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The role of *Staphylococcus aureus* in allergic disease and cross-reactivity in fish allergy.

Studies in children and adolescents

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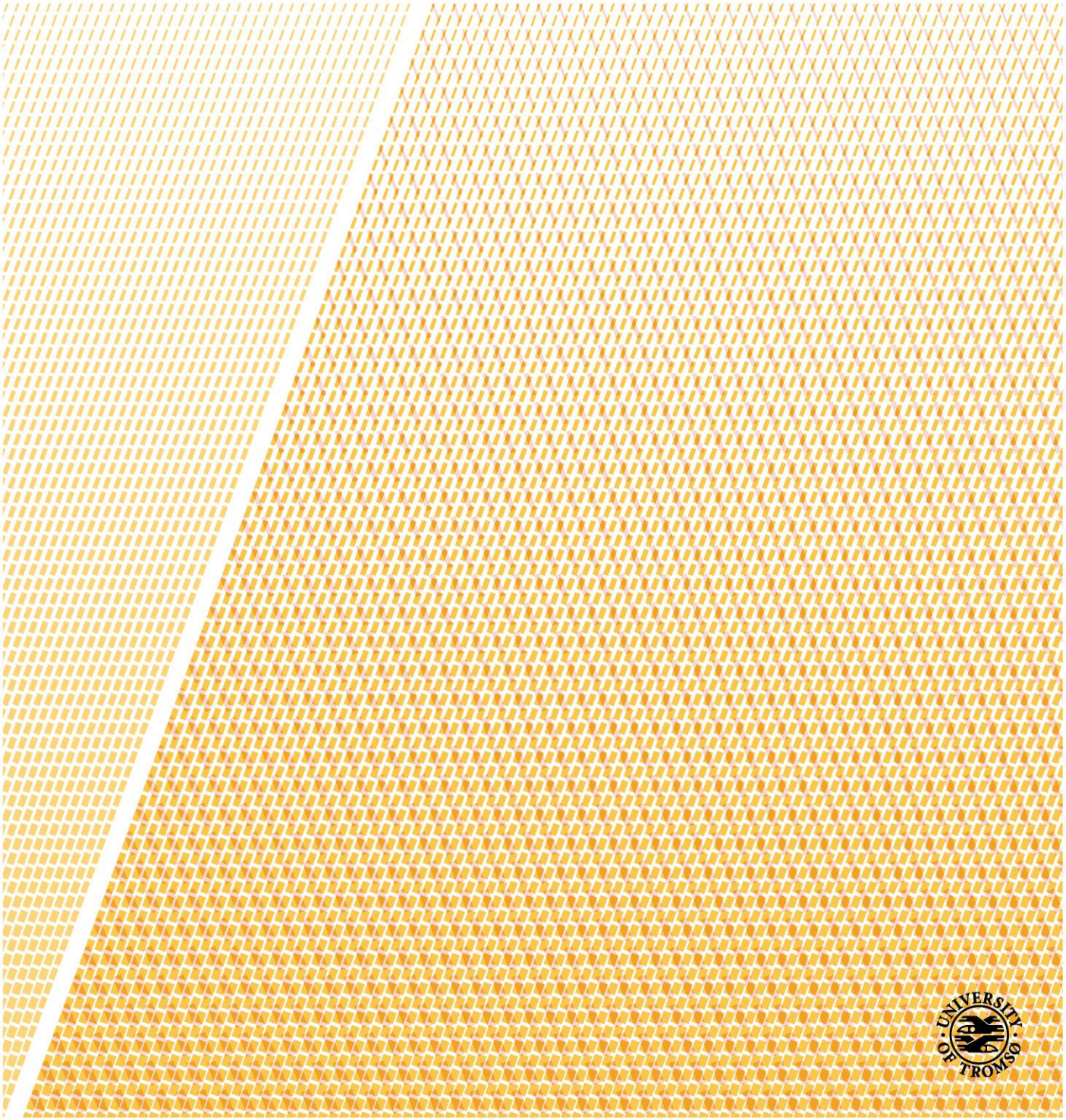

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List of papers

Paper I

Sørensen M, Wickman M, Sollid JU, Furberg AS, Klingenberg C. Allergic disease and *Staphylococcus aureus* carriage in adolescents in the Arctic region of Norway.

Pediatr Allergy Immunol. 2016; 27:728-735.

Paper II

Sørensen M, Klingenberg C, Wickman M, Sollid J.U.E, Furberg A-S, Bachert C, Bousquet J. *Staphylococcus aureus* enterotoxin-sensitization is associated with allergic poly-sensitization and allergic multimorbidity in adolescents.

Allergy. 2017 Apr 5. doi: 10.1111/all.13175. [Epub ahead of print]

Paper III

Sørensen M, Kuehn A, Mills C.E.N, Costello C.A, Ollert M, Småbrekke L, Primicerio R, Wickman M, Klingenberg C. Cross-reactivity in Fish Allergy: A Double-Blind Placebo-Controlled Food Challenge Trial.

J Allergy Clin Immunol. 2017 May 4. pii: S0091-6749(17)30741-8. doi: 10.1016/j.jaci.2017.03.043. [Epub ahead of print]

Abbreviations

AD; Atopic dermatitis

BAMSE; Swedish abbreviation for Children, Allergy, Milieu, Stockholm, Epidemiology

CI; confidence interval

DBPCFC; double-blind, placebo-controlled food challenge

ECA; The environment and childhood asthma study in Oslo

ED_{10}; Dose at which an allergic reaction would be elicited in 10% of the population

FE_{NO}; Exhaled Nitric Oxide

GA^2LEN; Global Allergy and Asthma European Network

IOW; The Isle of Wight study

IgE; immunoglobulin E

LOAEL; Lowest Observed Adverse Effect Level

MAS; The German Multicenter Allergy Study

NOAEL; No Observed Adverse Effect Level

OR; odds ratio

SAgs: Staphylococcal enterotoxin-like molecules

SE; Staphylococcal Enterotoxin

TFF; Tromsø Study Fit Futures

TRO-FAST; Tromsø Fish Allergy Study

Summary

Background and Aims:

Allergic diseases are common and the prevalence has been increasing worldwide over the past decades. Knowledge about aetiology, pathogenesis and risk factors are still lacking and diagnostic tools are suboptimal. The microbiota of the mucosa and skin is important for the development of the immune system, and *Staphylococcus aureus* colonization has been linked to the development of allergic diseases. Fish is a healthy nutrient and consumption of fish is increasing, but fish is also one of the most common food allergens. Cross-sensitization and cross-reactivity to multiple fish species are common among fish allergic patients, but some patients may be tolerant to one or more species.

The overall aim of this thesis is to contribute to the understanding of the development of multiple allergic diseases and multiple allergies in children and adolescents. My first aim was to gain novel insight in the epidemiology of allergic diseases in adolescents in the Arctic region of Norway. Moreover, I aimed to investigate how allergic diseases and multiple allergen sensitizations are associated with *S. aureus* carriage and enterotoxin-sensitization. My second aim was to contribute to novel insight in cross-reactivity between fish species in fish allergic children and adolescents sensitized to multiple fish species. Finally, I aimed to study the utility of existing and novel specific IgE (sIgE) tests in the diagnostic work-up of fish allergy, and to estimate threshold doses for allergic reactions to different fish species.

Material and Methods:

Prevalence`s of allergic diseases and associations to *S. aureus* were studied in a cross-sectional study including 868 third year high-school students in the municipalities of Tromsø and Balsfjord using a standardized questionnaire, clinical examinations, measurements of sIgE to multiple food and inhalant allergens, nasal *S. aureus* carriage and sensitization to staphylococcal enterotoxins (Tromsø Study Fit Futures 2). Cross-reactivity in fish allergy was studied in a clinical trial with double-blind, placebo-controlled food challenges with cod, salmon and mackerel, a questionnaire and measurement of sIgE to traditional allergen extracts and novel allergen molecules in 35 fish allergic children and adolescents (Tromsø Fish Allergy Study).

Results:

In the "Tromsø Study Fit Futures 2", the prevalence of current asthma, atopic dermatitis and allergic rhinitis were 11.9%, 10.4% and 26.0%, respectively. Around one in 10 had more than one allergic disease and the lifetime prevalence for any allergic disease was 45.1 %. More than 4 out of 10 participants were sensitized to at least one food or inhalant allergen. Nasal *S. aureus* carriage was found in half of the participants and was associated with the severity of allergic disease and allergic multimorbidity. Sensitization to staphylococcal enterotoxins was found in one fourth of the participants and was associated with allergic multimorbidity and poly-sensitization to food and inhalant allergens. In the "Tromsø Fish Allergy Study", we found tolerance to at least one fish species in 30% of fish allergic children regarding any allergic symptoms and in more than half regarding only objective allergic symptoms. Sensitization to standard fish allergen extracts and fish parvalbumins was found in nearly all participants, including participants with tolerance to certain fish species. Sensitization to species-specific enolase or aldolase was predominantly found in patients with objective allergic symptoms. However, sIgEs to enolase or aldolase were also negative in several patients with fish allergy. Specific IgE to cod extract >8.2 kU_A/L or salmon extract >5.0 kU_A/L discriminated best between non-tolerance and tolerance to at least one fish species. Estimated threshold doses for cod were in line with previously published data, and estimated threshold doses were higher for salmon and mackerel compared to cod.

Conclusions:

Allergic diseases are common among adolescents in the Arctic region of Norway. *S. aureus* carriage may play a role in disease severity and allergic multimorbidity, whereas sensitization to staphylococcal enterotoxins may play a role in poly-sensitization to food- and inhalant allergens and allergic multimorbidity. Cross-reactivity between different fish species is common among fish allergic patients, but tolerance to some species exists in around one third and should be identified in order to avoid unnecessary food restrictions. A combination of clinical history and sIgE to fish-allergen extracts and molecules may reduce the number of food challenges needed for specific diagnosis of fish allergy. Threshold doses for allergic reactions seem to be lowest for cod, compared to salmon and mackerel.

1 Introduction

1.1 Preface

The overall topic of this thesis is allergic diseases in children and adolescents. More specifically, a major issue is to study associations to the development of multiple allergic diseases and sensitization to multiple allergens. Patients with multiple allergies may either be sensitized to several structurally unrelated allergens, be primary sensitized to one key allergen and cross sensitized to one or more structurally related allergen(s) or a combination of these two mechanisms may exist. In paper I and II, we studied associations between the bacterium *Staphylococcus aureus* and allergic diseases and sensitization to multiple allergens. The study also provides valuable novel knowledge about the prevalence of allergic disease among adolescents in the Arctic region of Norway.

In paper III, we studied cross-sensitization and cross-reactivity to multiple fish species in children and adolescents with fish allergy. Fish allergic patients are commonly advised to avoid all species of fish. However, cross-sensitization may not necessarily result in clinical cross-reactivity. Furthermore, the gold standard in diagnosing food allergy, double-blind placebo-controlled food challenge (DBPCFC), is both time-consuming and potential harmful to the patient. Measurements of specific IgE (sIgE), together with skin prick test, are the primary methods for diagnosing sIgE-sensitization but these tests are less reliable than DBPCFC in diagnosing food allergy. In paper III, we also studied how sIgEs to fish allergen extracts and molecules perform in diagnosing fish allergy and/or tolerance compared to the results from DBPCFC. The study design also included a low dose DBPCFC protocol to allow us to estimate threshold doses for allergic reactions to different fish species. In the following part of the Introduction I will define and describe allergic diseases and concepts important for this thesis.

1.2 Atopy

"Atopy is a personal and/or familial tendency, usually in childhood or adolescence, to become sensitized and produce specific immunoglobulin E (sIgE) in response to ordinary exposures to allergens."[1]. Atopy describes the genetic predisposition to become sIgE-sensitized to allergens commonly occurring in the environment, but to which the majority do not produce a prolonged sIgE response. Common atopic diseases are asthma, rhinoconjunctivitis and atopic dermatitis.

1.3 Asthma

Asthma is a heterogeneous disease, usually characterized by chronic airway inflammation. It is defined by the history of respiratory symptoms such as wheeze, shortness of breath, chest tightness and cough that vary over time and intensity, together with variable expiratory airflow limitation [2]. Although asthma is a chronic inflammatory disease, the natural course is characterized by intermittent exacerbations and reversibility of symptoms and airway obstruction. Viral infections, cold air, exercise, allergens, psychological stress, and air pollutants including tobacco smoke typically trigger exacerbations [3]. The pathogenesis of asthma is complex and incompletely understood. Different phenotypes have been suggested [4, 5], but it is unclear whether they represent different subgroupings of a single disease or are separate diseases within the syndrome of asthma. Allergic asthma is related to IgE-sensitizations to inhaled allergens, whereas non-allergic asthma is also coined "intrinsic asthma". Asthma may result in chronic persistent airway inflammation unrelated to allergen contact and has features of autoimmunity. Chronic inflammation has been associated with airway remodelling with fixed airflow limitation as a result of "scarring" of the airways [6].

There is no cure for asthma, but patients benefit from inhalation therapy with little side effects. In spite of available good therapy, many patients with more severe asthma or failure to adhere to treatment remain uncontrolled. Mild asthma attacks usually respond well to the inhalation of short acting beta2-agonists and persistent asthma to inhaled corticosteroids. Leukotriene-antagonists, long acting beta2-agonists, anticholinergic drugs, theophylline and anti-IgE-antibodies can be added in more severe or therapy refractory cases [3]. Asthma treatment is monitored by symptoms and use of reliever medication, exacerbation history, measures of airflow obstruction and biomarkers such as exhaled nitric oxide [2, 6]. Education of children with asthma and their parents is effective in improving clinically relevant outcomes. Adherence to maintenance medication and correct use of the inhaler device are key factors in obtaining asthma control. To monitor asthma, monitoring symptoms appears to be sufficient, whereas home monitoring of lung function does not improve health outcomes in asthmatic children [7].

1.4 Allergic rhinitis

Allergic rhinitis is an inflammatory disorder of the nasal mucosa induced by an allergic immune response to inhaled allergens in sensitized individuals. The allergic immune cascade in the nasal mucosa may give rise to the following symptoms in a variable degree of severity

and duration: sneezing, itching, rhinorrhoea, or nasal congestion/obstruction, which frequently occur in conjunction with itchy, red and watery eyes (rhinoconjunctivitis). Symptoms may also affect the ears and throat and include postnasal drainage [8]. General symptoms like fatigue, impaired concentration and reduced productivity are all associated with allergic rhinitis. Eliciting allergens are most often pollens, animal dander, dust mites and mold spores, and symptoms may be seasonal (i.e. pollen allergy) or perennial (i.e. house dust mite allergy) [9]. Symptoms can be controlled by avoidance of the eliciting allergen(s) and by pharmacological therapies such as oral, intranasal and topical antihistamines, intranasal corticosteroids and leukotriene-antagonists, as mono-therapy or in combination [10]. Allergen immunotherapy is an additional potential treatment option, particularly for patients with more troublesome disease who remain inadequately controlled due to the allergen(s) being difficult to avoid and/or despite regular pharmacotherapy [10]. Allergic rhinitis and non-allergic rhinitis are independent risk factors for asthma, with the rhinitis frequently appearing prior to the asthma [11]. New methods for monitoring allergic rhinitis and asthma are now focusing on the implementation of emerging technologies for individualized and predictive medicine [12]. Mobile technology is developed for the management of rhinitis and asthma by a multi-disciplinary group and by patients [13].

1.5 Atopic dermatitis

Atopic dermatitis is a common skin disease often associated with other atopic disorders, such as allergic rhinitis and asthma. The clinical manifestations vary with age. It is a chronic, relapsing skin inflammation with disturbance of epidermal barrier function, dry skin and IgE-mediated sensitization to food and environmental allergens. Itching, that worsens at night causes sleep loss and impaired quality of life, leads to scratching and crusted erosions [14]. Multiple triggers, i.e. allergens, climate, infections, psychological stress, irritants and others, influence on the course of the disease. Typically, symptoms arise during the first year of life with great tendency to outgrow the disease during childhood, but some patients continue to have atopic dermatitis during adolescence and adulthood [15].

The lesions of atopic dermatitis can affect any part of the body but typically show age-related morphology and distribution. No specific laboratory or histological findings are typical for atopic dermatitis, and thus the diagnosis relies exclusively on clinical features. Children with atopic dermatitis have increased risk of developing cutaneous *S. aureus* infections and cutaneous viral infections such as disseminated herpes simplex infections and molluscum

contagiosum [16]. Up to 90% of patients with atopic dermatitis are colonized with *S. aureus* on their skin surface [17].

Filaggrin is a filament-associated protein that binds to keratin fibers in epithelial cells. It is important in the epidermal homeostasis, and filaggrin deficiency plays an important role in the pathogenesis of atopic dermatitis [18]. Filaggrin genotype, the skin microenvironment and environmental factors may contribute to decreased levels of filaggrin. Filaggrin deficiency increases the risk for microbial infection and development of other atopic diseases as asthma and rhinitis. Furthermore, cutaneous cytokine milieu or environmental influences including low humidity or mechanical damage are capable to secondarily modulate filaggrin expression [19].

At present, there is no cure for atopic dermatitis. Thus, the aim of management is to improve symptoms and achieve long-term disease control with a multistep approach. The main principles are continuous epidermal barrier repair with emollients, avoidance of individual trigger factors, and anti-inflammatory therapy with topical corticosteroids or calcineurin inhibitors [20]. In severely affected cases, phototherapy or systemic immunosuppressants are indicated. Treatment failure due to poor adherence is common and doctors should spend sufficient time to explain the disease and its treatment. Written action plans might be helpful.

A systematic review showed no preventive clinical benefit of anti-staphylococcal interventions such as antiseptic bath additives or soaps, or the addition of antimicrobial agents to topical therapies in non-infected atopic dermatitis [21]. However, in clinically superinfected atopic dermatitis, antiseptics may be sufficient to treat superinfected small areas, and are preferable to topical antibiotics with regard to the development of antibacterial resistance. More extensive superinfections often requires treatment with short courses of systemic antibiotics [21].

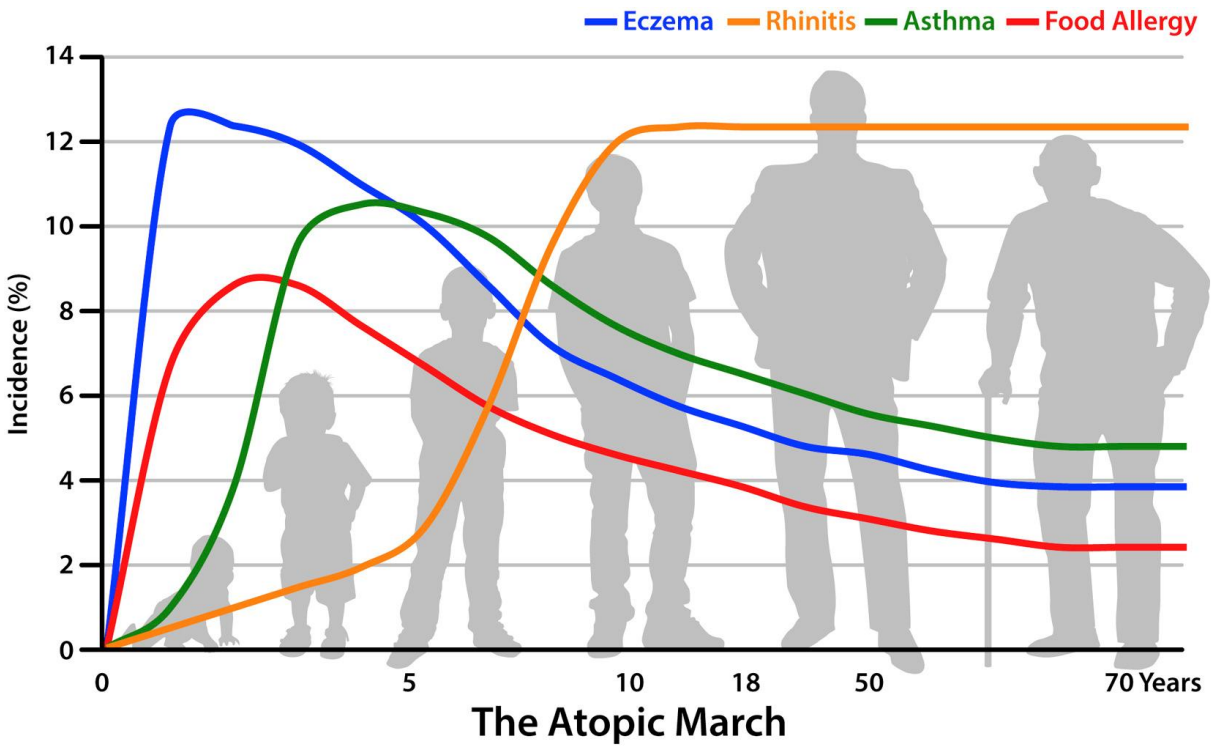
1.6 Allergic co-morbidity and the atopic march

Atopic dermatitis, asthma and allergic rhinitis develop dynamically throughout childhood, and allergic co-morbidity is common [15, 22-25]. Having atopic disease in the family and/or having one atopic disease, greatly increases the risk of co-morbidity with other atopic diseases. A large number of studies from different continents have presented data on atopic co-morbidity. In the BAMSE birth cohort study in Stockholm, co-morbidity increased with age. At 12 years of age, at least two allergy-related diseases affected 7.5% of all the children

[22]. Parental allergy was associated with increased atopic co-morbidity and persistent atopic disease. Moreover, it increased the risk of having any allergy-related disease up to 12 years of age. These findings indicate that allergy-related diseases should be neither seen nor studied as isolated entities [22].

Patients with atopic dermatitis may develop a typical sequence of food allergy, rhinitis, and asthma, at certain ages, known as the atopic march (Figure 1) [16, 26, 27]. Atopic dermatitis and food allergies often start in the first year of life, followed by asthma triggered by viral infections, allergic asthma and allergic rhinitis by school age. IgE sensitization, early onset and severity of atopic dermatitis are the main risk factors for progression and persistence of asthma [26]. In a Norwegian study, participants with atopic dermatitis at two years had more asthma at six years of age compared to participants without atopic dermatitis at two years of age (OR 1.80, 95% CI 1.10-2.96) [28]. A defect in the epithelial barrier integrity may contribute to the onset of atopic dermatitis and progression of the atopic march [29].

Figure 1. The atopic march.



The atopic march. Atopic dermatitis prevalence peaks early in infancy, probably opening the door for consequent development of the atopic march. Development of food allergy, asthma, and allergic rhinitis in the young toddler age group is common after cutaneous manifestations. The figure is printed with permission from Copyright Clearance Center's RightsLink® service.

1.7 Staphylococcus aureus

1.7.1 Bacterial characteristics and virulence factors

Staphylococci are Gram-positive, facultative anaerobic bacteria that grow most rapidly under aerobic conditions and in the presence of CO_2. The *Staphylococcus* genus includes at least 40 species, of which most are harmless and commensal bacteria on the skin and mucous membranes. Some staphylococci are able to produce the enzyme coagulase, an important feature used in classifications schemes of staphylococci. The by far most important and clinically relevant coagulase-positive *Staphylococcus* is *S. aureus*; a bacterium well equipped with potent virulence factors, survival fitness, and antimicrobial resistance determinants [30]. Some of the important virulence factors are staphylococcal enterotoxins (SEs); proteins with a common phylogenetic relationship, structure, function, and sequence homology. Over 20 SEs have been identified, including SE-A, SE-B, SE-C, SE-D, and SE-E [31]. SEs and staphylococcal enterotoxin-like molecules (collectively known as SAgs) are major virulence factors of *S. aureus*. Most *S. aureus* strains encode for and can produce SAgs when the opportunity arises [32].

1.7.2 C*linical aspects; infections and toxin-related disease*

S. aureus colonizes the nose and skin of approximately 50% of healthy individuals. Around 80% of *S. aureus* infections are caused by the carrier strain already present on the skin or mucosa of the patient [33, 34]. The clinical presentation of *S. aureus* infections may vary from minor self-limiting soft tissue infections to a life threatening systemic disease. Infections encompass skin and soft tissue infections, muscle and visceral abscesses, septic arthritis, osteomyelitis, endocarditis, pneumonia, brain abscesses, meningitis and bacteremia, as well as toxinoses with toxic shock syndrome (TSS), scalded skin syndrome, and food poisoning [35]. *S. aureus* is the cause of a large proportion of bloodstream infections worldwide [36]. Decreased susceptibility to antibiotics is a major concern and methicillin resistant *S. aureus* (MRSA) is associated with higher mortality, morbidity and financial costs compared to methicillin-sensitive *S. aureus* [37].

S. aureus is a major food-borne pathogen worldwide and a frequent food contaminant causing food poisoning by secretion of SE's [38]. Common symptoms of food poisoning are nausea, vomiting, diarrhoea and abdominal cramps [31]. SE-A is most frequently involved in food poisoning [39], whereas SE-B also is identified as a potential biological weapon of war and

terrorism [40]. Most outbreaks of food poisoning are due to improper food handling either in the food industry or in the home.

SAgs do not only cause vomiting and diarrhoea, but may also be highly lethal in humans [41]. They contribute to the pathophysiology in life-threatening infections with *S. aureus*, including sepsis, infective endocarditis, and necrotizing and haemorrhagic pneumonias. These infections have high mortality rates. Mortality is partly also dependent on the development of TSS, mediated by a complex interaction of SAgs with the host and resulting in extensive immune dysregulation and multi-organ dysfunction [41].

1.7.3 *S. aureus* carriage and allergic disease

The nose, throat and skin are the major sites of *S. aureus* carriage [42]. In the majority of patients with atopic dermatitis the skin is colonized with superantigen-encoding *S. aureus.* Furthermore, *S. aureus* abundance fluctuates and parallels clinical symptoms of atopic dermatitis. These observations have led to the "outside-inside model", stating that genetic skin barrier defect compounded by a skin microbiota dysbiosis is the primary pathogenic event of atopic dermatitis [43].

The role of infections and bacterial carriage in the pathogenesis of allergic rhinitis and asthma is less clear. Patients with allergic rhinitis have a high nasal carriage rate of *S. aureus* associated with aggravation of symptoms, possibly by promoting local IgE production to staphylococcal superantigens [44, 45]. There are conflicting data on a possible association between *S. aureus* carriage and asthma. In infants and preschool children, no clear association between *S. aureus* carriage and wheeze or airway inflammation has been shown so far [46]. However, in older children and adolescents *S. aureus* nasal carriage has been associated with increased risk of asthma and asthma exacerbations [47].

1.7.4 Sensitization to *S. aureus* enterotoxins (SEs) and allergic disease

SEs modulate the IgE isotype-switching leading to IgE sensitization to SEs [48]. IgE sensitization to SEs is associated with allergic disease. Local and/or systemic sIgE to SE may play a role in the development and/or disease severity of allergic disease [49-53]. SE-sensitization is associated with asthma in adults and in the elderly, but an association has not yet been clearly demonstrated among children and adolescents [49, 50, 54, 55]. In the German MAS study they found a possible relationship between sensitization to SE and asthma at the age of 20 years (OR 2.5, 95% CI 1.3-4.7), but the difference between SE-sensitized and non-

sensitized was no longer statistically significant after adjusting for potential confounders (OR 1.6, 95% CI 0.80-3.4) [50]. In a GA^{2}LEN study SE-sensitization was associated with asthma in adults (OR 2.10, 95% CI 1.60-2.76) and total IgE concentrations were higher in SE-sensitized compared to non-sensitized [49]. In a systematic review and meta-analysis, SE-sensitization was associated with asthma (pooled OR 2.95, 95% CI 2.28-3.82). Both children and adults were included in the review and rates of SE-sensitization increased with age and severity of asthma [56]. Some studies indicate an association between SE-sensitization and polyclonal allergen sensitization, reflected by higher total IgE levels among SE-sensitized individuals [49, 50]. However, the patterns and the magnitude of allergen poly-sensitization related to SE-sensitization have previously not been studied.

1.8 Allergen

An allergen is "a protein or glycoprotein capable of binding IgE". Most allergens are derived from naturally occurring allergen sources. Allergenicity is related to the conformational structure of the folded protein recognized by the Fab part of the IgE molecule [57]. A protein consists of a defined amino acid sequence and a three-dimensional structure, but may have several variant proteins with slight differences in the amino acid sequence or with other small modifications [58]. Antigen determinants (epitopes) are localized regions on the surface of an antigen that are capable of binding IgE. Drugs, chlorhexidine and other pharmacological compounds are examples of non-protein allergens. The mucosal surfaces of the airways (inhalant allergens) and the digestive tract (food allergens) are the two most common sites of entry into the body.

Allergen sources vary from highly complex structures such as pollen, animal hair and dander, house dust mites or foods to single molecules such as chemicals or drugs [59]. Protein families share common evolutionary origin reflected by similarity in the overall structure, topology and amino acid sequences. Allergen proteins are limited to a small number of protein families. Common examples are the *2S albumins* in tree nuts, peanuts, legumes and seeds, the *profilins* in pollen from tree, grass and weed, fruits, vegetables and latex, the *tropomyosins* in crustaceans, molluscs and mites and the *parvalbumins* in fish.

The nomenclature of allergens are standardized and based on the scientific name of the plant or animal species from which the allergen originates [60]. For example, the major allergen from Atlantic cod, Gad m 1, is named after the scientific name of the fish Gadus morhua, in which Gadus is the genus and morhua the species. The first three letters of the genus (Gad)

and the first letter of the species (m) together form the allergen name, followed by a number given in order of discovery of the allergen from the same allergen source. Different allergens from the same allergen source may represent different protein families; Gad m 1 is a parvalbumin, Gad m 2 an enolase, and Gad m3 an aldolase.

In allergy testing the sensitization of a patients is often assessed according to whether sIgE binds to an allergen. These allergens may contain a mixture of many, and not completely purified allergens; in this thesis coined **allergen extracts.** Over the last two deacdes it has been possible to extract native allergens (components) from allergen sources, and to produce recombinant high purity allergens in the laboratory . These purified recombinant allergens, in this thesis coined **allergen molecules**, are currently used in the new diagnostic concept termed component resolved diagnosis (CRD).

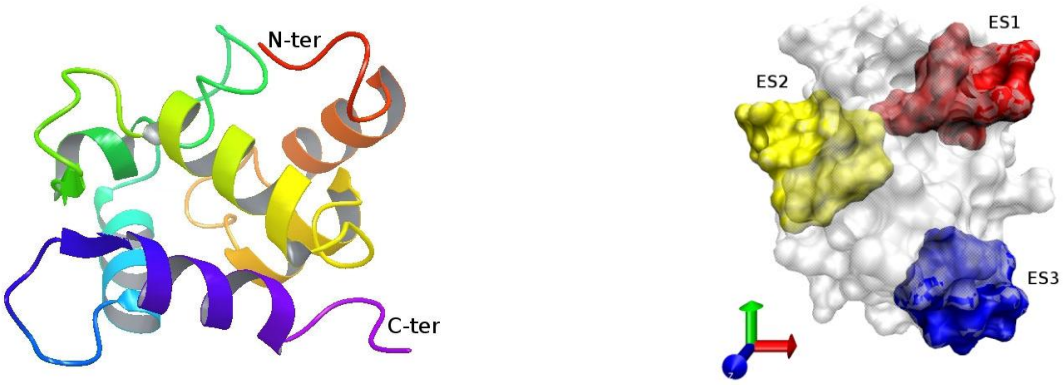
Cross-reactivity occurs when sIgE antibodies originally raised against one key allergen bind to a similar protein in another allergen, whereas co-sensitization describes multiple, unrelated sensitizations to several structurally unrelated allergen groups [61, 62]. Sensitizing allergens have the ability to induce allergen-specific IgE antibodies, whereas non-sensitizing allergens only can cause allergic symptoms if previous contact with a related (cross-reactive) allergen has caused sensitization. The birch pollen allergen Bet v 1 is a common sensitizer whereas the homologous apple allergen Mal d 1 is a non-sensitizer [63].

1.9 Fish Allergens

Parvalbumin

Parvalbumin, the major fish allergen, is a stable calcium binding fish muscle protein of low molecular weight, resistant to heat, chemical denaturation and proteolytic enzymes [64-67]. Aas et al identified the first fish parvalbumin, Gad c 1, in Baltic cod in the late 1960`s [64]. Subsequently, parvalbumins from many fish species have been identified and described in detail [68-71]. By 2014, the official database of allergens, contained 21 parvalbumins from 12 fish species and the Allergome database lists more than 100 entries for fish parvalbumins and their isoforms [72]. Only parvalbumin allergen molecules from carp and cod (Cyp c 1 and Gad c 1) are commercially available for sIgE testing (Figure 2).

Figure 2. Cod parvalbumin with epitopes. Crystallographic illustrations kindly provided by Annette Kuhn.



Immunological cross-reactivity between parvalbumins

Only beta-isoforms of fish parvalbumins are allergenic. High amino acid sequence homology in different fish species is the cause of cross-reactivity between species [73, 74]. However, some fish allergic patients may tolerate certain fish species [75-77], reflecting that heterogeneity in parvalbumin structure may cause differences in clinical allergic reactions to different fish species. In patients with cod allergy, sensitization to cod, salmon and pollock occur more frequently than sensitization to halibut, flounder, tuna and mackerel. IgE binding patterns are more similar among phylogenetic closely related fish species with parvalbumins of high amino acid sequence homology [73]. Even minor differences in amino acid sequence in salmon and trout parvalbumin, compared to other fish species, may result in mono-sensitization to salmonid fish species [78]. Furthermore, regional differences in food culture may lead to different patterns of sensitization and different species of fish responsible for allergy [79].

Parvalbumin content in different fish species

The amount of parvalbumin in fish muscle is related to the allergenic properties. Parvalbumin levels differ considerable between fish species. In raw fish, parvalbumin levels decreases significantly in the following order: herring > carp > redfish > salmon/trout > cod > mackerel > tuna. The difference between herring and tuna vary by a factor of 100 [80]. Parvalbumin content also differs in different parts of a fish. Parvalbumin content in dark muscle is significantly lower than in white muscle, and dark fish muscle is much less allergenic than the white muscle [81]. Tuna parvalbumin is only measurable in the white tissue muscle [80]. White muscle is used for rapid movements and dark muscle for continues swimming. Active

fish species, such as tuna and mackerel, have a higher proportion of dark muscles than bottom dwelling fish species, such as cod and flounder that have high contents of white muscle.

Stability of parvalbumins

The stability of proteins is a characteristic that affects the allergenic properties of an allergen. Unstable proteins are denatured during processing of food or soon after ingestion. Consequently, they are less allergenic than stable proteins that conserve their structure during processing and digestion. Parvalbumins are highly stable proteins resistant to heat, chemical denaturation and proteolytic enzymes [64, 73]. However, parvalbumin levels are lower in processed food such as pickled, canned or smoked fish due to protein denaturation caused by processing conditions such as low pH, high pressure and high temperature. Simple boiling is a mild food processing reaction that does not change parvalbumin content considerably [80]. As a result, most fish-allergic patients have allergic reactions after ingestion of cooked fish. Allergens are even conserved and transported as airborne particles in vapour from cooking and fish allergic patients may experience allergic reactions after inhalation of allergen aerosols [82].

Parvalbumin and specific allergen immunotherapy

So far, no allergen immunotherapy is available for treating fish allergy. Recombinant carp parvalbumin is by some researchers regarded as a major cross-reactive fish allergen and a possible tool for both diagnosis and immunotherapy of fish allergy [74]. A hypoallergenic drug product with conserved immunogenicity based on recombinant carp parvalbumin (rCyp c 1) has been developed, and from 2015 studied in a safety phase I/IIa clinical trial on immunotherapy in fish-allergic patients [83].

Enolase and aldolase.

Kuehn et al identified enolases and aldolases, native oligomers which are labile to thermal treatment, as important fish allergens in cod, salmon and tuna [84]. She also suggested that fish-allergic patients may be divided into three clusters based on their IgE sensitization patterns to fish allergen molecules. In the first cluster (58.1 %), patients were sensitized to all three parvalbumins, and a significant proportion to enolase (80.6 %) and aldolase (58.3 %). These patients were allergic to multiple fish species. In the second cluster (14.5%), most patients were sensitized only to salmon parvalbumin. These patients reported clinical reactivity exclusively to salmon and with milder symptoms than patients in the first cluster. In

the third cluster (27.4 %), patients were not sensitized to parvalbumins, but reported clinical reactivity to one or several fish species. Specific IgE's to enolase, aldolase or fish gelatin were detected in 70% of patients in the third cluster. Their allergic symptoms ranged from mild to severe and 76% reported tolerance to single fish species. Interestingly, five patients in this study [84] reacted to still unidentified allergens assumed by the presence of IgE-reactive bands in immunoblot. The authors concluded that a high proportion of fish-allergic patients are not sensitized to parvalbumin and the use of additional allergens such as enolase, aldolase and possibly fish gelatin may be of great importance. However, the clinical significance of these allergens should be further assessed in studies were sIgE reactivity is compared with results from oral food challenges with different fish species [84].

Collagen/Gelatin.

Hamada et al identified collagen as a possible important fish allergen [85]. Native collagen is composed of three α-chains twisted together in a triple helix and is found as a large extracellular matrix protein in animals. Collagen is denatured to a mixture of protein fragments (gelatin) by heating and digestion with muscle proteases. There is no antigenic cross-reactivity between collagens from fish and other animals, indicating that fish collagen contains IgE-binding epitopes with amino acid sequences, which are not found in collagen molecules from other animals. However, IgE cross-reactivity to heated extracts from five species of fish indicates that collagen is commonly allergenic regardless of fish species. Sakaguchi et al also found that some fish-allergic patients showed IgE reactivity to fish gelatin and concluded that fish gelatin might be an allergen in fish allergy [86]. In another study, only three of 100 serum samples from fish-allergic or fish-sensitized patients gave evidence of reactivity to gelatin extracted from tuna skin [87]. In a randomized, double-blinded, placebo-controlled oral challenge with fish gelatin, none of 30 fish allergic patients reacted adversely to a cumulative dose of 3.61 g gelatin [88]. The relevance of fish gelatin as a food allergen is therefore still controversial and the results from studies are diverging. Meanwhile, fish gelatin used in the food industry is increasing since bovine and porcine gelatin used as additives in vaccines have been linked to anaphylactic reactions. In a case-report, a 12-year-old boy had a severe anaphylactic reaction after ingestion of marshmallows containing fish gelatin. He was allergic to different fish species and sensitized to tuna, salmon and cod. Extensive in vivo and in vitro testing proved that anaphylaxis was elicited by fish gelatin [89].

Other fish allergens.

Vitellogenin is identified as an allergen in Beluga caviar allergy and several case-reports on allergy to fish roe has been reported [90].

Fish allergens; diagnose and treatment of fish allergy.

The allergenic properties of a wide number of fish allergens are characterized in detail. However, this knowledge has not yet resulted in significant improvements when it comes to diagnosing and treating fish allergy. We still depend on oral food challenges to establish a clear diagnosis in many patients and avoidance of the offending allergen is still the only clinical approach available. The ongoing trials with specific allergen immunotherapy with hypoallergenic carp parvalbumin may be a step forward in the treatment of fish allergy [83]. More research is needed to address the role of parvalbumin, enolase, aldolase, gelatin and other possible fish allergens. One aim is to develop more precise diagnostic tools to better discriminate between allergy and tolerance to different fish species, and thereby avoid unnecessary food restrictions. Another aim is to develop effective and safe allergen immunotherapy that can induce tolerance to fish in fish-allergic patients.

1.10 Food allergy

Adverse reactions to foods include different reactions with different mechanisms including toxic, enzymatic and hypersensitivity reactions. Food allergy refers to the subgroup of food hypersensitivity reactions in which immunologic mechanisms have been demonstrated. An US expert panel defined food allergy as ‘‘an adverse health effect arising from a specific immune response that occurs reproducibly on exposure to a given food’’, and food intolerance as “non-immune reactions that include metabolic, toxic, pharmacologic, and undefined mechanisms” [91]. A food intolerance occurs when a person has difficulty digesting a particular food. This can lead to symptoms such as intestinal gas, abdominal pain or diarrhoea. Food intolerance is sometimes confused with or mislabelled as food allergy. However, food intolerances mainly involve the digestive system whereas food allergies involve the immune system. With a food allergy, even a very small amount of the food has the potential to lead to anaphylaxis. The most common food intolerances are lactose intolerance and gluten intolerance. Symptoms from food intolerance and allergy may still be overlapping, creating diagnostic dilemmas [91].

Food allergies may be IgE mediated, non-IgE mediated, or a combination of both. IgE mediated food allergies most often cause immediate symptoms and have the potential to cause anaphylaxis. Non-IgE mediated food allergies are cell mediated with more delayed symptoms. The pathophysiology of both mechanisms is through food antigen sensitization and Th2 skewing of the immune system [92]. Food allergies are believed to be a result of either loss of oral tolerance or the failure to induce tolerance. The mechanisms of the development of oral tolerance is a complex interaction between ingested food proteins and antigen-presenting cells leading to suppression of cellular and humoral immune responses [93]. Non–IgE-mediated immunologic reactions include food protein–induced enterocolitis, proctocolitis, and enteropathy syndromes that primarily affect infants or young children. The symptoms are vomiting, abdominal cramps, diarrhoea, and occasionally blood in the stool and failure to thrive or poor weight gain. Eosinophilic esophagitis and atopic dermatitis are examples of co-morbidities with mixed IgE- and non–IgE-mediated causes.

The clinical presentation of food allergy may involve symptoms from the skin (urticaria, angioedema, and atopic eczema), the gastrointestinal tract (vomiting, abdominal pain, diarrhoea, and constipation), the respiratory tract (rhinorrhoea, sneezing, cough, and dyspnoea) and the circulation (hypotension and cardiac collapse). Reactions are elicited by food ingestion, inhalation of vapour from cooking, or skin contact [94]. The most common food allergen sources are egg, milk, peanuts, tree nuts, soy, wheat, shellfish, and fish [95]. Childhood food allergies to egg, milk, wheat and soy typically resolve during childhood, whereas allergies to tree nuts, peanuts, shellfish and fish most often are persistent [96].

Depending on the route of sensitization, food allergies are either a result of reactivity to ingested allergens or the result of secondary sensitization to cross-reactive inhalant allergens due to primary sensitization to homologous pollen allergens via the respiratory tract [97]. The former group of allergens are often resistant to heat, degradation and digestion, whereas the latter group are mainly labile and easily degradable. The type of allergens to which an individual is sensitized influence the clinical manifestation. Primary sensitization to food allergens has the potential to induce more severe reactions compared to food allergies due to primary sensitization to pollen allergen, which often induces symptoms restricted to the oral cavity.

1.11 Prevention of food allergy

Different strategies for prevention of food allergy have been recommended. The American Academy of Paediatrics recommended in 2000 that infants at higher risk of allergy should delay the introduction of 'more allergenic' foods in their diet, including avoidance of eggs until two years, fish and nuts until three years of age [98]. Since then, increasing evidence has shown that early introduction of solid food in fact may prevent development of allergies [99-102], whereas late introduction is associated with increased risk of allergy [99, 100]. In a recent review the authors concluded that the findings of dietary interventions to prevent allergy are unconvincing, inconsistent or not adequately tested and that numerous questions remain about how to implement early food introduction, and which groups of infants should be targeted [105]. However, it is clear that the paradigm has shifted from recommending avoidance of common food allergens in infancy, to consideration of early consumption strategies to prevent allergy development.

Several studies have also tried to restore a more "healthy" gut microbiota by giving probiotics, either as a supplement or in infant foods in order to reduce allergy [103]. A moderate benefit has been reported for the prevention of atopic dermatitis, but no preventive effect has been shown for other allergic disease [103] . So far, no expert bodies do generally recommend probiotics for allergy prevention. However, a recent meta-analysis shows some protective effect on developing atopic disease (RR 0.71; 95% CI 0.57-0.89) and food hypersensitivity (RR 0.77; 95% CI 0.61-0.98) if probiotics were administered prenatally to the pregnant mother and postnatally to the child. When probiotics were administered either only prenatally or only postnatally, no effects on atopy and food hypersensitivity were observed [104].

1.12 Fish allergy

Fish is an important healthy human nutritive but also one of the most common food allergen sources, together with milk, egg, peanuts, tree nuts, soy, wheat, and shellfish [106]. Sensitization to fish starts during childhood and patients often remain allergic throughout their life. Fish and shellfish are among the food groups most commonly provoking severe food anaphylaxis [107]. Fish allergy is especially prevalent in fish-eating, fish-processing and coastal countries like Norway, Greece, Spain and Japan. The prevalence of fish allergy varies between 0.1% and 2.2% in different countries measured with different methods [106, 108]. In one survey of parents in Norway, adverse food reactions were reported in 35% of their

children, nearly 3% of whom had a reported reaction attributable to fish by the age of two years [109]. Studies based on sensitization to allergens and in particular studies based on self-reported allergy, tend to overestimate the prevalence of clinical allergy.

Symptoms of fish allergy range from oral pruritus, urticaria, angioedema, nausea, abdominal pain, vomiting, diarrhoea, and asthma to systemic anaphylaxis in some cases. Symptoms can be elicited by ingestion, inhalation of vapour from cooking and skin contact. Exposure to high concentrations of fish allergens in the fish industry is a high-risk factor for occupational fish allergy. IgE mediated fish reactions are most common and appear within minutes to an hour after exposure. Fish can also be the cause of food-dependent exercise-induced anaphylaxis [110], and may be the cause of food-protein-induced enterocolitis syndrome (FPIES), a non IgE-mediated reaction mainly in young children [111].

Clinical cross-reactivity between fish species is very common and patients with fish allergy are often advised to avoid all fish species. In a Norwegian study, ten adult patients with a clear history of fish allergy and sensitization to fish allergens, were tested regarding sensitization to recombinant parvalbumin from cod, salmon and mackerel. The study showed that cod, salmon, pollock, herring and wolfish contained the most potent cross-reacting fish parvalbumins, whereas halibut, flounder, tuna and mackerel were the least allergenic. No food challenges were performed because of previous severe reactions/anaphylaxis [73].

Only a few small clinical trials have studied cross-reactivity using DBPCFC with different fish species. Bernhisel-Broadbent studied eleven children and young adults, and only two (18%) had objective reactions to all three fish species tested [75]. In this study, the utility of fish extracts in the diagnosis of fish allergy was investigated both by using skin prick test (SPT) and by measure of specific IgE to fish allergen extracts. They concluded that SPT and in vitro evidence of IgE-specific cross-reactivity not necessarily correlate with symptomatic fish allergy. They did not use the new fish allergen molecules for sIgE testing. Helbling studied nine participants with fish allergy, and only three (33%) experienced objective symptoms to all three fish species [77]. Compared to the positive challenges, predictive accuracy of skin prick test and specific IgE with fish allergen extracts was 84% and 78%, respectively. They concluded that clinically relevant cross-reactivity among various species of fish might exist, and advised fish-allergic subjects to avoid all fish species until tolerance to specific species can be proven safe by food challenge.

In a study based on self-reported fish allergy, Swoboda et al have shown that recombinant parvalbumin from carp fish reacted with IgE from all fish-allergic patients tested (n=60), induced specific and dose-dependent basophil histamine release, and contained most of the IgE epitopes present in natural allergen extracts from cod, tuna and salmon. They therefore concluded that carp parvalbumin can be used to identify patients suffering from IgE-mediated fish allergy in general [74]. However, the high degree of cross-sensitization with parvalbumins from other fish species makes it a poor test in order to identify clinical tolerance to some fish species. As for other food allergies, there is no cure for fish allergy, and patients have to avoid fish in their diet.

Threshold dose distributions for fish species have only been published for cod in the EuroPrevall project [112]. Estimated dose eliciting a reaction in 10% of the study population (ED_{10}) for cod protein was 0.2 mg (95% CI 0.005-8.2) for subjective symptoms and 27.3 mg (95% CI 5.3-171.2) for objective symptoms. No estimates have so far been published for other fish species, thus data are lacking as to whether threshold doses may vary between different fish species.

1.13 Anaphylaxis

Anaphylaxis is a severe, life-threatening generalized or systemic hypersensitivity reaction, affecting around 1 in 300 of the European population at some time in their lives. A consensus clinical definition states that anaphylaxis is highly likely when any of the following three criteria is fulfilled [113]:

1. Acute onset (minutes to several hours) with involvement of the skin, mucosal tissue, or both (e.g. generalized hives, pruritus or flushing, swollen lips–tongue–uvula), and at least one of the following:

a. Respiratory compromise (e.g. dyspnoea, wheeze, stridor, reduced peak expiratory flow (PEF), hypoxemia)
b. Reduced blood pressure (BP) or symptoms of end-organ dysfunction (e.g. syncope)

2. Two or more of the following that occur rapidly after exposure to a likely allergen:
a. Involvement of the skin–mucosal tissue (e.g., hives, flushing, swollen lips–tongue)
b. Respiratory compromise (e.g. dyspnoea, wheeze, stridor, reduced PEF, hypoxemia)
c. Reduced BP or associated symptoms (e.g. collapse, syncope, incontinence)

d. Persistent gastrointestinal symptoms (e.g. crampy abdominal pain, vomiting)

3. Reduced BP after exposure to known allergen for that patient (minutes to several hours):
a. Infants and children: low systolic BP (age specific) or > 30% decrease in systolic BP
b. Adults: systolic BP of < 90 mmHg or > 30% decrease from that person's baseline.

In a systematic review on anaphylaxis in Europe, the key triggers were foods, medications, stinging insects and latex [114]. However, in up to 20% of cases the elicitor is not identified. Foods are the most frequent cause of anaphylaxis in children, with pollen allergy and asthma being important risk factors [114]. In central Europe the most common elicitors in a mixed child- and adult population were insect stings (50%), food (24%) and drugs (17%) [115]. Among food elicitors, the most common were peanuts and legumes (21.5%), followed by animal derived food (20.3%; cow`s milk, hen`s egg, fish, shellfish and meat), tree nuts (19.7%), vegetables (7.8%) and fruits (7.4%). Co-factors may increase the risk of an allergic reaction occurring or the severity of the reaction. Co-factors have been described in nearly 20% of young patients in a prospective register-based study [116]. They include exercise, fever, acute infection, premenstrual status and emotional stress. Non-steroid anti-inflammatory drugs (NSAIDs) and alcohol also seem to enhance some food allergic reactions. Exercise is a mandatory factor in exercise-induced anaphylaxis (EIA) and food-dependent, exercise-induced anaphylaxis (FDEIA) which are more common in adults than in children. The association with exercise is crucial for the onset of symptoms or signs, but EIA is not fully reproducible so that the same exercise may not always result in anaphylaxis [114].

Intramuscular adrenaline is the first-line-intervention for anaphylaxis, followed by oxygen, fluid support, inhaled beta2-agonists, systemic antihistamines and oral or intravenous glucocorticosteroids [117].

1.14 Atopic disease, heredity and epigenetic regulation

The risk of a child developing an IgE-mediated allergy is 40–60% if both parents have atopic disease compared to 5–10% if neither of the parents have atopic disease. Associations between several gene loci and asthma, high IgE levels, and other allergic conditions have been reported. However, no single specific genetic marker for atopy has been identified, indicating that atopy is a complex polygenic disorder, influenced by multiple disease genes [59, 118]. Atopic diseases are the result of complex interactions between genetic predisposition and environmental factors. Epigenetic regulation is the exposures to

environmental, nutritional, and lifestyle factors that contribute to either disease development or protection by directly or indirectly modulating and modifying the accessibility of the gene transcription machinery. One such mechanism is CpG methylation directly occurring on the DNA sequence, whereas other mechanisms operate on the chromatin structure, such as biochemically modified histone protein tails which subsequently allow or deny access of transcription factors to gene promoter regions [119]. Microbes play an important role early in life, and allergy protection has been associated with exposure to or growing up in traditional farming environment [120]. Exposure to microbes in early childhood is also associated with lower risk of respiratory allergic disease later in life. These protective effects are most likely partly mediated through epigenetic modifications.

1.15 Prevalence of atopic disease

In recent decades, there has been a worldwide increase in prevalence of asthma and allergic diseases with different regional patterns. The highest prevalence's are seen in western, high-income countries. Worldwide time trends indicate that international differences are decreasing with increase in low-income countries in Africa, Latin America and parts of Asia and decrease in some western countries [121]. However, asthma continues to be a major public health concern worldwide [122]. In the BAMSE cohort study in Stockholm, 58% of the children had had atopic dermatitis, asthma and/or allergic rhinitis at some time by the age of 12 years [22]. Between four and eight years of age the proportion of children sensitized to any of eight inhalant allergens increased from 15% to 25% [123]. In a recent study using population based incidence rates in Denmark and Sweden, atopic dermatitis, asthma and allergic rhinoconjunctivitis affect nearly one third of the children at five years of age. Time trends showed the incidence of atopic dermatitis to be stable, the incidence of asthma increasing in Sweden, but decreasing in Denmark, and the incidence rate of allergic rhinoconjunctivitis to be decreasing [124]. In a Dutch population-based cohort study, the incidence rate of physician-diagnosed asthma in children was 6.7 per 1000 person year from 2000 to 2012. The asthma incidence was increasing until 2008, but showed a non-significant decrease from 2008-2012 [125]. In a Norwegian birth cohort study, the lifetime prevalence of asthma was 20.2% and allergic sensitization 29.3% [126]. In the northern subarctic region of Norway, increasing prevalence of asthma, allergic rhinoconjunctivitis and atopic dermatitis among school children were shown in three surveys during the period 1985-2008. Prevalence of asthma increased from 7.3% to 17.6% and allergic rhinoconjunctivitis from 15.9% to 24.5% [127, 128]. Within this region, major differences were shown between Russian and

northern Norwegian school children [129]. Atopic diseases were reported in 38.7% of Norwegian children versus 24.2% of Russian children, atopic dermatitis in 23.9% versus 7.9%, allergic rhinitis in 20.6 % versus 14.7% whereas self-reported asthma was similar in both areas, 12.3% versus 13.1%.

Prevalence's of allergy-related diseases tend to decrease during adolescence. In the Norwegian birth cohort study there was a decrease in bronchial hyper-responsiveness measured with metacholine challenges between the ages of 10 and 16 years [130]. However, in Uppsala the estimated prevalence of exercise-induced bronchoconstriction in a cross-sectional study among 12-13 years old adolescents was as high as 19.2% [131]. Despite increasing incidence of asthma, hospital admissions due to asthma exacerbations decrease, probably due to improved care of children with asthma or a real reduction in asthma exacerbations [132].

Food allergy varies by age groups and regions and there is a marked difference between self-reported and challenge-verified prevalence of food allergy. In a prospective study from birth to the age of two year, the cumulative incidence of parent reported adverse reactions to food was 35% [109]. In a systematic review and meta-analysis of food allergy in Europe the overall lifetime prevalence of self-reported food allergy was 17.3% [106]. Point prevalence for self-reported food allergy (5.9%), positive skin prick test to at least one food (2.7%), positive sIgE (10.1%) but challenge-verified food allergy (0.9%) were lower. The highest prevalence was seen in North Western Europe and in children compared to adults. Low prevalence of self-reported and confirmed food allergy was found in Southern Europe, while sensitization was similar to other regions. Highest prevalence in children is for cow´s milk where the pooled lifetime self-reported prevalence is 6% and the point self-reported prevalence 2.3 %. Prevalence of allergy to cow`s milk and egg is higher in younger age groups and allergy to peanut, tree nut, fish and shellfish higher in older age groups and in Northern Europe. Although data on the time-trends of food allergy were weak, the prevalence appeared to be increasing [106]. Moreover, progression of sensitization may shift from foods of animal and plant origin over to pollen and animal allergens during childhood [123].

1.16 Diagnostic work-up of food allergy

1.16.1 Clinical history and examination

Knowledge about route of exposure, type and severity of symptoms and a careful dietary history is fundamental for the diagnosis of food allergy [94]. The history may identify the potential allergen source and indicate whether an IgE mediated or non-IgE mediated mechanism is involved. The clinical examination should include nutritional status and growth, especially in children, as well as examination for other atopic diseases. In relation to an ongoing allergic reaction, attention should be on examination of the skin and oral cavity, the gastro-intestinal, respiratory and circulatory systems.

1.16.2 Skin prick test and serum specific IgE tests.

Skin prick test and serum specific IgE are the first-line tests to assess IgE sensitization. However, these tests cannot accurately diagnose food allergy since sensitization to an allergen may exist without the patient experiencing allergic symptoms after exposure to the same allergen. It is of crucial importance that the results of these tests always are interpreted in relation to the clinical history, and the diagnosis of food allergy should not be based on the results from these tests only. The skin prick test is performed on the forearm or on the upper back. Negative and positive controls are required and the maximum wheal diameter is reported with a positive cut-off diameter ≥ 3 mm after 15 min [133]. There are numerous variables to be considered (e.g. lancet type, recording of wheal diameter, age, sex, site of testing), and only trained health care professionals should perform and interpret the results of the skin prick test [133].

The discovery of IgE in 1967 [134], allowed researchers and allergists to improve the diagnosis of IgE-mediated diseases. The first generations of IgE-assays were based on allergen extracts, which is a mixture of allergenic and non-allergenic compounds derived from natural sources (e.g. a pollen extract). They are still widely used in the diagnostic work-up of allergies. However, because extracts contain a mixture of allergens from an allergen source, they cannot tell to which specific allergen an individual is sensitized. Over the last two decades, an increasing number of allergen molecules have been identified and used in the new diagnostic concept termed component resolved diagnosis (CRD) of allergy. CRD provides a more individualized and stratified diagnosis and improves the sensitivity, specificity and clinical performance of the laboratory assay. It has the potential to better select patients for immunotherapy, to predict the risk for severe allergic reactions and to monitor

patients for immunotherapy outcome. During the last decade, parallel diagnosis on hundreds of allergen molecules from a large number of allergenic sources has also become available as a multiplex microarray test [135].

1.16.3 Diagnostic sIgE work-up strategies [136].

From symptoms to molecules: The "top-down approach":

Current guidelines recommend that the diagnostic work-up should be primarily guided by the clinical history, rather than to start with random screening for IgE sensitization. Allergen extract-based skin prick testing and/or serum sIgE-testing are usually enough to identify or exclude sIgE-sensitizations to potentially involved allergen sources. This may give either a negative result, a restricted sIgE antibody response sufficient to identify the underlying allergen source or many positive results to inhalant or food allergens extracts. In case of many positive results, further work-up ("top-down approach") with specific allergen molecules may identify the most important molecular IgE sensitizations.

From molecules to symptoms: The "bottom-up approach":

In this approach the diagnostic work-up start with analysing the entire IgE repertoire and then ask the patient for symptoms in order to identify the relevant sensitizations. However, for several reasons, this is currently not an appropriate diagnostic approach; not all allergens are available for diagnostic purposes, it will be too expensive, give too much information to process and interpret, and it will generate a large number of positive test results without clinical relevance.

"Top down and bottom up", the "U-shaped approach":

If the "top-down approach" leaves open questions regarding the implications of potentially cross-reactive allergens after one key allergen has been identified, the "bottom-up approach" can be applied only for relevant cross-reacting protein families depending on the initial findings. This approach explores the degree and potential clinical relevance of cross-reactivity to related molecules of a protein family [136].

1.16.4 Elimination diet.

A diagnostic elimination diet is avoidance of the food(s) suspected of triggering allergic reactions based on the clinical history, diet history, and allergy testing such as skin prick testing and sIgE. The duration of the avoidance is usually 2–4 weeks for IgE-mediated symptoms and longer for non-IgE mediated symptoms. Symptoms are monitored and results

evaluated to establish or refute the food allergy diagnosis. The avoidance phase should be followed by a planned reintroduction of the eliminated food(s). A reported clinical reaction should be confirmed by oral food challenge (OFC) under medical supervision [117].

1.16.5 Oral food challenges (OFC)

Oral food challenges are used to confirm the diagnosis of food allergy, to monitor food allergy, or to prove oral tolerance to a given food. Guidelines include patient selection, safety criteria, type and quantity of the food allergen to be administered, timings between doses, outcome criteria, observation periods, and recipes to be used [137]. Challenges can be performed open or blinded. Blinded challenges can be single or double-blinded and also include placebo [117]. In most clinical settings, an open OFC with an objective allergic reaction is sufficient for the diagnosis of food allergy. DBPCFC is the gold standard diagnostic test for the diagnosis of food allergy. If negative, the result has to be confirmed by a negative open OFC of a regular age-appropriate serving or the cumulative dose of the previous challenge.

DBPCFC is better than open OFC in patients with atopic dermatitis and only subjective or suspected psychological symptoms. DBPCFC is also the gold standard in research settings [138]. During a DBPCFC the food is blinded for taste, smell and texture, and the appearance of placebo and the active food should be indistinguishable from each other. The food is served in titrated doses, at set intervals and challenges stopped if objective clinical reactions are observed or the last dose is consumed without clinical symptoms. Immediate reactions usually appear within 2 h after the last food intake, but atopic dermatitis may worsen several hours or days following an oral challenge. The most common objective signs are urticaria and angioedema, but gastrointestinal, respiratory or cardiovascular system involvement is also common. Vital signs should be closely monitored during both open OFC and DBPCF. Necessary equipment and drugs, and appropriately trained staff should be in place to deal with allergic reactions including anaphylaxis [117].

1.16.6 Cellular allergy testing.

Cellular testing is used when the patient history and specific IgE or skin prick tests are discordant, when there is no reliable specific IgE or skin test, or if the patient history indicates that oral challenges or skin tests may elicit a systemic response [59]. The most common test is the basophil activation test (BAT) with determination of basophil sensitivity with serial

dilutions of allergen to measure the point of inflection of an allergic response [139]. Basophil sensitivity can be used to identify food allergens, the primary sensitizer amongst cross-reacting allergens or allergen preparations and to monitor progress of allergen immunotherapy and anti-IgE therapy. Basophil activation is measured as either histamine release or as up-regulation of granule proteins to the cell surface in response to allergen exposure.

1.17 Precautionary allergen labelling

Precautionary allergen labelling (PAL) was introduced by the food industry to help manage and communicate the possibility of reaction from the unintended presence of allergens in foods. Common labels are "may contain" or "may contain trace amounts of allergen". However, in its current form, PAL may be confusing for consumers with food allergies. Knowledge about threshold doses needed for eliciting allergic symptoms are lacking, resulting in inconsistent application of PAL, giving a poor relationship between the presence or absence of PAL and the actual risk of allergic reaction. Consequently, food allergic patients do not know if they can trust in PAL, reducing the possibility to make informed choices. The result has been reduced avoidance, reduced quality of life and increased risk-taking by consumers who often ignore PAL [140].

1.18 Hypotheses of allergy development

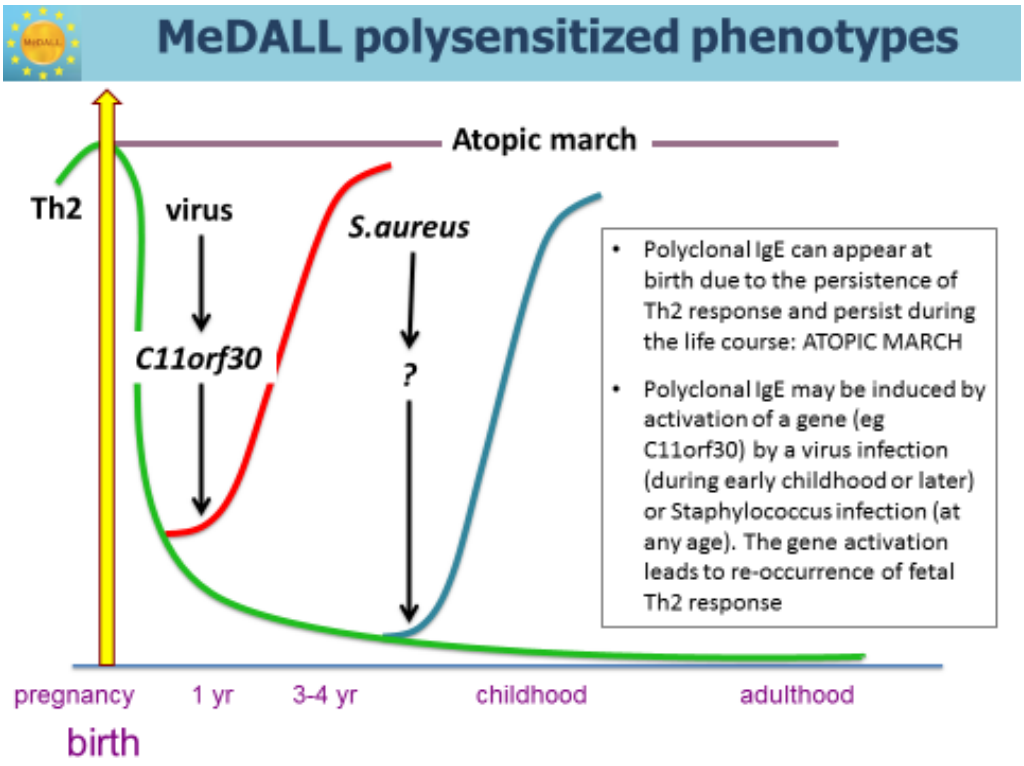
Strachan proposed the **hygiene hypothesis** in 1989 based on a national British birth cohort study, following 17 414 children to the age of 23 years [141]. He suggested that allergic diseases were prevented by infections in early childhood. Later infection or re-infection by younger siblings might result in additional protection against hay fever. Declining family size, improvements in household amenities, and higher standards of personal hygiene reduced the opportunity for cross-infection in young families. The result being more widespread clinical expression of atopic disease [141]. Later studies have shown protection against asthma and allergies after exposure to traditional farming environment in childhood [120, 142, 143].

The **biodiversity hypothesis** extends on the hygiene hypothesis. Biodiversity is defined as the variability among living organisms from all sources, both the macro- and micro-levels. Loss of macro-diversity, give also a lesser micro-diversity, which is associated with alterations of the microbiota. Reduced biodiversity and alterations of the gut and skin microbiota are associated with inflammatory diseases, including asthma, allergic and inflammatory bowel disease, type I diabetes and obesity. The cross-talk between human DNA and environmental

DNA determines the capability of the human immune system to make the difference between danger and non-danger, and the difference between self and non-self [144]. Ongoing and future studies will give more knowledge about the gut microbiota and the role of microbes in health and disease. Future guidelines for disease prevention and treatment of allergic diseases will also address how this may be achieved by influencing the gut microbiota [145].

Recently, Bousquet et al published **"The MeDALL" hypothesis** [146]. Allergic multimorbidity is associated with IgE poly-sensitization. The authors hypothesized that persistence or re-occurrence of foetal type 2 signalling genes plays an important role in the development of allergic multimorbidity and IgE poly-sensitization. Epigenetic mechanisms induced by environmental factors activate genes regulating these processes. Allergens and environmental cofactors (nutritional, bacterial and viral infections, microbiota) act at different times in pregnancy and the life cycle in subjects with a variable genetic predisposition to develop an IgE-mediated disease (Figure 3). Of special interest for this PhD-thesis is the fact that staphylococcal carriage/infection and sensitization to SEs are suggested to be one of many environmental factors that can play a role in children and adolescents.

Figure 3. The MeDALL hypothesis of poly-sensitization



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1.19 Impact of atopic disease

1.19.1 Impact of atopic disease on quality of life

Symptoms and signs from allergic diseases vary from very mild to life threatening. Moderate to severe symptoms affect the quality of life for a large number of patients. Patient-assessed severity of atopic dermatitis correlates with lower quality of life, indicating a greater impact with greater disease severity [147]. There is no cure for food allergy and so far, avoidance of the offending food is the only way to avoid allergic reactions. Anxiety for hidden allergens in food is common, especially when eating outside home [148]. In fact, the anxiety associated with the risk of a potential reaction has more profound effects on emotional and social aspects of a child's everyday life than clinical reactivity induced by food intake [149]. Most patients and parents to children with food allergy are aware of what to eat and not to eat, resulting in low morbidity due to their food allergy. However, there is strong evidence that food allergy has an impact on psychological distress and on the quality of life of children and adolescents, as well as their families [150, 151]. Asthma also has a significant impact on quality of life, both for children, adolescents and parents. A recent meta-analysis of 15 quantitative studies with 1797 asthma patients showed lower overall quality of life, physical functioning, psychological functioning and social functioning in asthma patients compared to controls [152].

1.19.2 Impact of atopic disease on health-related costs

The World Allergy Organization (WAO) have made a report (WAO White Book on Allergy) updated in 2013 and summarizing the burden of allergic diseases worldwide, referred in an editorial article by Pawankar [153]. According to WAO, 300 million patients have asthma and it is expected to increase to 400 million, by 2025 [153]. Direct and indirect costs are high (Table 1), in particular in severe or uncontrolled asthma. Paediatric asthma results in 14 million missed days of school each year in USA and high numbers of lost workdays for care-givers. An increase of asthma in children in lower-income countries, will lead to long-term consequences for their education and perpetuation of their poverty. The costs for treating rhinitis in the USA have doubled in 5 years to 11 billion US$ in 2005. In the developed countries, the financial burden of asthma ranges from US$ 300 to 1300 per patient per year annually. In developing countries, like Vietnam it is estimated to be US$184 per patient per year and in India, the monthly cost of medication for an asthmatic child can amount to one third of an average family's monthly income [153].

Table 1. The economic burden of allergy – annual costs

Country	Year	Population	Disease	Direct costs*	Indirect costs**	Total costs
Australia	2007	23 million	All allergies	Aus$ 1.1 billion	Aus$ 8.3 billion	Aus$ 9.4 billion
Finland	2005	5.3 million	All allergies	€ 468 million	€ 51.7 million	€ 519.7 million
South Korea	2005	50 million	Asthma			US$ 1.78 billion
South Korea	2005		Allergic rhinitis			US$ 226 million
Israel		7.5 million	Asthma			US$ 250 million
Mexico	2007	103 million	Asthma			US$ 35 million
USA	2007	310.2 million	Asthma	US$ 14.7 billion	US$ 5 billion	US$ 19.7 billion
USA	2005		Allergic rhinitis	US$ 11.2 billion	Up to US$ 9.7 billion	Up to US$ 20.9 billion

A few global facts and figures for two common allergic diseases: asthma and rhinitis. *Direct costs: Expenditure on medications and health care provision. **Indirect costs: Cost to society from loss of work, social support, loss of taxation income, home modifications, lower productivity at work, etc. Extracted from Ref [153].

1.20 Study research questions and hypotheses

How frequent are allergic diseases and allergic sensitization in adolescents in the Arctic region of Norway (Tromsø and Balsfjord)? Are allergic diseases and sensitization to multiple allergens associated with *S. aureus* carriage or sensitization to staphylococcal enterotoxins? These research questions were addressed in a cross-sectional study; The Tromsø Study Fit Futures 2 (TFF2). We hypothesized that allergic diseases are common among adolescents in Tromsø and Balsfjord and that the prevalence would be similar to comparable countries in northern Europe. Furthermore, we hypothesized that allergic diseases and multiple allergen sensitizations are associated with *S. aureus* carriage and/or sensitization to staphylococcal enterotoxins.

Can fish-allergic children and adolescents tolerate some fish species? How useful are specific IgE measurements to fish allergen extracts and molecules in the diagnosis of fish allergy and tolerance? What are the threshold doses for allergic reactions in fish allergy? These research questions were addressed in a DBPCFC-trial; The Tromsø Fish Allergy Study (TRO-FAST). We hypothesized that clinical cross-reactivity between cod, salmon, and mackerel is common in children and adolescents with fish allergy, but that tolerance to one or more species exists in a considerable proportion of the participants. We suspected that most participants would react to cod. Due to the homogeneity between parvalbumins of different species, we expected to find widespread cross-sensitization between fish allergen extracts and parvalbumins that would limit their utility in the diagnosis of clinical reactivity in fish allergy. Due to less cross-sensitization between enolase and aldolase in different fish species, we hypothesized that sIgE to enolase and aldolase may add some information in the diagnostic work-up of fish allergy. Finally, we hypothesized that threshold doses for allergic reactions are lower for cod compared to salmon and mackerel.

1.21 Aims of the thesis

The overall aim of this thesis is to contribute to the understanding of the development of multiple allergic diseases and multiple allergies in children and adolescents in the Arctic region of Norway.

My first aim was to gain novel insight in the epidemiology of allergic diseases in late adolescents and to investigate how allergic diseases and multiple allergen sensitizations are associated with *S. aureus* carriage and enterotoxin-sensitization, in order to expand our understanding of allergy development and multimorbidity.

My second aim was to gain novel insight in cross-reactivity between different fish species in children and adolescents with allergy to multiple fish species, in order to improve diagnosis and management of fish allergy.

The specific objectives were:

- To describe prevalence`s of asthma, allergic rhinitis, atopic dermatitis and allergic multimorbidity in adolescents and compare with prevalence trends from national and international studies (Paper I).
- To describe sensitization to common food and inhalant allergens and its association to allergic disease and compare with trends from national and international studies (Paper II).
- To study associations between allergic diseases and nasal *S. aureus* carriage (Paper I) and between allergic diseases/allergic sensitization and staphylococcal enterotoxin sensitization (Paper II).
- To assess cross-reactivity between cod, salmon and mackerel in fish allergic children and adolescents, and in particular describe the rate of participants who are tolerant to one or two of the three fish species tested (Paper III).
- To assess the utility of specific IgE to fish extracts, parvalbumins, aldolases and enolases in the diagnostic work-up of fish allergy (Paper III)
- To estimate threshold doses for subjective and objective allergic symptoms for cod, salmon and mackerel (Paper III).

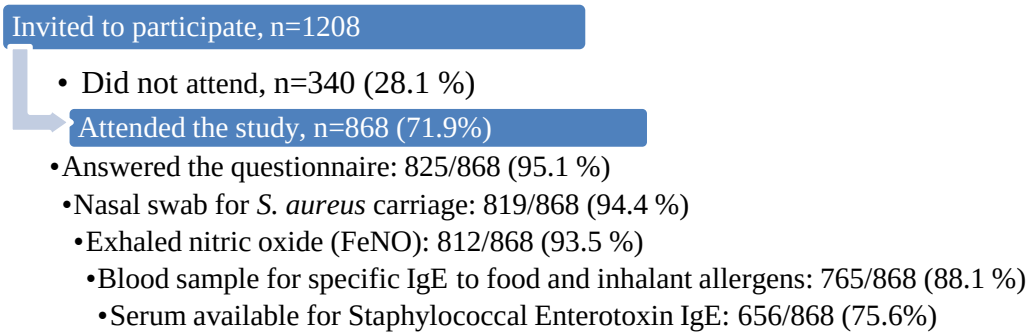
2 Material and Methods

2.1 Samples

The Tromsø Study Fit Futures (TFF) cohort was initiated in 2010-11. All first-year high school students in both academic and vocational educational programs from all eight high schools in the municipalities of Tromsø and Balsfjord were invited to participate (TFF1) and 92.8 % attended [154]. In this region, more than 90% of the population in the age group 16-19 years attend high school. In the second wave of the study (2012-13), all third-year high school students, including all participants from TFF1, were invited for follow up (TFF2). Among 1208 invited students 868 (71.9%) participated in TFF2. ”Atopic disease in adolescents in Northern Norway”, is one of 17 study projects in TFF2. It is a population based cross-sectional study.

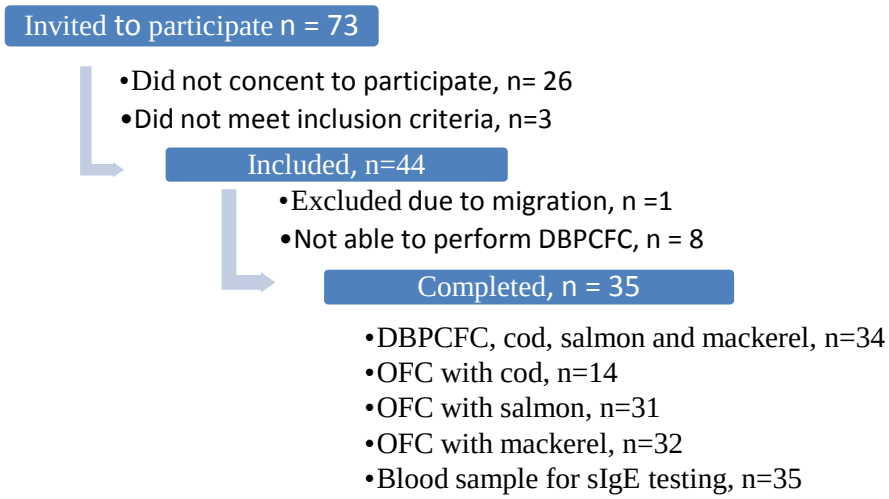
Each participant completed a web-based general health and lifestyle questionnaire (http://www.questback.com) and underwent clinical examination during a 1-day session, between November 2012 and June 2013. Among the 868 participants, 844 (97.2 %) answered the questionnaire. A total of 825 (95.1%) underwent clinical examinations, 812 (93.5 %) measured fractional exhaled nitric oxide (FeNO) and 804 (92.6 %) performed spirometry with reversibility test. Another 819 (94.4%) participants had a nasal swab done for analysis of *S. aureus* colonization. Moreover, samples for identification of *S. aureus* were successfully taken from eczematous skin in 46 of 63 participants with eczema on the day of visit.

Figure 3. Flow diagram with completed tests. Tromsø Study Fit Futures 2



Tromsø Fish Allergy Study (TRO-FAST) is a cross-sectional diagnostic study on fish allergic children and adolescents recruited from outpatient clinics at the University Hospital of North Norway. The study was conducted at the Research Unit, University Hospital of North Norway between September 2014 and August 2015. Seventy-three children and adolescents, between 5 to 20 years of age, with a clinical history of previous allergic reaction to fish and a positive sIgE/skin prick test to fish, were invited to participate. Forty-seven consented to participate. Three had outgrown their fish allergy, and 44 were enrolled in the study. Nine participants did not complete the study; eight due to problems with eating the test food and one due to migration out of Tromsø. One participant participated only in the open OFC.

Figure 4. Flow diagram with patient inclusion and completed tests. Tromsø Fish Allergy Study.



2.2 Methods

2.2.1 Questionnaire

The MeDALL (Mechanisms of the Development of Allergy) core questionnaire was used in both TFF2 and TRO-FAST to collect information about allergy, atopic dermatitis, asthma and risk factors [155]. The questionnaire was translated from Swedish to Norwegian and back translated in good agreement with the original Swedish and English versions. In TFF2, the adolescent version (appendix 9.1) was incorporated in a common web-based questionnaire for all studies in TFF2. In TRO-FAST, separate versions were used for parents (appendix 9.2) and adolescents (12-18 years) (appendix 9.1).

2.2.2 Assessment of *S. aureus* carriage – nose and skin (TFF2)

Both anterior nares were swabbed with a NaCl-moistened sterile rayon-tipped swab in 819 participants and eczematous skin areas in 46 participants with active eczema. The eczematous skin was rubbed with a NaCl-moistened compress prior to swabbing. *S. aureus* was identified using methods previously described [156] with a few modifications; all swabs were submerged into Bacto® m Staphylococcus medium broth (Difco laboratories, Sparks, MD, USA) for enrichment and incubated for 18-24 hours at 37 °C before plating. The liquid culture was then plated on blood agar (Oxoid, Cambridge, UK) and *S. aureus* ID agar (SAID, bioMérieux, Marcy I′ Etoile, France), incubated for 18-24 hours at 37 °C. The most dominating colony was selected and confirmed as *S. aureus* by the Staphaurex Plus (Remel, Lenexa, KS, USA) agglutination test. Only observations of bacterial growth on blood agar (Oxoid, Cambridge, UK) and/or SAID agar (BioMérieux, Marcy I′ Etoile, France) plates were included. We defined a positive *S. aureus* culture result as *S. aureus* carriage. We did not perform quantitative analysis of bacterial load.

2.2.3 Lung function test (TFF2)

Lung function was measured with spirometry with reversibility test using Easy on-PC (Medizintechnik AG, Zürich, Switzerland), according to guidelines from American Thoracic Society/European Respiratory Society (ATS/ERS) [157]. The best result of three approved tests was used. Reversibility was tested using 0.2 mg Salbutamol inhalation aerosol as bronchodilator (Airomir Autohaler® 0.1 mg/dose, 0.2 mg x 1, Teva Norge AS/IVAX Norge NUF). Post-bronchodilator spirometry was performed 15-25 min after inhalation of salbutamol. Pre- and post salbutamol values for forced vital capacity (FVC), forced expiratory

volume the first second (FEV1) and FEV1/FVC were recorded and expressed as % predicted according to reference values. The Global Lungs Initiative (GLI 2012) equations were used as reference for spirometry [158]. Spirometry data were anonymized at analysis.

2.2.4 Exhaled nitric oxide (TFF2)

Fractional exhaled nitric oxide (FENO) was measured with one measurement in every participant using NIOX MINO® (Aerocrine AB, Solna, Sweden). All measurements were performed before performing spirometry [159-161].

2.2.5 Serum sIgE (TFF2 and TRO-FAST)

Specific IgE were measured both in TFF2 and TRO-FAST with three mixes of allergens; rx6, containing extracts from birch, timothy, mugwort, alternaria and cladosporium, rx7 containing extracts from cat, horse, dog, house dust mite, and rabbit and fx5 containing extracts from egg white, milk, cod, peanut, wheat, and soy (ImmunoCAP, Phadia-Thermofisher, Uppsala, Sweden). To assess number of allergen sensitizations in TFF2 we divided participants, separately for inhalant and food allergens, in non-sensitized, mono-sensitized (1 allergen), and poly-sensitized groups (>1 allergen) [162]. To assess the total levels of specific IgE values in TFF2, all values from either food or inhalant allergens were added and reported as median sum of inhalant or food allergen values with 95 % confidence intervals. In addition, IgE against SE-A, SE-B, SE-C and TSST (Toxic Shock Syndrome Toxin) were analysed in TFF2 in spring 2016 in serum samples stored at minus 70°C. Specific IgE values < 0.35 kU_A/L for a screening panels (rx6, rx7 and fx5) were interpreted as negative for all included allergens. If screening panels had IgE ≥ 0.35 kU_A/L, then specific IgE was measured to all allergens included in the screening panel. In line with previous publications, a lower cut-off level for SE-sensitization, IgE > 0.1 kU_A/L, was chosen [35, 49, 50, 54]. In TFF2, all serum analyses were performed at the Department of Laboratory Medicine, University Hospital of North Norway. Specific IgE to cod, salmon and mackerel allergen extracts were determined by ImmunoCAP (Phadia-Thermofisher, Uppsala, Sweden). Sensitization was defined by sIgE > 0.10 kU_A/L. sIgE to the fish-allergen molecules parvalbumin, enolase and aldolase from cod, salmon and mackerel were determined at the Department of Infection and Immunity, Luxembourg Institute of Health, using enzyme linked immunosorbent assays, as previously reported [84]. Parvalbumins, enolases and aldolases from the three fish species were purified by column chromatography to homogeneity followed

by purity and identity check [84], and classified according to the International Union of Immunological Societies [136].

2.2.6 Patient Oriented Eczema Measure (POEM score, TFF2)

For monitoring disease severity of atopic dermatitis the Patient-Oriented-Eczema-Measure (POEM, appendix 9.3) was used in participants with ongoing eczema. It is a practical self-assessed measurement tool for monitoring aspects of atopic dermatitis that are important to patients in routine clinical practice or in the clinical trial setting [163].

2.2.7 Outcome definitions (TFF2)

The classification of allergic diseases was based on the standardized self-reported questions (MeDALL) used by European population-based birth cohort studies on asthma and allergy, and validated in the International Study of Asthma and Allergies in Childhood (ISAAC) [155]. Outcome definitions of allergic diseases are listed in Table 2.

Table 2. Clinical outcome definitions, The Tromsø Study Fit Futures 2.

	Current Disease	Disease Ever	Severe Disease
Atopic Dermatitis	Dry skin, itchy rashes with age-specific location (antecubital or popliteal fossae, wrists, ankles, neck or face) for 2 weeks or more in the past 12 months, or self-reported atopic dermatitis combined with us of topical corti-costeroids in the past 12 months (4).	Current atopic dermatitis or self-reported doctor-diagnosed atopic dermatitis ever.	Current atopic dermatitis and duration more than 1 month last 12 months or kept awake due to itchy rash during nights
Asthma	At least two of the following 3 criteria: 1) self-reported doctor-diagnosed asthma ever, 2) any indicative symptom in the last 12 months (wheezing, shortness of breath, dry cough at night) and 3) use of asthma medication in the past 12 months (3).	Current asthma or self-reported doctor-diagnosed asthma ever	Current asthma and FE_{NO} >25 ppb or breathing difficulties last 12 months graded 7-10 on a scale from 0 (no complaints) to 10 (the hardest to imagine) and more than 12 attacks with wheeze/breathing difficulties last 12 months.
Allergic Rhinitis	Symptoms of sneezing, a runny or blocked nose, or itchy, red and watery eyes after exposure to furred pets or pollen the last 12 months (4).	Current rhinitis or self-reported doctor-diagnosed allergic rhinitis ever	Current allergic rhinitis and use of nose spray last 12 months or nose symptoms more than 4 weeks in a row last 12 months or nose complaints graded 7-10 on a scale from 0 (no complaints) to 10 (the hardest to imagine) or having to stop activity due to nose problems or disturbed sleep due to nose problems
Multi-Morbidity	Having at least two of the diseases current atopic dermatitis, current asthma or current allergic rhinitis (4).	Current multimorbidity or at least two of self-reported doctor-diagnosed atopic dermatitis or asthma or allergic rhinitis ever	

2.2.8 Double-blind, placebo-controlled food challenge (TRO-FAST)

Double blind, placebo-controlled food challenges (DBPCFC) with cod, salmon, mackerel and placebo were performed according to guidelines from the European Academy of Allergy and Clinical Immunology (EAACI) [94]. A low-dose protocol with standardized test-food developed for multicenter studies was used to examine allergic reactions to low doses of allergen [164]. Test-food for DBPCFC was developed in cooperation with the National Food Research Institute in Norway (Nofima) and the Molecular Allergology Group, Manchester Institute of Biotechnology, UK. Nofima made dried powders from cooked, boned and skinned cod, salmon and mackerel filets, measured protein content and examined for microbiological contamination. The Molecular Allergology Group made chocolate dessert matrixes containing the dried fish powders and placebo based on a recipe used in EuroPrevall studies on food

allergy [164]. Chocolate dessert matrixes were stored frozen at -20°C, thawed and reconstituted with bottled water with additive of sugar and rum flavour 24 hours before use. Reconstituted desserts were stored in refrigerator at 4°C.

Participants underwent four separate challenge days in randomized order, with at least 6 weeks between each challenge day. Trained study nurses, blinded to the test food, recorded subjective and objective allergic symptoms based on previous published approaches [165]. The participants graded subjective symptoms of allergic reaction on a Visual Analogue Scale (VAS). A research nurse blinded to the content of the test food graded objective signs of allergic reactions from 0-3. One paediatric allergist (Martin Sørensen), blinded to the test food, classified all DBPCFC as negative, positive with objective symptoms or positive with subjective symptoms only. DBPCFC was stopped and classified as positive when predefined stop criteria were reached. Allergic reactions were treated according to EAACI guidelines for Food allergy and anaphylaxis [117]. Escalating doses of fish protein given in the DBPCFC were; 3 µg, 600 µg, 12.5 mg, 120 mg and 1 g. The cumulative dose of 1.13 g fish protein corresponds to about 5 g fish filet.

2.2.9 Open oral food challenge (TRO-FAST)

Participants with negative DBPCFC, or positive DBPCFC only with subjective symptoms, were tested with higher doses of the same fish species in open OFC. Fish burgers for open provocations were produced from minced filets from cod, salmon or mackerel filets, potato flour, water, salt and pepper. Fish burgers of 60 gram were oven baked at 90°C for ten minutes, vacuum packed and frozen at -20°C. They were served after being thawed over night at 4°C and heated in vacuum packing for 3 min in water with 80°C. Participants could choose to eat the fish burgers together with tomato ketchup. Nofima measured the protein content of the burgers. Open challenges were given with doses of 2 g, 6 g and 12 g of protein which correspond to a cumulative dose of approximately 100 g fish filet. Challenges were performed, symptoms and signs recorded and allergic reactions treated with the same procedures as for DBPCFC. The same paediatric allergist (MS), again blinded to the test food, classified all challenges as negative, positive with objective symptoms or positive with subjective symptoms only. The participants were not told which fish species they were served. After completed provocation, they were asked to guess which species they had been served.

2.3 **Statistical analyses**

We performed statistical analyses with IBM SPSS® statistics, version 23. The characteristics of the study participants and the prevalence of symptoms of allergic diseases were described with summary statistics. Pearson's chi-square test was used in comparisons of categorical variables and Student's t-test (normally distributed data) and Mann-Whitney U test (non-normally distributed data) were used for comparisons of continuous variables. Multivariable logistic regression models were used to analyse associations between *S. aureus* carriage or SE sensitization with allergic disease. Separate analyses were performed with each allergic entity as dependent variable and with *S. aureus* carriage or staphylococcal enterotoxin (SE)-sensitization as independent variables, adjusting for gender, body mass index (BMI), smoking, use of snuff tobacco and physical activity. Comparison between *S. aureus* carriage or SE-sensitization with food and inhalant allergen sensitization were performed with multinomial logistic regression models, comparing poly-sensitized or mono-sensitized groups with the non-sensitized group as reference category. A two-way between-groups analysis of variance was conducted to explore effect modification of SE-sensitization on *S. aureus* carriage. Statistical significance was assumed at a 5% level. Receiver operating characteristic (ROC) curves were used to analyse the performance of fish-allergen sIgE to discriminate between partially tolerant and non-tolerant participants. To estimate threshold doses, data were analysed using an interval censoring survival analysis approach [112]. Dose intervals were arranged from low to high doses by ordering lower and upper bounds of the intervals, and the step intervals were calculated according to the expectation-maximization algorithm. Dose distributions for objective and subjective reactions to each fish species were fitted by log-normal, log-logistic and Weibull distribution functions using Maximum Likelihood Estimation. As the three distributions were comparable in goodness of fit, the log-normal distribution was used to estimate the eliciting dose predicted to provoke an allergic reaction in 10% of individuals with fish allergy (ED10). Confidence bands for the fitted distribution functions, and 95% confidence intervals of the ED10 values, were calculated by bootstrapping (1,000 resamples). The analyses were conducted with the statistical software R using the "fitdistrplus" package (version 1.07) for interval censoring survival analysis (https://cran.r-project.org/web/packages/fitdistrplus/index.html).

2.1 Ethical approval and trial registration

The Regional Committee for Medical and Health Research Ethics approved TFF2: REK 2012/1350, and TRO-FAST: REK 2013/757. All participants and parents (if participants were less than 16 years of age) signed informed consent.

TRO-FAST is registered in ClinicalTrials.gov with Identifier: NCT02365168.

3 Summary of results

Paper I and II.

We found asthma (10.4%), atopic dermatitis (11.9%) and allergic rhinitis (26.0%) to be very common diseases among adolescents in Tromsø and Balsfjord. Asthma (55%) and atopic dermatitis (57%) were more common in conjunction with other allergic diseases than occurring as single entities. In contrast, allergic rhinitis (62%) more frequently occurred as a single entity. At least one of asthma, atopic dermatitis or allergic rhinitis were found in 36.5% of the participants, whereas 45.1 % had experienced at least one of these allergic diseases during their lifetime. Allergic multimorbidity, defined as more than one of these diseases was found in 10.2 % of the participants, more prevalent in women (12.7%) compared to men (7.9%), p=0.024. Atopic dermatitis was also more common among women (14.0 % vs 6.8%), p=0.001. Among participants with asthma (N=85) we found reduced lung function and increased FeNO in 11.6% and 22.1%, respectively.

More allergy were reported and more participants were sensitized to inhalant allergens compared to food allergens. For all allergens, sensitization was considerable more frequent than reported allergic symptoms. We found specific IgE reactivity to at least one food or inhalant allergen in 319/765 (41.7 %) participants, more frequently in men (47.7 %) than in women (36.7 %), p=0.003. Specific IgE reactivity to at least one allergen was found in 54/85 (63.5%) participants with asthma, 48/79 (60.8%) participants with atopic dermatitis, 149/189 (78.8%) participants with allergic rhinitis and 66/75 (88%) participants with allergic multimorbidity.

Nasal *S. aureus* carriage was found in 420/819 (51.3%) of the participants, with no gender difference, and was twice as prevalent as sensitization to at least one SE 173/656 (26.4%). Of 173 SE-sensitized participants, 102 (59.0%) were sensitized to one SE, 36 (20.8%) to two SEs, 19 (11.0%) to three SEs and 16 (9.2%) to all four SEs. There was no statistically significant association between *S. aureus* carriage and SE-sensitization (p=0.062), and 56 % of SE-sensitized participants were current nasal *S. aureus* carriers.

Nasal *S. aureus* carriage was associated with atopic dermatitis (OR 1.79, 95% CI 1.11-2.89), but not asthma and allergic rhinitis, in multivariable logistic regression models. *S. aureus* carriage was also associated with severe atopic dermatitis (OR 2.40, 95% CI 1.25-4.58), severe asthma (OR 3.37, 95% CI 1.34-8.51), severe allergic rhinitis (OR 1.70, 95% CI 1.09-

2.66) and allergic multimorbidity (OR 1.64, 95% CI 1.02-2.64). After stratification, SE-sensitization was only associated with allergic multimorbidity (OR 2.34, 95% CI 1.37-4.0).

S. aureus carriage was more frequent in participants with polysensitization to inhalant allergens (OR 1.65, 95% CI; 1.19-2.31) compared to non-sensitized participants, but the difference was not statistically significant when SE-sensitized participants were excluded from the analysis. There was no difference between *S. aureus* carriers and non-carriers in sensitization to food allergens.

Sensitization to SEs was more frequent in participants with polysensitization to food and inhalant allergens, compared to non-sensitized participants. The largest difference between polysensitized and non-sensitized was seen for SE-A; inhalant allergens (OR 7.89, 95% CI 3.97-15.65) and food allergens (OR 16.76, 95% CI 7.77-36.14). In contrast, there was no difference between monosensitized and non-sensitized to inhalant allergens. The strongest association was seen in participants sensitized to all four SEs with OR 50.5 (95% CI 6.6-389.3) for polysensitization to inhalant allergens compared with non-sensitization.

Among participants with inhalant allergen sensitization, participants sensitized to at least one SE had significantly higher median sum of specific IgE values to inhalant allergens (41.4 kU_A/L, IQR 10.1-118.4) compared to non SE-sensitized participants (18.0 kU_A/L, IQR 5.5-48.6), p=0.004. In contrast, no statistically significant difference was seen between *S. aureus* carriers (22.1 kU_A/L, IQR 5.7-53.3) and non-carriers (16.4 kU_A/L, IQR 5.1-48.3), p=0.104, and when SE-sensitized participants were excluded from the analysis the p-values were almost identical (14.8 kU_A/L vs 14.3 kU_A/L), p=0.893. Moreover, there were no differences in median sum of IgE values to food allergens between SE-sensitized and non SE-sensitized participants (2.33 kU_A/L vs 1.30 kU_A/L), p=0.166, or between *S. aureus* carriers and non-carriers (1.5 kU_A/L vs 1.7 kU_A/L), p=0.529.

Overall, SE-sensitized participants had higher median total IgE levels (113.0 kU_A/L, IQR 42.0-116.3) compared to non-sensitized participants (24.1 kU_A/L, IQR 9.2-60.0), p <0.001. However, no difference was seen between *S. aureus* carriers (34.5 kU_A/L, IQR13.0-116.3) and non-carriers (34.0 kU_A/L, IQR 13.2-91.5), p=0.744.

Paper III.

The mean (SD) age among the 35 participants (22 boys) who completed the study was 11.6 (3.0) years. Around 2/3 of the participants had also asthma, atopic dermatitis and/or allergic rhinitis, and 31/35 (89%) had also other food allergies than fish allergy.

Any allergic symptoms were observed/reported from 33 (cod), 28 (salmon) and 28 (mackerel) participants, whereas objective symptoms were observed in 32 (cod), 23 (salmon) and 19 (mackerel) participants. Five participants reported mild, transient subjective symptoms on placebo challenge, but had unequivocal findings during the DBPCFC-active arm and are thus included in the analysis.

We found tolerance to at least one of three fish species (defined as partially tolerant) in 10 (29%) participants regarding any symptoms and in 19 (54%) regarding objective symptoms. There was no difference between non-tolerant (reacting to all three fish species), and partially tolerant participants regarding age, sex, other allergies, parental allergy, asthma or atopic dermatitis.

Oral itching and swellings/blisters were the most frequent subjective and objective symptoms, respectively, followed by itching and erythema/urticaria of the skin. Involvement of more than one organ was seen in 14/35 participants. Upon challenge, two participants received adrenaline, but none had severe anaphylaxis requiring further observation or treatment.

Sensitization to all three fish-allergen extracts and parvalbumins was found in nearly all participants, including participants without clinical reactivity, whereas sensitization to enolases/aldolases was predominantly found in participants with objective symptoms. However, many participants with objective symptoms were not sensitized to the corresponding enolase/aldolase.

Non-tolerant participants had higher sIgE levels to fish-allergen extracts and parvalbumins than partially tolerant participants (Table 3), whereas no difference was seen for enolase and aldolase.

Table 3. Median sIgE in partially tolerant vs non-tolerant participants regarding objective and subjective symptoms.

Allergen (IUIS code)	Objective symptoms			Subjective symptoms*		
	Partially Tolerant N=19 Median (IQR)	Non-Tolerant N=15 Median (IQR)	p-value	Partially Tolerant N=10 Median (IQR)	Non-Tolerant N=24 Median (IQR)	p-value
Cod Extract sIgE	8.8 (1.6-18.9)	19.5 (6.0-49.3)	0.064	4.0 (1.1-13.2)	15.0 (6.0-36.0)	**0.017**
Cod Parvalbumin sIgE (Gad m 1)	8.0 (1.5-20.6)	13.4 (5.5-41.1)	0.103	5.1 (0.7-13.6)	12.5 (5.5-36.9)	**0.034**
Cod Enolase sIgE (Gad m2)	0.8 (0.3-1.4)	0.9 (0.1-1.6)	0.792	0.4 (0.3-1.2)	0.9 (0.1-1.6)	0.478
Cod Aldolase sIgE (Gad m 3)	0.4 (0.1-1.5)	1.3 (0.1-1.8)	0.217	0.4 (0.1-0.9)	1.2 (0.1-1.8)	0.144
Salmon Extract IgE	4.7 (0.9-13.1)	13.5 (6.7-23.6)	0.052	1.8 (0.4-6.8)	12.2 (5.7-25.3)	**0.003**
Salmon Parvalbumin IgE (Sal s 1)	4.7 (2.3-12.1)	12.4 (4.9-30.3)	0.052	4.5 (1.2-11.1)	11.2 (2.7-26.6)	0.085
Salmon Enolase sIgE (Sal s 2)	0.1 (0.1-0.1)	0.1 (0.1-0.4)	0.076	0.1 (0.1-0.1)	0.1 (0.1-0.1)	0.125
Salmon Aldolase sIgE (Sal s 3)	0.1 (0.1-0.1)	0.7 (0.1-2.1)	**0.015**	0.1 (0.1-0.4)	0.1 (0.1-1.4)	0.288
Mackerel Extract sIgE	2.1 (0.4-4.7)	5.5 (1.7-9.7)	**0.026**	0.6 (0.1-2.4)	5.4 (1.8-9.6)	**0.002**
Mackerel Parvalbumin sIgE (Sco s 1)	3.4 (1.9-13.4)	13.1 (5.6-49.2)	**0.029**	3.1 (1.1-9.3)	12.9 (2.7-26.3)	**0.039**
Mackerel Enolase sIgE (Sco s 2)	0.1 (0.1-0.1)	0.1 (0.1-0.1)	0.922	0.1 (0.1-0.1)	0.1 (0.1-0.3)	0.273
Mackerel Aldolase sIgE (Sco s 3)	0.1 (0.1-0.1)	0.1 (0.1-0.1)	0.853	0.1 (0.1-0.1)	0.1 (0.1-0.1)	0.813

Comparison of sIgE (kU_A/L) between groups are performed with Mann Whitney U test.

Partially tolerant: No symptoms to at least one fish species (cod, salmon or mackerel).

Non-tolerant: Symptoms to all three of cod, salmon and mackerel.

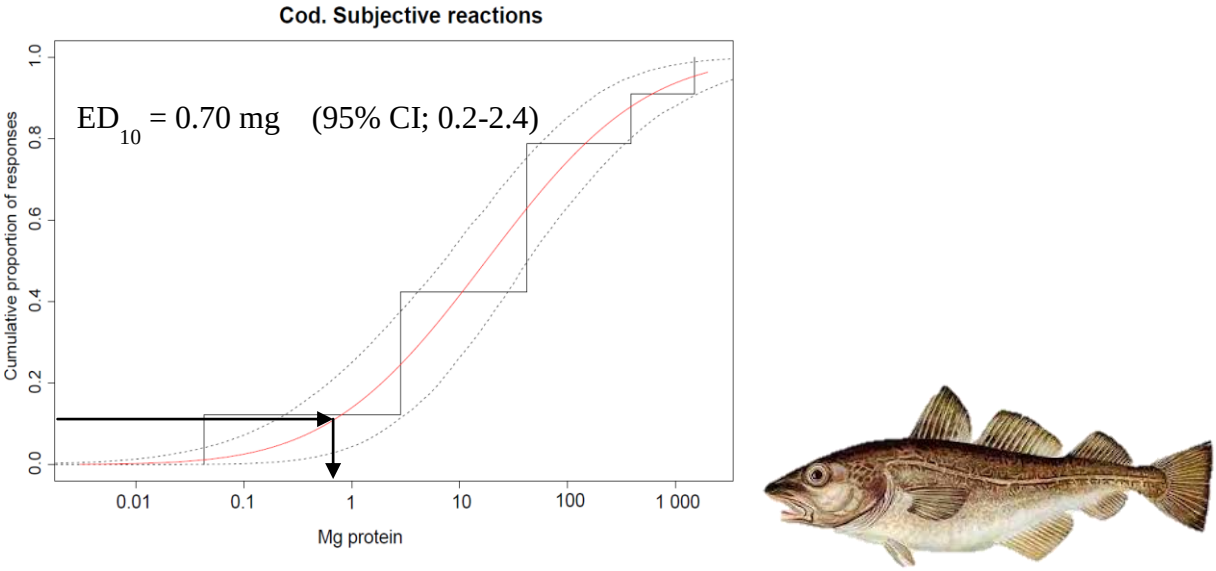
*All participants with subjective symptoms, including participants with objective symptoms.

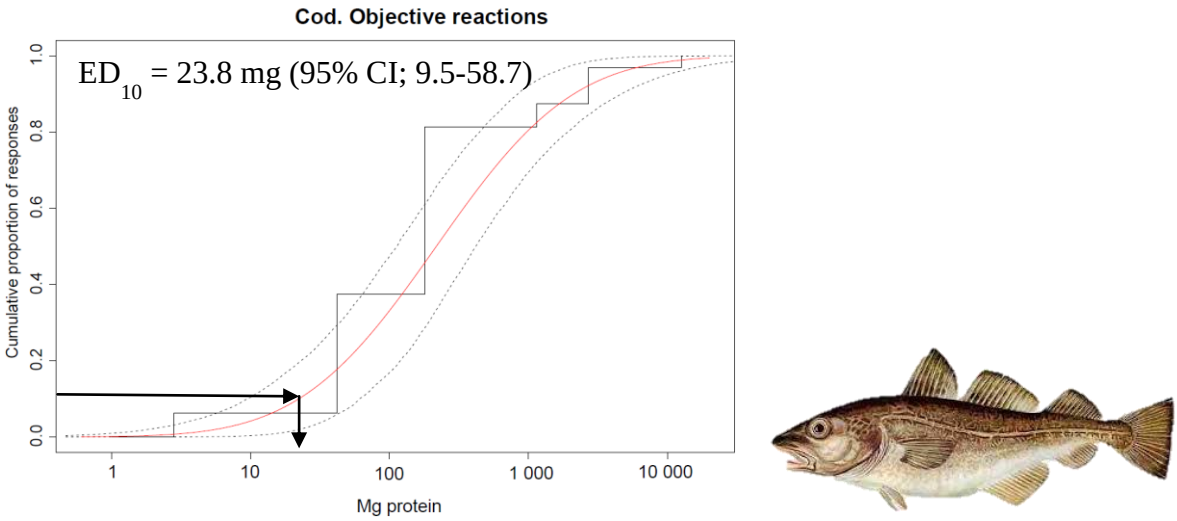
Significant differences in bold. One participant did not show any allergic reaction during fish challenge, and is excluded from the analysis. IUIS = International Union of Immunological Societies, IQR = Interquartile Range.

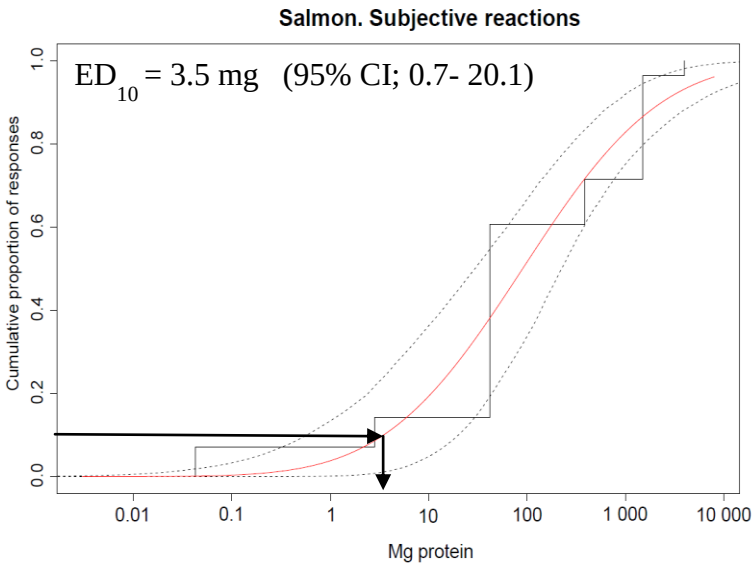
Specific IgE to extracts discriminated best between the non-tolerant and partially tolerant groups. sIgE to cod extract >8.2 kU_A/L or salmon extract >5.0 kU_A/L identified 18/24 and 19/24 non-tolerant participants, respectively, whereas below these cut-off values, 8/10 and 9/10 partially tolerant participants were identified.

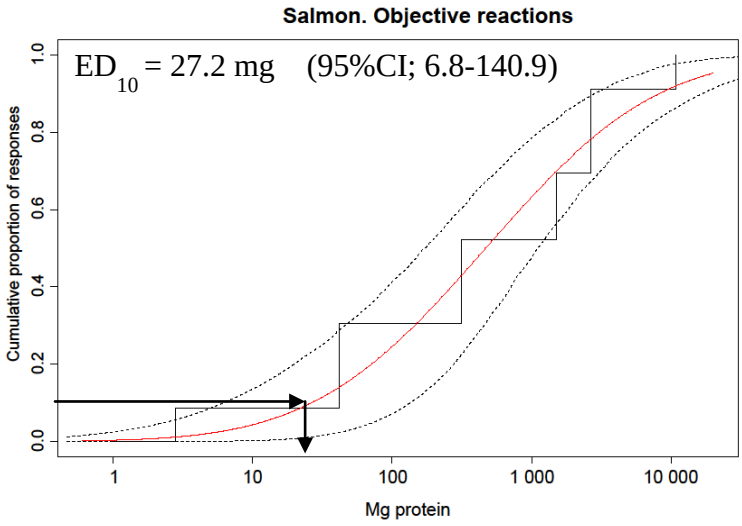
Finally, we estimated the eliciting dose predicted to provoke an allergic reaction in 10% of individuals (ED_{10}), based on dose distribution curves (Figure 5).

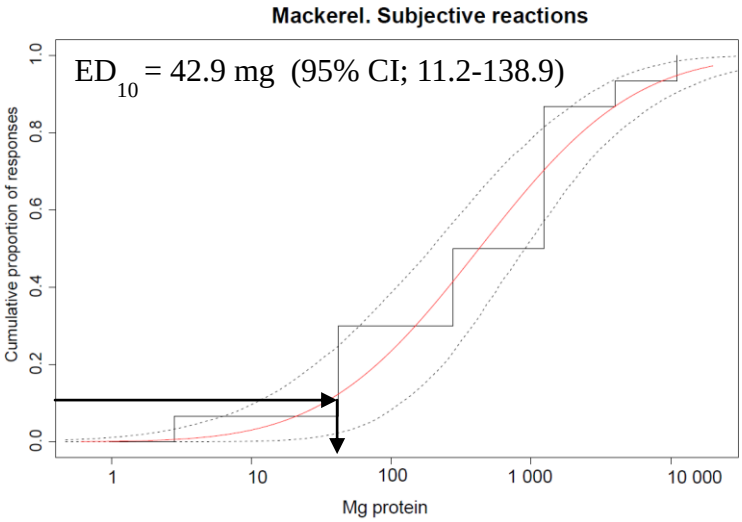
Figure 5. Dose-distribution curves and ED_{10} for allergic symptoms to cod, salmon and mackerel.

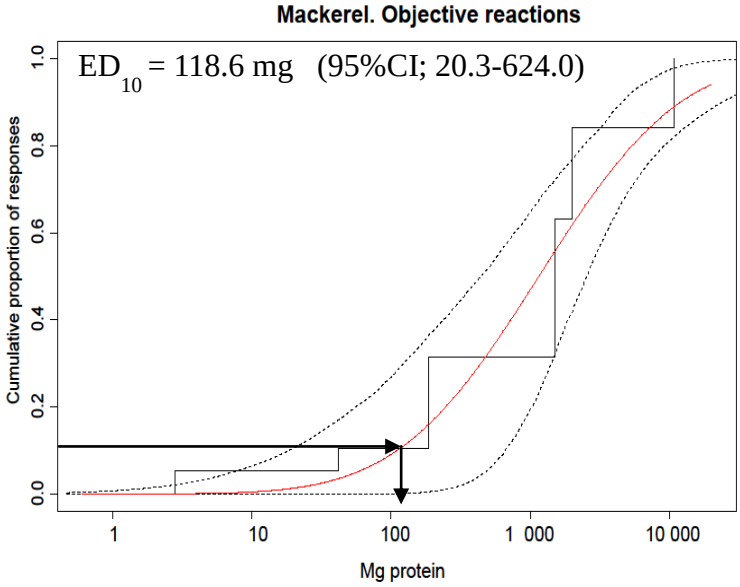





Salmon. Subjective reactions
ED10 = 3.5 mg (95% CI; 0.7- 20.1)
Cumulative proportion of responses
0.0
0.2
0.4
0.6
0.8
1.0
0.01
0.1
1
10
100
1 000
10 000
Mg protein


Salmon. Objective reactions
ED10 = 27.2 mg (95%CI; 6.8-140.9)
Cumulative proportion of responses
0.0
0.2
0.4
0.6
0.8
1.0
1
10
100
1 000
10 000
Mg protein





We found ED_{10} for cod protein in line with previously published data [112]. ED_{10} was lowest for cod and highest for mackerel, but the low number of participants with objective reactions to salmon and mackerel reduces reliability of these estimates. However, comparison of lowest observed adverse effect levels (LOAEL) for objective symptoms to cod, salmon and mackerel shows more participants with low LOAELs for cod compared to salmon and mackerel.

4 Discussion

4.1 Discussion of results

In this thesis, we have studied important issues as epidemiology, aetiology, cross-sensitization, cross-reactivity and diagnostic work-up in the field of allergic disease in children and adolescents, with special attention to the development of multiple allergies. We found allergic disease to be common among adolescents in the Arctic region of Norway and in line with comparable countries in northern Europe. We found atopic dermatitis and severe asthma, severe allergic rhinitis and allergic multimorbidity to be associated with nasal *S. aureus carriage,* whereas SE-sensitization was associated to polysensitization to food- and inhalant allergens and allergic multimorbidity. Cross-sensitization and cross-reactivity to multiple fish species were common among children and adolescents with fish allergy, but around one third could tolerate at least one fish species without any reactions and around half could tolerate at least one fish species without objective reactions. IgE measurements to fish allergen extracts and molecules cannot substitute oral food challenges in the diagnosis of fish allergy and tolerance, but combined use of clinical history and IgE measurements may reduce the number of challenges needed for a precise diagnosis. Finally, threshold doses for eliciting allergic reactions seem to be lower for cod, compared to salmon and mackerel.

Compared to the Swedish BAMSE study [166] , the British IOW study [167] and the German MAS study [15], the prevalence of allergic disease in our population is close to the findings in Sweden and Germany, except for asthma, which is lower in Sweden. In the UK, there are higher prevalence for asthma, allergic rhinitis and multimorbidity [167]. The prevalence of sensitization to at least one food or inhalant allergen in our study is in line with findings from the UK [167], but slightly lower than reported for 16 year old adolescents in Southern Norway [168] and Sweden [166]. Allergenic exposure in the Arctic region is probably quite similar to Sweden, Germany and UK.

We found a female predominance of atopic dermatitis and multimorbidity. It has been hypothesized that sex hormones play a role in the pathogenesis of allergic diseases [169, 170]. Androgens appear to have immunosuppressive effects while oestrogens are pro-inflammatory and may increase the susceptibility to atopy. Influence of sex hormones may thus explain the gender difference in our results.

S. aureus carriage was twice as common as sensitization to SE. This may partially be due to carriage of *S. aureus* strains not producing enterotoxins and partially due to SE-sensitized participants no longer being *S. aureus* carriers. In adults, only on fourth had nasal *S. aureus* carriage in a previous population based study from the same region [156]. The reason for strikingly higher nasal *S. aureus* carriage among adolescents is not clear.

Both *S. aureus* carriage and SE-sensitization have previously been shown to be associated with allergic disease [42, 44-47, 49-55]. Our results indicates that *S. aureus* carriage may influence the severity of allergic diseases, whereas SE-sensitization may influence on poly-sensitization to food and inhalant allergens. Both *S. aureus* carriage and SE-sensitization were associated to allergic multimorbidity.

In line with a study among adult patients with allergic rhinitis, we found no association between *S. aureus* carriage and poly-sensitization to food and inhalant allergens [45]. In contrast to our findings, among the adult patients with allergic rhinitis, poly-sensitization was not associated with sensitization to SE-A and SE-B, using a higher cut-off for sIgE (0.35 kU_A/L) than we did (0.10 kU_A/L) [45]. This difference may partly be due to the difference in cut-off values for SE-sensitization but also due to more enterotoxins tested in our study. We found higher sIgE levels to inhalant allergens in SE-sensitized participants compared to participants not sensitized to SE, whereas no association was found for food allergens. No association was found between *S. aureus* carriage and level of sIgE food or inhalant allergens. In addition, SE-sensitization, but not *S. aureus* carriage, was strongly associated to the level of total IgE.

In contrast to many studies, we found no association between SE-sensitization and asthma or allergic rhinitis. However, the statistically non-significant effect estimates (i.e. odds ratios) in our study are comparable to the effect estimates in other studies. This indicates that our findings may be in line with other studies, but due to smaller sample size, the differences in our study are non-significant. In the German MAS study, a moderate relationship between SE-sensitization and asthma was found at age 20, but not after adjusting for potential confounders. In a GA^2LEN study on adult patients, SE-sensitization was associated with asthma in the general population [49, 50]. Stronger associations to SE-sensitization have been shown for adult [35] and elderly [54] patients with asthma and severe asthma, indicating that the influence of SE may increase with age. In asthmatic patients, SE-sensitization is commonly detected in patients with severe asthma [49, 51]. In contrast, in a recent Brazilian

cross-sectional study on asthma patients there was no difference in SE-sensitization between patients with mild and severe asthma [171]. In this Brazilian study, 43.7% of asthma patients were sensitized to SE, but they did not include healthy controls for comparison.

In our study, both *S. aureus* carriage and SE-sensitization are strongly associated with an increase in FeNO > 25 ppb. Thus, both may influence the pathogenesis and severity of allergic sensitization and disease. Possibly, *S. aureus* carriage may induce inflammation through other pathways than SE-sensitization, which may explain the associations to severe allergic diseases and other airway diseases such as chronic rhinosinusitis. A link has been shown between *S. aureus* biofilms and skewing of the T-cell response toward the Type 2 pathway that is independent of superantigen activities [172]. It is possible that *S. aureus* can induce release of epithelial derived cytokines that may contribute to the inflammation.

The role of *S. aureus* skin carriage contributing to the exacerbation of atopic dermatitis is well established [43, 173-175], and SE sensitization is also shown to be strongly associated with atopic dermatitis [176-178]. The majority of participants colonized with *S. aureus* on eczematous skin were also colonized in the nose, pointing to the nose as the source of *S. aureus* in patients with atopic dermatitis. In our study, the role of *S. aureus* carriage in atopic dermatitis exacerbation was supported by increasing eczematous skin carriage rates with increasing atopic dermatitis severity, as previously reported in other studies [175, 179].

Finally, our study supports the MeDALL hypothesis, suggesting that *S. aureus* may induce re-occurrence of foetal type 2 signalling, resulting in polyclonal allergen sensitization and allergic multimorbidity [146]. It is possible that the activation of Type 2 immune responses by *S. aureus* leads to the re-occurrence of Type 2 foetal pathways. Type 2 immunity is involved in IgE production, polyclonal activation, the cellular inflammation of atopic dermatitis, asthma and allergic rhinitis [180] as well as in the regulation of the epithelial barrier function in the skin [181], the airways [182] and Type 2 responses [183]. *S. aureus* can induce sIgE class switching in nasal polyps [184]. IL-33 may be of great importance in the understanding of multimorbidity and poly-sensitization [185] as it modulates the expression of human β-defensin 2 in human primary keratinocytes and may influence the susceptibility to bacterial superinfection in acute atopic dermatitis.

Our epidemiological data does not prove causation and the observed associations could also be the result of reverse causation; inflammation due to allergic disease makes the mucosa

more susceptible to *S. aureus* carriage. Many studies suggest that *S. aureus* carriage play a role in the severity of established allergic diseases. This is supported by our findings, but pathophysiological mechanisms and putative therapeutic or prophylactic consequences need to be addressed in future studies.

Spirometry showed signs of current obstruction in 11.6% of asthmatic participants, and only around 5% showed reversibility after inhalation of salbutamol. Spirometry has a low sensitivity to diagnose asthma [186, 187]. This may be due to the intermittent course of asthma and good asthma control in treated patients. In line with others [160], we also found that only one fifth of asthmatic participants had increased levels of FeNO > 25 ppb. Furthermore, in a Norwegian study on adolescents with bronchiolitis in infancy, exhaled nitric oxide was related to atopy, but not to asthma [188]. Some international guidelines recommend using FeNO in phenotyping airway inflammation and monitoring of severe asthma [160] while recent international guidelines on severe asthma suggest that clinicians should not use FeNO to guide therapy in adults or children [189]. We found that FeNO > 25 ppb was associated to asthma only in participants with nasal *S. aureus* carriage. A possible explanation for this finding is nasal eosinophilic inflammation due to staphylococcal superantigens.

In line with our hypothesis, clinical experience and previous small studies, we found a high degree of clinical cross-reactivity between fish species in fish allergic children and adolescents. However, more than half of all participants had objective tolerance, and around one third had subjective tolerance to at least one fish species. We also found that we could predict the outcome at challenge in the majority of patients by combining information from traditional fish allergen extracts and new fish allergen molecules.

Two small studies from the last century compared clinical cross-reactivity between fish species using DBPCFC, both reporting higher frequency of partial tolerance compared to our study [75, 77]. In our study, reactions to cod were more serious and more prevalent and with lower threshold doses for reactions, compared to salmon and mackerel. This may be due to high consumption of cod and cod being the primary fish allergen sensitizer in our region.

We found high rates of sIgE sensitization to cod, salmon and mackerel extracts and parvalbumins, in line with previous studies [68, 69, 190, 191]. More than 90% of participants were sensitized to all three parvalbumins, a higher proportion than in a recent European study

[84]. The ability of fish allergen extracts and parvalbumins to identify tolerance to specific fish species in fish allergic patients was poor. This may be due to cross-reacting parvalbumins [73, 80]. Sensitization to cod enolase/aldolase was more prevalent compared to salmon and mackerel enolase/aldolase. This finding may be due to less inter-species IgE cross-reactivity between enolases/aldolases, compared to parvalbumins [84], and cod being the primary cause of sensitization.

We believe it is important to identify partial tolerance in order to avoid unnecessary food restrictions. At study entry, already three quarters of the participants avoided more than one food. Indeed, two of the invited patients were diagnosed with hypothyroidism due to iodine depletion, most probably caused by avoidance of iodine containing foods as fish and dairy products.

We also believe it is desirable to substitute time-consuming food challenges, with risks of serious allergic reactions, with less invasive diagnostic procedures in diagnosing fish allergy. However, the specificity of sIgE extracts or parvalbumins in order to identify tolerance was low, as nearly all participants tolerant to salmon and mackerel were still sensitized. In contrast, participants with tolerance were not sensitized to enolase/aldolase to the respective fish species. However, many participants with confirmed allergy were not sensitized, in particular to salmon and mackerel enolase/aldolase. Useful in the diagnosing process of fish allergy is the finding that sensitization to enolase/aldolase most probably represents allergy with objective symptoms, and food challenge may be unnecessary.

Furthermore, we analysed how these tests could discriminate between the non-tolerant and partially tolerant groups, irrespective to which fish species they were tolerant. We found that sIgE to salmon extract at a cut-off level around 5 kU_A/L and cod extract at a cut-off level around 8 kU_A/L, discriminated rather well between non-tolerant and partially tolerant participants. Using these cut-off values will identify around 75% of the non-tolerant patients. It would have been preferable to use lower cut-off values and identify more non-tolerant patients (i.e. 95%), but this would give more partially tolerant patients being classified as non-tolerant. Furthermore, there may be variable allergen levels in different allergen batches. Suggested cut-off levels based on allergen extracts therefore need cautious interpretation [80]. In our study, both children and adolescents were studied. IgE levels and cut-off values may differ between different age groups and give further uncertainty to the estimated cut-off values. Based on the above limitations, the estimated cut-off values have to be used with

caution. Still, in a clinical setting, patients with an obvious history of self-reported fish allergy and salmon extract sIgE clearly above 5 kU_A/L or cod extract above 8 kU_A/L, may be advised to avoid all fish species, and food challenges seem unnecessary. The only risk with this approach is that a few patients with partial tolerance are advised to avoid all species of fish. Most participants with salmon sIgE ≤ 5 kU_A/L and cod extract t sIgE ≤ 8 kU_A/L tolerated at least one fish species. Some of these patients are non-tolerant, of which a proportion could avoid food challenge if confirmed sensitization to enolase and/or aldolase. In contrast, patients with sIgE < 0.1 kU_A/L to enolase/aldolase may still have fish allergy and must undergo challenge with the respective species in order to identify or rule out partial tolerance. Thus, our results indicate that sIgE to enolase/aldolase may have a specific role in diagnosing or ruling out fish allergy when it comes to species such as cod, salmon and mackerel.

Participants with only subjective symptoms did not report symptoms to placebo. However, many participants with only subjective symptoms had troublesome oro-allergic or abdominal subjective symptoms. Food challenges are usually rated positive only if objective reactions are observed [94]. Although the symptoms were not serious, the discomfort of the subjective symptoms restricted them from eating fish. Based on these observations, we consider participants with only subjective symptoms as fish allergic patients, but with a milder allergy phenotype compared to patients with objective symptoms. This is supported by no significant difference in IgE titres between participants with objective symptoms and only subjective symptoms. This is also in line with "A new framework for the documentation and interpretation of oral food challenges in population-based and clinical research" [192].

Previously published ED_{10} values for objective and subjective symptoms to cod are in line with our findings [112]. The estimated ED_{10} values for objective symptoms to salmon and mackerel were based on limited number of observations and have to be interpreted with caution. However, ED_{10} for subjective symptoms to salmon and mackerel were higher for salmon and highest for mackerel, compared to cod. Comparison of lowest observed adverse effect levels(LOAEL) for objective symptoms to cod, salmon and mackerel also shows more participants with low LOAELs to cod compared to salmon and mackerel, indicating that ED_{10} is likely to be higher for salmon and mackerel. However, it will require additional challenges in salmon and mackerel allergic patients to confirm this. Another limitation of the estimated threshold doses is the variation in age among the participants. Threshold doses may vary with age, especially between younger children and adolescents. However, the small sample size restricted us from estimating threshold doses in subgroups of age.

Despite being the largest DBPCFC fish allergy study ever performed, the relatively low number of participants limits the power to detect small differences between groups. This is discussed in more detail in chapter 4.2.7. In spite of these limitations, we still believe the data represent good estimates of the patterns of cross-reactivity and severity in the fish allergic population in the region of North Norway, but results may not be directly applicable to other regions with other food traditions and sensitization patterns. Furthermore, children of lower age had higher dropout rates due to poor palatability of the test food. None dropped out due to allergic reactions. A methodological limitation is that the production of test food for challenge involved thermal processing of the fish fillets, which reduces the allergenicity of many allergens. Cooking of fish marginally diminishes parvalbumin content and thus serological recognition, but may affect parvalbumins from various fish species differently [80, 190]. Thermal treatment may also reduce the allergenicity of enolase/aldolase [84, 193]. Other processing methods may either reduce or increase the allergenicity of parvalbumins [194].

The main strength of our study is the randomized, placebo-controlled design using standardized test food, followed by open oral food challenge with higher doses of protein. Six weeks intervals between the challenges reduce the possibility of transient tolerability, possibly affecting the results of the next challenge. Furthermore, blinding of the participants and the paediatric allergist reduces biases known to occur in open food challenges.

4.2 Methodological considerations

4.2.1 Selection bias

In TFF1, 92.8 % of the invited students attended the study, whereas 71.9% attended TFF2. Dropout from the study was partially due to migration, dropout from school and educational practice in other parts of Norway. This may have caused a selection bias in our sample. Dropout from school and education requiring practice periods are more common among students from low-income families with less education, which is associated with higher prevalence of many diseases. Thus, selection bias in our sample would most probably have underestimated the prevalence of allergic disease and sensitization. More participants were analysed for sIgE to food and inhalant allergens compared to SE because sIgE to SE was measured later in frozen stored sera and one box of the stored boxes with sera was missing. Thus, the difference in sIgE analyses is random and should not be a cause of selection bias.

The participants in TRO-FAST were recruited from outpatient clinics and may have more severe allergies compared to the total fish allergic population. This may lead to a selection bias with subsequent overestimation of the severity of fish allergy and underestimation of partial tolerance. In a recent EuroPrevall study in the Netherlands, there were large differences in self-reported food allergy between community and outpatients [195]. However, when selecting only those with a probable food allergy (i.e. symptoms of allergy and elevation of sIgE to the respective food), no major differences were observed with respect to severity, causative foods, sensitization and DBPCFC-results between community and outpatients. According to these results, choosing outpatient fish-allergic participants should not result in significant selection bias.

There may also be difference in disease severity between those who consented to participate and those who not consented. Patients with the most severe reactions might not have consented because they were afraid of the reactions provocations with fish would elicit. On the other hand, they might have consented because they then had the opportunity to be tested in a safe setting in the hospital.

Finally, there may be difference between dropouts and participants that completed the study. Eight of nine dropouts were among the youngest participants. They did not manage to eat the test food due to taste or volume. Compared with the total fish allergic population, our sample is therefore somewhat biased regarding age.

We found no differences in sensitization to cod or salmon, age, sex or doctor diagnosed allergic diseases between participants and non-participants and age was the only characteristic that differed between dropouts and those who completed the study. Except from infants and small children, our sample should be representative for the fish allergic population of children and adolescents in our region.

4.2.2 Questionnaire

The MeDALL core questionnaire [155] for adolescents and parents is developed and validated by European experts in the field of asthma and allergy research. It is used in large European birth cohort studies, which makes comparison between studies and countries possible. Large questionnaires may lead parents and participants not to answer all questions with the same awareness. By giving time to answer the questionnaire between clinical examinations and measurements at the hospital, this kind of bias was minimized. Questions

about the past are prone to recall bias whereas recent history is easier to remember. In the MeDALL questionnaire, most questions focus on the past 12 months and the past week, thereby reducing this kind of bias.

4.2.3 Outcome definitions

There is lack of simple diagnostic tests with high sensitivity and specificity to diagnose asthma, atopic dermatitis and allergic rhinitis. The patient's history is important, supplemented with clinical examination and other measurements. In research, the history is collected with questionnaires and outcome variables are constructed by combining variables from the questionnaire. However, operational definitions of asthma in recent epidemiological studies are inconsistent, even among studies based on the ISAAC questionnaire [196]. Using more or less strict outcome variables will either underestimate or overestimate the prevalence of the disease. When using six different outcome definitions of asthma used in different studies [197-202], all based on the ISAAC questionnaire, the prevalence of current asthma in our study varied from 6.2 % to 20.0%. To obtain comparable prevalences with a European study of about the same size and with participants at the same age, we chose to use the same outcome definition for asthma as the German MAS study [15]. With this definition, the prevalence for asthma was 11.9%, whereas prevalence with the definition used in the Swedish BAMSE study was 10.7% [166]. For atopic dermatitis and allergic rhinitis, we used the same outcome variables as in the BAMSE study [166].

4.2.4 Spirometry with reversibility test

Spirometry measures how an individual inhales or exhales volumes of air as a function of time [157]. With reversibility testing, a determination of airflow limitation is done by performing spirometry before and 15 min after administration of a bronchodilator. The spirometry test procedure is complicated and requires cooperation between the subject and the examiner. The quality of the obtained results depends on technical as well as personal factors with many possibilities for bias. Instruction by trained staff is especially important to reduce intra-observer variability, and a restricted number of instructors to reduce inter-observer variability. To reduce bias, one trained paediatric allergist, one trained assistant, or two trained study nurses performed all spirometry measurements in TFF2.

In patients with asthma, spirometry between exacerbations may be normal and the sensitivity of the test may be low [203]. Reversibility test with bronchodilator or provocation with

exercise may increase sensitivity, but not sufficiently to be a good screening tool for asthma. Asthma diagnosis is based on the clinical history and the results of clinical examination and lung function tests. Even in children with severe asthma, reduction in lung function is an insensitive measure for identification of problematic severe asthma in unselected asthmatic patient populations [186]. In a recent study from our region, they found good agreement between the questionnaire responses and clinical assessments. The questionnaire had good validity and served as a useful epidemiological tool. Detailed clinical testing added little additional information [204].

4.2.5 Serum specific IgE

A good allergy test of low cost and low risk with high sensitivity and specificity is lacking. The patient history is important, and in some cases, enough to diagnose allergy. In food allergy, the performance of tests is often compared with the results from DBPCFC as the gold standard. Serum specific IgE is the most widely used laboratory test. It is measured in kU_A/L as a continuous variable with a cut-off value often set to 0.35 kU_A/L or 0.10 kU_A/L. A positive test is interpreted as sensitization to that specific allergen. Sensitization is not the same as allergy and it is possible to be tolerant and sensitized to the same allergen. As a test of allergy, the specificity is low with many false positive tests. Studies use receiver operating characteristic (ROC) curves to find allergen specific cut-off values, to which allergy is present with 95% certainty. However, there are large variations between allergens and there is often no association between sIgE levels and seriousness of allergic reactions. The sensitivity of the test varies between different allergens, but is usually as high as 90-95% or even higher.

Despite low specificity, sIgE is an important test in the diagnosis of allergy, and sensitization itself an important risk factor for allergic disease. In clinical practice, sensitization together with a clear history of allergic reactions is often enough to diagnose allergy. However, often the history is not clear, and the only way to confirm allergy or tolerance is to perform challenges. ROC curves for sIgE can be used to find the most optimal cut-off values for performing food challenges.

An allergen source like fish, consist of many proteins of which only a few may act as allergens and elicit allergic reactions. With a conventional sIgE test, serum from the patient is exposed to an extract of many allergens (allergen extracts) and a positive test does not identify to which allergen(s) the patient is sensitized. The last 20 years, an increasing number of specific allergen molecules have been identified from many different allergen sources.

With component resolved diagnostics (CRD), each allergen molecule (highly purified proteins) can be tested separately for sensitization. Allergens may have different ability to elicit allergic reactions depending on the protein structure and the stability of the protein. For some allergies (e.g. peanut and hazelnut), CRD allows us to differentiate between primary sensitization and cross-sensitizations and to assess the seriousness of an allergy without performing challenges [205, 206]. Recently, sIgE to the peanut component Ara h2 in infants was shown to predict later peanut allergy in adolescents [207]. Increasing number of tests are available and routinely used in clinical practice. For fish, the only allergen molecule (component) available for clinical use is parvalbumin to cod and carp. Enolase and aldolase have been identified as important new fish allergens [84], and in our study they seem to have a role in confirming fish allergy in situations where the results from other fish allergens are unequivocal.

4.2.6 Oral food challenges

DBPCFC is the gold standard in diagnosing food allergy. The procedure is time-consuming with risk for serious allergic symptoms [94]. The suspected allergen is given in escalating doses and the patient is observed for allergic symptoms. If objective allergic symptoms evolve, the test is stopped and interpreted as positive. Standardized procedures performed by trained staff in a location equipped for immediate intensive care are essential. The test result may be biased due to inadequate test food or performance and interpretation of the test. Use of placebo is important because subjective symptoms may be due to psychological mechanisms. However, some patients may have a mild allergy with only subjective symptoms as itching in the mouth, mild stomach pain or nausea. If no symptoms on the placebo day, subjective symptoms on active substance are likely to represent an allergic reaction rather than psychological mechanisms. Although the observer is blinded, interpretation of the test may be biased. Objective symptoms may not be obvious if rashes or blisters are subtle. Standardized stop criteria are important. We used a grading system of symptoms used in other European studies; "DBPCFC – Clinical manifestations and grading" from the University Hospital of South Manchester, UK [165]. The participants VAS-graded subjective symptoms. DBPCFC was stopped when predefined stop criteria were reached or all doses completed. Stop criteria are also important for the safety of the participants. The observer dilemma is to continue the challenge until reliable objective signs are observed, but to stop the challenge before serious reactions evolve. Interpretation of symptoms is prone to bias due to both inter-

and intra-observer variability. In our study, we aimed to minimize bias by using only one trained paediatric allergist in the observations and interpretations of all challenges.

In the DBPCFC test food, high volumes of dessert matrix were needed to disguise small doses of fish, resulting in a cumulative dose of fish protein of only 1.13 mg (equals approximately 5 g fish filet). Participants with mild allergies may not react to this low dose. To avoid false negative tests due to low doses, all participants without symptoms or only subjective symptoms with DBPCFC, continued with open oral food challenges with higher doses of fish allergen after the randomization had been broken.

Unfortunately, many of the youngest children did not like the dessert and refused to continue. However, by adding sugar and flavour, most children were able to complete the challenges.

It has been questioned if DBPCFC may induce a transient tolerance to the tested allergen in allergic patients. If so, false negative DBPCFC could be the result of this effect. It is not known if this effect is present with fish allergens. To reduce or eliminate the effect of a potential transient tolerance induction, DBPCFC was performed with six weeks intervals between each challenge day.

In summary, by using high quality test food in standardized test procedures, performed by trained staff and interpreted by one paediatric allergist, we believe bias due to DBPCFC was reduced to a minimum.

To avoid false negative tests due to low doses, DBPCFC negative participants underwent open OFC with higher doses of fish protein. The result may be biased by psychological symptoms since there is no possibility to compare with placebo. The observer's interpretation may be biased due to lack of blinding. To reduce bias, it is important to continue the test until objective signs of allergic reactions appear or top dose is reached. Open OFC was performed with fish burgers. To avoid confounding by other allergens, ingredients were restricted to water, potato flour, salt and pepper. Participants with no history of reaction to tomato were given the opportunity to use tomato ketchup. To maintain some blinding, participants were not told which fish species they were challenged with in the open challenges. Although fish burgers from cod, salmon and mackerel look different and have different taste, we expected that children having minimal experience with eating fish would not be able to identify which fish species the burgers contained. After completed challenge, participants were asked to guess which fish they had been challenged with. Participants correctly guessed which fish

species they had been served in the open OFC in the following ranking order mackerel (31%), cod (46%) and salmon (56%). Observer bias was reduced by blinding the paediatric allergist who examined the participants and interpreted the symptoms.

Exposures other than fish allergens may influence the outcome of a challenge, by either confounding or interaction. Symptoms may be elicited, reinforced or reduced by non-allergic mechanisms. Controlling for these exposures can either be done by statistical methods or by eliminating the exposures. It is important to eliminate such exposures to reduce this type of bias. Some exposures may increase allergic symptoms and give serious reactions and harm for the participants. Thus, elimination of these exposures is necessary due to safety and ethical reasons.

Several medications influence the outcome of oral food challenge, most of them by reducing allergic symptoms. To control for these effects, ongoing medication is registered and medication that influence symptoms have to be ceased for a predefined period before the food challenges (i.e. antihistamines have to be ceased at least 72 hours before the food challenge). Ongoing infections may increase the symptoms of an allergic reaction. All participants were examined before food challenges were started. If symptoms or signs of infections were present, the food challenges were postponed. Recent or ongoing allergic reactions make the interpretation of the food challenges impossible and may contribute to elicit more serious reactions. Participants were asked about recent allergic symptoms and examined for allergic signs before each food challenge. If symptoms or signs of allergic reactions were present, challenges were postponed. Pollen allergic patients were not challenged during the pollen season. It is also important that participants are not exposed to other allergens during challenge days. They were allowed to eat a light breakfast to which they knew they were tolerant. During the challenge, they were not allowed to eat other foods, but could drink water or lemonade.

Co-morbidity with allergy and asthma is common and asthma is one of the most serious allergic symptoms in an allergic reaction. Uncontrolled asthma is a risk factor for anaphylactic reactions to an allergen. For safety reasons, it is of crucial importance that participants with asthma are well controlled before food challenges are performed. Before inclusion, all participants underwent a clinical examination, including auscultation of the lungs and lung function test with spirometry. Uncontrolled asthma was treated with adjustment of asthma medication. Before each food challenge, participants were asked about

asthma symptoms and examined with lung auscultation. If symptoms or signs of asthma were present, challenges were postponed and asthma treatment adjusted. Exercise may reinforce the effect of an allergen and even lead to anaphylaxis for some allergens (Exercised Induced Anaphylaxis). Participants were told not to do exercise before, during or after the food challenges on challenge days.

4.2.7 Statistical methods including sample size

We used multivariable logistic regression models to study associations between *S. aureus* carriage/SE-sensitization and allergic disease/allergic sensitization. These models are used to show statistical associations but cannot be used to prove causality. Furthermore, statistical associations may be biased by other variables not corrected for in the model (confounders).

To estimate threshold doses, data were analysed using an interval censoring survival analysis approach described in the method section. This is considered the best statistical method to estimate threshold doses. However, with low numbers, the estimated threshold doses are less reliable. In our study, this was the case for the estimated threshold doses for objective reactions to salmon and mackerel, reflected by wide confidence intervals for these estimates.

In TFF2, the study sample was the complete population of adolescents in the geographic area of the study. Thus, a formal calculation of power was not regarded as necessary for this study. Selection bias due to non-responders are discussed in chapter 4.2.1 (selection bias).

The primary purpose of the TRO-FAST study, as reported prospectively before study start in ClinicalTrials.gov (NCT02365168) was "to determine to which degree fish allergic children and adolescents can tolerate some species of fish, and to find the minimal eliciting allergen dose to which only 10% of participants get allergic reaction." We aimed to include 40 patients. We anticipated that some patients would drop-out due to different reasons, and therefore 44 patients were included. Prior to study start, other researchers had reported that up to, or more than, 50% of fish allergic patients would be tolerant to some other fish species ("partially tolerant"). However, previous studies using "gold standard" investigations with DBPCFC were limited and very small. We considered our study sample large enough to describe the distribution of partially tolerant and non-tolerant participants. No formal power analysis was applied for the sample size, and the sample size was clearly also a pragmatic choice based on number of available patients and the very large work-load with both blinded and open food challenges on several occasions. However, at least 30 observations is needed to

estimate reliable threshold doses for allergic reactions using dose-distributions curves [112]. Thus, we expected to have enough power to be able to calculate estimated threshold doses for subjective reactions to at least cod. Indeed, we were able to calculate estimated threshold doses for subjective reactions to all three fish species, although the difference in age between participants may add uncertainty due possible differences in threshold doses in different age groups. The estimated threshold doses for objective reactions to salmon and mackerel are less reliable and should be interpreted with caution due to less than 30 observations.

We also investigated secondary outcomes including comparisons of cut-off levels for different sIgE's in the partially tolerant versus the non-tolerant group. Our sample size was not powered for these secondary outcomes. We still found significant differences between the two groups in several sIgE values indicating that these two groups were different fish allergic phenotypes, with different levels of sIgE response. However, we certainly acknowledge that a larger sample size would have been an advantage when analysing the sIgE responses. However, inclusion of more participants was not possible due restricted number of eligible patients and a very costly and time-consuming design of the study. A researcher should consider to not perform a study without enough statistical power. Participants may have to go through potential harmful procedures without the study being able to answer the study questions. However, our sample size was three times larger than the largest study sample previously used in a comparable study and we find our sample size to be adequate to answer the main purpose of the study. We therefore found it ethical justifiable to perform the study. These considerations were also conveyed to the regional ethical committee (REC) in 2013 prior to study start. We stated clearly that we considered our sample size large enough to find and describe the distribution of partially tolerant and non-tolerant participant. Moreover, we stated that secondary outcomes would be explored, but that a formal sample size calculation could not be based on these secondary outcomes. The study was approved by REC.

4.3 **Ethical considerations**

Ethical issues are relevant in all research on humans and The World Medical Association has developed the Declaration of Helsinki as a statement of ethical principles for medical research involving human subjects, including research on identifiable human material and data [208].

Due to the vulnerability of children, research on children has been restricted in many countries to protect them from negative effects of research. Researches should be vigilant to expose vulnerable subjects to risks and burdens. Traditionally there should be no or very

small risk and risks should be justified by a direct benefit to the research subject. A consequence of less research is use of non-evidence based treatment to children. It is an ethical dilemma that protection from harm due to research, may produce harm due to lack of research. This problem has been addressed in the book ”The ethics of paediatric research” [209]. In conclusion, the author states that clinical research on children is necessary, even if there is a small risk and no benefit for the participant. Bos et al. have explored the legal regulation and ethical guidance for paediatric clinical research in the European Union and discussed the future challenges in this field [210]. Less research, especially trials with new medications, is a consequence of strict regulations for research on children. Therefore, the European Union have ordered the pharmaceutical industry to plan trials with children for all new medications going into the marked.

Valid decisions to participate in research must be made voluntarily by legally competent adults, after being informed of the nature, significance, implications, risks and burdens of the research. Children are not old enough to give legally valid consent and their capacity to understand and assess information is limited. As a result, parents are almost always involved in decisions to enrol a minor in a clinical trial [210]. This is problematic from an ethical point of view, especially if the research involves painful or potentially harmful procedures. However, decision-making strategies aim at encouraging shared decision making between the parent and the minor research subject. This may include dual consent, consent by the parent and agreement of the minor to participate, and respect for the dissent of the child [210]. Ondrusek et al concluded that most children younger than 9 years of age cannot be expected to consent or assent to clinical research in a meaningful way [211].

In TRO-FAST, we recruited children and adolescents. To facilitate shared decision-making we adapted separate written information about the study to children, adolescents and parents. Under the age of 16, parents’ consent is mandatory. All participants between the age of 12 and 16 also gave written consent to participate and children between 8 and 12 years of age were given the opportunity to sign an agreement to participate. However, in spite of shared decision-making, the children could have consented because they felt pressure to do so, either from their parents, from the researchers or both. In the study from Ondrusek et al, they showed that voluntariness may have been compromised in many subjects by their belief that failure to complete the study would displease the parents or the researcher [211].

The Oviedo Convention is binding EU member states and specifically addresses the issue of paediatric research in Article 17 [212], supplemented with an additional protocol on biomedical research [213]. Important conditions in this article are that the research should have potential benefit for the child and should implement minimal risk or burden to the research subject. Research on minors should not be done if comparable effectiveness can be carried out on individuals capable of giving consent or if the child concerned object to participate.

In TRO-FAST, blood sampling and performing food challenge may have been a burden for the participants and the food challenge elicited allergic reactions in many participants. In the beginning of the study, some of the youngest children refused to eat the test-food. Some parents wanted to persuade the children to continue. This is not in line with the Oviedo convention and these children were excluded from the study as dropouts. Still, many participants experienced the challenge and the allergic reactions as a burden. However, they wanted to continue because they (or their parents) considered it as a benefit to get a specific diagnose of their fish allergy in a safe setting. Although the test entails burdens and risk of allergic reactions, it is the gold standard in diagnosing food allergy and it is routinely used in clinical practice [138]. In spite of risks and burdens, based on these facts, we found it ethically justifiable to perform the study among children. Considerations regarding sample size and the TRO-FAST study are discussed in 4.2.7.

5 Conclusions

- Asthma, atopic dermatitis and allergic rhinitis are common chronic diseases among adolescents in the Arctic region of Norway. Nearly half of the adolescent population has experienced one or more of these diseases by the age of 18-19 and multimorbidity of allergic disease exists in 10 % of all adolescents.

- The severity of allergic disease is associated with *S. aureus* carriage, but its role in the pathogenesis and severity is not established.

- Polysensitization to food and inhalant allergens and allergic multimorbidity are associated with sensitization to *S. aureus* enterotoxins. Thus, sensitization to *S. aureus* enterotoxins may play a role in the development of polysensitization to food and inhalant allergens and allergic multimorbidity in adolescents.

- Allergy to multiple fish species due to cross-sensitization to multiple fish species are common in fish allergic children and adolescents, but among participants challenged with three different fish species, half of them had no objective symptoms and around one third of them had no subjective symptoms to at least one fish species (partial tolerance).

- Fish allergic patients with partial tolerance should be identified to avoid unnecessary food restrictions. Measurement of sIgE against fish allergen extracts and molecules cannot substitute oral food challenges, but a combination of clinical history and specific IgE measurements may reduce the number of food challenges needed for specific diagnosis of fish allergy and tolerance.

- Threshold doses for eliciting allergic reactions seem to be lower for cod, compared to salmon and mackerel.

6 Future research questions.

Does *S. aureus* biofilm production play a role in the development of allergic disease and sensitization? If the observed associations between *S. aureus* carriage/SE-sensitization and allergic disease/allergic sensitization represent a causal mechanism, does the ability of *S. aureus* to produce biofilms and create a persistent niche for colonization play a role in this mechanism? Analysing *S. aureus* colonies from the TFF2 cohort with regard to biofilm production could contribute to further understanding of the impact of *S. aureus* on allergic disease and sensitization.

Do environmental pollutants play a role in the development of allergic disease and sensitization? There has been increasing focus on toxins from environmental pollutants and their impact on human health. In TFF2, toxin levels in blood from environmental pollutants were measured in all participants. Analysing associations between toxins from environmental pollution and allergic disease could contribute to further understanding of risk factors for allergic disease.

Can the basophil activation test (BAT) with fish allergens substitute oral food challenges in the diagnosis of fish allergy? BAT is a flow cytometry-based assay where the expression of activation markers is measured on the surface of basophils following stimulation with allergens [139]. Maybe this test (BAT) may have better test properties to identify the phenotype of fish allergic patients than skin prick test and serum sIgE? Maybe the use of BAT (exposing basophils to fish allergens in a test tube) can further reduce the need for exposing the "whole patient" to fish allergens in an OFC? For peanut allergy, BAT can discriminate between tolerance and allergy among sensitized individuals and reflect the severity and threshold of reaction to peanut [214, 215]. BAT has not been studied with use of different fish allergens in diagnosing fish allergy. In a future study, we will compare BAT results with the results from DBPCFC.

Can OFC s improve quality of life in fish allergic children and adolescents? OFC is a diagnostic procedure. Can the test also contribute to reduce anxiety related to food allergy and thereby contribute to increase quality of life in food allergic children? Many participants and parents in TRO-FAST reported that the OFC s made them feel safer and contributed to reduce anxiety for allergic reactions, also when they were intolerant. They gained knowledge about how they react and how much fish they had to eat before allergic reactions evolved. With the

“Food Allergy Quality of Life Questionnaire” (FAQLQ), this effect on quality of life could be measured in a follow-up of the participants.

Does diagnosing tolerance lead to changes in the diet? From clinical experience, we know that many patients with previous food allergy do not implement the food in their diet after tolerance has been demonstrated with OFC. In a follow-up questionnaire, questions about implementation of fish species to which they were tolerant in their diet can answer this question.

Can specific oral allergen immunotherapy be used to treat fish allergy? Specific immunotherapy for inhalant allergies and insect venom allergies has existed for several decades. So far, no safe and efficient immunotherapy is ready for clinical use to treat food allergies. Research on food allergy has focused on oral immunotherapy and there is also an ongoing project with subcutaneous immunotherapy with fish allergens [83]. An alternative route of allergen exposure in specific immunotherapy is transdermal immunotherapy. So far, transdermal immunotherapy has not been studied with fish allergens, but may be an option for future research.

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