From THE INSTITUTE OF ENVIRONMENTAL MEDICINE
Karolinska Institutet, Stockholm, Sweden

# DIET AND THE RISK OF LATENT AUTOIMMUNE DIABETES IN ADULTS (LADA)

# STUDIES ON THE ASSOCIATION WITH FISH AND SWEETENED BEVERAGES

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Stockholm 2019

Published by Karolinska Institutet.
Printed by Universitetsservice US-AB

ISBN 978-91-7831-442-3

# Diet and the risk of latent autoimmune diabetes in adults (LADA)
# Studies on the association with fish and sweetened beverages

## THESIS FOR DOCTORAL DEGREE (Ph.D.)

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# ABSTRACT

Diabetes is an increasing public health problem affecting a breathtaking number of people worldwide. The knowledge about modifiable lifestyle factors influencing diabetes risk is extensive for type 2 diabetes, but limited for autoimmune forms of diabetes such as type 1 diabetes. Latent autoimmune diabetes in adults (LADA) is a hybrid form of diabetes with characteristics of both type 1 and type 2 diabetes. Diet has an important role in the development of type 2 diabetes but its role in autoimmune diabetes is largely unknown. The aim of this thesis was to study the risk of LADA in relation to intakes of fish and sweetened beverages, two commonly consumed foods hypothesized to play a role in the development of both type 1 and type 2 diabetes.

Analyses were based mainly on ESTRID, a Swedish case-control study with incident cases of LADA and type 2 diabetes, and population-based controls. All participants in ESTRID were aged ≥ 35 years and LADA was defined by the presence of glutamic acid decarboxylase autoantibodies (GADA) and a level of C-peptide indicating remaining insulin secretion. Data on intakes of fish and sweetened beverages were available from questionnaires and investigated in relation to risk of LADA and type 2 diabetes. One study was based on data from EPIC-InterAct, a case-cohort study with prospective data from eight European countries. In EPIC-InterAct, we assessed the interaction of baseline GADA positivity and self-reported dietary fish or plasma n-3 PUFA in relation to the risk of adult onset diabetes.

Based on ESTRID, weekly fatty fish intake was associated with 49% reduced risk of LADA (odds ratio [OR] 1.51, 95% confidence interval [CI] 0.30-0.87), whereas no association was found for type 2 diabetes (OR 1.01, 95% CI 0.74-1.39). These findings were supported by the results from EPIC-InterAct; low fatty fish intake was found to interact with GADA positivity on the risk of adult onset diabetes (attributable proportion due to interaction [AP] 0.48, 95% CI 0.24-0.72), and findings were similar for plasma n-3 PUFA. In ESTRID, sweetened beverage intake was positively associated with both LADA and type 2 diabetes. For LADA, the increased risk was evident only among carriers of low/intermediate risk HLA genotypes (OR per one daily serving 1.32, 95% CI 1.06-1.64). BMI was suggested to only partly mediate the associations between sweetened beverages and risk of LADA and type 2 diabetes, indicating that there may also be direct effects on glucose homeostasis.

In conclusion, these results suggest that long-chain n-3 PUFAs from fish may decrease the risk of LADA whereas intake of sweetened beverages may increase the risk. Importantly, these findings indicate that diet may be a modifiable lifestyle factor influencing the development of LADA and hence be a target for preventive strategies.

# SAMMANFATTNING PÅ SVENSKA

Diabetes är en kronisk sjukdom och ett stort folkhälsoproblem. Idag beräknas 1 av 11 vuxna vara drabbade av sjukdomen världen över och siffran förväntas öka. Det finns olika former av diabetes men gemensamt för alla är att blodsockernivån är för hög, detta på grund av att kroppens eget system förlorat förmågan att genom hormonet insulin hålla blodsockret på rätt nivå. Personer med diabetes löper även högre risk att drabbas av flera allvarliga sjukdomar såsom hjärtinfarkt och stroke. Den vanligaste formen är typ 2-diabetes (vanligaste formen hos vuxna), som kännetecknas av att kroppens celler har minskad förmåga att känna av insulinets signaler om att ta upp socker från blodet, så kallad insulinresistens. Typ 1-diabetes är en autoimmun form av diabetes (vanligaste formen hos barn), vilket innebär att kroppens immunförsvar av okänd anledning attackerar och förstör cellerna som ska producera insulin vilket leder till insulinbrist. Latent autoimmun diabetes hos vuxna (LADA) har drag av både typ 1- och typ 2-diabetes och kännetecknas av både autoimmunitet och insulinresistens. Kunskapen om vad som påverkar risken för LADA är mycket begränsad. I den här avhandlingen undersöks om konsumtion av fisk och sötade drycker påverkar risken att utveckla LADA, något som inte studerats tidigare. Avhandlingen innefattar fyra delstudier, där tre baseras på svensk data från ESTRID-studien och en baseras på europeiska data från studien EPIC-InterAct.

I **delarbete I** undersökte vi sambandet mellan konsumtion av fet fisk och risken att utveckla LADA och typ 2-diabetes. Fisk innehåller särskilda omega-3-fettsyror (EPA och DHA) som har visats vara fördelaktiga för många olika processer i kroppen. I vissa studier har man sett en minskad risk för typ 1-diabetes bland barn med ett högt intag av omega-3-fettsyror. Att äta fisk har även föreslagits minska risken för typ 2-diabetes, men studier har visat blandade resultat. I ESTRID-studien fann vi att personer som uppgav att de åt fet fisk minst en gång i veckan löpte omkring 50% lägre risk för LADA jämfört med dem som åt fet fisk mer sällan. Vi såg inget samband mellan fet fisk och typ 2-diabetes. Dessa resultat skulle kunna tyda på att fet fisk kan minska risken för LADA genom effekter kopplade till autoimmunitet.

I **delarbete II** använde vi data från EPIC-InterAct-studien för att forsätta studera sambandet mellan fiskkonsumtion och autoimmun diabetes. Vi fann att personer som vid studiens start hade GAD-antikroppar i blodet (påvisar autoimmunitet och är en riskfaktor för diabetes) löpte särskilt hög risk om de samtidigt hade lågt intag av fisk. Liknande samband sågs för dem med låga nivåer av omega-3-fettsyror (särskilt DHA) i blodet. Dessa resultat ger ytterligare stöd för att omega-3-fettsyror från fisk minska risken för diabetes, även hos personer som redan utvecklat autoimmunitet.

I **delarbete III** undersökte vi sambandet mellan konsumtion av sötade drycker (främst läsk) och risken för LADA och typ 2-diabetes baserat på ESTRID-data. Sötade drycker har kopplats till ökad risk för typ 2-diabetes i många studier, och ett par studier har även visat en koppling till typ 1-diabetes. Läsk innehåller vanligtvis stora mängder socker som dels kan öka risken för övervikt men även bidra till insulinresistens. Läsk som innehåller sötningsmedel (light-läsk) innehåller inga kalorier men det finns hypoteser kring att light-läsk ändå skulle kunna bidra till

diabetesrisk. Vi fann att hög konsumtion (mer än två glas/dag) av sötade drycker var kopplat till en fördubblad risk för både LADA och typ 2-diabetes. För varje extra glas läsk per dag ökade risken med 15-20 %. Detta gällde både ”vanlig läsk” och light-läsk, men den ökade diabetesrisken kopplad till light-läsk har eventuellt andra förklaringar än att den drycken i sig skulle öka risken. Dessa resultat talar för att sötade drycker ökar risken för LADA och att LADA delvis har samma riskfaktorer som typ 2-diabetes.

I **delarbete IV** ville vi ta reda på hur stor del av sambandet mellan sötade drycker och LADA och typ 2-diabetes som förklaras av effekter på BMI, samt om sambandet påverkas av riskgener för diabetes. Baserat på ESTRID-studien fann vi att BMI förklarar ungefär hälften av sambandet mellan sötade drycker och typ 2-diabetes, och 17% av sambandet för LADA. När vi delade upp studiedeltagarna med avseende på genetik, såg vi att den ökade risken för LADA kopplad till sötade drycker endast fanns bland personer *utan* högrisk-varianter av HLA-gener (kopplade till autoimmunitet). Däremot verkade inte genen *TCF7L2* (riskgen för typ 2-diabetes) påverka sambanden. Dessa resultat föreslår att konsumtion av sötade drycker ökar risken för både LADA och typ 2-diabetes genom mekanismer kopplade till ökad kroppsvikt men även att andra mekanismer har betydelse.

Sammanfattningsvis tyder resultaten på att omega-3-fettsyror från fisk minskar risken för LADA medan läskkonsumtion är förenat med en ökad risk. Kostfaktorer tycks alltså kunna påverka utvecklandet av LADA. Kunskap om riskfaktorer är viktigt eftersom den kan användas för att förebygga diabetes. Fler studier behövs dock för att bekräfta de samband som påvisats här.

# LIST OF SCIENTIFIC PAPERS

This thesis is based on the four papers listed below. These will be referred to as Paper I-IV in the text and are reproduced in full at the end of the thesis.

I. **Löfvenborg JE**, Andersson T, Carlsson PO, Dorkhan M, Groop L, Martinell M, Tuomi T, Wolk A, Carlsson S. Fatty fish consumption and risk of latent autoimmune diabetes in adults. Nutr Diabetes 2014;4:e139

II. **Löfvenborg JE**, Carlsson S, Andersson T, Hampe CS, Sharp SJ, Wareham NJ, Rolandsson O for the InterAct Study Group. Interaction between GAD65 antibodies and dietary fish or plasma n-3 PUFA on the risk of adult onset diabetes: The EPIC-InterAct Study [manuscript]

III. **Löfvenborg JE**, Andersson T, Carlsson PO, Dorkhan M, Groop L, Martinell M, Tuomi T, Wolk A, Carlsson S. Sweetened beverage intake and risk of latent autoimmune diabetes in adults (LADA) and type 2 diabetes. Eur J Endocrinol 2016;175:605-614

IV. **Löfvenborg JE**, Ahlqvist E, Alfredsson L, Andersson T, Dorkhan M, Groop L, Tuomi T, Wolk A, Carlsson S. Genotypes of HLA, *TCF7L2*, and *FTO* as potential modifiers of the association between sweetened beverage consumption and risk of LADA and type 2 diabetes. Eur J Nutr 2019 Jan 17 [Epub ahead of print]

# CONTENTS

# LIST OF ABBREVIATIONS

| | |
|---|---|
| ALA | α-Linoleic acid |
| ANDIS | All New Diabetics in Scania |
| ANDiU | All New Diabetics in Uppsala |
| AP | Attributable proportion |
| BMI | Body mass index |
| CI | Confidence interval |
| DHA | Docosahexaenoic acid |
| EIRA | Epidemiological Investigation of Rheumatoid Arthritis |
| EPA | Eicosapentaenoic acid |
| EPIC | European Prospective Investigation into Cancer and Nutrition |
| ESTRID | Epidemiological Study of Risk Factors for LADA and Type 2 Diabetes |
| FFQ | Food frequency questionnaire |
| GADA | Glutamic acid decarboxylase autoantibody |
| GWAS | Genome-wide association study |
| $HbA_{1c}$ | Glycated hemoglobin |
| HLA | Human leucocyte antigen |
| HOMA | Homeostatic model assessment |
| HR | Hazard ratio |
| HUNT | The Nord-Trøndelag Health Study |
| IA-2A | Islet antigen-2 autoantibody |
| IAA | Insulin autoantibody |
| IU | International units |
| LADA | Latent autoimmune diabetes in adults |
| MHC | Major histocompatibility complex |
| n-3 PUFA | Omega-3 polyunsaturated fatty acid |
| OR | Odds ratio |
| SNP | Single nucleotide polymorphism |
| WHO | World Health Organization |
| ZnT8A | Zinc transporter 8 autoantibody |

# 1 INTRODUCTION

Diabetes is a common, chronic disease that manifests when the pancreatic beta cells can no longer meet the body's need for insulin to maintain glucose homeostasis, resulting in hyperglycemia. Since there is no cure for diabetes, prevention is key to reduce the number of affected individuals. Knowledge about risk factors is necessary for effective disease prevention. Lifestyle modifications have been shown to be effective in prevention of type 2 diabetes [1, 2]. For autoimmune forms of diabetes, such as type 1 diabetes, there is a lack of established risk factors suitable as targets for preventive actions [3] and the role of lifestyle is largely unknown. For latent autoimmune diabetes in adults (LADA), an autoimmune form of diabetes with adult onset, studies are scarce.

Diet constitutes a major component in primary prevention of type 2 diabetes [2, 4] and several dietary risk factors have been identified including sweetened beverages [5]. The role of diet in the etiology of autoimmune diabetes is less clear. However, in children, a reduced risk of type 1 diabetes has been reported in relation to intake of fish, including fish-originated fatty acids [6, 7], whereas intake of sweetened beverages has been associated with excess risk [8, 9].

Latent autoimmune diabetes in adults (LADA) is a common (9-12% of all adult onset diabetes [10, 11]), autoimmune form of diabetes with features of both type 1 and type 2 diabetes. Risk factors are largely unknown and the aim of this doctoral thesis was to investigate, for the first time, the risk of LADA in relation to dietary fish and sweetened beverage consumption. Analyses were based on data from the Swedish ESTRID Study, which is the largest population-based study of LADA to date, and the European EPIC-InterAct Study, which is a prospective study with data on dietary factors, autoantibodies, and incident adult-onset diabetes. The overall aim was to contribute to an increased understanding of the etiology of LADA.

# 2 BACKGROUND

## 2.1 DIABETES

Diabetes is one of the most common chronic diseases and among the top ten causes of deaths worldwide [12]. The prevalence is estimated at 9% in the adult population globally and 7% in Sweden [13, 14]. The number of affected individuals has increased tremendously over the past decades, from 151 million to 425 million adults with diabetes since the year 2000, and the prevalence is projected to continue to rise [13]. The increase is explained by a combination of several factors, including changes into a more sedentary lifestyle combined with unhealthy dietary habits [15] but also increased life expectancy [16] and improved diabetes survival [14]. Diabetes often poses a heavy burden on the individual and also constitutes large healthcare costs for society, primarily due to risk of common complications such as cardiovascular diseases and problems related to the kidneys (nephropathy), eyes (retinopathy), and the nervous system (neuropathy) [15]. Better understanding on how modifiable lifestyle factors, such as diet, may impact the development of diabetes is crucial for primary prevention.

### 2.1.1 Diagnosis

Diabetes is characterized by hyperglycemia as a consequence of insulin resistance, insulin deficiency, or a combination of the two [17]. The inability to maintain normal blood glucose levels may be due to autoimmune destruction of beta cells, or that the insulin production is unable to meet the increasing demands due to insulin resistance [18]. Diagnosis is based on measurements of fasting plasma glucose, 2-hour plasma glucose after oral glucose tolerance test, random plasma glucose together with symptoms of hyperglycemia, or $HbA_{1c}$ [19-21].

### 2.1.2 Subtypes

Diabetes is a disease with broad manifestation, all characterized by raised blood glucose levels but with different pathogenesis. There has traditionally been a division into type 1 diabetes and type 2 diabetes, but its heterogeneity has been increasingly recognized and about 25 years ago, the term LADA was introduced to describe a subgroup of type 2 diabetes patients with autoantibodies [22], similar to type 1 diabetes. Since then the field has been evolving further and new diabetes classifications addressing the heterogeneity of type 2 diabetes have recently been proposed [23]. In this thesis, I will refer to type 1 diabetes, type 2 diabetes, and LADA as the three main subtypes of diabetes. In addition to these, there are a number of monogenic diabetes subtypes (e.g. forms of MODY; maturity onset diabetes of the young), gestational diabetes, and secondary diabetes which will not be further discussed in this thesis.

#### *2.1.2.1 Type 1 and type 2 diabetes*

Type 2 diabetes is the most common subtype of diabetes and constitutes about 75-85% of all patients [23, 24]. Type 2 diabetes used to be a disease almost exclusively occurring in adults, but has become an increasing health concern also among younger age groups [25]. Type 1

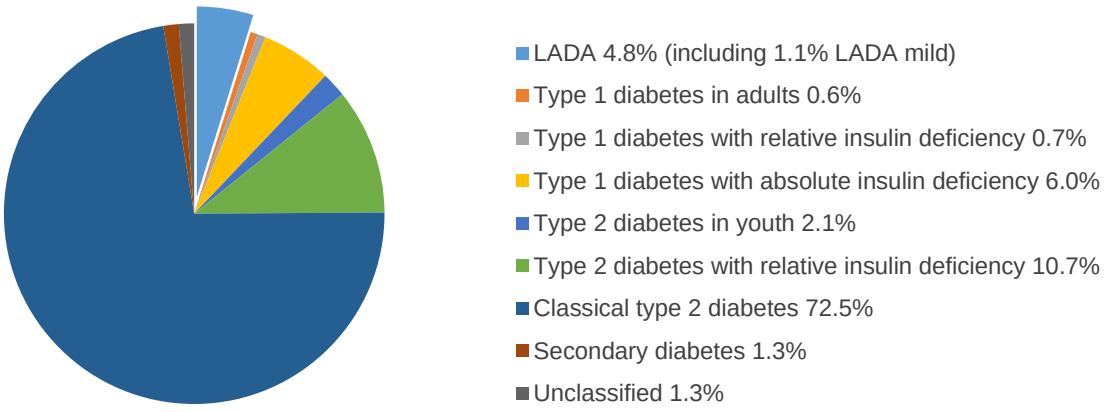
diabetes is an autoimmune form of diabetes most commonly thought of as affecting children and adolescents, but it may also occur in adulthood.

#### *2.1.2.2 Latent autoimmune diabetes in adults (LADA)*

The term LADA was first presented in 1993 by Tuomi et al [22], although presence of autoantibodies in adult patients diagnosed with type 2 diabetes was described already in 1977 [26] and a latent form of adult type 1 diabetes was described in 1986 [27]. LADA is by many considered to be a hybrid form of diabetes because of the presence of autoantibodies similar to type 1 diabetes but presenting with a phenotype more similar to type 2 diabetes [18]. LADA constitutes about 9-12% of all adult-onset diabetes in Europe [10, 11] and may be the most common form of autoimmune diabetes in adults. According to data from the ANDIS (All New Diabetics in Scania) study, where newly diagnosed diabetes patients are classified into different subtypes based on genetic and clinical features, LADA accounts for about 5% of all incident cases (Figure 2.1) [24]. Among all autoimmune diabetes with an adult onset, more than 80% may be classified as having LADA [28].

There are no unified criteria for LADA classification, but three criteria commonly applied at diagnosis are i) adult onset (usually ≥30 or 35 years), ii) autoantibody positivity (predominantly GADA), and iii) remaining insulin secretory capacity (absence of insulin therapy 6-12 months after diagnosis) [18]. However, the insulin therapy criterion has been questioned as it is open to subjectivity [29] and an alternative approach is to use C-peptide levels above a certain cut-off, as a measure of remaining insulin secretion [23]. The antibody criterion separates LADA from type 2 diabetes and the criterion about remaining insulin secretion distinguishes LADA from type 1 diabetes. The World Health Organization (WHO) and American Diabetes Association however consider LADA as a slowly progressing form of type 1 diabetes [30].

**Figure 2.1.** Distribution of different subtypes of diabetes in the ANDIS registry, adapted from the ANDIS website [24].



### 2.1.3 Pathogenesis

#### *2.1.3.1 Type 2 diabetes*

Type 2 diabetes is characterized by insulin resistance of skeletal muscle, liver, and adipose tissue [17], a metabolic condition also related to obesity [31]. Insulin resistance imposes an increased insulin demand and hyperglycemia manifests when the capacity of the pancreatic beta cells to increase insulin secretion is compromised. Insulin resistance in the liver leads to increased gluconeogenesis resulting in fasting hyperglycemia. This, in combination with muscle insulin resistance and other impairments in glucose metabolism such as beta cell resistance to incretin hormone signaling [32], results in postprandial hyperglycemia [17]. Furthermore, the presence of systemic inflammation mediated by pro-inflammatory cytokines and macrophage infiltration in adipose tissue is an additional contributor to insulin resistance [17].

#### *2.1.3.2 Type 1 diabetes*

Type 1 diabetes is a disease where autoimmune processes, mediated by T cells [33], lead to destruction of the pancreatic insulin-producing beta cells. This results in insulin deficiency and subsequent hyperglycemia. For most patients, the situation rapidly becomes acute and lifelong insulin therapy (injections) is needed [34]. Presence of autoantibodies is a hallmark of type 1 diabetes, but seroconversion, i.e. development of detectable autoantibodies in the blood, may have started already earlier in life [35]. There are four major types of autoantibodies related to type 1 diabetes; autoantibodies against insulin (IAA), glutamic acid decarboxylase (GADA), a tyrosine phosphatase-like protein (islet antigen-2 [IA-2A]), and zink transporter 8 (ZnT8A) [33]. Recently, also autoantibodies against tetraspanin 7 (TSPAN7A) has been described [36]. Positivity to multiple autoantibodies is associated with a markedly increased risk of progression to type 1 diabetes compared to single autoantibody positivity [37]. IAA usually appears as primary antibody (the first occurring autoantibody) among patients who become antibody positive early in life, whereas GADA is more frequent as primary antibody in children with later seroconversion [38].

#### *2.1.3.3 LADA*

Similar to type 1 diabetes, LADA is characterized by the presence of autoantibodies. The predominantly occurring autoantibody is GADA which is present in 90% of all autoantibody-positive adult patients [18]. Many LADA patients are positive for a single autoantibody, whereas in type 1 diabetes multiple autoantibodies are usually present at time of diagnosis [37]. The autoimmune processes are progressing more slowly in LADA than in classic type 1 diabetes and the patient's own insulin production is preserved for a longer period of time [11, 29]. Autoantibodies are prevalent also in the diabetes-free population and positivity confers an increased risk of diabetes [39].

Despite autoantibody positivity, patients with LADA are phenotypically similar to those with type 2 diabetes. In populations where LADA is not routinely diagnosed, about 5-14% of adults

diagnosed as type 2 diabetes are autoantibody positive and would fall within the framework of LADA [18]. The most important shared feature is insulin resistance, although this may be less pronounced than in type 2 diabetes patients [40, 41]. The relative contribution of insulin resistance is likely to differ depending on the degree of autoimmunity; those with high GADA levels tend to be less insulin resistant whereas those with low GADA levels display a higher degree of insulin resistance [28]. The presence of both autoimmunity and insulin resistance is likely to have implications for the etiology of LADA and risk factors may be related to either one, or both, of these characteristics.

#### *2.1.3.4 Diabetes treatment*

The aim of diabetes treatment is to target hyperglycemia in order to minimize the risk of complications. Common treatments for type 2 diabetes include lifestyle interventions, oral glucose-lowering agents and injectable medications [42]. For type 1 diabetes patients, daily injections of insulin forms the basis of therapy [43]. For patients with LADA however, there are currently no specific treatment guidelines. Hence, patients may not receive the optimal treatment, which may lead to adverse effects such as more rapid beta cell loss [44].

### 2.1.4 Genetics

#### *2.1.4.1 Type 2 diabetes*

More than 400 susceptibility loci have been associated with type 2 diabetes [45], most of them related to insulin secretion rather than insulin resistance [46]. The strongest genetic determinant of type 2 diabetes is *TCF7L2* [47]. *TCF7L2* encodes transcription factor 7-like 2 which is a key effector in the Wnt signaling pathway and suggested mechanisms for its role in type 2 diabetes development include impaired insulin secretion and enhanced rate of hepatic glucose production [48]. Another important risk gene is the fat-mass and obesity-associated (*FTO)* gene, for which carriers of common risk variants are predisposed to develop obesity and type 2 diabetes [49]. Underlying mechanisms are believed to include changes in satiety perception and energy intake [50, 51]. Despite the large number of identified type 2 diabetes susceptibility genes, they collectively still only explain a small part of the heritability [46].

#### *2.1.4.2 Type 1 diabetes*

The strongest genetic determinants for type 1 diabetes risk are located in the human leukocyte antigen (HLA) region on chromosome 6. The system covers several genes related to the immune system and is highly polymorphic and HLA genotypes are estimated to be responsible for about half of the genetic risk in type 1 diabetes [52]. The strongest association with type 1 diabetes is found with HLA DR and DQ, which encode cell surface receptors involved in antigen-presentation for T-lymphocytes. HLA DR and DQ are tightly linked and risk is conferred by specific combinations, or haplotypes. The haplotypes most strongly associated with type 1 diabetes are DRB1*04:xx-DQA1*03:01-DQB1*03:02 also referred to as DR4-DQ8, and DRB1*03:01-DQA1*05:01-DQB1*02:01 also referred to as DR3-DQ2. The heterozygous DR4-DQ8/DR3-DQ2 genotype confers the highest risk with odds ratio (OR) of

16. Some HLA haplotypes are associated with protective effects, such as DRB1*15:01 (DR2)-DQA1*01:02-DQB1*06:02 [53].

In addition, there are more than 50 non-HLA genes associated with type 1 diabetes. Important non-HLA polymorphisms include the insulin gene (*INS*), protein tyrosine phosphatase non-receptor type 22 gene (*PTPN22*), interferon-induced with helicase C domain 1 (*IFIH1*) [54], and cytotoxic T-lymphocyte associated protein (*CTLA-4*) [53]. The risk variant of *TCF7L2*, conferring the strongest genetic risk of type 2 diabetes, is more frequently found among non-carriers of high risk HLA genotypes compared to risk genotype carriers among childhood type 1 diabetes patients [55].

#### *2.1.4.3 LADA*

Studies conducted to date indicate that LADA shares most of its genetic background with type 1 diabetes. This was highlighted in the first genome-wide association study (GWAS) of LADA published recently [56]. The strongest genetic determinants for LADA are the HLA genes [57] and, similar to type 1 diabetes, the haplotypes conferring the strongest risk are DRB1*04-DQB1*03:02 (DR4-DQ8) and DRB1*03:01-DQB1*02:01 (DR3-DQ2), albeit with attenuated effect size [57, 58]. In addition, high risk HLA genotypes seem to correlate with GADA level [59]. The protective HLA-DQB1*06:02/X and HLA-DQB1*06:03/X seem to be more frequent among individuals with LADA compared to type 1 diabetes [57, 58]. A number of non-HLA type 1-related genes have also been associated with LADA, including variants in *PTPN22* and *INS* [57]. The LADA GWAS also confirmed a genetic overlap with type 2 diabetes, but less clear than for the autoimmune-related genes [56]. An association with the type 2 diabetes risk gene *TCF7L2* could not be confirmed at the genome-wide significance level, but has been demonstrated in several other studies [60] including two based on Swedish data [58, 61]. With regard to the *FTO* gene, one study based on data from the Norwegian HUNT Study have reported an association for LADA, especially among patients with low GADA levels [62], but replication is needed for verification. Based on the genetic background, autoimmunity seems to be the major driving force in the development of LADA. In line with this, family history of type 1 diabetes is a stronger risk factor for LADA than family history of type 2 diabetes [63].

### 2.1.5 Environmental and lifestyle risk factors

#### *2.1.5.1 Type 2 diabetes*

The contribution of environmental and lifestyle factors in the development of type 2 diabetes is substantial and much is known when it comes factors affecting risk of disease. Lifestyle plays an important role and excess adiposity, indicated by high body mass index (BMI), is the strongest single risk factor by its contribution to insulin resistance. In addition, increased abdominal fat has been shown to be a risk factor independent of BMI [15]. Other important risk factors include low quality diet which will be further discussed below (Section 2.2.1.1), but also low physical activity [64], sedentary lifestyle [65], cigarette smoking (in a dose-dependent manner) [67], air pollution [67], sleep duration [68], and psychological stress [69]. All of these risk factors are thought to influence risk of type 2 diabetes through mechanisms

contributing to impaired insulin sensitivity. Lifestyle interventions including modifications in diet and physical activity have been shown effective for weight loss and subsequent reduction in type 2 diabetes risk [2, 70].

#### *2.1.5.2 Type 1 diabetes*

Europe has the highest incidence of childhood type 1 diabetes, where Finland is in the top and Sweden comes second with an annual incidence of 40 new cases per 100,000 children aged <15 years [71]. A dramatic increase in incidence has been seen over the past decades; a doubling of childhood cases in Europe during the past 20 years [72]. This increase is unlikely to be explained by genetic changes but rather by an increased prevalence of environmental risk factors. Such factors could potentially act by triggering autoimmunity or promote the progression from islet autoimmunity to type 1 diabetes, or both.

Established environmental risk factors are lacking despite large efforts. However, exposure to enteroviruses is the factor most consistently associated with type 1 diabetes risk [73-75]. Other factors that have been proposed to increase the risk are respiratory infections [76], maternal age at delivery, high birthweight [77], accelerated weight gain early in life [78], and childhood adiposity [54, 79], but also serious life events and psychological stress [80]. Furthermore, there has been an increasing interest in the gut microbiota and its potential role in type 1 diabetes development [81], and is even discussed as a potential target for preventive interventions [82]. Alterations in gut microbiota (e.g. lower diversity) may be induced by several factors including diet (further discussed in Section 2.2.1.2), birth delivery mode (Ceasarian section) [83], antibiotics use, low exposure to viral infections, or excessive hygiene ('the hygiene hypothesis') [84, 85], which are all candidate factors for modulating type 1 diabetes risk [75]. It has proven difficult to replicate findings across studies and so far, attempts to prevent type 1 diabetes through lifestyle modification has been unsuccessful [3].

#### *2.1.5.3 LADA*

Given the hybrid nature of LADA, risk factors may have underlying mechanisms related to autoimmunity or/and insulin resistance. At present, there is a paucity of studies on environmental and lifestyle risk factors for LADA. One probable reason is that most studies with incident cases lack information about antibody status which is required to distinguish LADA from type 2 diabetes. Antibodies are not routinely measured in health care. The few studies investigating risk of LADA in relation to lifestyle factors conducted to date have all been based on data from two population-based studies in Norway and Sweden; the prospective HUNT Study [86] and the case-control study ESTRID [28]. Findings from HUNT and ESTRID suggest that LADA, despite its autoantibody positivity, may share several risk factors with type 2 diabetes; increased age, overweight, physical inactivity [87], family history of diabetes [88, 63], low birthweight [89], heavy smoking [90], sleep disturbances and low psychological well-being [91], which supports the importance of insulin resistance in the development of LADA. Some of these studies also highlight that the group of LADA patients is heterogeneous; for some of these factors, the associations vary depending on degree of autoimmunity [63, 90].

## 2.2 DIETARY RISK FACTORS

### 2.2.1 Overview

#### *2.2.1.1 Type 2 diabetes*

Diet has been shown to play an important role in the development, and hence also for preventive actions, of type 2 diabetes [2, 92]. Lower risks are generally seen with adherence to a Mediterranean diet which is characterized by high intakes of nuts, vegetables, legumes, fruits, fish and seafood, and moderate alcohol intake, and often limited intakes of red and processed meat, (high fat) dairy products, and either high olive oil intake or high monounsaturated fatty acid (MUFA) to saturated fatty acid (SFA) ratio [93, 94]. Contrary, positive associations have been observed for a Western diet which include high consumption of red and processed meat, fried products, high fat dairy products, refined grains, and sweets [95]. The beneficial effects of the Mediterranean diet have been attributed to cardiovascular improvements and reductions in fasting levels of glucose and insulin, insulin resistance, and inflammatory markers [96] whereas the Western diet has been related to increases in inflammatory markers [97]. Dietary components associated with lower risks are whole grain [5, 98], total dairy products [5, 99] which may be attributed to low-fat dairy products and/or yoghurt intake [99] although controversies exist [100], fruits [5, 101], vegetables [5] – specially green leafy vegetables [101], coffee [102], low to moderate alcohol consumption [103], and fatty fish intake (further discussed in Section 2.2.2.4) [104]. Increased risks have been proposed for intakes of sweetened beverages (further discussed in Section 2.2.3.3) [5, 105], red and processed meat [5], and diets high in glycemic load [106].

A role of total protein intake in the development of type 2 diabetes has also been discussed, with increased risks in relation to total protein which may be attributed to protein from animal sources [107, 108] whereas inverse associations for have been suggested for plant-based proteins [109]. A high PUFA:SFA ratio has been associated with lower risk of type 2 diabetes and *trans* fatty acids have been positively associated [110], but more recent findings suggest that the potential role of specific fatty acids may also vary by their carbon length; in EPIC-InterAct, plasma phospholipid even-chained SFAs were positively associated but odd-chained SFAs were inversely associated with type 2 diabetes [111]. Furthermore, the degree of unsaturation may affect the roles of specific fatty acids; among n-6 PUFAs the direction of association with type 2 diabetes was different for fatty acids with two, three, or four double bonds [112]. Taken together, a lot is known when it comes to dietary factors and their association with type 2 diabetes, but the specific components in foods and the underlying mechanisms explaining these associations are not fully elucidated.

#### *2.2.1.2 Type 1 diabetes*

Several dietary factors have been hypothesized to influence development of islet autoimmunity and type 1 diabetes but results have been inconsistent and no associations have been firmly established [113]. Dietary factors proposed to increase the risk include cow's milk [114-117], high sugar intake [9], low omega-3 PUFA intake and status [7], low vitamin D intake [118]

and 25-hydroxyvitamin D status [119, 120], and intake of nitrate and nitrite [121]. The timing, with regard to age but also to cessation of breastfeeding, for infant introduction to solid foods or specific foods such as gluten/cereal products [122, 123], fruits [122, 124], and root vegetables [124], may be of importance. The notion that risk factors for type 1 diabetes may be related to the gut microbiota is highly relevant when it comes to diet since the gut microbiota composition is vastly influenced by environmental factors such as diet [125, 126]. In support of an important role of gut microbiota, early supplementation of probiotics have been inversely associated with islet autoimmunity [127], however probiotic supplementation during the first six months in life was not associated with islet autoimmunity and type 1 diabetes [128]. Importantly, the vast majority of studies have been carried out in children, or siblings of children, and often in those with genetic susceptibility. Studies in adults, with and without genetic risk, are lacking.

#### *2.2.1.3 LADA*

To hypothesize that diet may influence the risk of LADA is not far-fetched, given the large number of dietary factors studied in relation to type 1 and type 2 diabetes discussed above. Despite this potential, the number of studies are few. In fact, by the start of this thesis project, only alcohol consumption had been assessed in relation to LADA risk; based on data from ESTRID and HUNT studies, an inverse association with moderate alcohol intake was found but suggestively only among patients with mild autoimmunity (low GADA levels) [129, 130], which is similar to observations in type 2 diabetes [103]. In addition, we later found coffee consumption to be positively associated with LADA but only in those with high GADA levels [131]. Interestingly, this contrasts previous findings in type 2 diabetes [102]. Along those lines, we recently showed that heavy coffee consumption may interact with high risk HLA genotypes on the risk of LADA [132], suggesting potential mechanisms related to autoimmunity.

This thesis project focuses on the role of dietary fish and sweetened beverage consumption in development of LADA. There are hypotheses relating dietary fish intake and related nutrients as well as sweetened beverage consumption to risk of both type 1 and type 2 diabetes (presented in the next section), but their association with LADA has not been previously investigated. This thesis is based primarily on data from the ESTRID Study since the dietary information in HUNT is limited to indicator questions on key dietary factors [133]. One of the included studies is however based on data from the prospective EPIC-InterAct Study [134]. Here, it is not possible to study incident LADA (requires known antibody status at diagnosis) but instead, GADA measured at baseline (i.e. before diabetes onset) could be explored as a risk factor including its potential interaction with other risk factors such as diet. A schematic summary of the background for this thesis work is presented in Figure 2.2 (page 14).

### 2.2.2 Fish consumption

Dietary fish is rich in several important nutrients and considered part of a healthy diet. The dietary guidelines in Sweden recommend consumption of fish and shellfish two to three times a week, and to alter between types of fish consumed (www.slv.se). Fish is the most important

dietary source of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA), which are long-chain polyunsaturated omega-3 fatty acids (n-3 PUFA), and one of the most important sources of vitamin D. However, dietary fish is also a source of environmental contaminants that may have adverse health effects [135, 136].

#### *2.2.2.1 Long-chain omega-3 fatty acids (n-3 PUFA)*

EPA and DHA are synthesized endogenously by elongation and desaturation of α-linoleic acid (ALA), an essential fatty acid with shorter carbon chain found in plant-based foods. However, this interconversion is very limited and may be as low as <1% for DHA [137]. For this reason, these fatty acids must be acquired through foods (or as dietary supplements). EPA and DHA have anti-inflammatory properties, which may be of relevance in diabetes prevention as both type 1 and type 2 diabetes have been described as having an inflammatory component [138, 139], and immunomodulatory properties through altered maturation and differentiation of T cells [140]. Also beneficial effects on insulin resistance has been demonstrated in animals [141] but findings are less consistent in humans [142]. The amount of n-3 PUFA is larger in fatty fish such as salmon and herring, and lower in lean fish such as cod and pollock. As an objective biomarker for dietary fish intake, measurements of EPA and DHA in various biological compartments is commonly used [143].

#### *2.2.2.2 Vitamin D*

Vitamin D is synthesized in the skin by sun exposure (ultraviolet-B radiation). The possibility to reach adequate vitamin D levels decreases with increasing latitude. In Sweden and other countries at a latitude above 40°, vitamin D synthesis cannot occur during the dark months of the year [144] and therefore it has to be acquired through food (as vitamin $D_3$ or $D_2$). After hydroxylation in the liver, into 25-hydroxyvitamin D [$25(OH)D_3$], and in the kidney, the biologically active form $1,25(OH)_2D_3$ is reached [145]. Dietary fish is one of the major dietary sources of naturally occurring vitamin D. Almost all immune cells have vitamin D receptors (VDR), which implies that vitamin D has an important role in immune function. Several diabetes-related health benefits have been proposed to be linked to vitamin D, including anti-inflammatory effects and preventive effects on autoimmunity through mechanisms such as reduced stimulation of T cells, but there may also be direct effects on pancreatic beta cells and insulin secretion [145].

#### *2.2.2.3 Environmental contaminants*

Dietary fish is also our primary source of exposure to persistent organic pollutants (POPs), especially polychlorinated biphenyls (PCBs) [135]. POPs have been shown to induce insulin resistance in rodents [146], and a positive association has been suggested with type 2 diabetes in humans [147]. Another contaminant present in fish in methyl mercury [136], which has been suggested to induce beta cell dysfunction [148].

#### *2.2.2.4 Dietary fish consumption and risk of diabetes*

The risk of type 2 diabetes in relation to dietary fish intake remains inconclusive and there are indications of regional differences with associations being positive for studies in North America, inverse for Asian and Australian studies, and no overall association in studies from Europe [149]. Differences in preparation methods or level of contamination in different species may partly explain the observed discrepancies [150]. Analysis of fish subtypes have suggested an inverse association for fatty fish based on data from European and Asian populations [104]. Moreover, serum levels of n-3 PUFA have been associated with lower risk of type 2 diabetes, with the strongest association seen for DHA [151].

Protective effects of fish intake and n-3 PUFA on islet autoimmunity or/and type 1 diabetes have been suggested as cod liver oil supplementation during first year of life was shown to be associated with decreased risk of developing type 1 diabetes [6]. Similarly, an American study reported reduced risk of islet autoimmunity in relation to total intake of n-3 PUFA with a similar tendency for marine-derived n-3 PUFA [7], but no association with progression to type 1 diabetes [152]. Moreover, a Finnish study found no association of serum EPA and DHA on islet autoimmunity [153]. Inverse associations for vitamin D and development of islet autoimmunity or/and type 1 diabetes are reported by some studies [118-120] but not others [154, 155]. Studies on dietary fish intake and objectively measured n-3 PUFA exposure are lacking with regard to autoimmune diabetes with an adult onset.

### 2.2.3 Sweetened beverage consumption

#### *2.2.3.1 Sugar-sweetened beverages*

The consumption of sugar-sweetened beverages has been increasing over the past decades [156]. Sugar-sweetened beverages contribute energy in the form of rapidly absorbed sugars but have low nutritional value. Consumption is associated with weight gain [158] with one explanation being that liquid calories are less likely to be fully compensated for in subsequent meals when compared to solid foods, possibly due to reduced satiety signaling [158, 159]. Apart from increased adiposity, there may also be independent increase in insulin resistance [160, 161], possibly through adverse effects of fructose-induced hepatic lipid synthesis [162, 163]. Furthermore, high sugar intake has been associated with increased inflammation [164] and demonstrated to induce beta cell apoptosis through oxidative stress [165] or beta cell overload [166]. Carbon-13 has been suggested as a biomarker for sugar-sweetened beverage intake but is not frequently used [167].

#### *2.2.3.2 Artificially sweetened beverages*

As an alternative to sugar, some beverages instead contain artificial (nonnutritive) sweeteners such as aspartame and acesulfame K. Stevia is another nonnutritive sweetener which is plant-based. In this text, all nonnutritive sweeteners will be referred to as artificial. Artificial sweeteners contribute with no or very few calories and that way do not directly add to the glycemic load or total daily energy intake. However, artificially sweetened beverages have

been suggested to enhance appetite due to distorted satiety signaling [168] and cause alterations in gut microbiota with subsequent deterioration in glucose tolerance [169, 170].

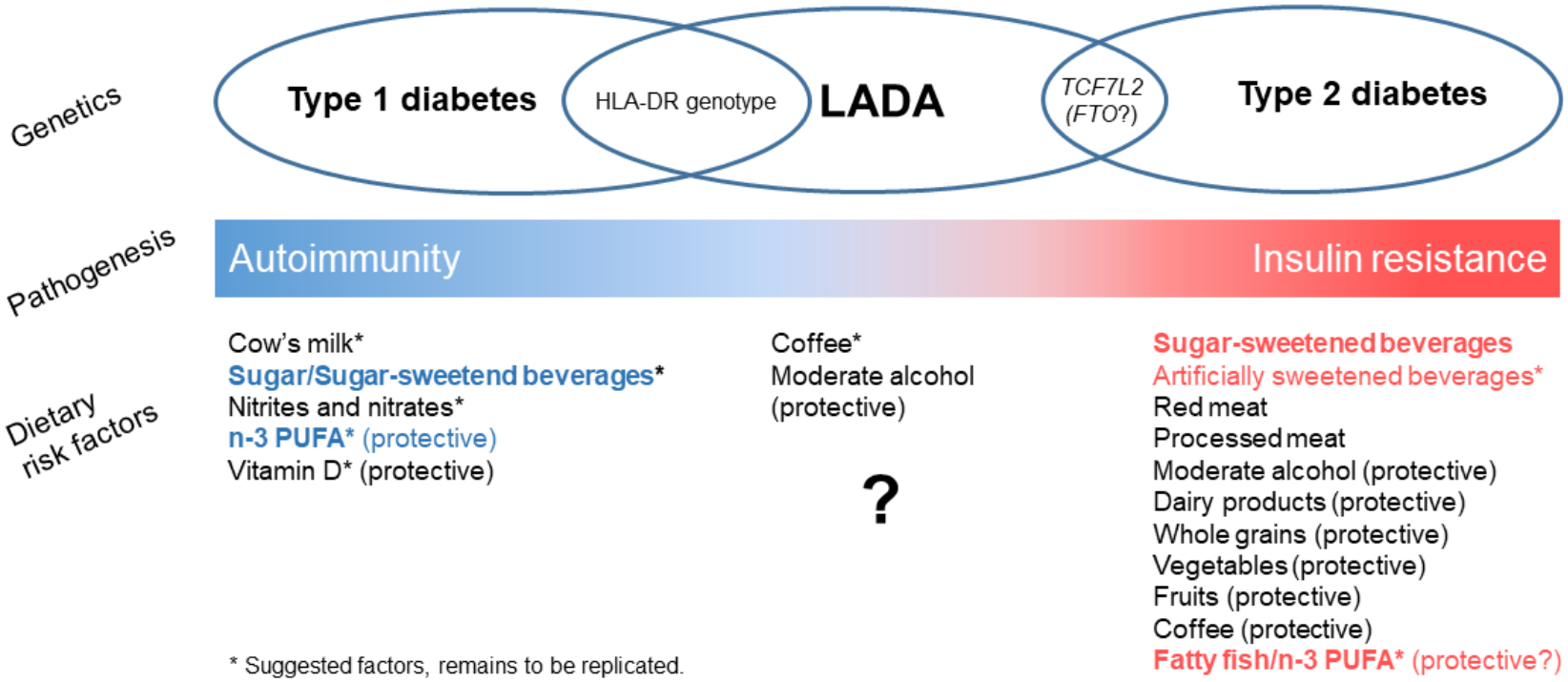
#### *2.2.3.3 Sweetened beverage consumption and risk of diabetes*

The large body of evidence linking sugar-sweetened beverage intake to type 2 diabetes is convincing [5]. The association may partly be mediated by BMI, but there also seems to be an association independent of adiposity [105]. The role of artificially sweetened beverages in the development of type 2 diabetes is controversial; a positive association has been suggested [105] but may be explained by BMI or confounding by other dietary or lifestyle factors [171-173]. The role of sweetened beverages in the development of autoimmune diabetes is less clear. Positive associations with sugar-sweetened beverage intake have been reported in two studies from North America [8, 9], but no association was seen in a Swedish study [175]. One study investigated islet autoimmunity and type 1 diabetes in relation to artificially sweetened beverages and found no indication of an association [9].

#### *2.2.3.4 The potential influence of genes*

Only one previous study has addressed the potential interaction between intake of sweetened beverage and genetic susceptibility on the risk of type 2 diabetes, which was done by means of a genetic risk score, but they found no evidence of interaction [175]. Gene-environment studies of individual genes are complex due to very modest effects on type 2 diabetes risk. Still, a positive association with *TCF7L2* (rs12255372) has been indicated to vary across levels of glycemic load [176]. The *FTO* gene has been demonstrated to influence nutrient intake preferences [177] and a lower intake of sugar-sweetened beverages, but not artificially sweetened, was reported among risk genotype carriers (of rs9939609) compared to those without the risk genotype [178]. Furthermore, *FTO* may interact with dietary intake as a study demonstrated that the risk variant was positively associated with type 2 diabetes only in combination with low adherence to a Mediterranean diet, but not among risk genotype carriers with high adherence (where one score is given for low intake of sugar-sweetened beverages) [179]. With regard to type 1 diabetes, one of the North American studies mentioned above found a positive association with sweetened beverages only among carriers of a high risk HLA genotype who had already developed islet autoimmunity [9]. Whether diabetes-related genotypes have a role in a potential association between sweetened beverages and LADA remains to be explored.

**Figure 2.2.** A schematic summary of the background for this thesis work. N.B. that this is not a complete list of associated genes and dietary risk factors.



# 3 AIMS

The overall aim with this thesis was to investigate consumption of fatty fish and sweetened beverages on the development of LADA, and compare to that of type 2 diabetes.

The specific aims were:

i. to study the risk of LADA and type 1 diabetes in relation to fatty fish consumption and dietary supplementation of fish oil and vitamin D (Paper I)

ii. to study the potential role of n-3 PUFA, by measures of dietary fish intake and plasma n-3 PUFA, on the progression from islet autoimmunity to diabetes onset in adults (Paper II)

iii. to study the risk of LADA and type 2 diabetes in relation to sweetened beverage consumption, including separate analysis of sugar-sweetened and artificially sweetened beverages (Paper III)

iv. to study whether risk genes for diabetes modify the associations between sweetened beverage consumption and risk of LADA and type 2 diabetes, and estimate the proportion mediated by BMI (Paper IV)

# 4 SUBJECTS AND METHODS

## 4.1 THE ESTRID STUDY (PAPER I, II, IV)

The ESTRID Study (Epidemiological Study of Risk Factors for LADA and Type 2 Diabetes; https://ki.se/imm/estrid) was initiated in 2010 with the overall aim to enable investigations of lifestyle risk factors for LADA, including their underlying mechanisms and interaction with genetic factors. Such studies were at the time scarce and could in fact be counted on the fingers of one hand, all based on the same cohort from Norway, the HUNT Study (The Nord-Trøndelag Health Study) [86].

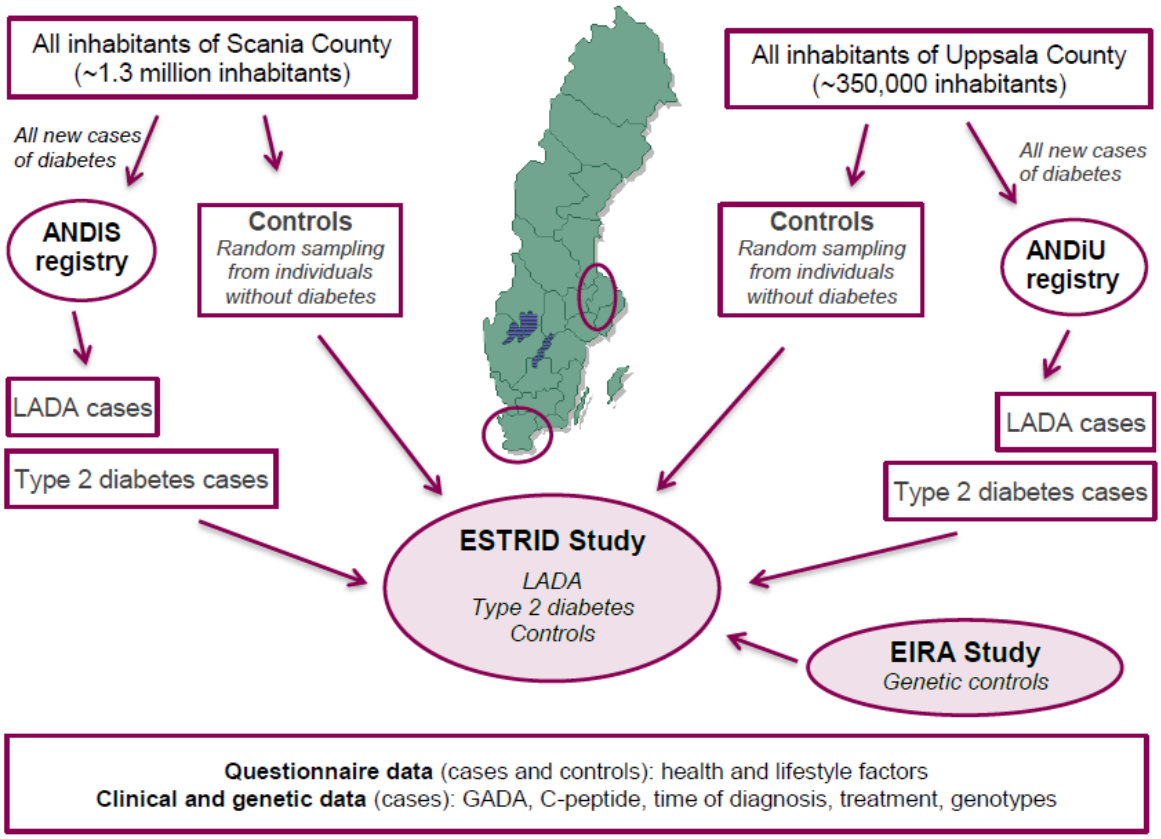
### 4.1.1 Study design

ESTRID is a Swedish case-control study with incident cases of LADA and type 2 diabetes recruited from two diabetes registries; ANDIS (All New Diabetics in Scania; http://andis.ludc.med.lu.se) and its sister-study ANDiU (All New Diabetics in Uppsala; http://www.andiu.se, recruitment since 2012). The aim of the registries is to include all new diabetes cases diagnosed in the regular health care system within the counties of Scania (ANDIS) and Uppsala (ANDiU) and classify them based on clinical and genetic characteristics. All incident cases of LADA and a random sample of type 2 diabetes cases (four per one LADA case) aged ≥ 35 years are invited to participate in ESTRID. Controls without diabetes aged ≥ 35 years are recruited as a random sample (six per one LADA case) from the Swedish Population Register, matched to the cases by county and calendar time (i.e. date of participation). ESTRID is an ongoing study which to date includes 584 cases of LADA, 2,033 cases of type 2 diabetes, and 2,349 controls.

### 4.1.2 Study population

The ESTRID dataset is updated annually. Analyses in **Paper I** were based on cases and controls recruited between September 2010 and July 2013 (only cases who responded to the questionnaire within six months of diagnosis to minimize influence of recall bias) including 89 cases of LADA, 462 cases of type 2 diabetes, and 1,007 controls. **Paper III** was based on cases and controls recruited between September 2010 and July 2015, including 357 cases of LADA, 1,136 cases of type 2 diabetes, and 1,371 controls with complete information on exposure and main covariates. **Paper IV**, in which we explored gene*environment interaction, was based on 386 LADA cases and 1,253 type 2 diabetes cases recruited between September 2010 and July 2017, together with 1,545 controls recruited between 2005 and 2014 within the EIRA Study (described in Section 4.1.6.2). The reason for this procedure was that controls within ESTRID contribute questionnaire data but no blood samples.

**Figure 4.1.** Schematic of the ESTRID study.



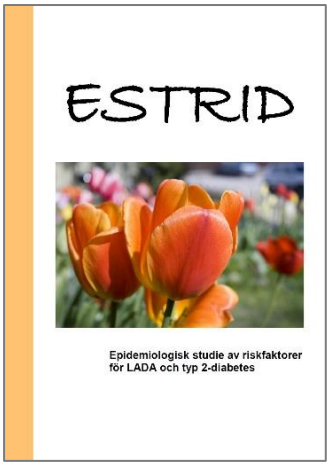

### 4.1.3 Clinical information

Fasting blood samples were collected at time of registration in ANDIS and ANDIU. Quantification of GADA was done with an enzyme-linked immunosorbent assay (ELISA), where values above 250 IU/ml were censored at 250. Autoantibody positivity was defined as ≥ 10 IU/ml. Sensitivity was 84% and specificity was 98% at a cut-off level of 10.7 IU/ml [180]. C-peptide concentration was measured using Cobas e601 analyzer (Roche Diagnostics, Mannheim, Germany) or IMMULITE 2000 (Siemens Healthcare Diagnostics Product Ldt., Llanberies, UK). Homeostatic model assessment for insulin resistance (HOMA-IR) and beta cell function (HOMA-B) was calculated based on values for fasting blood glucose and C-peptide in the HOMA2 calculator [181].

### 4.1.4 Classification of diabetes

Classification of diabetes subtype was based on age at diagnosis, GADA, and C-peptide. LADA was defined as age ≥ 35 years, GADA ≥ 10 IU/ml (i.e. autoantibody positivity), and C-peptide ≥ 0.2 nmol/L (IMMULITE) or ≥ 0.3 nmol/L (Cobas e 601). Similarly, type 2 diabetes patients were aged ≥ 35 years, but had GADA < 10 IU/mL (i.e. negative for autoantibodies), and C-peptide ≥ 0.60 nmol/L (IMMULITE) or ≥ 0.72 nmol/L (Cobas e 601). In Paper I, which was one of the first publications based on ESTRID, we used a cut-off of 20 IU/ml instead of 10 IU/ml for GADA (classified as 'LADA mild' in the ANDIS registry). In all later publications based on ESTRID, we instead used the cut-off of 10 IU/ml and addressed the heterogeneity of LADA by stratifying the patients by median GADA level.

### 4.1.5 Questionnaire data




All participants fill out a self-administered questionnaire with a wide range of questions including education, height, weight, physical activity, smoking, and diet. The cases were specifically instructed to report their habits as they had been prior to diagnosis.

#### *4.1.5.1 Dietary intake estimations*

Dietary habits were assessed by means of a validated [182-184] semi-quantitative food frequency questionnaire (FFQ) including 132 food items intended to cover all parts of the person's diet. For commonly consumed foods and beverages such as coffee, dairy, and bread, participants were asked to report the number of predefined servings per day or per week. For other food items, the participants were asked to choose from eight predefined frequency categories ranging from never to three times per day or more, to indicate intake as the average over the past year. Diabetes patients were instructed to report their diet as it had been the year preceding diagnosis. Reported intake frequencies were converted to servings per day by taking the midpoint of the marked frequency category and used together with age- and sex-specific portion sizes to estimate daily intake in grams. Based on these daily intakes, estimations of nutrients and total energy intake were calculated by food composition values from the Swedish National Food Agency database. Each item in the FFQ represent several specific foods, and the relative contribution of each specific food was determined by distribution of consumption in the population according to national surveys. Nutrient intakes were energy-adjusted according to the residual method [185].

For Paper I, the above mentioned data from nutrient calculations were not available. The EPA and DHA intake was instead estimated based on EPA and DHA content in the most commonly consumed types of fish and shellfish, with nutrient data from the Swedish National Food Agency Database.

#### *4.1.5.2 Fish consumption*

The ESTRID FFQ contains eleven items concerning seafood intake, of which four were regarded as fatty fish (herring/Baltic herring/mackerel, salmon, sardines, smoked fish), four about lean fish (cod/pollock/plaice/blue hake, tuna, pike/pike perch/perch, fish fingers), and the remaining three about other unspecified fish, shellfish, and roe (Figure 4.2).

#### *4.1.5.3 Dietary supplements*

The questionnaire also asked various dietary supplements. Participants were asked to report if each of the stated dietary supplements had ever been consumed (yes/no) and if yes, to specify the time period (in years), and to indicate current use. There was one question specifically asking about fish oil and one about vitamin D. There were also an open-ended question and the respondents reporting any supplement that would without doubt contain fish oil was considered

exposed. In addition, there was a question on multivitamin use but this was not considered in the analysis of vitamin D.

**Figure 4.2.** ESTRID FFQ questions about dietary fish intake: "How often do you on average consume the following? Mark only one alternative on each row."

**5. Hur ofta brukar du i genomsnitt äta följande?** Sätt endast **ett** kryss på **varje** rad

| | gånger per månad | | eller | gånger per vecka | | | eller | gånger per dag | | |
|---|---|---|---|---|---|---|---|---|---|---|
| **Fisk/fågel/ägg** | 0 | 1-3 | | 1-2 | 3-4 | 5-6 | | 1 | 2 | 3+ |
| Rökt fisk | ☐ | ☐ | | ☐ | ☐ | ☐ | | ☐ | ☐ | ☐ |
| Sill/surströmming/makrill | ☐ | ☐ | | ☐ | ☐ | ☐ | | ☐ | ☐ | ☐ |
| Lax | ☐ | ☐ | | ☐ | ☐ | ☐ | | ☐ | ☐ | ☐ |
| Sardiner | ☐ | ☐ | | ☐ | ☐ | ☐ | | ☐ | ☐ | ☐ |
| Torsk/sej/spätta/hoki | ☐ | ☐ | | ☐ | ☐ | ☐ | | ☐ | ☐ | ☐ |
| Tonfisk | ☐ | ☐ | | ☐ | ☐ | ☐ | | ☐ | ☐ | ☐ |
| Gädda/gös/abborre | ☐ | ☐ | | ☐ | ☐ | ☐ | | ☐ | ☐ | ☐ |
| Fiskpinnar | ☐ | ☐ | | ☐ | ☐ | ☐ | | ☐ | ☐ | ☐ |
| Annan fisk | ☐ | ☐ | | ☐ | ☐ | ☐ | | ☐ | ☐ | ☐ |
| Rom (t ex stenbit) | ☐ | ☐ | | ☐ | ☐ | ☐ | | ☐ | ☐ | ☐ |
| Skaldjur (räkor, kräftor etc) | ☐ | ☐ | | ☐ | ☐ | ☐ | | ☐ | ☐ | ☐ |

### *4.1.5.4 Sweetened beverage consumption*

Sweetened beverage consumption were covered by four question in the FFQ of the ESTRID questionnaire; two about sugar-sweetened and two about artificially sweetened (Figure 4.3). These were part of the section where the respondent was asked to report the number of 200 ml glasses consumed per day or per week. In the EIRA questionnaire, sweetened beverages were covered by one only question. For this reason, it was not possible to analyze sugar-sweetened and artificially sweetened beverages separately in Paper IV.

**Figure 4.3.** ESTRID FFQ questions about sweetened beverage consumption: "How much do you drink of the following? 1 glass = 200 ml"

**1. Hur mycket dricker/äter du av följande?**
(1 glas=2 dl, 1 kopp=1,5 dl)

| | **per dag** | | ***eller*** | **per vecka** | |
|---|---|---|---|---|---|
| Coca Cola/Pepsi, *light* | _____ | glas/dag | | _____ | glas/vecka |
| Coca Cola/Pepsi | _____ | glas/dag | | _____ | glas/vecka |
| Annan läsk/saft, *light* | _____ | glas/dag | | _____ | glas/vecka |
| Annan läsk/saft | _____ | glas/dag | | _____ | glas/vecka |

### 4.1.6 Genetic information

#### *4.1.6.1 Cases*

Genotyping was performed at the Clinical Research Center in Malmö, Sweden, using iPlex Gold Technology (Sequenom, San Diego, CA, USA). Imputation for missing genotypes were done on a subset using Infinium CoreExome v1.1 (Illumina, San Diego, CA,USA), which was based on the Haplotype Reference Consortium (http://www.haplotype-reference-consortium.org/; version r1.1 2016) reference panel. Genotyping for HLA was based on three SNPs within the major histocompatibility complex (MHC) class II gene region; rs3104413, rs2854275, and rs9273363, combined to indicate HLA genotypes or haplotypes according to previously described methodology with an accuracy of 99.3% [186].

#### *4.1.6.2 Genetic controls*

For the genetic analyses in Paper IV, we used data for diabetes-free controls collected within the Swedish EIRA (Epidemiological Investigation of Rheumatoid Arthritis; http://www.eirasweden.se) Study. The EIRA study design is very similar to that of ESTRID, including the recruitment of randomly selected population-based controls. Controls included in the analysis were aged 35 years or older, adhering to the age criteria in ESTRID. SNP data for HLA genotyping (rs3104413, rs2854275, rs9273363) was generated from an Infinium Illumina 300K immunochip custom array (Illumina, San Diego, CA, USA). Genotypes of *TCF7L2* rs7903146 and *FTO* rs9939609 were based on GWAS data derived from an Illumina Global Screening array.

## 4.2 THE EPIC-INTERACT STUDY (PAPER II)

### 4.2.1 Study design

The EPIC-InterAct Project started in 2006 with the overall aim to elucidate the role of diet and physical activity, and their interaction with genes, on the risk of type 2 diabetes [134]. InterAct is a nested-case cohort consisting of data from eight European countries within the already existing EPIC (European Prospective Investigation into Cancer and Nutrition) Study [187], in which a total of 340,234 participants were followed for 3.99 million person-years between 1991 and 2007 (median follow-up 10.9 years). During this time, 12,403 individuals were diagnosed with type 2 diabetes. These individuals, together with a subcohort of 16,154 individuals free of diabetes at baseline, forms the InterAct case-cohort (Figure 4.4). After exclusions due to missing information on covariates, the analyses in Paper II were based on 11,247 diabetes cases and 14,981 subcohort participants (including 693 of the cases).

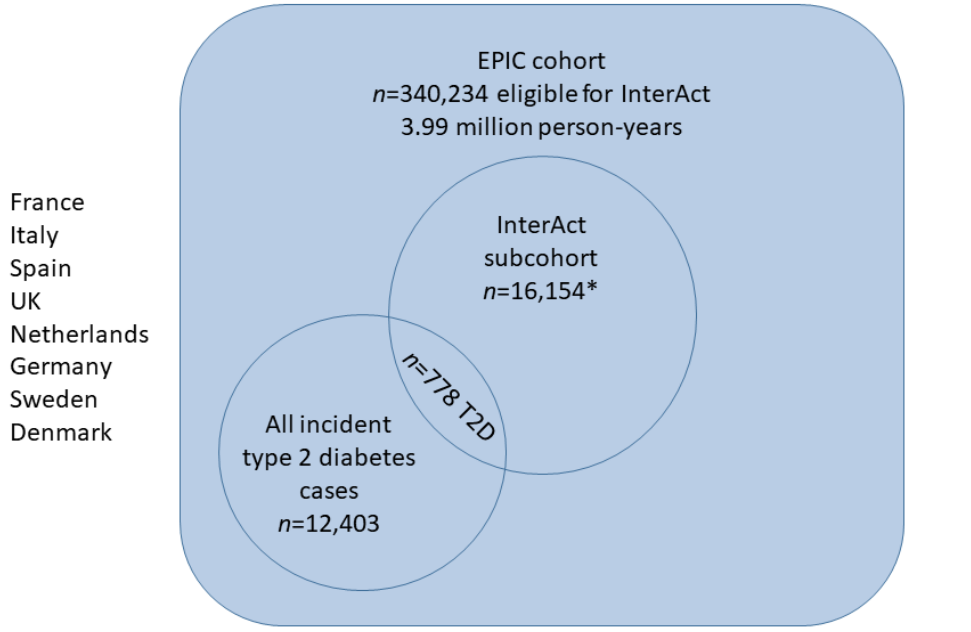
### 4.2.2 Blood samples

Blood samples for all participants were drawn at baseline, i.e. at time of inclusion in EPIC and analyzed for autoantibodies against the 65kD isoform of glutamic acid decarboxylase (GADA; but referred to as GAD65 antibodies in Paper II) using a radioligand binding assay [188, 189].

Units (U) were expressed relative to the WHO standard [190]. The cut-off for GADA positivity was set at ≥ 65 U/ml, determined through a competition assay using recombinant human GAD65 (Diamyd Medical, Sweden), as previously described [191]. This corresponds to sensitivity of 85% and specificity of 99% [192].

The samples were also analyzed for plasma phospholipid fatty acids through solid phase extraction followed by hydrolysis and methylation into fatty acid methyl esters which were subsequently separated by gas chromatography [193]. Commercial standards were used as comparison and the different fatty acids were identified through their retention time and finally expressed in mol%, which represents the percentage of total phospholipid fatty acids.

**Figure 4.4.** Schematic of the EPIC-InterAct case-cohort study design.




* After exclusion of prevalent diabetes (*n*=548) and unknown diabetes status (*n*=133) at baseline.

### 4.2.3 Ascertainment of diabetes

All incident cases of diabetes classified as type 2 occurring during the follow-up period were ascertained and verified through a minimum of two out of the following potential sources: self-report, primary-care registers, secondary-care registers, use of diabetic medication, hospital admissions and mortality data. In Denmark and Sweden, all cases were identified through linkage to local and national registers for diabetes and prescribed drug registers, for which no further verification was considered to be needed. Prevalent cases of diabetes at baseline were identified via self-report, doctor's diagnosis, or use of diabetic medication, and excluded from the study. All cases were diagnosed with type 2 diabetes and since no antibody measurements were taken at diagnosis, we are unable to distinguish LADA. These cases will for the remainder of this text be referred to as 'diabetes'.

### 4.2.4 Questionnaire data

Information on education, occupation, and lifestyle including physical activity and smoking status was collected for all participants at baseline in a standardized manner across study centers. Habitual diet was assessed through self-administered or interviewer-administered questionnaires. Development and validation of the dietary questionnaires were performed within each country [194, 195]. The dietary intake data was standardized with regard to food items and individual nutrients through the use of the EPIC Nutrient Database [196].

#### *4.2.4.1 Fish consumption*

Information on dietary fish intake among the EPIC-InterAct participants was divided into fatty fish, lean fish, total fish intake (which is the sum of fatty and lean fish), shellfish, and combined fish and shellfish intake (also including "other" types of fish such as fish products/fish in crumbs, and nonspecific or combined fish). In Paper II, analyses were based on information on total fish intake, fatty fish intake, and lean fish intake.

## 4.3 STATISTICAL ANALYSIS

### 4.3.1 The ESTRID Study (Paper I, III, IV)

In Paper I, III, and IV, conditional logistic regression was used to estimate odds ratios (OR) and 95% confidence intervals (CI) of LADA and type 2 diabetes in relation to the exposure of interest. Since the cases are incident and the controls are sampled from the population at risk, i.e. they are representative of the source population and eligible to become a case, this is also called incidence-density sampling. As an implication, the odds ratio may be interpreted as the incidence rate ratio or relative risk [197]. In Paper IV, controls from the EIRA Study was used and post-matched to the ESTRID cases by age (in 5-year strata) and sex, due to an over-representation of women in EIRA (as a consequence of the controls originally being matched to cases of rheumatoid arthritis; a disease predominantly affecting women).

In Paper IV, we used causal mediation methodology [198] to estimate the natural direct effect and natural indirect effect of high sweetened beverage consumption, and proportion of the association between high intake and risk of diabetes that is mediated by BMI. In order for a causal interpretation of the effect estimates to be made, there are four assumptions that need to be met, namely that there should be no unmeasured confounding between 1) exposure and outcome, 2) mediator and outcome, and 3) exposure and mediator, and also 4) there should be no mediator – outcome confounder that is affected by the exposure [198].

In Paper IV, we also investigated the potential effect modification by genotypes of HLA, *TCF7L2* rs7903146, and *FTO* rs9939609 on the association between sweetened beverage intake and diabetes. We did so by stratifying the analyses on genotype to obtain OR per 1 daily serving increment in intake specific for each genotype. The genotypes were categorized into high risk (high risk HLA; ≥ 1 risk allele [T] for rs7903146; ≥ 1 risk allele [A] for rs9939609) or low risk (low/intermediate risk HLA; CC in rs7903146; TT in rs9939609). We also assessed

the presence of interaction defined as departure from additivity of effects by estimating the attributable proportion (AP) due to interaction for the combined effect of high sweetened beverage intake (> 2 servings per day) and high risk genotype, compared with non-risk genotype carriers with lower intakes (≤ 2 servings per day). AP was based the following formula [199]:

$$\frac{RR_{11} - RR_{10} - RR_{01} + 1}{RR_{11}}$$

where RR denotes the relative risk, but in this study it is replaced by OR because of the case-control design with binary outcome measure. $OR_{11}$ represents the doubly exposed, i.e. to both high sweetened beverage intake and high risk genotype, and $OR_{10}$ and $OR_{01}$ are those exposed to one of the factors but not the other. AP > 0 indicates positive interaction, which is considered significant when the confidence interval does not include 0.

### 4.3.2 The EPIC-InterAct Study (Paper II)

In Paper IV, Cox proportional hazards regression models were used in the main analyses due to the prospective nature of the data. The models were Prentice-weighted as a way to account for the over-representation of cases following the case-cohort design [200]. Hazard ratios (HR) and 95% CI of incident diabetes in relation to baseline GADA status, plasma levels of n-3 PUFAs, or dietary fish intake, and for mutually exclusive combinations of antibody status and n-3 PUFA/fish were estimated. Age was used as the underlying timescale, and person-years of follow-up were calculated from baseline (time of participation) until diabetes diagnosis, death, or December 31 2007, whichever occurred first. All models were conditioned on study center to handle potential differences between centers or countries.

The main focus of Paper IV was the interaction analysis in which we wanted to investigate the risk of diabetes in relation to the combination of antibody positivity and low dietary fish intake or plasma levels of n-3 PUFA. Interaction was defined as departure from additivity and expressed as attributable proportion due to interaction (AP). Calculations were based on the same formula as presented above, but with HR replacing RR. In these analyses, the double exposed ($HR_{11}$) were those with GADA positivity (or high GADA level) and low dietaryfish/plasma n-3 PUFA level. Antibody negativity and high fish/n-3 PUFA was used as reference, as the combination representing the lowest risk should be used as reference [201]. AP with 95% CI was estimated using the freely available EpiNET tool [202].

## 4.4 ETHICAL CONSIDERATIONS

All participants in ESTRID, EIRA, and EPIC-InterAct provided informed consent. Ethical approval for ESTRID, including the addition of EIRA controls, were obtained from the Ethics committee at Karolinska Institutet, Stockholm, Sweden. EPIC-InterAct was approved by the IARC Institutional Review Board Committee and local approvals were obtained in each country.

# 5 RESULTS

## 5.1 CHARACTERISTICS OF STUDY PARTICIPANTS

In ESTRID, individuals with LADA were younger and leaner at diagnosis, had worse beta cell function but better insulin sensitivity compared to individuals with type 2 diabetes (Table 5.1). Furthermore, high risk HLA genotypes were more frequent in LADA than in type 2 diabetes but no difference was seen with respect to risk genotypes of *TCF7L2* and *FTO*. Median time since diagnosis was 6.7 months for LADA and 5.1 months for type 2 diabetes. Characteristics of study participants in EPIC-InterAct are presented in the manuscript.

## 5.2 PAPER I: FATTY FISH CONSUMPTION AND LADA

Weekly intake of fatty fish was associated with a reduced risk of LADA (OR 0.51, 95% CI 0.30-0.87) compared to less frequent consumption (Figure 5.1) whereas no association was found for type 2 diabetes (OR 1.01, 95% CI 0.74-1.39). Estimations of total EPA and DHA intake from seafood sources supported the associations observed for fatty fish. In addition we investigated the risk of LADA in relation to dietary supplementation (ever vs. never use) of fish oil and vitamin D. These were hampered by very small numbers but the ORs, although with wide confidence intervals, were compatible with a reduced risk of LADA in supplement users (Figure 5.1).

**Figure 5.1.** OR of LADA and type 2 diabetes in relation to fatty fish consumption and dietary supplementation of fish oil and vitamin D. The models were adjusted for age, sex, education, smoking, physical activity, family history of diabetes, and intakes of alcohol, red meat, fruit and vegetables.

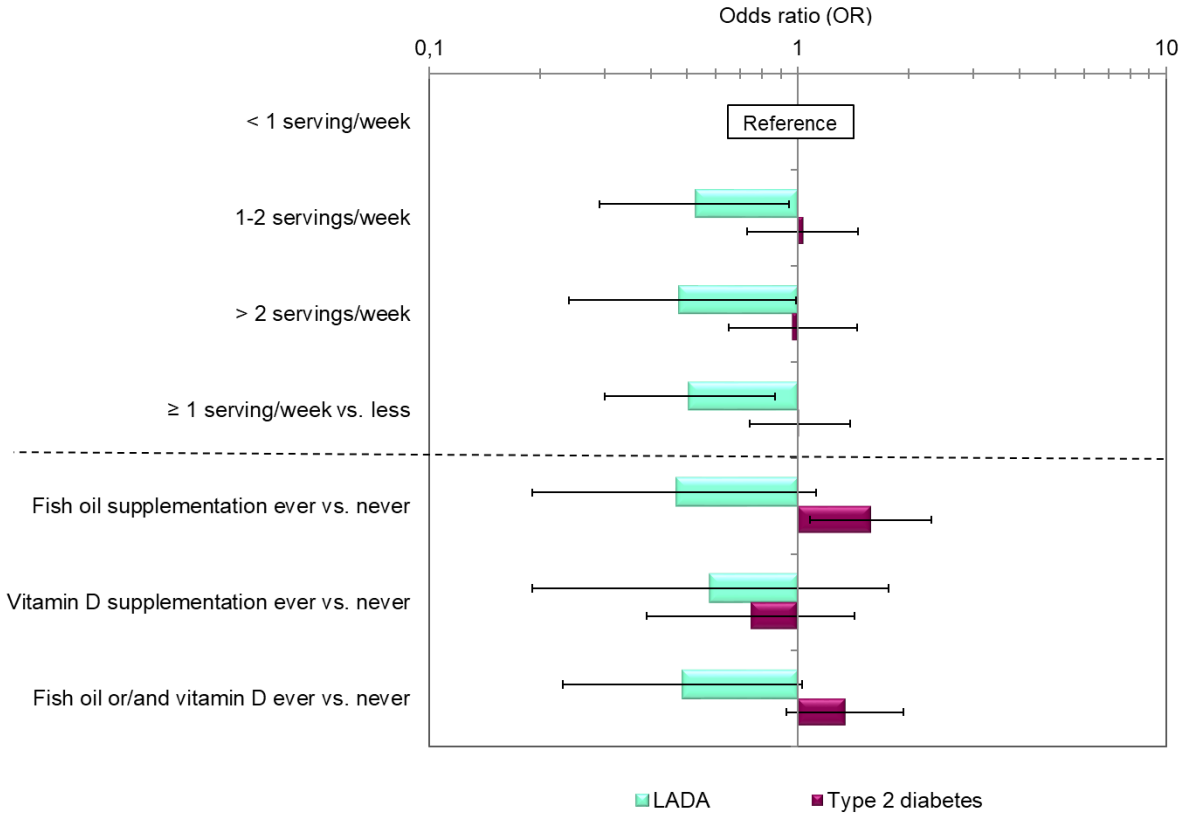


**Table 5.1.** Characteristics of patients with LADA or type 2 diabetes, and diabetes-free control subjects included in the ESTRID dataset used for the latest paper included in this thesis (Paper IV).

| Characteristic | Controls | LADA | Type 2 diabetes | *p*[a] | Genetic controls[b] |
|---|---|---|---|---|---|
| **No. of individuals** | 1,790 | 386 | 1,253 | | 1,545 |
| **Age (years), mean (SD)** | 59 (14) | 59 (12) | 63 (10) | <0.0001 | 58 (10) |
| **Women, %** | 52 | 47 | 40 | 0.0064 | 74 |
| **BMI (kg/m$^2$), mean (SD)** | 25.9 (4.2) | 27.9 (5.3) | 31.1 (5.3) | <0.0001 | 25.4 (4.1) |
| **Overweight (BMI ≥ 25), %** | 54 | 70 | 93 | <0.0001 | 47 |
| **Obese (BMI ≥ 30), %** | 15 | 31 | 51 | <0.0001 | 12 |
| **With family history of diabetes, %** | 25 | 42 | 50 | 0.0046 | - |
| **High risk HLA genotype, %** | - | 61 | 31 | <0.0001 | 32 |
| **≥1 risk allele *TCF7L2* rs7903146, %** | - | 52 | 53 | 0.8225 | 46 |
| **≥1 risk allele *FTO* rs9939609, %** | - | 66 | 68 | 0.5561 | 64 |
| **GADA (IU/ml), median (IQR)** | - | 240 (29-250)[c] | - | | - |
| **C-peptide (nmol/l ), mean (SD)** | - | 0.80 (0.52) | 1.35 (0.59) | <0.0001 | - |
| **HOMA-IR, median (IQR)** | - | 2.7 (1.8-4.4) | 3.6 (2.7-4.8) | 0.0014 | - |
| **HOMA-B, median (IQR)** | - | 33 (13-65) | 68 (42-94) | <0.0001 | - |

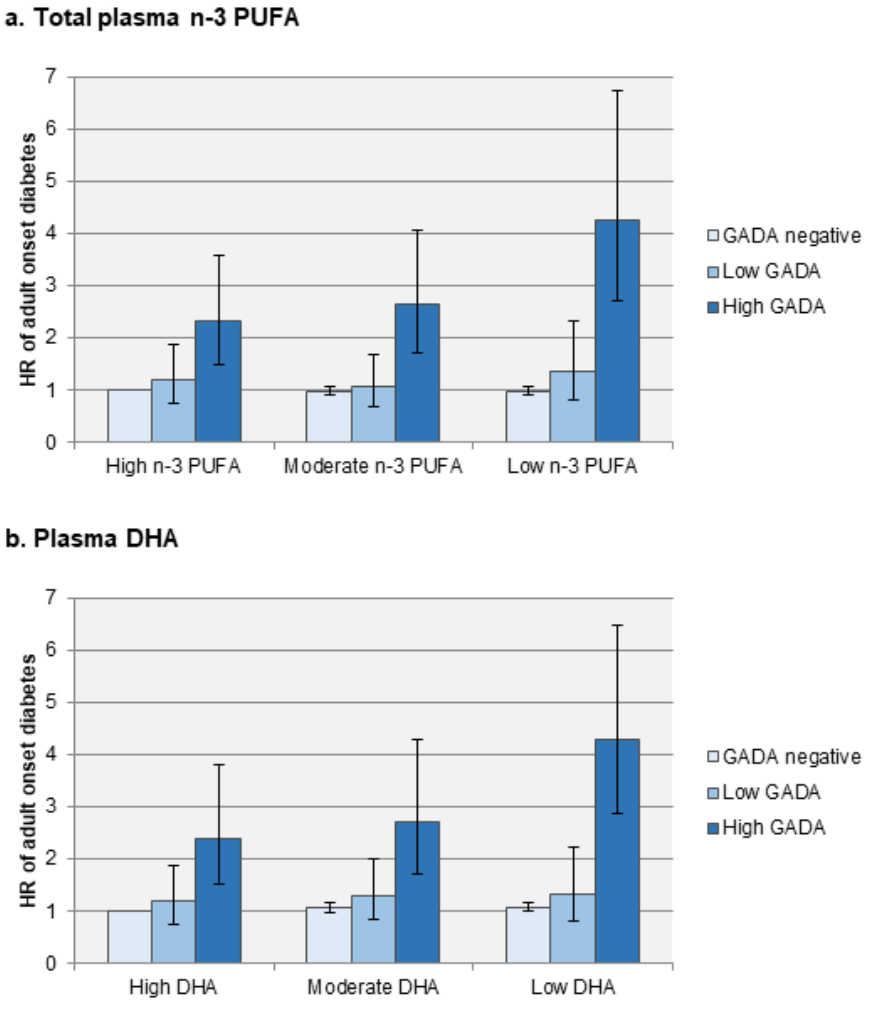
[a] p for the comparison between LADA and type 2 diabetes.
[b] Genetic controls collected within the EIRA Study.
[c] High values are censored at 250 IU/ml.

## 5.3 PAPER II: GADA, FISH/N-3 PUFA, AND DIABETES

In EPIC-InterAct, GADA positivity vs. negativity at baseline conferred a nearly 2-fold increased risk of diabetes (HR 1.81, 95% CI 1.49-2.20), which was even more pronounced in those with high GADA levels defined as ≥ 167.5 U/ml (HR 2.93, 95% CI 2.27-3.79). Neither total fish intake nor plasma n-3 PUFA was associated with the risk of diabetes (highest vs. lowest tertile: HR 1.03, 95% CI 0.93-1.15, and HR 1.01, 95% CI 0.93-1.11, respectively).

Despite the lack of overall association, consumption of both total fish and fatty fish was found to interact with GADA status in relation to the risk of diabetes; HR for positivity combined with low intake was 2.52 (95% CI 1.76-3.63) and 2.48 (95% CI 1.79-3.45), respectively. Estimations of AP indicated that 44-48% (95% CI 16-72%) of the doubly exposed cases could be attributed to the interaction between the two exposures. The highest risk of diabetes was seen in individuals with high GADA levels combined with low levels of total n-3 PUFA (HR 4.26, 95% CI 2.70-6.72, with AP 0.46, 95% CI 0.12-0.80), and plasma DHA (HR 4.30, 95% CI 2.86-6.47, with AP 0.43, 95% CI 0.08-0.77) (Figure 5.2).

**Figure 5.2.** Multivariable adjusted HR (95% CI) of adult onset diabetes by mutually exclusive combinations of GADA status and plasma levels of a) n-3 PUFA and b) DHA measured at baseline in the EPIC-InterAct Study. The models were adjusted for age (as underlying time-scale, center, sex, education, smoking, physical activity, BMI, alcohol, fruit and vegetable intake.



## 5.4 PAPER III: SWEETENED BEVERAGE CONSUMTION AND LADA

In Paper III we investigated the association between sweetened beverage intake and LADA or type 2 diabetes. High consumption (> 2 servings per day) was positively associated with both LADA (OR 2.12, 95% CI 1.20-3.75) and type 2 diabetes (OR 3.31, 95% CI 2.07-5.31) compared to non-consumption. The associations remained also after inclusion of BMI in the models (LADA: OR 1.99, 95% CI 1.11-3.56, and type 2 diabetes OR: 2.39, 95% CI 1.39-4.09). In separate analysis by beverage type, OR per one additional daily serving of sugar-sweetened beverages was 1.18 (95% CI 1.00-1.39) for LADA and 1.21 (95% CI 1.05-1.41) for type 2 diabetes after adjustment for BMI. The corresponding ORs for artificially sweetened beverage intake was 1.12 (95% CI 0.95-1.32) and 1.18 (95% CI 1.01-1.38) for LADA and type 2 diabetes, respectively.

## 5.5 PAPER IV: SWEETENED BEVERAGES, GENOTYPES, AND LADA

In Paper IV, we show that HLA was strongly associated with LADA but not type 2 diabetes (Figure 5.3) and furthermore that the association with LADA pertained only to carriers of low/intermediate risk HLA genotypes but not to high risk carriers (Figure 5.4). *TCF7L2* was associated with both LADA and type 2 diabetes, but did not seem to modify the association between sweetened beverages and neither LADA not type 2 diabetes. *FTO* was associated with type 2 diabetes but not with LADA, and *FTO* showed indications of being effect modifier in the association between sweetened beverage intake and type 2 diabetes.

**Figure 5.3.** Age- and sex-adjusted OR with 95% CI of LADA and type 2 diabetes in relation to genotypes of HLA, *TCF7L2* and *FTO*.

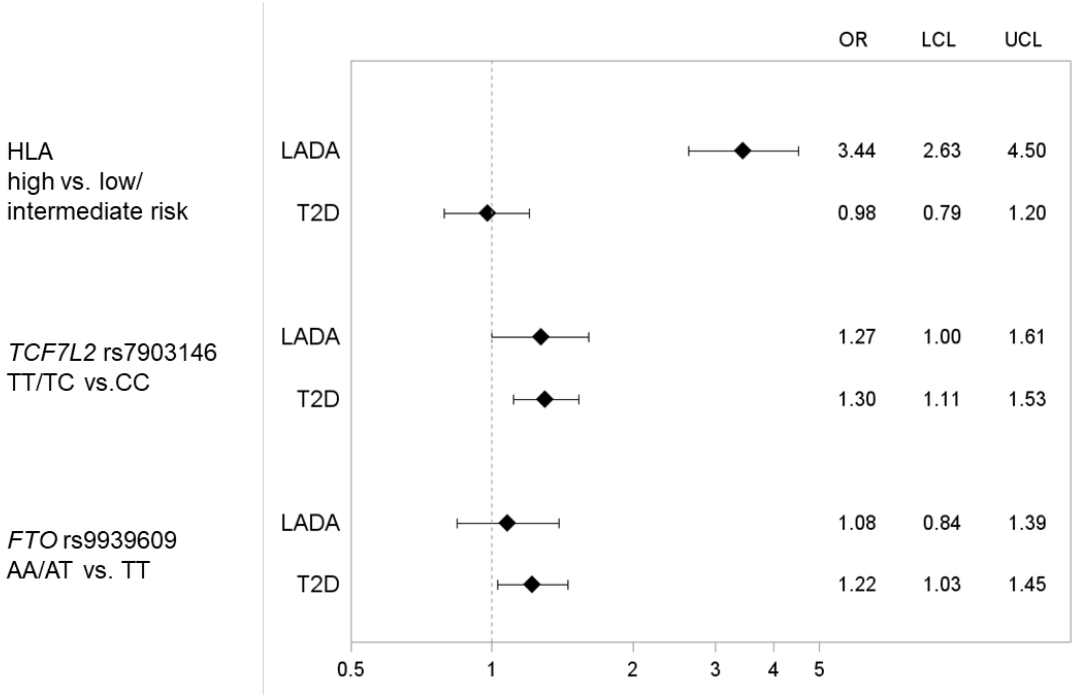
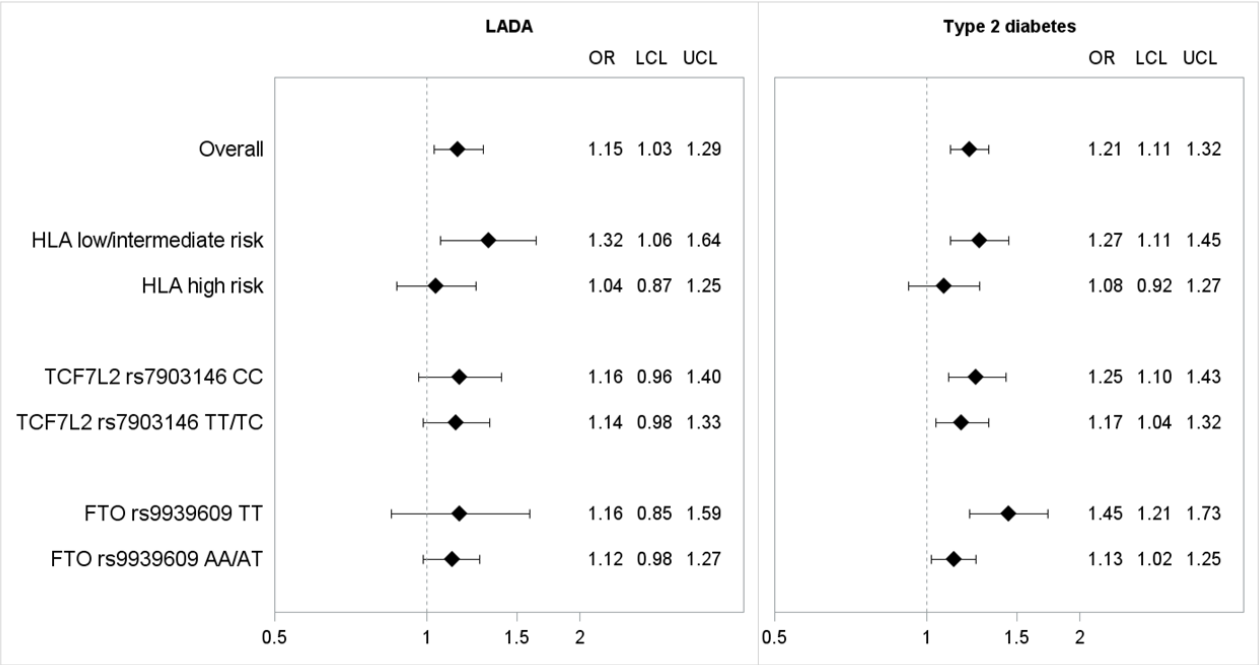


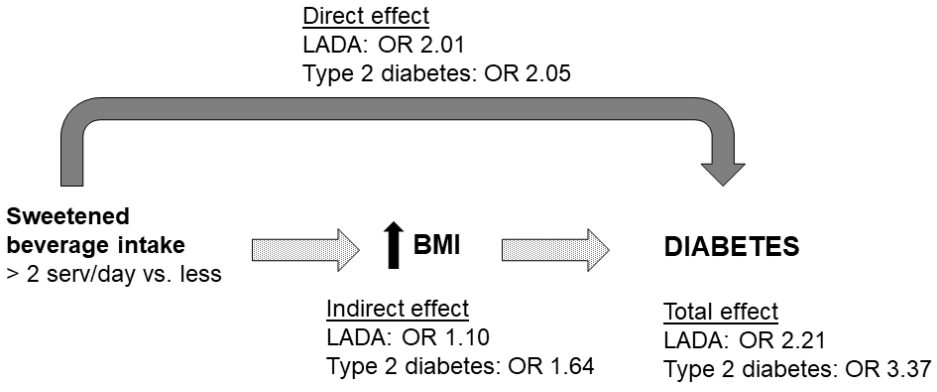
**Figure 5.4.** Multivariable adjusted OR of LADA and type 2 diabetes per 1 daily serving increment in intake of sweetened beverages, overall and by genotypes of HLA, *TCF7L7* rs7903146, and *FTO* rs9939609. The models were adjusted for age, sex, education, physical activity, smoking, and alcohol intake.




Sweetened beverage intake was positively associated with insulin resistance, measured as HOMA-IR, among individuals with type 2 diabetes both before (β=0.035, $p < 0.001$) and after (β=0.033, $p < 0.05$) adjustment for BMI. For LADA, similar tendencies were observed overall (β=0.051, $p = 0.09$, and β=0.043, $p = 0.14$), but stratification by HLA genotype indicated that the association was present only among carriers of low/intermediate risk HLA genotypes (β=0.162, $p < 0.05$, and β=0.122, $p < 0.05$). No associations were found with HOMA-B as an indicator of beta cell function.

By means of causal mediation analysis, BMI was estimated to mediate 17% of the association with high sweetened beverage consumption for LADA and 56% for type 2 diabetes, while the estimated direct effect was of similar magnitude for both LADA and type 2 diabetes (Figure 5.5).

**Figure 5.5.** Estimated natural indirect effect, natural direct effect, and total effect of high sweetened beverage intake on the risk of LADA and type 2 diabetes.



# 6 DISCUSSION

## 6.1 MAIN FINDINGS AND INTERPRETATIONS

This doctoral thesis aimed to elucidate the risk of LADA in relation to consumption of fatty fish and sweetened beverages. The findings suggest that fatty fish is associated with reduced risk of LADA, possibly by beneficial effects of marine-originated n-3 PUFAs on progression from autoimmunity to diabetes onset. In contrast, sweetened beverage consumption was associated with an increased risk of LADA, with indications of mechanisms including detrimental effects on bodyweight and insulin resistance. Stratification by genotypes suggested that the positive association with sweetened beverages pertains only to individuals without HLA-conferred genetic susceptibility but that neither *TCF7L2* nor *FTO* modified the association with LADA. The findings of this doctoral thesis clearly suggest that dietary factors may play a role in the development of LADA. These are the first studies investigating consumption of fish and sweetened beverages in relation to risk of LADA, thus confirmation and replication is warranted.

### 6.1.1 Fish consumption

The findings based on the ESTRID Study suggest that weekly fatty fish consumption is associated with a reduced risk of LADA. With data from EPIC-InterAct including both reported fish consumption and nutritional biomarkers, we found further support for a reduced risk of autoimmune diabetes in relation to the long-chain n-3 PUFA in fish. This is in line with previous reports in children [6, 7], although not with all [152, 153]. The mechanistic support for beneficial effects of EPA and DHA on autoimmunity and inflammation includes incorporation of these fatty acids into the cell membranes of immune cells, partly at the expense of arachidonic acid leading to a reduction in proinflammatory eicosanoid production [140]. Interestingly, there are potential links between n-3 PUFA and HLA-DR molecules, a type of cell surface receptors of antigen-presenting cells in the immune system encoded by the HLA genes. EPA and DHA have been shown to decrease HLA-DR concentration by down-regulating gene expression of HLA-DR and costimulatory proteins in dendritic cells (type of immune cell) [203]. It may be hypothesized that lower levels of the HLA-DR receptors would lead to a less intense immune response and lower rate of beta cell destruction. Hence, EPA and DHA may potentially interact with HLA genotype on the risk of autoimmune diabetes. Potentially modifying effects of HLA and other genotypes in the association between n-3 PUFA and LADA is thus an important topic for future studies. However, beneficial effects of vitamin D on development of autoimmune diabetes should not be ruled out as a potential explanation for our findings [118-120].

In EPIC-InterAct, we are assessing the risk of diabetes in relation to the interaction between baseline GADA positivity and dietary fish intake or plasma n-3 PUFA levels. The only previous study specifically addressing the potential role of n-3 PUFA on the progression from islet autoimmunity to diabetes onset found no association but was based on a limited number

of children and all of them were at increased genetic risk [152]. Of note, plasma phospholipid EPA and DHA concentration has been positively associated with fasting C-peptide in youth newly diagnosed with type 1 diabetes, which may suggest a preserving effect on beta cells [204]. We are unable to disentangle whether our findings based on ESTRID may be related to development of autoimmunity or progression to clinical diabetes. Autoantibodies may be present several years prior to diabetes onset [205] but there is no information on antibody status prior to diagnosis for the cases in ESTRID.

We did not observe an association between dietary fish intake and type 2 diabetes, which is in line with previously published data [149, 150]. Preparation method and exposure to contaminants such as PCBs and methyl mercury have been suggested to play a role in the relationship between fish intake and type 2 diabetes [150], but unfortunately we were not able to take these factors into account. It is however unclear to what extent these factors would differentially affect LADA and type 2 diabetes.

### 6.1.2 Sweetened beverage consumption

Consumption of sweetened beverages was found to be positively associated with both LADA and type 2 diabetes. This is in concordance with previous studies in type 2 diabetes [5] and two previous studies on type 1 diabetes in children [8, 9]. BMI has previously been suggested to mediate the association between sweetened beverage intake and type 2 diabetes [105]. We addressed this hypothesis by means of the causal mediation framework [198] and estimated that BMI mediates about half of the observed association between high intake of sweetened beverages and type 2 diabetes and 17% of the association with LADA. This fit with previous findings indicating that overweight is a less strong risk factor for LADA than for type 2 diabetes [28]. Furthermore, this speaks in favor of a direct effect of sweetened beverage consumption which may be of equal magnitude for LADA and type 2 diabetes. The overall positive associations with sweetened beverage intake, even after adjustment for BMI, found for HOMA-IR but not HOMA-B among type 2 diabetes patients and with similar tendencies for LADA provide support for a direct effect of sweetened beverages involving insulin resistance, which has been previously suggested. In addition, sweetened beverages may act through increased inflammation [164] and oxidative stress [165].

We observed a positive association with type 2 diabetes for both sugar-sweetened and artificially sweetened beverages, with similar indications for LADA although based on small numbers. Consumption of artificially sweetened beverages has previously been associated with type 2 diabetes [105, 171-173]. Although there are animal studies indicating a causal link between artificial sweeteners and impairments in glucose metabolism [169, 170], alternative explanations for the observed association between artificially sweetened beverages and diabetes risk need to be considered. It may be speculated that those consuming artificially sweetened beverages may have changed from previous consumption of sugar-sweetened beverages to prevent further weight gain [206]. In support hereof, we observed the highest BMI at present and at age 20 among high consumers of artificially sweetened beverages.

We also wanted to investigate whether genetic factors modify the association between sweetened beverages and LADA since it has been reported that the positive association between sugar-sweetened beverage intake and type 1 diabetes pertains only to children with high risk HLA genotypes [9]. Data from ESTRID and other studies [57, 58, 60, 61] show that HLA but also *TCF7L2* are associated with increased risk of LADA, but our findings do not suggest that high risk genotype carriers of either genotype are particularly susceptible for adverse effects of sweetened beverage intake. Contrary, we found a positive association with sweetened beverage intake only among carriers of low/intermediate risk genotypes. This may be explained by differences in the pathophysiology of childhood type 1 diabetes and adults with LADA and may suggest that the relative contribution of environmental factors to risk of LADA is greater in individuals with lower genetic susceptibility. Interestingly, a rise in the frequency of low/moderate risk HLA genotypes in newly diagnosed type 1 diabetes patients have been observed over time, suggesting an increased importance of environmental factors in the development of disease [207].

## 6.2 METHODOLOGICAL CONSIDERATIONS

### 6.2.1 Random errors

The findings presented in Paper I were among the first analyses based on data from the ESTRID Study and included only 89 cases of LADA. Hence, these findings of an inverse association with fatty fish intake need to be interpreted with caution. A strength is that our findings based on EPIC-InterAct provides further support of an association between fatty fish intake and autoimmune diabetes. However, since GADA was measured at baseline and not at time of diagnosis in EPIC-InterAct, we could not address the risk of LADA per se, but rather the interplay between dietary fish/plasma n-3 PUFA levels and GADA positivity in relation to incidence of diabetes (either type 2 diabetes or LADA). The number of LADA patients in the ESTRID dataset is now considerably larger (n≈400) which means that we will have the opportunity to re-run the fatty fish analyses in ESTRID using four times as many cases. At the same time, further confirmation in independent data is essential. With regard to the sweetened beverages analyses, these have only been carried out in ESTRID so far, thus confirmation using a different population is of importance.

### 6.2.2 Systematic errors

#### *6.2.2.1 Selection bias*

In ESTRID, controls were selected randomly and continuously from the same population in which the cases were generated. This method has high probability of providing a sample that is representative of the target population with regard to exposure prevalence [197]. Bias may be introduced if study participants differ from non-participants with regard to the exposure under study. The response rate among the ESTRID controls was 62% and in order to assess whether they are representative of the target population, we compared their consumption of fish and sweetened beverages to national intake level data. The agreement was high for mean sweetened beverage intake among both men (0.44 vs. 0.42 servings/day) and women (0.26 vs.

0.28 servings/day) [208]. The genetic controls (EIRA) had sweetened beverage consumption that was somewhat higher in males but lower in females compared to the national averages (0.47 and 0.21 servings/day, respectively). This does not seem to have influenced the results since the overall associations between sweetened beverages and LADA and type 2 diabetes were similar irrespective of whether ESTRID or EIRA (genetic) controls were used. National data on intake of dietary fish is not very detailed but 30% of adults consume fish at least twice weekly [209] and consumption increases with age [208]. The ESTIRD controls are largely in agreement with these numbers; 25% reported having fatty fish had more than twice weekly. Furthermore, the ESTRID controls have comparable education level as the general Swedish population (www.scb.se).

Selection bias may also be discussed in relation to case recruitment. In the county of Scania, from where the vast majority of ESTRID cases is recruited, > 90% of all eligible patients were included in the ANDIS registry [23]. Almost all LADA patients (approx. 95%) are invited to ESTRID, and 83% of those choose to participate. For type 2 diabetes, the response rate is similar at 79%. In all, the impact of selection bias with regard to cases should be limited.

For the EPIC-InterAct prospective case-cohort study, loss to follow-up may be an issue if it is differential with regard to level of dietary fish intake and probability of being detected as a case. A large number of sources were used for case ascertainment, including sources that do not rely on self-report, which would minimize loss to follow-up and increase the likelihood that any incomplete follow-up would be non-differential with regard to fish intake and plasma n-3 PUFA level and would, if anything, lead to dilution of the observed associations.

#### *6.2.2.2 Misclassification of outcome*

In ESTRID, all diabetes cases were identified within the regional health care system and diagnosed according to national criteria. This means that undiagnosed cases will be missed and also that such cases may be found among the controls. Inclusion of controls with diabetes will make them more similar to the cases and consequently lead to dilution of the studied associations. In EPIC-InterAct, several sources of information, both self-report and objective sources, were used to identify and verify incident cases, which would minimize the number of unidentified cases and the number of false positive cases. Undiagnosed diabetes will however be present among the non-cases and potentially lead to bias if the incidence is related to dietary fish intake.

GADA was the only antibody measured to indicate autoimmunity, and used to separate LADA from type 2 diabetes. Thus it is possible that individuals were positive for other antibodies (e.g. IAA, IA-2A, zinc transporter 8 antibody), which we did not have information on, and consequently some patients with autoimmune diabetes may have been missed. Importantly, GADA is present in 90% of adult patients with autoimmune diabetes [11, 205]. In ESTRID, the sensitivity of the GADA assay used was 84%, which means that some patients with LADA were erroneously classified as autoantibody negative. The specificity of 98% implies that type 2 diabetes patients may be incorrectly classified as having LADA and this could contribute to

the similar associations with sweetened beverage intake seen for LADA and type 2 diabetes. Notably, the positive association with sweetened beverage intake was found also in analyses restricted to LADA with high GADA levels (i.e. above the median). Moreover, false positive LADA cases could not explain the differences in associations with fatty fish intake for LADA and type 2 diabetes found in Paper I. Importantly, we found that high risk HLA genotypes are associated with LADA but not type 2 diabetes, indicating that the GADA assay did identify two distinct patient groups.

In the studies based on ESTRID, HOMA-IR was used to indicate degree of insulin resistance. Hence, we are not assessing insulin resistance per se. HOMA is widely used and has shown high validity as a proxy for insulin resistance in diabetes patients when compared to the "gold standard" tests of hyperinsulinemic-euglycemic and hyperglycemic clamps [210, 211].

#### *6.2.2.3 Misclassification of exposure*

The FFQ used in ESTRID has been extensively validated against repeated 24-h recall interviews [182], weighed diet records [183], and adipose tissue n-3 PUFA content [184]. Yet, self-reported dietary intake is inherently afflicted with some degree of misreporting, often related to different characteristics such as bodyweight; in general, underreporting is more common among overweight individuals while underweight individuals are more likely to overreport [212]. Misreporting is especially problematic when dietary data are collected retrospectively, as in the ESTRID Study. Diet is essential in diabetes management and bias would be introduced if cases have changed their dietary intake after diagnosis and reported accordingly. To minimize bias, cases were specifically instructed to report diet as it was prior to diagnosis. In addition, we restricted the analyses of Paper I to cases who responded to the questionnaire within six months of diagnosis, and in Paper III, sensitivity analyses were conducted based on time since diagnosis. Notably, such bias would explain our findings only if cases would have decreased their fatty fish intake and increased their sweetened beverage consumption after diagnosis, which seems unlikely. Furthermore, it would not explain the observed differences between LADA and type 2 diabetes in relation to fatty fish consumption as we have no reason to believe that potential bias in the reporting would differ by diabetes type. Our findings for type 2 diabetes in relation to intakes of sweetened beverages and dietary fish concur with findings in prospective studies [5], which provide further support for the validity of our findings. Nevertheless, the potential issues with misreporting make the use of biomarkers as objective indicators of dietary intake appealing. In Paper II based on prospective data from EPIC-InterAct, exposure to n-3 PUFA was assessed by self-reported fish consumption but also by plasma phospholipid levels of n-3 PUFA. These findings were in line with the associations found in Paper I, supporting the hypothesis that n-3 PUFAs may have a role in the development of autoimmune diabetes.

In EPIC-InterAct, dietary habits and plasma n-3 PUFA were assessed at baseline but participants may have changed their intakes of dietary fish during follow-up. Repeated measurements throughout follow-up would have been a way of minimizing bias due exposure misclassification, but no such information was available. Any misclassification of dietary

habits or n-3 PUFA levels could however be assumed to be non-differential with regard to diabetes status and hence lead to dilution of associations rather than spurious excess risks related to low fish/n-3 PUFA, but may distort potential dose-response relationships. Sensitivity and specificity of the GADA assay was high (85% and 99%, respectively). However, GADA status was assessed at baseline, which means that some of those classified as antibody negative may in fact have seroconverted during follow-up and this misclassification may lead to dilution and potential underestimation of the interaction between dietary fish/plasma n-3 PUFA and GADA positivity.

BMI is an essential covariate closely linked to both dietary intake and diabetes risk, which is why it is of interest to consider its role as a potential mediator. BMI is based on self-reported data in ESTRID and in general, weight tends to be underreported and height tends to overreported [213]. However, correlation with BMI based on clinical measurements is high (r=0.92) for the cases in ESTRID. Still, BMI is a crude measure of body fat [214] and it is possible that we have underestimated the proportion of association mediated by BMI in the causal mediation analysis. Notably however, this may not speak against a common underlying mechanism for LADA and type 2 diabetes since the estimated direct effect of high sweetened beverage consumption was equal for both diabetes subtypes.

#### *6.2.2.4 Confounding*

A strength in the present studies was the detailed information on a large number of characteristics and lifestyle factors, such as education, smoking habits, physical activity, BMI, alcohol and other dietary components which could be included as covariates in the main statistical analyses. In Papers II and IV, it was not possible to adjust for family history, which is an important risk factor for both type 2 diabetes [215] and LADA [63]. However, the interactions between fish/n-3 PUFA and GADA on the risk of adult-onset diabetes reported in Paper II remained after additional adjustment for family history in sensitivity analysis. Furthermore, family history did not appreciably affect the association between sweetened beverage intake and LADA or type 2 diabetes in Paper III. We had detailed dietary data and the possibility to adjust for a large number of potential confounders including intakes of red/processed meat, sweet/salty snacks, coffee, whole grain, fruits, and vegetables. Still, we cannot exclude that our results are influenced by residual confounding, e.g. from inaccurately measured dietary confounders.

# 7 CONCLUSIONS

This thesis aimed to explore the role of diet in the development of LADA, and more specifically the risk of LADA in relation to dietary fish and sweetened beverage consumption. The findings indicate that n-3 PUFAs, acquired predominantly through fatty fish intake, may decrease the risk of LADA. Ensuring adequate levels of long-chain n-3 PUFAs by regular consumption of fatty fish may be particularly important when autoantibodies are already present in order to delay the progression to diabetes in adults. Furthermore, high sweetened beverage consumption seems to increase the risk of LADA, possibly through mechanisms promoting insulin resistance. The increased risk may be limited to individuals with low HLA-conferred genetic susceptibility.

These findings are well in line with the notion of LADA as a hybrid form of diabetes with risk factors related to both autoimmunity and insulin resistance. These were the first studies of the risk of LADA in relation to dietary fish and sweetened beverage consumption and it is important to confirm the associations in other populations. Still, these results add to the limited by growing body of evidence suggesting that lifestyle factors play a role in the development of LADA. Increased knowledge about modifiable lifestyle factors for LADA and their interaction with diabetes-related susceptibility genotypes may aid in the prevention and be a step towards reducing the burden of autoimmune diabetes.

# 8 FUTURE PERSPECTIVES

The work of identifying lifestyle factors contributing to the development of LADA is still in its infancy and a lot remains to be explored. Diet has a large impact on health and disease and there is no shortage in factors hypothesized to have role in processes related to autoimmunity and insulin resistance.

There are currently no prospective studies of LADA with dietary intake data available. Such studies would add valuable information on the role of diet in the development of LADA. The use of biomarkers and repeated measurements of exposures and autoantibody status would enable even more detailed analyses.

The potential interaction between genetic and dietary factors is an interesting area that needs to be further explored, especially in times when increased attention is given to precision medicine.

Exploring the roles of individual foods and nutrients is of importance for increased understanding of possible routes of action, but diet is likely to be a complex interplay and it is of equal importance to study dietary patterns to account for synergistic effects.

The role of diet in the prognosis of LADA including potential diet–drug interactions is another unexplored area.

# 9 ACKNOWLEDGEMENTS

I wish to thank all the people who have contributed to this thesis in different ways, for all your support along the way and for making this an enjoyable, inspiring, and unforgettable journey. I would especially like to express my gratitude to:

First and foremost, my main supervisor *Sofia Carlsson*, I don't know where to start! Thank you for the opportunity to outline this thesis work according to my preferences and interest in nutritional science, and for initiating ESTRID that has provided such unique data. Also, thank you for your extensive trust and support, professional guidance through coaching conversations, for sharing your excellent writing skills, and for always being available for questions and discussions about epidemiology, diabetes, and everyday life.

*Tiinamaija Tuomi*, my co-supervisor, for sharing your profound knowledge in diabetes and genetics, and for your instrumental inputs on the thesis and manuscripts.

*Alicja Wolk*, my co-supervisor, for enabling the use of nutritional data in ESTRID, for your profound expertise in nutritional epidemiology, and your valuable comments on the manuscripts.

*Mozhgan Dorkhan*, my co-supervisor, for excellent work in the early history of ANDIS and ESTRID, for your expertise in diabetes medicine, and your valuable comments on the manuscripts.

*Maria Feychting* and *Anders Ahlbom*, present and former head of the Unit of Epidemiology at IMM, for your support and the opportunity to work and grow within a stimulating research environment, and for sharing your outstanding expertise in epidemiology.

*Tomas Andersson*, co-author and invaluable statistical support whenever needed, for patiently explaining and discussing complex (and the most basic) statistical issues.

*Leif Groop* for your outstanding contributions to the advancements in diabetes research including the initiation of ANDIS, and for generously sharing your data and expertise. Thank you also to *Ylva Wessman*, *Johan Hultman*, *Petter Storm*, *Anders Rosengren*, and *Emma Ahlqvist* for your tremendous work in ANDIS that has enabled the existence and progression of ESTRID.

*Per-Ola Carlsson* and *Mats Martinell* for sharing the data collected within ANDiU and for your contributions to manuscripts.

*Lars Alfredsson* for generously sharing data for the EIRA controls, and also to *Boel Brynedal* and *Leonid Padyukov* for enabling the data transfers.

*Niclas Håkansson* and *Alice Wallin* for your contributions to the nutrient intake estimations in ESTRID.

*Olov Rolandsson* for initiating new and exciting projects and for sharing your expertise on autoimmunity and diabetes. I am also very grateful to *Nicholas Wareham* and colleagues at the MRC Epidemiology Unit, University of Cambridge, for generously sharing the EPIC-InterAct data.

The ESTRID research group, everyone involved has been instrumental and this thesis would not have existed without your remarkable efforts and contributions to the data collection – a true team effort! I am very fortunate to have all of you as colleagues, many thanks to:

*Rebecka Hjort*, for your great contributions to the data collection, for keeping track of all genetic data, for all our fruitful discussions on work and everyday life, and for being such a generous and supportive friend.

*Jessica Edstorp*, for your work and development of the data collection, for sharing your excellent way with words, for your enthusiasm and support, and for all the everyday life discussions.

*Bahareh Rasouli*, for your brilliance and never-ceasing energy in the work with the data collection, for your invaluable efforts with the datasets, for always being supporting, generous and kind, and for answering all my questions even from the other side of the Atlantic.

*Jenny Sundqvist*, for your past contributions in the data collection and for sharing your energy and positive attitude. I would also like to thank all the *present and past part-time co-workers* with your contributions in punching the collected data.

*Anna Karin Lindroos*, my mentor, for your interest and support during these years.

*Valdemar Grill*, you never cease to impress with your profound knowledge within all aspects of diabetes.

I would like to express my gratitude to *Anita Berglund*, *Karin Leander*, *Matteo Bottai*, and all other lecturers at IMM and KI for your dedicated work with the doctoral courses and for sharing your vast knowledge in epidemiology and biostatistics.

All my colleagues at the epidemiology unit and IMM, especially *Hanna Mogensen*, *Giorgio Tettamanti*, *Anna Meyer*, *Mats Talbäck*, and *Karin Modig* – thank you for your contributions to a warm and friendly atmosphere, always being there to answer questions, and for all the enjoyable lunch breaks.

I would also like to acknowledge *Karin Fremling* – thank you for being such a generous and supportive friend, and all other former colleagues in the epi unit including *Hannah Brooke* (thanks for the quick whatsapp survey!), *Maral Adel Fahmideh*, *Håkan Malmström*, *Lena Holm*, *Korinna Karampampa*, *Lisa Berg*, *David Pettersson* and *Annika Gustavsson*.

To fellow present and former PhD students at IMM and KI for fruitful discussions in journal clubs as well as by the coffee machine, especially *Camilla Olofsson*, *Cecilia Orellana*, *Sandra Ekström*, *Jessica Magnusson*, *Anna Ilar*, *Oscar Javier Pico Espinosa*, *Germán Carrasquilla*, *Ayman Alhamdow*, *Alva Wallas*, *Jesse Thatcher*, and *Otto Stackelberg*.

To everyone in the "fika group", thank you for all the nice and refreshing discussions on Tuesday mornings – I will turn up more often from now on! I would especially like to thank *Lena Nise* – for all the assistance with EIRA data, *Ida Palmqvist* – my (lab)partner in crime throughout the years studying nutrition, *Edit Ekström*, *Caroline Öfverberg*, and *Amanda Swanemar*. I would also like to acknowledge other present and past colleagues in at the former cardiovascular unit, especially *Federica Laguzzi*, *Xia Jiang*, *Anna Peterson*, and *Anette Linnersjö*.

I am incredibly thankful for the valuable contributions from all study participants in ESTRID and EPIC-InterAct, without you this thesis would not have been possible!

I would also like to thank everyone around me outside the research community; my family and friends. It is unfortunately impossible to mention all of you, but I would especially like to acknowledge:

*Johanna* och *Cicci*, ert stöd i vått och torrt betyder oerhört mycket för mig! Med en vänskap som hållit i närmare trettio år känner man sig trygg och jag ser fram emot många framtida upplevelser tillsammans. *Christine*, *Dina*, *Louise*, *Mariah*, *Hanna*, ni förgyller mitt liv mer än ni anar och varje gång vi ses laddas batterierna! Ni är bäst!

Och såklart, min familj! *Mamma* och *pappa*, för allt ert stöd i vad jag än tagit mig för, för allt ni gett mig, för all er kärlek. *Vicki*, *Karro* och *Lovisa*, det bästa systergänget man kan tänka sig, för allt vi delat, för att ni alltid finns där. *Inger* och *Peter*, för att ni finns där och alltid ställer upp! *Johan*, *Emmy*, *Wilmer* och *Ivar*, *Marcus*, *Billie* och *Frej*, *Tina* med familj, *Gunilla* med familj, *mormor* – för alla släktträffar som förgyller tillvaron och bidrar med energi, för allt ert stöd genom åren.

*Morfar*, *Oscar*, *farmor* och *farfar* – ni finns alltid nära.

Och till sist, *Anders*, för allt vi delat och kommer att dela, för all din kärlek, för att du är världens bästa make och pappa till våra underbara döttrar *Ellen* och *Matilda* som visar vad som är viktigt i livet.

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