Have you made any changes in the child's diet during the intervention (apart from the increase in fish intake) as compared to the family's dietary habits at the start of the intervention? If yes, what changes have you made to the child's diet?	In general, 50.8% (32)of parents responded that they have made positive dietary changes to their child's diet with most parents belonging to the intervention group (30) as compared to the control (2). For the intervention group, 82.1% (25) of parents replied that they included fatty fish in their weekly family menu, which was consumed occasionally pre-intervention. Two families (7%) increased salad intake and one parent more vegetables, fruit and traditional Greek Mediterranean meals in children's diet. Conversely, for the control group, one parent admitted adding more fruit and vegetables to children's meals and another any fish and legumes.
QA4. Do you believe that this intervention improved your child's health and asthma status? If yes, indicate how	49.2% (31) of parents believed that the intervention improved their child's health with most of the improvement occurring in the intervention group (21) vs control (10) *Reasons mentioned***:** Parents in the intervention group reported that the dietary intervention resulted with a reduction in respiratory infections and asthma exacerbation during the winter months. Also that children had more endurance and energy to participate in physical activity as

	compared to pre-intervention period. Specifically, one parent noted that there was a reduction in coughing during the day and when playing sport.
QA5. Do you believe that this intervention improved your child's well-being and quality of life (able to engage better in daily activities-sport, play, study etc.)	43.5% (27) of parents believed that the intervention improved their child's quality of life, of which 18 were in the intervention group and 9 in the control. Marked improvements were witnessed in the intervention group. Children had more stamina, were able to participate more in physical activities than previously and had no asthma attacks during physical exertion. Two parents noticed that their children had less cough during the day and when playing sport.
QB1. Would you be interested in taking part in other dietary interventions in the future?	52.5% (32) of parents reported 'Yes', 42.6% (26) 'Maybe' and 4.9% (3) 'No'
QB2. Do you think that we can improve this intervention in anyway? If yes, please suggest how:	72.1% (44) of parents replied 'No' and 27.9% (17) 'Yes' Reasons suggested were: that parents would have liked more feedback from the Pneumologist regarding blood test results. Another parent suggested that information sessions regarding the impact of diet on asthma should be undertaken at the school level.
Questions referring to the intervention group only:	
QA6. How was the child's attitude regarding fish consumption during the 6 month period?	77.4% (24) of parents in the intervention group responded that children had a positive attitude, 19.4% (6) negative and 3.2%(1) indifferent.
QA7. Was the child's attitude a barrier for regular fish consumption?	25.8% (8) of parents in the intervention group responded that the child's attitude pose no barrier to fish consumption during the study period and 70.9% (22) 'sometimes'/or 'occasionally'.

QA8. At any time was there a problem in purchasing fatty fish due to availability or cost?	60% (18) reported 'never', 20% (6) 'occasionally' and 20% (6) 'sometimes'. For those parents reporting sometimes, one parent mentioned that there were times when the supply of fish in markets were scarce due to overconsumption by the public during the 40-day pre-Easter fasting period which is a customary practice in the Greek Orthodox religion.
QA9. Was the preparation of fish meals a problem due to lack of time?	50% (15) of parents reported 'never' and 50% (5) 'occasionally/or 'sometimes'.
QA10. Did you encounter any problems during this intervention? If yes, please indicate	86.7% (26) reported 'no' problems, however, 13.3% (4) 'yes'with the main reason being that by the end of the six month period children got tired of consuming fatty fish.
QA11. Do you feel that this dietary intervention was difficult to apply in your daily family life?	36.7% (11) responded 'never', 33% (10)'sometimes' and 30% (9) 'occasionally' because children either got sick of consuming fatty fish or disliked the taste of fatty fish, due to work commitments and lack of time to prepare fish meals. Nevertheless, parents agreed that they would try to maintain fish intake once per week in family meals.
QA12. Now that this intervention has ended, do you intend to maintain 2 fish meals per week as part of your family menu?	80% (24) of parents in the intervention group responded 'yes' that they intended to maintain 2 fish meals per week as part of the family menu, although 20% (6) replied 'no' because children got tired of consuming 2 fatty fish meals per week and they found that children could tolerate fatty fish once a week and intended to maintain one fish meal/week as part of family meals.
QA13. Would you have preferred to give your child an Omega -3 supplement daily as an alternative to feeding your child fatty fish twice weekly?	66.7% (20) of parents replied 'No', that they did not prefer omega 3 supplements to dietary sources and 26.7% (9) ' I don't know'

In brief, based on parents responses in the questionnaire, the following keypoints were highlighted:

1. The dietary intervention was ‘easily integrated’ into daily family life and affordable
2. Weekly consumption of fish meals were well-tolerated by children
3. No problems were anticipated in assessment tools
4. Sufficient support and motivation was provided by the candidate and researchers that enhanced good compliance and perseverance by parents and participants during the six month period.
5. In parents’ opinion compared to baseline, regular fatty fish intake as part of the Greek Mediterranean diet seems to have improved participants’ health, well-being, asthma status (less coughing at nite, exacerbations during sport), endurance and sport performance. This is consistent with six month assessments showing normal spirometry, well-controlled asthma control, good quality of life and improved FeNO values.
6. Another key point of this intervention is that nutrition education and dietary advice on the Mediterranean diet/ healthy eating practices for children provided by the candidate throughout the six month study duration, resulted in parents’ adoption of healthy eating practices such as increasing intake of fruit, vegetables, fish, legumes and Traditional Greek Mediterranean dishes in family meals. Improvement in diet quality for children in the intervention group was evident from the increase in KIDMED score by 1 unit.

SYNOPSIS

Baseline findings:

In the present study 72 children (54% (39/72) male, 46% (33/72) female, mean age 8 years old) were recruited and 64 completed baseline and six month assessments. At baseline, approximately 40% (28/72) of children were overweight/obese, 50% (34/70) exercised regularly and 64% (41/64) were insufficient in vitamin D(25(OH) D < 25 ng/mL) with girls more insufficient than boys (61% (25/64) vs 39% (16/64) respectively; p = 0.01). Children in both groups had normal lung function, well-controlled asthma and good quality of life. A positive correlation was found between BMI and FVC (p = 0.01) and BMI and FEV_1 (p = 0.03), but not between BMI and FeNO. Sex differences showed a significant linear association between BMI and FEV_1 including FVC in girls (p = 0.03, < 0.001 respectively); and negative between BMI and Peak Expiratory Flow (PEF) in boys, although borderline (p = 0.05). FeNO was lower in the overweight/obese group than in normal weight children (p = 0.03). In the linear regression model, $FEF_{25-75\%}$ was higher by 11.65 units for the overweight/obese group as compared to normal weight children after controlling for age, gender, height, regular physical activity, medication and KIDMED score (p = 0.04).

Dietary habits

Data from conventional dietary assessment tools (FFQs, 24 hr Food recalls and the KIDMED questionnaire) showed that children had poor dietary habits and low adherence to the Mediterranean dietary pattern. Energy intake (EI) exceeded the Estimated Average Requirement (EAR) for children (5-12 years old). In general, all children had low intake of milk products, fruit, vegetables, salads, fish, cereals, rice/pasta, legumes, nuts and high intake of red meat and sweets (> once per week). Children's diets were low in carbohydrate intake (< 50% of daily EI), high in fat (> 35%) and moderate in protein (16% to 20%). As for saturated fat, monounsaturated fatty acids (MUFA) and cholesterol, all exceeded the daily Recommended Nutrient Intakes (RNIs) of 11% and 13% of total EI and 300 mg/day respectively. Sodium consumption was above the recommended intake of 5 g/day indicating overconsumption of sodium-rich foods.

At six-month follow-up

By the end of six months there was a significant increase in fatty fish intake in the intervention group from 17 g/day to 46 g/day ($p < 0.001$) as well as a modest improvement in Mediterranean diet adherence from KIDMED score of 5 at baseline to 6 at six months ($p = 0.01$), due to regular fatty fish consumption. More specifically, fatty fish intake increased plasma DHA concentration in the intervention group by 119.8% and vitamin D levels by approximately 21%. Linear regression model showed that children consuming two meals of fatty fish/week (at least 150 g/meal) in addition to the Mediterranean diet, significantly decreased FeNO by 14 ppb as compared to the control after adjusting for age, gender, regular physical activity and BMI ($p = 0.04$). In contrast, FeNO increased in the control group by ~80% (from 10 ppb at baseline to 18 ppb at six months ($p = 0.11$). With respect to asthma therapy, there was a significant downwards trend (reduction) in medication use for the intervention group as compared to the control (Intervention group $p < 0.001$; control $p = 0.07$). There was no effect of the intervention on spirometry, asthma control or quality of life, possibly due to the short time period. With respect to plasma vitamin D concentrations, there was a marked improvement in FEV_1/FVC and $FEF_{25-75\%}$ by 4.89 units and 12.83 units respectively for the intervention group with sufficient plasma vitamin D status ($25(OH)D \geq 25$ ng/mL) at baseline ($p = 0.01$, $p = 0.01$ respectively). No effect on FeNO was observed for children insufficient/ sufficient in vitamin D.

Exploratory to this thesis was the use of targeted metabolomics to analyse associations between urinary organic acids and pulmonary function tests as well as asthma control scores. Thirty four (34) unique organic acids were detected in asthmatic children. In a simple correlation analysis, eight significant correlations were identified between lactic acid, 4-hydroxyphenylacetic acid, 5-hydroxyindolacetic acid, glycolic acid, and pulmonary function tests (FEV_1, FVC, FEV_1/FVC, PEF); malic, 2-hydroxyisobutyric and asthma control scores. This is clinically significant because pulmonary function tests do not always detect the underlying inflammation associated with asthma.

Evaluation of this clinical trial using the participant evaluation questionnaire and from telephone interviews showed that parents in the intervention group noticed a reduction in exacerbations and asthma symptoms as well as a substantial improvement in

children's health, endurance, ability to participate in sporting activities, general well-being and quality of life.

CHAPTER 7 Discussion

7.1 Efficacy of a Mediterranean diet enriched in fatty fish in childhood asthma

The aim of this PhD clinical study was to investigate the prophylactic potential of a Mediterranean dietary pattern enriched with fatty fish on pulmonary function and asthma symptoms in a RCT of Greek children (5-12 years) with 'mild asthma'. The key finding of this project was that by the end of the six-month study, FeNO an established measure of bronchial inflammation, decreased by about 18% in the intervention group as compared to 78% increase in the control group consuming their habitual diet. Specifically, consumption of two meals of fatty fish weekly (at least 150 g cooked filleted fish per meal) as part of the Greek Mediterranean diet reduced FeNO by 14 units for children in the intervention group compared to the control after controlling for age, sex, regular physical activity and BMI. According to ATS guidelines, a reduction in FeNO by at least 10 units for values lower than 50 ppb as the cut-off point indicates a significant response to anti-inflammatory therapy [(257)]. As noted in the published main result manuscript in Section 6.5, there was a significant reduction in medication use for the intervention arm as compared to the control. It seems that this intervention resulted in reduced symptoms and improved overall quality of health and well-being in asthmatic children. Furthermore, this study suggests the potential for complementarity between pharmacotherapy and dietary choices such as increased intake of omega-3 fatty acids from fatty fish that could lead to drug-sparing effects. A diet rich in omega-3 fatty acids and low in omega-6 fatty acids provides the optimal physiological background in which drugs function optimally [(204)]. Therefore, future studies might need to consider the fatty acid composition of the diet and the issue of concurrent drug use in childhood asthma management.

The beneficial effect of this dietary intervention was also expressed by parents in the patient evaluation of the six-month study. Parents in the intervention group reported that in comparison to baseline, they believed that this dietary intervention resulted in an improvement in children's health and endurance. The majority of children were able to participate more in physical activities without having an asthma attack. Parents reported

that children experienced less exacerbations and respiratory illness during the winter months as compared to pre-intervention. Therefore, the findings of this project support the primary hypothesis that consumption of fatty fish in the context of the Mediterranean diet improves pulmonary function in asthmatic children (5-12 years old) as reflected by the reduction in bronchial inflammation biomarker FeNO. To the candidate's knowledge this is the first RCT study to report a protective effect of 'fatty fish' intake on asthma in school-children and extends the current literature [344].

With respect to the secondary hypotheses whether consumption of a Mediterranean diet enriched with fatty fish improves asthma control and quality of life in asthmatic children, no effect was observed on spirometry, asthma control or on quality of life. The same trend was reported by Rice et al in a case-control study including 287 asthmatic children and 96 controls (9-19 years old) [386]. Adherence to the Mediterranean diet was assessed using the modified Mediterranean diet score based on Psaltopoulou [320], and asthma was diagnosed by the physician, Asthma Control Test and spirometry (FEV_1). Data analysis revealed no difference in asthma control as assessed by the asthma Asthma Control Test score and FEV_1. In the present thesis study, a plausible explanation for null findings between Mediterranean diet compliance and asthma control, quality of life and spirometry might be because at baseline the majority of children in both groups were taking asthma medication, had normal lung function, well-controlled asthma and no limitations in daily activities. The beneficial effects of this dietary intervention on asthma control and quality of life could be greater in children suffering with 'severe asthma' than in 'mild asthma'.

From another point of view, children had low adherence to the Mediterranean dietary pattern as assessed by the KIDMED index. Only 17% of children had optimal adherence to the Mediterranean diet (KIDMED score $\geq$ 8). Nevertheless, nutrition education provided by the candidate during telephone interviews did result in an improvement in KIDMED score by one unit in the intervention group (from a KIDMED score of 5 to 6, maximum 16) by the end of the six-month period, due to increased fatty fish intake (from 17 g/day to 46 g/day). This small but significant finding supports another secondary hypothesis that compliance to the dietary intervention improves adherence to the Mediterranean diet. As indicated in the published main result paper in Chapter 6.5, the KIDMED questionnaire showed that the frequency of fatty fish intake increased

from 13% at baseline to 84% in the intervention group at follow-up. Increased fatty fish intake was further validated by the 119% increase in plasma DHA levels and decrease in omega-6 to omega-3 fatty acid ratio for participants in the intervention group. It has been reported that fish consumption can significantly increase the serum levels of DHA in humans compared to fish oil supplementation [387]. Another advantage of regular fish consumption as opposed to fish oil supplements, is improvement in diet quality due to its high nutritional value. Fatty fish contains a variety of nutrients contributing to positive health benefits [388]. It is rich in high-quality protein, omega-3 fatty acids, selenium, iodine, potassium, vitamins B, A and D, antioxidants and low in saturated fats and cholesterol [388]. Incorporating food sources rich in nutrients and bioactive molecules as part of the usual diet rather than consumption of supplements are positive behavioural changes towards healthier eating practices.

The importance of nutrition education in influencing Mediterranean diet adherence in adolescents and children was demonstrated in a prospective study undertaken by Calatayud-Saez et al [389]. This was a 12-month programme designed to promote the adoption of the Traditional Mediterranean diet in 104 asthmatic children (1-5 years old) and to assess the effect on asthma. The authors found that adherence to the Mediterranean diet reduced bronchial hyperreactivity from the first weeks of the dietary intervention, 32.2% of patients did not have an asthma episode, 35.5% had only one attack during the year and 24.9% had two episodes compared to 4.73 episodes on average in the previous year. Moreover, the use of inhaled corticosteroids and bronchodilators markedly decreased from 3.92 ± 1.61 to 1.11 ± 1.09 times per patient per year ($p < 0.001$) and 4.14 ± 1.61 to 1.12 ± 1.40 ($p < 0.001$) respectively. It is worth noting that significant improvements in respiratory health were observed within the first four months of the dietary intervention. However, a limitation of this study is the young age group of participants, since 'true asthma' is diagnosed at about 6 years old [111].

The positive effects of the Mediterranean diet on asthma symptoms in children were documented earlier in the published systematic review of observational studies presented in Chapter 2.3 [390] and by others [391]. Recently, Castro-Rodriguez et al (2017) performed a systematic review that included 22 observational studies (cross-sectional, cohorts, case-control) with 17 studies in Mediterranean countries [391]. Mediterranean diet adherence

was evaluated from dietary details in FFQs. Critical appraisal of the literature revealed that adherence to the Mediterranean diet by children seemed to have a protective effect on asthma/wheezing symptoms after adjusting for age, sex, BMI, parental atopy, smoking, physical activity and maternal education. However, there was no effect on lung function and bronchial hyperresponsiveness. In particular in one cross-sectional study, the PANACEA (Physical Activity, Nutrition and Allergies in Children) study which included 700 Greek children (10-12 years), one unit increase in the KIDMED score was associated with 14% lower likelihood of having asthma symptoms after adjusting for age, sex, BMI, physical activity and energy intake [326]. No significant findings were associated between asthma symptoms and the consumption of individual food groups: fruit, vegetables, legumes, cereals, dairy, salty snack and margarine, while increased fish and meat intake was associated with less symptoms. Hence the authors of this systematic review concluded that it is unknown whether an intervention promoting the Mediterranean diet could reduce the prevalence of asthma and allergic disease in children. This PhD study is unique because it is the first clinical trial testing the feasibility of the Mediterranean diet in childhood asthma and adds to the literature by providing some evidence that not only the Mediterranean diet but adding fatty fish to the Mediterranean diet substantially improves lung inflammation in paediatric asthma. Nonetheless, more clinical studies are needed to assess whether a Mediterranean diet can prevent onset of asthma in children.

7.2 Dietary habits and physical activity level in Greek asthmatic children

Regarding the secondary hypothesis that the dietary intervention improved dietary habits in children, to a certain extent it did. Data from FFQs, KIDMED questionnaire and plasma DHA concentrations at six months verify the increase in regular fish intake. Indeed the KIDMED score increased by 1 unit after the six-month study. In addition, according to data from 24 hr recalls, daily energy intake in both groups decreased from overconsumption at baseline to within the EAR for this age group by the end of six months [293]. Hence, nutrition education on healthy eating according to the Traditional Greek Mediterranean prototype involving the family and grandparents could be a successful strategy in improving children's dietary habits and weight status. As

aforementioned, the 'Learning to eat from the Mediterranean' 1-year intervention study conducted by Calatayud-Saez (2016) was designed to promote adoption of the Mediterrranean diet in Spanish asthmatic children [389]. According to the investigators, by the end of the programme patients' dietary habits had improved. More specifically, there was an increase in the number of children consuming breakfast, fruit, vegetables, fish, wholegrains and fermented milk, whereas sweets and eating bakery products for breakfast decreased [389]. Therefore, an intervention of 12 to 18 months duration focusing on promotion of the Mediterranean diet in Greek asthmatic children could improve dietary habits and deserves future investigation.

With respect to diet quality of patients in this PhD study, dietary details recorded in FFQs and 24 hr food recalls showed that energy intake of children in both groups exceeded the EAR for children aged 5-12 years according to the Hellenic dietary recommendations for children and adolescents [293], thus justifying the high percentage of overweight/obese (~40%) children in this sample. Generally, children had low intake of fruit, vegetables, salads, fish, cereals, milk, rice/pasta, legume and nuts; food groups that form the basis of the Traditional Greek Mediterranean diet; whereas a high intake of red meat and sweets that are rich in saturated fats and cholesterol. Indeed, macronutrient analysis revealed that saturated fat, MUFA and cholesterol intake exceeded the daily RNIs of 11%, 13% of the total energy intake and 300 mg/day respectively [293]. The same pattern was observed for sodium intake which surpassed 5 g/day indicating over-consumption of sodium-rich foods and addition of table salt to meals [293]. Poor dietary habits were also apparent from the low KIDMED score. The low intake of fruit, vegetables, cereals and high intake of salt in Greek children is in line with other studies conducted in Greece [237; 392; 393]. On the other hand, promotion of vegetables, fruit and wholegrain cereals intake was beyond the scope of this PhD study.

As for meal time behaviour, breakfast is considered to be the most important meal of the day. In children, studies have shown that breakfast consumption improves diet quality, micronutrient intake, is associated with lower BMI, higher levels of physical activity as well as improved attention, memory and academic performance [394]. In this PhD study, 75% of parent's responded 'yes' that children consumed breakfast (at least a glass of milk). For those that replied 'no', breakfast skipping was associated with consumption of a small snack during school recess that included both healthy and unhealthy choices such as a toasted sandwich with cheese/turkey, croissant or cheese pie. Frequency of

consumption of breakfast in this study is in agreement with data from the ENERGY study that reported that 79% of children in Greece consumed daily the breakfast meal [395].

Furthermore, telephone conversations with parents revealed that children's eating habits were influenced by parents' and siblings' food choices. Some parents admitted that they did not consume fruits and vegetables with meals, thus explaining children's low intake of fruit and vegetables. Extensive research undertaken by Birch et al on the determinants of child eating behaviours and diet quality have demonstrated that 'good dietary habits start at home' [396]. Parental and sibling eating patterns mould children's dietary habits and food preferences via role modelling, availability of food in the home, mealtime structure and portion size, parents' beliefs and feeding practices, personal likes/dislikes including socio-economic, media influence (TV viewing) and cultural practices [396; 397]. Child feeding practices such as restriction, reward, coercion and forced-feeding counteract healthy eating habits in children and are positively associated with increased BMI, weight gain and eating disorders [396]. In some cultures 'more' is better and a fatter child is considered to be 'healthier'. In Greece it is common practice for grandparents to be involved in the care of grandchildren and being responsible for the preparation of family meals. Hasapidou (2009) documented that Greek grandparents involved in childcare tend to overfeed grandchildren by offering meals that are rich in fat as well as large portion sizes, thinking that a 'fat child is a healthy child' [398]. Similarly, Moschonis et al (2010) in a study of Greek school-children found a positive association between grandmothers as child-carers and the odds of overweight and obesity [399]. Many grandparents due to food deprivation during the Second World War possess the 'war syndrome' believing that food should be consumed in abundance when available [398]. Thus motivating them to overfeed grandchildren and consequently contributing to the obesity epidemic in Greek schoolchildren. In this PhD study telephone conversations with families confirmed that some grandparents were actively involved in the care of grandchildren during parents' working hours. According to data from a recent systematic review, children in the care of grandparents tended to have higher bodyweight and consume more unhealthy snacks and sugar-added drinks than children cared by parents, another adult or in childcare centres [400].

Despite the existing evidence about the health benefits of the Mediterranean diet, this healthy eating pattern is being abandoned among the younger generation and substituted

by the Western-type diet in Greece and other Mediterranean countries [5]. Modernization of the society is responsible for a progressive shift away from healthy Traditional dietary habits such as the Mediterranean diet and modification of food preferences toward energy-dense 'junk foods' and sedentary behaviour (computer, television viewing and video games), leading to an overall imbalance between energy intake and expenditure [401]. Lack of physical activity was evident in this PhD study as only 50% of children exercised at least three times per week. This is significant because a sedentary lifestyle and low physical activity level are risk factors that promote asthma development in children [21]. According to WHO global recommendations for physical activity in 5-17 years old, children and adolescents should engage in at least 60 minutes of moderate-vigorous intensity exercise daily [402].

Tambalis et al (2018), in the EYZHN study (National Action for Children's Health Program) that included 232,401 Greek school children (8-17 years), documented that 40% of children had optimal adherence to the Mediterranean diet (KIDMED score ≥ 8), and 10% of children in both genders had low adherence [238]. For each 1-year increment in the age of children the odds of low adherence to Mediterranean diet increased by 11% in both genders, while boys had almost 6% increased probability to low adherence than girls. Furthermore, insufficient sleeping hours (>2 h/day) and inadequate physical activity status were related to higher odds of low adherence to the Mediterranean diet [238].

Similarly, the Greek Childhood Obesity (GRECO) study reported that about 50% of children (10-2 years old) were classified as low adherers to the Mediterranean diet [237]. The overall KIDMED score was 3.65 ± 2.2 for the entire sample; only 4.3% of children had optimal adherence (≥ 8). Children with higher KIDMED scores reported following a healthier diet and had higher physical activity levels. In particular, they were more likely to consume frequently starchy foods, cereals, fruit/fruit juice, vegetables, dairy products, legumes, nuts, red meat, poultry, eggs, fish, seafood and less frequently ice-cream, traditional Greek foods with meat (for example souvlaki and gyros), burgers, salty snacks, and sweets.

Regarding factors that influence food choice, apart from the family environment, parent's education level and the cost of food are important determinants [396; 397];[403]. In the current PhD study, 45% of mothers had completed tertiary education and 34% of

fathers. A positive relationship was found between mother's education level and the KIDMED score. Studies have shown that in families with mothers of high education level, children consumed more fruit, vegetables and were more likely to consume breakfast daily than children with mothers of low education level [404] . Higher education level may be related to higher income and health literacy, thus, greater availability of healthy foods, increased nutrition knowledge and motivation to follow a healthy lifestyle such as the Mediterranean diet [300; 405]. In particular, Papadakis et al (2015), reported that mother's education level and living with both parents were factors that influenced adherence to the Mediterranean diet in adolescents [405]. Comparable findings were reported by Antonogeorgios et al (2013) [338]. According to the authors, parental education status played a mediating role in Mediterranean diet adherence and obesity status in Greek children aged 10-12 years old [338]. Adherence to the Mediterranean diet was inversely associated with children's obesity status only in families in which at least one parent was of high education level as compared to parents of low education level.

In a critical analysis of obesity in the United States, Drenowski et al (2005) identified that healthy diets were costly and in contrast, energy-dense, poor quality foods were of low cost [403]. More specifically, Lopez et al (2009) documented that the Mediterranean diet was more expensive to follow than a Western-type diet [406]. Healthy foods such as vegetables and fruit were found to be costly compared to convenient foods. With respect to SES, studies have shown that diets of individuals in relatively low socioeconomic groups tend to be characterized by high intake of meat products, full cream milk, fats, sugars, preserves, potatoes and cereals, and relatively low intake of vegetables, fruit, and wholewheat bread [407]. Moschonis et al (2010) in a study examining social-economic determinants correlated with overweight/obesity prevalence in Greek school children, found that annual family income of 12,000-20,000€ was associated with higher odds of overweight/obesity in children [399]. In this PhD study, the average annual income was 19,000€.

Recent surveys undertaken by the Hellenic Statistical Society have revealed that the economic crisis, unemployment and the high cost of food are factors for the decrease in consumption of healthy foods such as fruit, fruit juice, vegetables, milk products (mainly milk and cheese), eggs, nuts, fish, olive oil and red meat [408], but increase in chicken (replacing red meat), refined bread and cereals (rice, pasta), legumes, alcohol, coffee

and cocoa [408]. The statistics showed that the cost of food items (due to the increase in food tax), play an important role in determining what is purchased by the consumer. In general, data suggest that consumers try to follow a healthy diet while balancing food choices according to income. Nonetheless, there seems to be a decreasing trend in compliance to the Traditional Mediterranean diet in the age group younger than 35 years old. Apart from cost, information on multiethnic cuisines from the internet, social media and TV advertising appear to be influencing dietary habits of the younger generation in Greece [408]. Other factors known to be barriers to healthy eating in children are more mothers in the workforce and time-constraints, single-parents and longer working hours, less time for shopping, preparation and cooking of home-made meals which has been replaced by convenient and pre-packed meals [407].

7.3 Childhood Obesity: Mastiga of the 21st century

The aforementioned findings of this PhD study on low adherence to the Mediterranean diet, poor dietary habits and lack of exercise in asthmatic children are relevant because these factors are well-recognized to have contributed to the increase in childhood obesity, the mastiga of the 21st century [337]. Conversely, high adherence to the Mediterranean diet that is characterized by high intake of seasonal fruits, vegetables, wild greens, fish, nuts and olive oil has a protective effect on obesity [409]. A large cross-sectional study of 16, 220 children aged 2-9 years old in eight different European countries showed that a high adherence to the Mediterranean diet was inversely associated with overweight, obesity and fat mass [409]. Suggested mechanisms were the low glycaemic effect of the Mediterranean diet and its high antioxidant content which could lead to better metabolic function and less weight gain [409]. In the current PhD study approximately 40% of asthmatic children were overweight/obese at baseline, with higher prevalence of obesity in boys than in girls (18% vs 6% respectively), thus signifying the magnitude of the problem of obesity in Greek asthmatic school-children. This is in accordance with data from previous studies undertaken in Greece reporting the alarming increase in child obesity prevalence [410; 411; 412].

The GRECO study showed that 29.5% of children (aged 10-12 years old) were overweight and 11.7% obese [237]. The same trend was reported from the Healthy Growth Study with 29.6% prevalence of overweight and 11.1% obese in 9-13 year old

school-children [399]. Tzotzas et al (2011) in a nationwide, cross-sectional epidemiological survey showed that the overall prevalence of overweight and obesity in children (6-12 years) was 31.2% in boys and 26.5% in girls, while obesity prevalence was 9.4% and 6.4% respectively [413]. In 2014 findings of the PANACEA study revealed that 34% of children (10-12 years) were overweight/obese, and 58% were least moderately active [325]. With respect to the higher prevalence of overweight/obesity in children (5-12 years) of this PhD study, one could speculate that the low physical activity level (only 50% of children exercised at least three times per week), the economic crisis and high cost of healthy foods are possible determinants [410]. Another discrepancy is the age range of children that were evaluated in this study as compared to the study by Tzotzas and in the PANACEA study.

According to WHO statistics, among the European countries, Greece has the highest level of childhood obesity [329]. More specifically, the COSI study (Childhood Obesity Surveillance Initiative) was designed to monitor trends of overweight in school-children aged 6-9 years old from fifteen European countries including Greece [329]. Data from 224, 920 children showed that in 7 year old, 48.9% of Greek boys were overweight and 23.9% obese, while 44.8% of Greek girls were overweight and 18.6% obese according to WHO definition [IOTF definition (overweight vs obese) Greek boys: 38.1% vs 13.6%. Greek girls: 39.9% vs 14.3%]. The same trend was observed in 9 year olds. Overweight in Greek boys increased to 57.2% and obesity to 30.5%. As for Greek girls, 50% were overweight and 20.8% obese [IOTF definition (overweight vs obese) Greek boys: 45.1% vs 14.7%; Greek girls: 42.3% vs 14.6%] [329].

Recently Hasapidou et al (2017), published the results of COSI Round 2 which included 5231 Greek children, aged 7-10 years old [410]. Data showed that in 7 years old, the prevalence of abdominal obesity was identical for boys and girls (25.2% and 25.3% respectively; $p > 0.05$), whereas in 9 year olds, abdominal obesity was more prevalent in boys than in girls (33.2% vs 28.2% respectively; $p = 0.005$). In contrast, among normal weight and overweight children, the prevalence of abdominal obesity was 1.6-6.8% and 21.8-49.1% respectively. The authors speculated that the increase in prevalence of overweight and abdominal obesity in Greek children might be the result of the economic recession that could lead to increased purchase of low-cost, processed and high-fat food [410].

7.4 Obesity-asthma link?

During the past 20 years the concurrent increase in paediatric obesity and asthma prevalence suggest that both conditions are interrelated. They represent two of the most significant paediatric health problems worldwide. Childhood asthma/wheeze and obesity, measured by BMI, have been linked in cross-sectional, case–control, and prospective epidemiologic studies [136]. If overweight/obesity and childhood asthma epidemics are causally related is poorly understood. Also, whether high BMI precludes asthma development or asthma increases the risk of becoming overweight is yet to be elucidated [414].

An increasing body of evidence implicates obesity as a major risk factor for asthma [21; 414]. Azizpour et al (2018), performed an up-to-date systematic review and meta-analysis of case-control studies to investigate the effect of BMI on asthma in children/adolescents (2-19 years) [138]. Pooled analysis of 11 relevant case-control studies showed that the odds ratio for asthma risk in overweight children/adolescents was 1.64 times more likely than in normal-weight, whereas in obese children/adolescents, 1.92 times more likely. Subgroup analysis performed for age, sex, sample size, year of publication and asthma diagnosis methods showed that overweight and obesity increased asthma risk in both sexes, although non-significant most likely due to small sample size and number of studies. A possible source of limitation in this study was the large age range (2-19 years) and high heterogeneity among study designs. Nevertheless, it was concluded that there was a significant relationship between BMI (overweight/obesity) and asthma among children and adolescents. In contrast, Papoutsakis et al (2013) in a systematic review of 48 epidemiological studies undertaken in infants, children and adolescents from 2006 to 2011, concluded that the current evidence supports a weak yet significant association between high body weight in children and asthma [136]. In addition, the link between high body weight and asthma was stronger in non-allergic asthma.

Obese-asthma may represent a unique phenotype that is more difficult to manage and worsens asthma severity. Strong evidence supports that asthma in overweight/obese children is associated with more daily symptoms, frequent exacerbations and hospitalizations, missed school days, increased use of reliever medication and higher

doses, resistance to steroid treatment, exercise-related asthma including impaired lung function and worse asthma control than asthmatics of normal weight [415; 416].

To date, little is known regarding the impact of overweight and obesity on pulmonary function in paediatric years and is worth exploring. Novel to this PhD study was the investigation of the effect of adiposity (high BMI) on pulmonary mechanics in asthmatic children in a cross-sectional study that was reported in the publication in Section 6.3. Unique to this thesis study was that high BMI appeared to affect small airway calibre as represented by $FEF_{25-75\%}$. Compared to normal weight asthmatic children, a positive effect of overweight/obesity (β= 11.65 units) was found on $FEF_{25-75\%}$ after adjusting for age, sex, height, regular physical activity, medication and KIDMED score. No effect of adiposity was found on FEV_1/FVC (marker of airway limitation). In addition, a significant positive correlation was found between BMI and ventilatory flow (FEV_1) and ventilatory capacity (FVC). In contrast, an inverse trend was found between BMI and FEV_1/FVC, PEF and FeNO, although non-significant probably due to small sample size. However, after assessing sex differences, a positive correlation was observed between BMI and FVC and FEV_1 in girls; and a borderline negative correlation between PEF and BMI in boys. With respect to eosinophilic inflammation, FeNO was found to be significantly lower in overweight/obese asthmatic children as compared to normal weight. The findings of this PhD study add to the gap in current literature. Further studies are recommended to clarify whether the observed anomalities in children with asthma are due to disproportionate lung growth or developmental effect. From the limited paediatric studies investigating the impact of adiposity on pulmonary mechanics in asthmatic children, a link between obesity and greater lung volumes, mild obstructive impairment in airway flow and low FeNO values have been documented [417; 418; 419; 420; 421; 422]. The above-mentioned findings have important implications in the interpretation of lung function tests because spirometry may have reduced specificity and sensitivity for asthma in overweight paediatric patients.

Yao et al (2017) in the PATCH study (Prediction of Allergies in Taiwanese Children) which was a cohort study of 1,717 children aged 5-18 years old investigating the effect of excess weight on lung function and FeNO, reported that BMI disproportionately increased FVC, FEV_1, PEF and $FEF_{25-75\%}$ but decreased FEV_1/FVC and FeNO [422]. Comparable results were noted in a case-control study of 188 asthmatic and non-

asthmatic children aged 8-16 years old undertaken by Jones et al, (2017) [420]. According to the authors there was a positive effect of BMI on FVC and $FEV_{1,}$ but negative on FEV_1/FVC in school-children. Compared to normal weight asthmatics, overweight was associated with an increase of 0.71 and 0.44 *z* scores in FVC and FEV_1 respectively, and reduction in FEV_1/FVC by 0.40 *z* scores ($p < 0.01$). Similarly, the BAMSE (Swedish abbreviation for Children, Allergy, Milieu, Stockholm, Epidemiology) population–based birth cohort that included 2889 children aged 8-16 years, revealed that obesity was associated with reduced FEV_1/FVC, but increased FVC (volume) and FEV_1 (airway flow) up to 16 years old [418]. In a German study that included 142 children (6-18 years) with asthma and healthy controls, normal weight children with asthma had lower FEV_1,VC, $MEF_{50\%}$ and higher FeNO than overweight/obese children and healthy controls [421]

Contradictory to these findings, Spathopoulos et al (2009) published that high BMI significantly reduced the percentage expected FVC, FEV_1, $FEF_{25-75\%}$ and FEV_1/FVC in overweight and obese Greek children (aged 6-11 years old) compared to normal weight ($p = 0.007$, $p < 0.001$, $p < 0.001$, $p < 0.001$ respectively) [146]. Potential explanations for discordance among studies could be heterogeneity in study methodologies, statistical tests applied and adjustment for confounding factors, population diversity (age, ethnicity), sample size, measurement of adiposity and environmental factors such as diet and air pollution.

According to extensive research undertaken by Forno et al on obesity and airway dysanapsis in childhood asthma, a feasible explanation for supra normal values observed in ventilatory flow (FEV_1) and capacity (FVC) could be due to dysanapsis or asymmetric growth of lungs and airways where there is faster growth in lung volume and airway length and slower increase in airway calibre [419]. It has been speculated that obese children with dysanapsis may have airway obstruction which is anatomical or developmental and not related to airway inflammation, thus explaining low FeNO measurements in this weight category. More specifically, the obese-asthma phenotype could be mediated by neutrophils and not eosinophilic airway inflammation [419]. However, in this PhD study blood neutrophils and eosinophils were not measured in participants which deserves further investigation. From another perspective, low FeNO values might be caused from limited production of NO in airways and increased NO

metabolism [423]. Additionally, consumption of high fat meals (containing saturated and trans fats) have been shown to cause neutrophilic inflammation in asthmatic patients [424].

With respect to no effect of overweight/obesity on airway limitation biomarker FEV_1/FVC in this PhD study, one reason could be from the lack of statistical power since only seven children were classified as obese according to IOTF cut-offs. Another possibility, as mentioned earlier, spirometry specificity and sensitivity for obstructive diseases may be reduced in populations with high prevalence of overweight [420]. Further studies in these patients are warranted to measure airway diameter and resistance in peripheral and central airways to confirm the findings derived from lung function testing. Ideally, airway imaging and impedance oscillometry [425] would clarify true obstructive disorder and the presence of dysanapsis as compared to spirometry [420].

As noted in Section 6.3 of this thesis in the published manuscript, airway limitation as expressed by FEV_1/FVC may be dependent on the degree of overweight [426]. Furthermore, in adults it has been suggested that obesity may affect spirometry measurements when BMI $\geq$ 40 kg/m^2 [426]. The highest value for BMI in our children was 30.61 kg/m^2. As a result potential impairment of lung function due to severe obesity would not have been possible. Consistent with our findings, no effect of BMI on FEV_1/FVC was observed in a cross-sectional study performed by Mahut et al (2012) involving 491 asthmatic children aged 6-15 years [427]. Reasons given by the authors were the small frequency of severe obese children (n = 7) and the limitation of BMI to distinguish between muscle and fat mass [427]. In adults, body fat distribution (as predicted by waist circumference, waist to height ratio and percentage body fat) whether central or abdominal alters the pressure-volume characteristics of the thorax and restricts the descent of the diaphragm limiting lung expansion [426]. Reduced lung ventilation and expiratory reserve volume as well as increased work of breathing leads to lower FVC and FEV_1 measurements [426]. A limitation of the paediatric studies mentioned including the present PhD study is that the effect of adiposity (central or abdominal fat) on spirometric measurements were not evaluated. Another factor, sex differences in fat distribution [426]. Females have a higher percentage of body fat for each unit of BMI than males [426]. This discrepancy could explain the correlation observed between high BMI and FEV_1 and FVC in girls and not in boys.

Apart from the effect of obesity on lung mechanics, obesity is regarded to be a state of low grade inflammation and oxidative stress that affects the cellular and molecular signalling pathways of the immune system [428]. It has been proposed that systemic inflammation could modulate airway inflammation thus promoting asthma development in obese subjects [428]. Obesity in asthmatic children skews CD4 cells towards Th1-polarization (mediated by systemic inflammation, insulin resistance and/or alterations in lipid metabolism), increased interferon-γ, interleukin IL-6, TNFα production and suppression of Th2 cells and decreases in T regulatory cells which are associated with worse asthma control and severity as well as abnormal lung function [428]. In addition, leukotriene molecules, adipokines and leptin that are pro-inflammatory mediators released by adipocytes could be involved in airway inflammation and in the development of hyperreactivity associated with asthma [429; 430; 431]. In obesity, serum concentrations of leptin and resistin are increased, while adiponectin is decreased [431]. Adiponectin has anti-inflammatory properties and is lower in obese children compared to non-obese[432]. Similarly, low adipokines and adiponectin have been associated with asthma in children [429; 431].

Also, truncal adiposity contributes to metabolic dysfunction in asthma [433]. High body weight is associated with hypertriglyceridemia, hypercholesterolemia, low HDL and insulin resistance that are involved in the development of airway inflammation and inversely related to pulmonary function [430]. Hyperglycemia and hyperinsulinemia can contribute to airway hyperresponsiveness and remodelling through epithelial damage and airway smooth muscle proliferation contributing to impaired lung function and the promotion of inflammation in airways and consequently asthma symptoms [433]. High levels of insulin resistance and low levels of HDL are associated with impaired FEV_1/FVC ratio [430].

Nonetheless, in the present PhD study, due to laboratory limitations, the relationship between adipokines, leptin, dyslipidemia, hyperinsulinemia and pulmonary function tests were not assessed and would be interesting hypotheses for further testing.

7.5 The role of vitamin D in childhood asthma

The role of vitamin D in respiratory health has received considerable attention over the recent years owing to its pleiotropic properties and downregulation of the immune response and inflammation**.** Vitamin D insufficiency is frequent among asthma sufferers. Decreased levels of serum 25(OH)D were correlated with increased asthma prevalence, exacerbations [209; 214] , hospitalization [212], emergency visits [214], along with declined lung function [209], asthma control [218] and medication use [212] in asthmatic children.

In Chapter 4 of this thesis, another secondary hypothesis under investigation was that there is an association between plasma vitamin D and lung function in children. The findings of this PhD study justify this hypothesis. More specifically in section 6.6.3 of this thesis, the impact of vitamin D status on lung function was examined in the manuscript that is currently under-review.

Briefly, 64% of children were insufficient in vitamin D (25(OH)D < 25 ng/ml), primarily in girls which corresponds with European data in children up to 14 years old [233; 364]. As outlined in the manuscript feasible reasons for hypovitaminosis D witnessed in participants are more time spent indoors (as confirmed by the low frequency of children exercising daily), low intake of fortified cereals, fish meals, sunscreen use and the nature of the disease [239; 364]. Most common barriers to physical activity and outdoor leisure-time were working parents, busy extra-curriculum activities, TV viewing and more computer-time [379]. Another potential limiting factor is reverse causation where children with poor asthma control are less likely to engage in outdoor activities due to difficulty in breathing and more respiratory infections during winter months and as a result are exposed to less sunlight [380]. Nonetheless, by the end of the six month follow-up vitamin D status increased by 21.4% in the intervention group most likely due to fatty fish intake which is a rich dietary source of vitamin D [239; 364]. However, we did not measure the amount of daily sun exposure due to outdoor play. It is worth noting that although follow-up was undertaken during the period of May-August which include spring and summer months with ample sun-exposure, children may not be conditioned to spend time outdoors as reflected by the low percentage of children exercising regularly (~50%). One more factor leading to hypovitaminosis D in patients is adiposity. In this PhD study BMI was found to be inversely correlated to vitamin D with

approximately 80% of overweight/obese children insufficient 25(OH)D < 25 ng/ml compared to normal weight. Obesity-associated vitamin D insufficiency is possibly caused by decreased bioavailability of vitamin D3 from cutaneous and dietary sources because of its accumulation in body fat stores [434].

Another exceptional finding and innovative to this thesis was that in asthmatic children following a Mediterranean diet intervention enriched with fatty fish, having sufficient serum vitamin D levels (25(OH)D ≥ 25 ng/ml) at baseline, reduced airway obstruction significantly as represented by an increase in FEV_1/FVC by 4.89 units and improved airway flow in small airways ($FEF_{25-75\%}$) by 12.83 units controlling for age, sex, regular physical activity and BMI. No significant effect was observed on bronchial inflammation biomarker, FeNO. This may have clinical significance in managing childhood asthma because both FEV_1/FVC and $FEF_{25-75\%}$ are sensitive indicators of airway obstruction [111]. Low levels of $FEF_{25-75\%}$ and FEV_1/FVC have been associated with steroid use, hospitalization, asthma exacerbations and asthma severity as compared to children with normal spirometry [435]. Given that the majority of children have normal FEV_1, finding that another spirometry parameter is associated with poor asthma outcome has important implications for health professionals performing spirometry in asthmatic children. The results of this study suggest the involvement of vitamin D in lung function (even in small airways) and in the development of airway limitation in asthmatic children. This is the first clinical trial to identify that sufficient serum vitamin D levels in children following a dietary intervention, improved $FEF_{25-75\%}$. Prior cross-sectional studies have reported low 25(OH)D levels in asthmatic children and a direct correlation between serum vitamin D and lung function test measurements (FEV_1, FVC, FEV_1/FVC) but not for FeNO [206; 218; 436] [437]. Regarding $FEF_{25-75\%}$, only one out of the four studies examined the effect of vitamin D level on $FEF_{25-75\%}$ in asthmatic children, reporting no effect [437]. A likely reason is that these studies focused on the function of large airway calibre as reflected by FEV_1 and FVC rather than in small airways. With respect to no significant effect of vitamin D status on bronchial inflammation biomarker (FeNO), this is not surprising because FeNO detects eosinophilic inflammation [258] and vitamin D may not be involved in eosinophilic asthma [437]. Nevertheless, the findings of these studies suggest an important role of vitamin D in paediatric respiratory health.

Another key-point unique to this PhD study is, that it is the first time that the potential of vitamin D in enhancing the effect of a dietary intervention on pulmonary function has been demonstrated and extends to the literature on the pleiotropic properties of vitamin D. Up to now, the threshold for vitamin D demonstrating health benefits is based on bone health [364]. Recommendations on the level of vitamin D considered to be beneficial for lung health is yet to be elucidated. In this study, the results propose that plasma 25(OH)D ≥ 25ng/ml may be required to improve lung function. Drawing on this, stratification of data according to the threshold for vitamin D deficient (25(OH)D < 10 ng/ml), insufficient (25(OH)D 21-29 ng/ml) and sufficient (25(OH)D ≥ 30 ng/ml) concentrations based on bone health [200] did not alter the results. It has been suggested that vitamin D levels above 30 ng/ml may be necessary for optimal respiratory health [364]. In our sample only six patients had levels above this threshold at baseline. Future robust clinical trials are recommended to replicate our findings in other dietary interventions and to test whether plasma (25(OH)D ≥ 25 ng/ml) has a positive effect on respiratory health.

On the topic of medication use, by the end of the six-month trial vitamin D levels increased by 21% in the intervention group. There was a significant decrease in medication use for the intervention group with insufficient vitamin D levels baseline (25(OH)D < 25 ng/ml) from 91% at baseline to 42% at six months. It can be speculated that regular fatty fish intake in combination with the Mediterranean diet improved overall health and lung function as evident from the decrease in need for asthma medication.

The ability of vitamin D to enhance the response to asthma therapy is remarkable and is consistent with earlier paediatric studies [212; 217]. Searing and Brehm documented an inverse relationship between vitamin D levels and use of steroids and anti-leukotriene therapy in asthmatic children [212; 217]. This could be an effective strategy especially in the case of corticosteroid resistance [438] and given that children's adherence to asthma therapy is poor [439]. Vitamin D enrichment of asthmatic children's diet could decrease the dose of medication required to relieve symptoms, hence limiting the systemic side-effects of pharmacotherapy. The findings of this PhD study suggest that small dietary modifications such as increased fatty fish intake and sun exposure could be an adjunct to asthma therapy. Fatty fish is a rich source of vitamin D and provides a variety of other

nutrients, antioxidants and bioactive molecules, including omega 3 fatty acids, selenium, iodine, potassium, B-vitamins, that might interact synergistically providing beneficial effects on pulmonary function [388].

Although the exact mechanisms underlying the link between vitamin D status and lung function are poorly understood, it is possible that vitamin D could improve lung function by influencing airway remodelling, smooth muscle cell movement, growth and contractility, collagen synthesis and by inhibiting matrix metalloproteinase, fibroblast proliferation as well as protection from respiratory infections and enhancing asthma therapy [415]. Other plausible mechanisms include the action of vitamin D on regulating airway inflammation, since it is a potent regulator of the immune response [230; 340].

7.6 Organic acids and pulmonary function in asthmatic children

Original to this PhD study was the application of targeted metabolomics (using GC-MS) to investigate the metabolic profile of asthmatic children. To the candidate's knowledge, a limited number of studies have explored urinary organic acids in asthmatic [276; 277; 440] and non-asthmatic children [441; 442; 443; 444] and in particular, no studies in Greece. As indicated in Section 6.7 of this thesis by the publication, 34 unique organic acids from urinary samples of asthmatic children were detected, which is consistent with previous literature [441; 442; 443; 444]. This study showed that urinary organic profiles can serve as diagnostic indicators of abnormal metabolism in relation to asthma in children. Feasible explanations proposed for the correlation between organic acids (lactic acid, 4-hydroxyphenylacetic acid, 5-hydroxyindoleacetic acid, 2-hydroxyisobutyric acid, glycolic and malic acid) and pulmonary function tests (as represented by FEV_1, FVC, FEV_1/FVC, PEF, FeNO) as well as asthma control include metabolic pathways involved in energy production, immune response, hypoxia, inflammation and oxidative stress in airways, changes in intestinal microflora along with detoxification systems [445; 446; 447; 448; 449; 450; 451; 452; 453; 454].

7.7 Strengths/Limitations

Pulmonary function testing is an important pathway to asthma diagnosis in children and diet should also be considered. The outcome of this thesis showed that a dietary intervention could be a valuable adjunct to conventional asthma therapy, given that medication adherence is poor in children. The use of FeNO in the management of paediatric asthma is debatable. This study is unique since it was able to assess bronchial inflammation reflected by FeNO in paediatric patients exhibiting normal spirometric results.

To date, no universal guidelines on nutritional recommendations in the management of asthma for children or adults exist. The findings of this clinical trial are significant since they will assist in the planning of these guidelines. This thesis adds to the evidence in support of a Mediterranean diet enriched with fatty fish in paediatric asthma management. To the candidate's knowledge, this is the first Mediterranean diet trial to test dose and quantity (≥ 150 g twice weekly) of fatty fish intake in childhood asthma as compared to the assessment of fish intake from FFQs in previous observational studies [455; 456]. Another strength is that the study protocol is easily reproducible in any primary care setting and can significantly contribute to a better understanding of the therapeutic effects of diet on respiratory disease. Also, the feasibility of the intervention, that is, it was of low-patient burden, affordable in most diets including Western, applicable to 'real life' situations and with minor dietary modifications can be implemented in non-Mediterranean settings.

The beneficial effects of a Mediterranean diet on asthma in children have been documented in observational studies which are limited in determining a causal relationship and can only be used to propose hypotheses. Given that the effect of omega 3 fatty acid as a nutrient alone did not clearly demonstrate a protective effect on asthma in children [457], the present study is innovative since it examined the combined effect of dietary Ω3 (from fatty fish) within a dietary pattern in speculation that the synergistic interaction between all components of the Mediterranean diet would confer protective effects on pulmonary function in asthmatic children [200; 296].

Apart from omega 3 fatty acids, the present clinical trial demonstrated for the first time the importance of plasma vitamin D in enhancing the effect of a dietary intervention and subsequent improvement in lung function in both central and peripheral airways. Up till now the evidence on the role of vitamin D in bone health has been well-established but its relation to asthma development is a matter of debate. Currently there is no universal consensus on the threshold for hypovitaminosis D in children and for optimal respiratory health. This PhD study found that plasma 25(OH)D ≥ 25ng/mL enhanced lung function in asthmatic children following a Mediterranean diet intervention and adds new evidence to the literature.

Another strength of this clinical trial is the study design, randomized controlled trials are considered to be the gold standard for identifying cause-effect relationships. Also, lung function, bronchial inflammation and omega-3 fatty acids intake and vitamin D status were assessed quantitatively, which improves accuracy and validity of the results, thus reducing errors and false inferences. Fish and fish products correlate positively with EPA and DHA in phospholipids in children and adults [458]. In addition, serum 25(OH)D is considered to be the most reliable measure of overall vitamin D status in children and adolescents [459]. The use of multiple metabolic biomarkers aids in the development of sophisticated, targeted and personalized treatment plan. Regular monitoring and support provided by the candidate to all participants throughout the six month study duration, contributed to the high participation rate (89%), motivation and good adherence to the dietary intervention. Patient satisfaction was evident from the positive feedback received at the end of the trial.

One more point that deserves mentioning, this is the first study in Greece to evaluate urinary organic acids and pulmonary function in Greek asthmatic children. Studies quantifying urinary organic acids in asthmatic children are sparse [276; 277; 440]. The findings of this clinical study support the potential clinical utility of organic acids as a screening tool for areas of abnormality that may not be detected from patient history and conventional pulmonary function tests, to monitor asthma control and lung function in a non-invasive manner. Organic acid concentrations might potentially serve as a quantitative index with which to guide asthma therapy in paediatric asthma. Determination of organic acids can be easily obtained from spot ‘urine specimens’

which is a non-invasive procedure as compared to blood sampling or sputum collection. Organic acid quantification was undertaken using validated GC-MS assay [460]. Given that this study was exploratory, further research is necessary to better elucidate how organic acid profiles might translate to clinical symptomatology and herald asthma exacerbations as well as clinical implications of inhibiting these pathways.

With respect to eating habits, evaluation of dietary intake in children using FFQs is substantially cheaper than other methods of dietary assessment [461]. A concise 27-item FFQ based on the PANACEA-FFQ, a validated semi-quantitative questionnaire designed for children, was used to collect dietary information in this trial. The advantage of the short FFQ was that it was simple, easy to understand, low-patient burden, minimum cost and entailed approximately 10 minutes for completion. Short FFQs have been used successfully to evaluate dietary intake in children in the renowned ISAAC study [8; 36; 323]. From responses to questionnaires nutrient intake was calculated based on known nutrient composition of foods [461]. It is well-known that brief FFQs are useful to analyse the association between nutrient intake and chronic disease, but they are subject to substantial measurement error, and dietary change [462]. Another drawback is that parents/guardians were used as proxies to estimate children's dietary intake which could have introduced a source of error due to social desirability, recall bias, under and over-reporting of dietary intake and misinterpretation of portion size and frequencies [463]. From another avenue, asthmatic children are susceptible to frequent respiratory infections, a known risk factor for asthma exacerbations which could be a reason for parents to overfeed children in an effort to optimize health status. These factors would explain the high energy and nutrient intake of participants in this PhD study. In comparison to 24 hr food recalls, the tendency for FFQs to overestimate dietary intake in the paediatric population has been reported in another study [463]. Additional disadvantages are misreporting, unreporting or forgotten foods (such as sweets, lollies) and reporting of foods not consumed. One more potential factor, parents may not be aware of foods consumed by children when they are working or during school hours. Perhaps nutrient analysis using biochemical biomarkers would have led to more accurate results [274]. Then again biochemical testing is expensive and laboratories are often limited in equipment required to assess all nutrients of interest.

Nevertheless, multiple 24 hr food recalls [315] as well as the KIDMED questionnaire were used to validate dietary information in FFQs. In comparison to doubly-labelled water, multiple 24 hr food recalls are considered to be the most accurate dietary assessment method to estimate total energy intake in children when conducted for at least 3 days, including weekdays and weekends and using proxies for reporting [463]. Additionally, when conducting 24 hr food recalls, relying on short-term memory (foods consumed over the last 24 hours versus previous month) and use of probing questions to retrieve dietary details, minimizes recall bias, is of low-respondant burden and does not require literacy as compared to FFQs [463]. However, 24 hr food recalls may not capture seasonal variation or reflect long-term intake [461]. Even so, the use of multiple 24 hr food recalls to assess habitual dietary intake in children is common practice [315].

Overall, this study should be considered in light of a few limitations. An issue identified during telephone interviews was that it was difficult to record day to day variations in children's dietary intake. On weekdays parents were absent from home because of busy work schedules and children's after-school activities. The majority of parents were available to conduct interviews on weekends from Friday to Sunday. One more drawback, a larger sample size would have increased the power to detect significant differences between the intervention and pulmonary function tests. A possible shortcoming was the short study duration in order to minimize participant burden and cost of the intervention. Since children had low adherence to the Mediterranean dietary pattern, perhaps a longer time frame (> 6 months) might have been necessary for differences to be seen between adherence to the Mediterranean diet and spirometry testing. It would be expected that the effect of the intervention would be greater in a non-Mediterranean setting. Another source of limitation was the inability to blind groups or investigators due to the nature of the study. Greater support could have been given to the intervention group as compared to the control which helped to enhance good compliance to the dietary intervention and positive results. Regarding statistical tests, in the regression model best efforts were made to control for potential confounders of pulmonary function, nevertheless other factors such as maternal educational level, socio-economic status, family income, parental allergy, smoking status, number of siblings in the family, breast-feeding duration, respiratory infections, birth-weight and gestation duration were not investigated. Given that adherence to the Mediterranean diet

was low in children, future research is warrented to elucidated whether high adherence to the Mediterranean diet improves lung function and prevents symptoms in asthmatic children.

One more discrepancy in this study, inflammatory markers (CRP, TNF a, IL-4, IL-5, IL-8, IL-13), cholesterol, LDL, HDL and blood glucose concentration, mediators in systemic inflammation are increased in children with asthma and were not measured due to laboratory limitations and expenses [114; 464; 465]. A recent study undertaken in Greek asthmatic children documented that adherence to the Mediterranean diet modulated the production of inflammatory markers [466].

SYNOPSIS

The main findings of this thesis were summarized, discussed and critically appraised in Chapter 7 and evaluated against the hypotheses stated earlier in Chapter 4. Strengths and limitations of methodological techniques were also included. Exploratory to this thesis, the relationship of obesity to pulmonary mechanics in asthmatic children and metabolomic profiling were briefly mentioned with plausible explanations given. Finally, future directions, conclusions and key messages are provided in Chapter 8.

CHAPTER 8 Conclusions and future research

8.1 Future Directions

This clinical trial has addressed gaps in the literature, and identified new areas for future research:

Obese-asthma phenotype

The increasing prevalence rates of obesity and asthma in children suggests that both disorders could be interrelated [136]. Longitudinal studies have found obesity to be an independent risk factor for asthma [21]. Obese-asthma is a distinct phenotype that is associated with deficits in lung function and suboptimal response to asthma medications [416]. Recent studies have identified that this phenotype is related to metabolic, hormonal and immune dysregulation along with systemic inflammation [430; 433]. Since no clinical trials have been undertaken in asthmatic children, more longitudinal studies are needed to elucidate the mechanisms that link metabolic dysregulation and systemic immune responses to lung function. Potential themes of interest for new research are:

a) The effect of a Mediterranean diet enriched with fatty fish on inflammatory markers (CRP, TNF-α, leukotrienes), cholesterol, HDL, LDL, triglycerides, and insulin resistance in asthmatic children.

b) The relationship between dyslipidaemia and hyperinsulinemia in normal weight and overweight asthmatic children.

c) The association of adipokines, adiponectin and pulmonary function in normo and overweight asthmatic children

d)The impact of the Mediterranean diet on normo and overweight asthmatic children.

Mediterranean diet

Given the magnitude of childhood asthma burden including individual and societal costs [26], then in regions where food is abundant and relatively inexpensive such as in Australia, the feasibility and sustainability of a ‘Mediterranean-type diet’ enriched with

fatty fish in Australian asthmatic children would test whether the benefits of the current intervention can be translated to a non-Mediterranean region.

The Australian guidelines for healthy eating are comparable to the Mediterranean-diet pyramid and with the addition of virgin olive oil as the main source of fat, such a diet plan could be easily adopted into daily family menus and culinary practices. Consumption of two serves of fish weekly is already recommended in the Australian dietary guidelines [467]. The benefits of a 'Mediterranean-type' dietary pattern extend beyond asthma and promises good health, well-being and protection against chronic diseases in adulthood [297];[199]. Another application of this dietary intervention study is to investigate whether pulmonary benefits and reduction in asthma symptoms can be translated to adults suffering with asthma, chronic obstructive pulmonary disease as well as children with cystic fibrosis. With respect to asthma phenotypes, the efficacy of a Mediterranean diet enriched with fatty fish in children with severe asthma who do not respond to glucocorticoid therapy would reap the benefits of an inflammation lowering diet such as this one.

The efficacy of a Mediterranean diet enriched with fish oil supplementation on pulmonary function in asthmatic children has yet to be challenged. This study would test the hypothesis whether omega-3 fatty acids from supplements within a dietary pattern could enhance metabolism, bioavailability and absorption of these fatty acids thereby conferring prophylactic and beneficial effects on pulmonary function and airway inflammation in children. Fish oil supplementation would be an alternative to fresh fish intake in regions where fish is not abundant and/or costly for families with economic hardship. However, supplementation should not replace fresh fish which apart from EPA/DHA contains a variety of bioactive molecules, protein of high biological value, vitamins, minerals and antioxidants with health beneficial effects that are important for child development [202]. Perhaps families with asthmatic children could be educated to better understand the benefits of regular fatty fish intake and supported in developing habits where few times in a month they are consuming at least one portion of fresh fish. With respect to cost, in most countries, the cheaper small varieties of fatty fish such as sardines, anchovies, trout and mackerel, fresh, frozen or farmed are rich in EPA/DHA [202] and affordable for most people. Nonetheless, plant-sourced omega-3 fatty acids are found in walnuts (1, 884 ALA mg/30g serve), chia seeds (2,685 ALA

mg/30 g serve) and flaxseeds (6915 ALA mg/30g serve) [204]. However, in humans, plant derived omega-3 fatty acid, ALA can be converted to long chain omega-3 fatty acids, EPA and DHA, although at a slow rate [204]. Thus, emphasizing the importance of regular fatty fish intake as a source of EPA/DHA with good bioavailability [204].

New targets for pharmacotherapy in asthma

Pharmacotherapy is the cornerstone of asthma management in children that focuses on the prevention of symptoms. Based on the findings of this PhD study, perhaps future therapies could focus on the prevention of inflammation and oxidative stress in airways by recommending an anti-inflammatory diet such as the Mediterranean diet enriched with fatty fish.

Vitamin D and childhood asthma

Hypovitaminosis D is common in asthmatic children. Vitamin D status may affect responsiveness to treatment and it is important to monitor in chronic diseases including asthma. It would be worth investigating the effect of vitamin D enrichment via dietary sources (such as fatty fish and fortified foods) and sunlight exposure [225] during outdoor play on asthma, pulmonary function and medication use in asthmatic children.
Given that vitamin D plays a pivotal role in immunity and inflammation [340], a study assessing the relationship between vitamin D status, inflammatory cytokines (IL3-5, TNF α, Th1/Th2), Ig E and airway function in paediatric asthma could provide insight on the underlying mechanisms of vitamin D in pulmonary disease.

Food Allergy and childhood asthma

Food allergy prevalence ranges from 3-35% in children [468] and is one of the factors known to increase asthma risk and is associated with increased daytime symptoms, hospitalization, emergency department visits, lung function deficits including high risk of anaphylactic symptoms [468]. Population studies have shown that early food sensitization/ or food allergy in the first year of life precedes the development of asthma [468]. Therefore early diagnosis, accurate identification of the offending food/ or food additive is necessary in order to avoid exposure. Intolerance to foods may be related to

naturally-occurring pharmacological agents, tyramine and histamine which are known to provoke Ig E-mediated immune reactions [469]. Tyramine and histamine are vasoactive amines found in a variety of foods including tuna, pickled herring, sardines, bananas, overripe fruits, fermented foods (cheese, alcoholic drinks, sausages, sauerkraut, canned fish), yeast extract (vegemite/marmite), chocolate, wine, spinach, and tomatoes as well as synthesized by gut bacteria [469]. A high intake of vasoactive amines tyramine and histamine can trigger acute respiratory symptoms of rhinitis, laryngeal edema, wheezing, bronchospasm and cough [468]. Hence, investigational strategies assessing the effect of elimination of these two amines from the diet of asthmatic children are worth pursuing. In terms of the Mediterranean diet, positive health outcomes with respect to chronic disease and longevity have been well-established [282], however the effect on food allergy in children is unknown. Novel to the literature and of interest would be a comparative study evaluating the prevalence of food allergy in children following a Mediterranean–style diet versus Western diet.

Microbiome and foetal nutrition

The importance of nutrition during pregnancy for 'early life programming' is well established and an important factor contributing to a favourable environment for the developing foetus including priming and regulation of immunity [470]. Promising data from observational studies has documented protective effects of the Mediterranean dietary pattern during gestation on asthma development in offspring [391]. However further exploration in well-designed randomized controlled trials are needed to provide more solid evidence before nutritional guidelines for the prevention of childhood asthma can be implemented. Recently, occurrence of atopy and asthma has been linked to the microbiome [128]. Diet can significantly impact the gut microbiota and metabolome. High-level consumption of plant foods consistent with the Mediterranean diet regime is associated with alterations in gut microbiota beneficial to overall good health [471]. Remarkably, microbiota modulation through consumption of diets rich in diverse plant foods such as the Mediterranean diet offers the prospect of improving health and mitigating disease risk. However, interplay of the Mediterranean diet, microbiome and respiratory health is less clear [472]. In light of this, more intervention studies investigating the strains of bacteria promoted by the Mediterranean diet regime and its effects on lung function in paediatric asthma are merited as well as the mode of action.

A feasible study would be the impact of the Mediterranean diet enriched with fatty fish on airway microbiome in asthmatic children. In addition, the utility of probiotics during pregnancy in primary prevention and as live bio-therapeutic agents in childhood asthma management is yet to be elucidated.

Application of 'omics' in asthma research

The implementation of the precision medicine in the management of asthma in clinical practice requires the detection of valid biomarkers, but currently available biomarkers are limited in number and precision [(275)]. The use of omics data appears a promising tool in asthma phenotyping [(275)]. Viewing the heterogeneity of asthma, the development of composite biomarkers from blood and urine could be an appropriate solution in clinical practice to predict therapeutic response. Further research and validation of emerging biomarkers are needed to define the molecular phenotype of asthma, to predict outcomes and therapeutic response to more specific targeted therapies.Topics for future investigation applying the 'omics' approach in asthma research are:

a) A comparison study of urinary organic acids and pulmonary function tests in asthmatic and non-asthmatic children.
b) A comparison study of serum fatty acids and pulmonary function in asthmatic and non-asthmatic children.
c) Determination of biomarkers associated with obese-asthma phenotype
d) The relationship between plasma folic acid and paediatric asthma

8.2 Conclusion

The findings of this clinical trial provide proof-of-concept evidence on the prophylactic role of a Mediterranean diet enriched with fatty fish on lung function and airway inflammation in children with mild asthma and adds to the current scientific knowledge. The Mediterranean diet enriched with fatty fish could be considered as an 'anti-inflammatory and anti-oxidant diet' for asthma. According to UNESCO, the Mediterranean diet is a sustainable, ecological-friendly dietary pattern promising optimum health, general well-being, free from future chronic disease. The present study has important public health implications because dietary habits are easily modified and this dietary intervention could be easily integrated into weekly family menus. In high risk families, simple dietary changes in early childhood would greatly support healthy eating habits, could reduce asthma symptoms in children and contribute to healthy weight gain. Following the Mediterranean diet enriched with omega-3 fatty acids from fatty fish might be a simple, cost effective non-pharmacological adjunct to conventional asthma therapy that could assist in reducing the burden of asthma in children, thus improving their quality of life and decreasing the societal financial burden. Collectively the data stipulate that vitamin D (25(OH)D $\geq$ 25 ng/mL) might support immune function as well as enhance the effect of a dietary intervention on pulmonary function in childhood asthma. Thus adding to the ever growing body of evidence on the importance of vitamin D in respiratory health. The magnitude of the overweight/obesity epidemic extending to Greek asthmatic children was emphasized in this clinical trial and underlines the need for urgent action from healthcare professionals in the development of public health strategies in promotion of the Mediterranean diet combined with daily physical activity in the battle against obesity along with asthma. Future robust clinical trials in Mediterranean as well as non-Mediterranean regions are recommended to replicate the promising findings documented in this PhD thesis as well as to provide evidence and the molecular pathways involved in preventing asthma onset in children with pre-existing asthma.

8.3 Key Message

Key message: *Given the overall health benefits of consuming fatty fish, the findings of this PhD clinical trial suggest that adoption of a healthy diet such as a Mediterranean-type diet combined with two meals of fatty fish per week could be an effective strategy in the management of asthma in children.*

What we don't know:

There are no universal dietary guidelines in the management of childhood asthma.

What this thesis adds:

Potential prophylactic effect of Mediterranean diet enriched with two meals of fatty fish per week (≥ 150 g fish fillet per meal) on bronchial inflammation associated with asthma in children. Could a Mediterranean diet enriched with fatty fish be the new 'anti-inflammatory and anti-oxidant diet for asthma?' Treating vitamin D insufficiency in asthmatic children could improve pulmonary function, reduce medication use and enhance the effect of dietary interventions. Abnormal spirometry and FeNO measurements are observed in overweight/obese asthmatic children.

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Appendices*

*Appendices are submitted as a separate file.