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Interplay between birthweight, family history, obesity, and genes in the development of latent autoimmune diabetes in adults (LADA) and type 2 diabetes

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To My Family

ABSTRACT

Diabetes is a chronic and serious public health concern affecting millions of people around the globe. For effective prevention understanding of modifiable risk factors is fundamental. For type 2 diabetes these risk factors are well-described. However, for autoimmune diabetes such as type 1 diabetes, information is essentially missing. LADA, latent autoimmune diabetes in adults is suggested to be a hybrid of type 1 and type 2 diabetes and despite being common, its risk factors are scarcely investigated. Thus, the aim of this thesis was to add new understanding to the aetiology of LADA in relation to birthweight, overweight, obesity, family history of diabetes (FHD) and genes as well as the interaction between these factors in relation to the risk of LADA and how this compares with type 2 diabetes.

The analyses were based on data from two large population based studies: ESTRID, an ongoing case-control study from Sweden including incident cases of diabetes and randomly selected controls (≥35 years of age) and HUNT, a prospective study from Norway with incident cases of diabetes and 22 years of follow-up. LADA was defined by the criteria that age at initial diagnosis is at least ≥35 years old and glutamic acid decarboxylase antibody (GADA) positivity. In ESTRID LADA patients also had C-peptide levels indicating residual insulin secretion. Patients ≥35 years of age with GADA negativity were considered to have type 2 diabetes. Information on birthweight, body mass index (BMI kg/m^2) and FHD was self-reported in ESTRID. In HUNT, information on BMI was collected at the baseline medical examination.

Our results indicate that heredity is primarily linked to FHD of type 1 diabetes (Relative risk [RR] 5.75; 95% confidence interval [CI] 3.23-10.25) but is also associated with FHD of type 2 diabetes (RR 1.89; CI 1.45-2.47). Furthermore, our findings suggest that obesity (BMI ≥30) increases the risk of LADA 3-6 fold, although the risk was not as marked as for type 2 diabetes (10-19 fold increased risk). In line with this, the risk of LADA was greater in individuals with low GADA but present also among those with higher degree of autoimmunity. Notably, our analyses indicate that 31-56% of all LADA patients may be prevented by keeping a normal weight (BMI <25). The greatest risk of LADA was seen in those with overweight in combination with HLA genes associated with autoimmunity (RR 7.59, CI 5.27-10.93), with attributable proportion due to interaction (AP) estimated to 0.29 (CI 0.10-0.47). Additionally, type 2 diabetes risk genes interacted with overweight in relation to LADA; *TCF7L2* (AP=0.31, CI 0.09-0.52) and *FTO* (AP=0.38, CI 0.15-0.61). Moreover, low birthweight was associated with a more than 2-fold increased risk of LADA and type 2 diabetes, with the strongest risk in those with low birthweight in combination with adult overweight; LADA, RR 3.26 (CI 1.69, 6.29); and type 2 diabetes, RR 39.93 (CI 19.27, 82.71). In conclusion, results from this thesis indicate that LADA is associated with risk factors linked to insulin resistance and type 2 diabetes while genetic susceptibility is linked foremost to genes associated with autoimmunity and type 1 diabetes. Importantly, our findings suggest that LADA in part may be prevented by keeping a healthy weight.

SAMMANFATTNING PÅ SVENSKA

Diabetes är en kronisk och mycket vanlig sjukdom som kännetecknas av förhöjda blodsockernivåer (hyperglykemi). Sjukdomen orsakas av total brist på, eller delvis minskad utsöndring av hormonet insulin från bukspottkörtelns beta-celler. Insulinet behövs för att kroppens celler ska kunna ta upp energi från födan. Andra faktorer bakom diabetes är minskad insulinkänslighet i viktiga metabola organ som muskler, fettvävnad och lever. Diabetes är kopplat till ökad risk för hjärtsjukdom och stroke. Även risken för synnedsättning, njursjukdomar samt andra komplikationer, som beror på defekter i små och stora blodkärl eller nervskador, ökar till följd av kronisk hyperglykemi.

Traditionellt sett brukar diabetes delas in i typ 1-diabetes (vanligast hos barn) och typ 2-diabetes (vanligast hos vuxna). Det som framförallt utmärker typ 1-diabetes är mer eller mindre total insulinbrist, orsakad av en autoimmun reaktion d.v.s. där kroppens eget immunförsvar av okänd anledning förstör de insulinproducerande beta-cellerna. Hos personer med typ 2-diabetes producerar bukspottkörteln för lite insulin eller att kroppen inte kan använda insulinet på ett tillräckligt effektivt sätt (insulinresistens). Ny forskning visar dock att denna indelning kan vara alltför grov. Diabetes är i själva verket en betydligt mer heterogen sjukdom med flera undergrupper. En av dessa är LADA, latent autoimmun diabetes hos vuxna. Man beräknar att ungefär 5-10 % av alla diabetespatienter i Europa har denna variant. LADA föreslås vara en blandning av både typ 1-diabetes (autoimmunitet) och typ 2-diabetes (insulinresistens). Hittills är forskning om riskfaktorer för LADA nästan obefintlig men hypotesen är att miljöfaktorer eller livsstil kan vara viktiga delar i etiologin. För att ta reda på mer om orsakerna bakom LADA var syftet med denna avhandling att undersöka risken i relation till födelsevikt, ärftlighet, övervikt/fetma och gener samt interaktion dem emellan. För att undersöka detta analyserades data från två stora befolkningsstudier, den svenska ESTRID-studien och den norska HUNT-studien.

I delarbete I var syftet att undersöka sambandet mellan födelsevikt och risken för LADA och typ 2-diabetes med data från ESTRID. Resultatet tyder på att låg födelsevikt (<3 kg) fördubblar risken för både LADA och typ 2-diabetes jämfört med dem som hade hög födelsevikt (≥4 kg). Studien indikerar att det framförallt är miljöfaktorer under fosterstadiet t.ex. mammans diet eller rökning och inte gener som ligger bakom den förhöjda risken. Detta kan leda till permanenta metabola förändringar hos fostret som får både minskad insulinfrisättning och insulinkänslighet. Låg födelsevikt har även föreslagits öka risken för övervikt. I linje med detta sågs i vår studie en 3 gånger högre risk för LADA hos personer med låg födelsevikt och övervikt i vuxen ålder, jämfört med personer som var normalviktiga och med en födelsevikt på minst 3 kg. Denna dubbla börda, låg födelsevikt och övervikt, var associerad med en 40 gånger ökad risk för typ 2-diabetes.

I delarbete II undersöktes om ärftlighet ökar sannolikheten för LADA och typ 2-diabetes. Resultaten tyder på att risken för LADA framförallt är kopplad till typ 1-diabetes i familjen; 6 gånger ökad risk sågs hos dem med mamma, pappa, syskon eller barn med typ 1-diabetes.

Risken var dock även förhöjd med typ 2-diabetes i släkten där en näst intill fördubblad risk sågs. Individer med LADA och ärftlighet för typ 1-diabetes hade även en högre grad av autoimmunitet och fler hade HLA-riskgener (högriskgener för typ 1-diabetes) jämfört med LADA-patienter med typ 2-diabetes i släkten. Risken för typ 2-diabetes var kopplad till typ 2 men inte till typ 1-diabetes i släkten, vilket är i linje med tidigare studier.

I delarbete III använde vi data från både ESTRID och HUNT för att utforska sambandet mellan LADA, övervikt och fetma. Resultaten visade att övervikt kan vara en stark riskfaktor för LADA. Individer med fetma (body mass index [BMI] ≥30) löpte en 3-6 gånger högre risk jämfört med normalviktiga (BMI <25). Risken var dock inte lika markant som för typ 2-diabetes där en 10-19 gånger ökad risk sågs hos dem med BMI ≥30. Vidare sågs den högsta risken för LADA och typ 2-diabetes hos dem med både övervikt och ärftlighet i släkten. I det här sammanhanget är det viktigt att påpeka att våra analyser indikerar att 31-56 % av alla LADA-patienter och 70-82% av alla med typ 2-diabetes kan förebygga sjukdomen genom att behålla en hälsosam vikt.

I delarbete IV fortsatte vi att undersöka risken för LADA och typ 2-diabetes i förhållande till den kombinerade risken av ärftlighet och övervikt. Här studerade vi interaktion med högriskgener för typ 1- diabetes (HLA) och typ 2-diabetes (*TCF7L2* och *FTO*). Risken var högst hos dem med HLA-gener (kopplade till autoimmunitet) där hela 8 gånger förhöjd risk sågs hos de som var överviktiga (BMI ≥25) jämfört med normalviktiga utan dessa riskgener. Beräkningarna tyder på att 29 % av alla LADA-patienter med både övervikt och HLA-gener kan tillskrivas växelverkan mellan dessa riskfaktorer. Vilket betyder att en lika stor andel LADA-fall kan förebyggas om övervikten inte uppstår. Även hos dem med övervikt i kombination med *TCF7L2* och *FTO* syntes en klar interaktionseffekt. Gällande resultaten för typ 2- diabetes observerades en tydlig samverkan mellan övervikt och *TCF7L2*, men inte med vare med sig *FTO* eller HLA.

Sammanfattningsvis stöder resultaten från avhandlingen att de faktorer som är kopplade till insulinresistens och typ 2-diabetes också utgör en ökad risk för LADA. Genetisk känslighet är däremot främst kopplad till autoimmunitet och typ 1-diabetes. Dessa fynd bidrar till det begränsade men gradvis ökande antalet studier som visar att livsstil spelar en viktig roll för förekomsten av LADA. Slutligen tyder våra resultat på att LADA delvis kan förebyggas med viktnedgång. Detta tycks även gälla för dem med genetisk känslighet. Andelen LADA-fall som kan förebyggas är sannolikt inte lika stor som för typ 2-diabetes.

LIST OF SCIENTIFIC PAPERS

I. **Hjort R**, Alfredsson L, Carlsson P-O, Groop L, Martinell M, Storm P, Tuomi T, Carlsson S. Low birthweight is associated with an increased risk of LADA and type 2 diabetes: results from a Swedish case–control study. Diabetologia, 2015, 58:2525–2532

II. **Hjort R**, Alfredsson L, Andersson T, Carlsson P-O, Grill V, Groop L, Martinell M, Rasouli B, Storm P, Tuomi T, Carlsson S. Family history of type 1 and type 2 diabetes and risk of latent autoimmune diabetes in adults (LADA). Diabetes & Metabolism, 2017, 43:536-542.

III. **Hjort R**, Ahlqvist E, Carlsson P-O, Grill V, Groop L, Martinell M, Rasouli B, Rosengren A, Tuomi T, Åsvold BO, Carlsson S. Overweight, obesity and the risk of LADA: results from a Swedish case–control study and the Norwegian HUNT Study. Diabetologia, 2018, 61(6):1333-1343

IV. **Hjort R**, Löfvenborg JE, Ahlqvist E, Alfredsson L, Andersson T, Grill V, Groop L, Sørgjerd EP, Tuomi T, Åsvold BO, Carlsson S. Interaction between overweight and genotypes of HLA, *TCF7L2*, and *FTO* in relation to the risk of Latent Autoimmune Diabetes in Adults and type 2 diabetes. J Clin Endocrinol Metab. 2019 May 24. pii: jc.2019-00183. doi: 10.1210/jc.2019-00183. [Epub ahead of print]

CONTENTS

LIST OF ABBREVIATIONS

ANDIS	All New Diabetics in Skåne
ANDiU	All New Diabetics in Uppsala
AP	Attributable proportion due to interaction
BMI	Body Mass Index
EIRA	Epidemiological Investigation of Rheumatoid Arthritis
ESTRID	Epidemiological Study of Risk Factors for LADA and Type 2 diabetes
FHD	Family history of diabetes
FHD-T1D	Family history of type 1diabetes
FHD-T2D	Family history of type 2 diabetes
GADA	Glutamic acid decarboxylase antibody
GWAS	Genome-wide association study
HLA	Human leucocyte antigen
HOMA	Homeostatic model assessment
HR	Hazard ratio
HUNT Study	Nord-Trøndelag Health Study
IA-2A	Islet antigen-2 autoantibody
IAA	Insulin autoantibody
LADA	Latent autoimmune diabetes in adults
OR	Odds ratio
PAR	Population-attributable risk
RERI	Relative excess risk due to interaction
RR	Relative Risk
SNP	Single-nucleotide polymorphism
WHtR	Waist-to-height ratio
WHR	Waist-to-hip ratio
ZnT8A	Zinc transporter 8 autoantibody

1 INTRODUCTION

Diabetes is a group of rapidly increasing metabolic disorders characterized by raised blood glucose levels (hyperglycaemia) [1]. The disease is caused by an inhibited or non-existing insulin secretion from the pancreatic β-cells, reduced insulin sensitivity in key metabolic organs (muscle, adipose tissue and liver) or both [2]. At the moment, diabetes is not possible to cure; primary prevention is therefore a prerequisite to reduce the diabetes burden. For effective prevention, knowledge of modifiable risk factors is essential. For type 2 diabetes such knowledge is extensive [3] and it has been shown that type 2 diabetes can be prevented through lifestyle modification [4, 5]. As for autoimmune diabetes, much research has addressed type 1 diabetes in children, but to date, only a few environmental risk factors have been identified [6]. For autoimmune diabetes with onset in adulthood, research is scarce. LADA, latent autoimmune diabetes in adults is the most common form of autoimmune diabetes in adults and accounts for 5-12% of all patients in Europe [7, 8]. LADA is a hybrid form of diabetes with a pathogenesis that includes autoimmune destruction of the β-cells, similar to type 1 diabetes, as well as features of type 2 diabetes, including insulin resistance. The aim of this thesis was to contribute to the limited knowledge of the aetiology of LADA by studying how low birthweight, family history, overweight/obesity and genes, as well as the interaction between these factors, affect the risk of LADA compared to type 2 diabetes. For this purpose we used data from two large population-based studies; the Swedish ESTRID study and the Norwegian HUNT study.

2 BACKGROUND

2.1 DIABETES

Diabetes is the fastest growing disease worldwide and a major threat to public health, ranked 7th on the list of leading causes of death [1, 9]. In 2017, the global prevalence of diabetes was estimated to be 8.4%, which is equivalent to 451 million affected individuals. This is estimated to rise to 693 million by the year 2045 [10]. The increase is expected to be greater in low- and middle-income in comparison with high-income countries [1]. Demographic and cultural changes, including an ageing population and increasing prevalence of obesity and sedentariness are believed to be the major forces behind this trend [11]. However, better healthcare leading to improved life expectancy for individuals with diabetes has also contributed to this increased prevalence [10, 12]. In Europe, the prevalence of diabetes is 8.8% in the adult population [1], while the prevalence in Sweden is slightly lower at 6.8% [12]. Despite of improved medical treatment, the burden of the disease is enormous, with large individual and societal consequences [13], primarily by disease related complications and comorbidities, and an increased risk of premature death [11]. Because diabetes cannot be cured, primary prevention strategies are key in reducing disease burden. Effective prevention requires detailed information on risk factors. This knowledge is comprehensive for type 2 diabetes but limited for autoimmune diabetes.

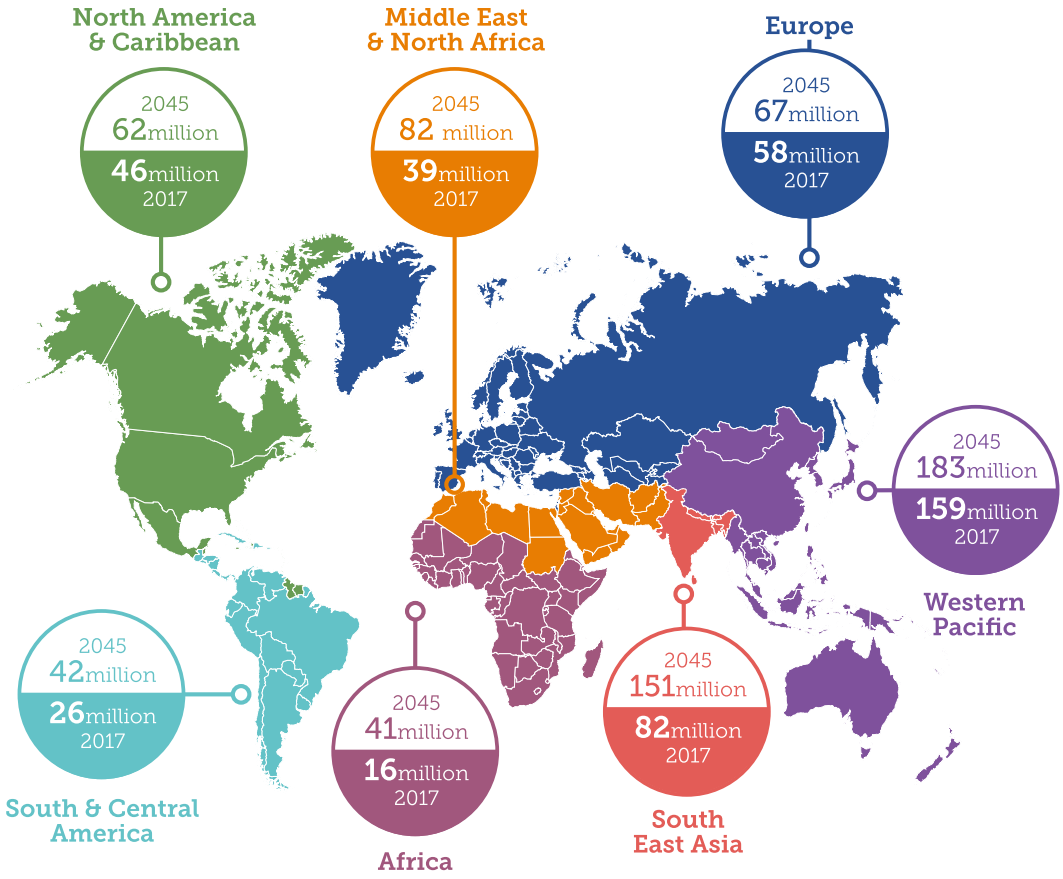

Figure 2.1 Number of people with diabetes in different regions of the World in 2017 and 2045 (20-79 years). Adapted with permission from the IDF diabetes Atlas (https://www.diabetesatlas.org/).

2.1.1 Diagnosis

Hyperglycaemia is the main symptom used to diagnose diabetes. According to guidelines from the American Diabetes Association (ADA) [14] and the World Health Organization (WHO) [15] hyperglycaemia is diagnosed on the basis of either a) fasting blood sugar levels ≥7 mmol/L, b) 2 hour oral glucose tolerance test ≥11mmol/L, c) Hb1Ac ≥6.5% (48 mmol/mol) or d) in patients with classic symptoms of hyperglycaemia or hyperglycaemic crisis, a random plasma glucose ≥11.1 mmol/L.

2.1.2 Diabetes subtypes

Diabetes is typically divided into two major subgroups: type 1 and type 2 diabetes. However, over the last decades it has become clear that diabetes is more heterogeneous than this sub-division implies [16]. The concept of LADA, latent autoimmune diabetes in adults, was introduced in the nineties [17] and is a form of diabetes which shares clinical and genetic features with both type 1 (autoimmunity, HLA-conferred genetic susceptibility) and type 2 diabetes (insulin resistance). It has been proposed that type 1 and type 2 diabetes can be regarded as two ends of a diabetes continuum containing several different phenotypes, distinguished only by various degrees of autoimmunity, insulin resistance, genetic susceptibility, age and obesity [16]. Recently, a completely new way of classifying the diabetes spectrum has been proposed, addressing foremost the diversity of type 2 diabetes [18]. This thesis focuses on LADA, which will be compared to type 1 and classical type 2 diabetes. Other types of diabetes will not be covered in the thesis.

2.1.2.1 Type 1 and type 2 diabetes

Type 1 diabetes accounts for 7-12% of all cases of diabetes [1, 8]. It is the most common form of diabetes in children but can occur at any age [1, 19]. The incidence has increased dramatically over the latest decades. Although the greatest rise has been in North America [20], the highest incidence (aged <15 years) is found in Finland followed by Sweden and Kuwait. By contrast, the disorder is rare in China and Venezuela [21]. Type 2 diabetes is the most common form of diabetes and accounts for 75-85% of all cases [8, 18]. The prevalence depends on ethnicity and geographical area [1, 22] and while the highest prevalence is seen in Pima Indians [23], China and India have the highest number of affected individuals [1]. Type 2 diabetes has traditionally been seen as a disease of the elderly but is unfortunately becoming more prevalent in adolescents and children [4, 28]. This trend has been linked to unhealthy lifestyle and the increasing childhood obesity [29, 30].

2.1.2.2 LADA

LADA may be the second most prevalent type of diabetes in adults, accounting for 3-12% of all diabetes patients [7]. The highest prevalence is seen in Europe where LADA is estimated to account for around one tenth of all patients initially classified as type 2 diabetes [24, 25]. Recent data from the Swedish ANDIS-study (All New Diabetics in Skåne) [8] including >10,000 incident diagnoses, indicates that LADA accounts for 5% of all patients (Figure 2.2).

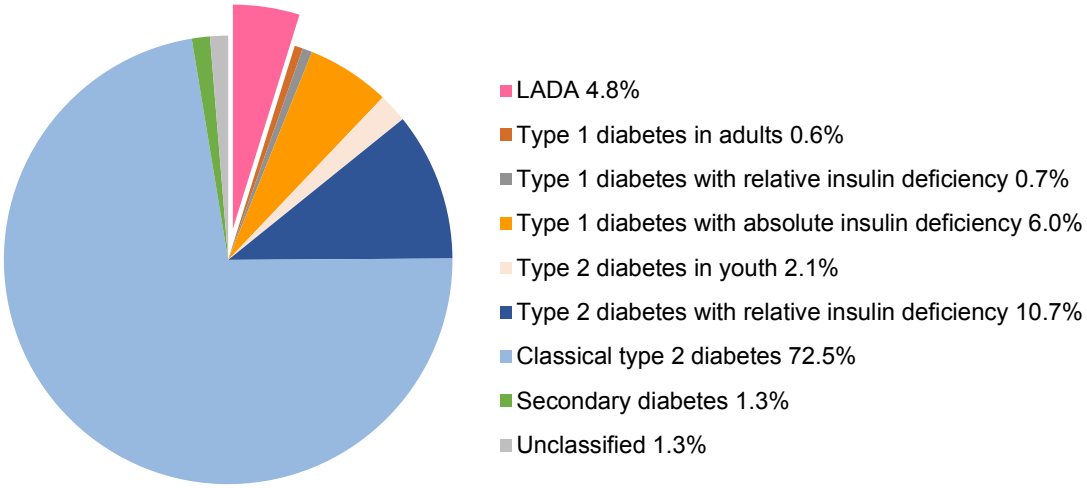

Figure 2.2 Subtypes of diabetes in the ANDIS registry (http://andis.ludc.med.lu.se/)

2.1.3 Pathogenesis

2.1.3.1 Type 1 diabetes

Type 1 diabetes is characterized by insulin deficiency caused by a T-cell mediated autoimmune reaction towards the insulin producing β-cells of the pancreas [26]. Onset is typically rapid with an acute and lifelong need of insulin treatment [27]. The autoimmune process can be detected by circulating autoantibodies, which may be present several years prior to diagnoses [28]. There are four autoantibodies commonly associated with autoimmunity in type 1 diabetes, namely: antibodies directed against insulin (IAA), glutamic acid decarboxylase (GADA), tyrosine phosphatase-like protein (islet antigen-2 [IA-2A]) and zinc transporter-8 (ZnT8A) [29]. Individuals with multiple antibodies are at especially high risk of type 1 diabetes, and these individuals commonly have an earlier age of onset [30, 31]. Type of antibody is also important for age at seroconversion with IAA usually being the first detected antibody in young children, while GADA is the primary autoimmune marker for onset at later ages [32].

2.1.3.2 Type 2 diabetes

Insulin resistance in muscle, liver and adipose tissue plays a key role in the pathogenesis of type 2 diabetes. Insulin resistance leads to increased glucose production in the liver and reduced glucose uptake in muscle and adipose tissue [2]. Many mechanisms are involved e.g. genetic susceptibility, ectopic fat accumulation and systemic inflammation are all associated with reduced insulin sensitivity [2, 33]. β-cell dysfunction is however also needed for onset of type 2 diabetes. Besides a genetic predisposition, chronic hyperglycaemia and pancreatic fat accumulation put additional stress on the β-cells which leads to insufficient insulin secretion [2, 33]. Hyperglycaemia becomes manifest when the β-cells are no longer able to compensate for the increased need of insulin driven by insulin resistance [2].

2.1.3.3 LADA

In 1977, Irvine and colleagues [34] identified a group of type 2 diabetes patients with pancreatic-islet-cell antibodies, which is indicative of an autoimmune pathogenesis. The term LADA was introduced by Tuomi et al. in 1993 [17] to better define this patient group that were autoantibody positive, but unlike type 1 diabetes patients, did not have a pronounced need for insulin at diagnosis. At the same time these patients had a clinical phenotype similar to that of type 2 diabetes, including adult onset and insulin resistance [17, 35]. Compared with type 1 diabetes patients that are often multi-antibody positive, individuals with LADA are typically positive for only one antibody, with GADA present in around 90% of the patients [25]. This results in a milder autoimmunity and a slowly progressive insulin-dependence. Whether LADA should be regarded as a separate subtype of diabetes is still debated. While WHO [15] suggests that LADA is a hybrid diabetes type, ADA [14] proposes that LADA is a mild form of type 1 diabetes.

Classification of LADA

There is no universal definition of LADA. The presence of circulating islet autoantibodies, most often GADA, is however an absolute criterion, which is also how LADA is distinguished from type 2 diabetes. To separate LADA from type 1 diabetes, age at onset, typically above 30-35 years, is used. As supplementary criteria, to distinguish LADA from type 1 diabetes with adult onset, patients should be insulin independent at least 6-12 months after diagnosis [16]. Since the insulin criteria is subjective and depends on the decision made by the physician [36], it has also been suggested to use an indicator of remaining insulin production e.g. C-peptide to indicate a slow “latent” autoimmune process [18].

Table 2.1 Clinical and genetic features of LADA in comparison with type 1 and type 2 diabetes

	Type 1 diabetes	**LADA**	**Type 2 diabetes**
Age at onset	Childhood (rare in adults)	>30 years	Adulthood (rare in children)
Autoimmunity	Strong	Mild	Absent
β-cell function	Absent/low	Present but declines	Present
Insulin resistance	Rare	Some	Increased
C-peptide	Non detectable	Decreased but detectable	Present
Insulin dependence	At diagnosis	>6 months after diagnosis	None or years after diagnosis
Genetic susceptibility	HLA	HLA *TCF7L2, FTO*?	*TCF7L2, FTO*

Adapted from Buzzetti et al. [7] and Tuomi et al. [16].

2.1.4 Co-morbidities and complications

There is a serious risk of co-morbidities and disease related complications in individuals with diabetes [1]. In type 1 and type 2 diabetes chronic hyperglycaemia is found to damage blood vessels, eyes (retinopathy), kidneys (nephropathy) and nerves (neuropathy) [11]. Lower limb amputation as a consequence from peripheral nerve damage is 10-20 times more common in those with diabetes [1]. There is also an increased risk of macrovascular outcomes such as heart disease and stroke, and an increased overall risk of dying [11]. In Sweden the risk of excess mortality was ~4-fold for individuals with type 1 diabetes [37, 38] and ~2-fold for those with type 2 diabetes [39], compared with the general population. However, the risk widely varies depending on age at onset and number of cardiovascular related risk factors [40, 41]. Studies of complications in LADA are scarce. Still, even if it has been indicated that LADA patients have healthier metabolic profile than those with type 2 diabetes [42, 43], the risk of cardiovascular events does not seem to differ [42-46]. In some studies, the prevalence of neuropathy has even been suggested to be higher in individuals with LADA [7]. It is presumed that this is a consequence of more severe insulin deficiency and worse glycaemic control in individuals with LADA [7].

2.1.5 Diabetes management

The purpose of diabetes management is primarily to keep blood glucose levels at an adequate level. For individuals with type 1 diabetes, lifelong insulin therapy is a necessity. For type 2 diabetes, lifestyle modification (diet and physical activity) is the first line treatment followed by oral glucose lowering agents and injectables [47]. Metformin is the first hand choice while commonly used add-ons to metformin include sulfonylurea, thiazolidinediones, GLP-1 receptor agonists and DPP-4 and SGLT2 inhibitors. However, if neither of these treatments suffice in stopping rising glucose levels, insulin injections may be required [47]. Alongside this, management of blood pressure and lipids may be needed to reduce macro and microvascular complications and mortality [47]. For LADA, the best treatment regimen is unclear. Most patients are initially treated with oral glucose lowering drugs [7] but because β-cell deficiency typically progresses faster in LADA than in type 2 diabetes [48] it is currently

unclear whether insulin should be initiated at diagnosis [49]. Several intervention studies with LADA patients have been conducted to try to understand this, but they have left inconclusive answers [50, 51]. However, this topic will not be further discussed in the thesis.

2.2 GENETICS

2.2.1 Type 1 diabetes

There is a known strong association between type 1 diabetes and DR and DQ genes within the human leukocyte antigen (HLA) complex on the short arm of chromosome 6 [52]. The HLA region is involved in the immune response; the DR and DQ encode cell-surface proteins which are involved in the presentation of antigens to T-lymphocytes. Polymorphisms are associated with insulin deficiency, presumably by an autoimmune-induced destruction of the β-cells [53]. HLA haplotypes DRB1*04:xx-DQA1*03:01-DQB1*03:02 (DR4-DQ8) and DRB1*03:01-DQA1*05:01-DQB1*02:01 (DR3-DRQ2) are the strongest predictors and the risk is particularly high when these are inherited together [52]. However, the frequency of these haplotypes in newly diagnosed patients decreases with age [54]. The HLA haplotypes are reported to account for approximately 50% of the familial aggregation of type 1 diabetes [55].There are also HLA haplotypes associated with protective effects including DRB1*15:01 (DR2)- DQA1*01:02-DQB1*06:02 [52]. Subsequent linkage and genome-wide association studies (GWAS) have found more than 60 loci outside the HLA system [56], such as the insulin gene (*INS*), *PTPN22*, *CTLA*-4, *IL2RA* and *SH2B3*, are associated with type 1 diabetes [52]. In all, results from genetic studies explain about 80% of the heritability in type 1 diabetes [57].

2.2.2 Type 2 diabetes

Following the introduction of GWAS technique in 2007 rapid breakthroughs in knowledge of the genes contributing to type 2 diabetes were made. Today, more than 400 genetic loci linked to type 2 diabetes have been identified [58]. Still, together these genes explain less than 15% of the heritability of type 2 diabetes [57]. The strongest genetic effect is conferred by variants in the transcription factor 7–like 2 (*TCF7L2*) gene encoding a protein involved in the Wnt signalling pathway, which induce the transcription of several other genes such as those for insulin [59-61]. The rs7903146 variant explains most of the association and carrying the T-allele is associated with a ~30% increased risk of type 2 diabetes [62]. The majority of genes identified to date, including variants of *TCF7L2*, affect type 2 diabetes by impaired insulin secretion [63]. However some genes, including variants of the fat mass and obesity-associated (*FTO*) gene, modify disease risk by insulin action [63]. In *FTO*, the risk is presumably mediated by greater overall energy intake and increased body fat [64].

2.2.3 LADA

LADA is, similar to type 1 diabetes, strongly associated with high-risk variants in the HLA region, in particularly with variants of the DR and DQ loci [48, 65-69], although, with weaker

effect estimates for LADA [70]. The frequency of these high-risk genotypes is further related to degree of autoimmunity with strongest associations in LADA patients with higher GADA levels [67, 71]. Although there are some conflicting results [70], non-HLA loci linked to type 1 diabetes including variants in *PTPN22*, *INS* and *SH2B3* have been associated with LADA [68]. Additionally, LADA has been linked to some genes associated with type 2 diabetes, including *TCF7L2* and *FTO* [70]. The association with *TCF7L2* has been reported in several European studies [66, 72, 73] including in a meta-analysis with nearly 1000 LADA patients [73]. However not in all studies [67, 68] including the first GWAS of LADA which was recently published [69]. This may be due to power issues; while there was no association on a genome-wide level the nominal p-value was significant. It may also depend on the heterogeneity of the LADA patients; the associations with the *TCF7L2* and *FTO* genes have been seen mainly in LADA with low GADA titre [67, 72].

2.3 ENVIRONMENTAL FACTORS

2.3.1 Type 1 diabetes

Few environmental risk factors for type 1 diabetes have been fully established. Study results have been difficult to replicate between populations and many contradicting findings have been presented [74]. Moreover, prevention studies have mostly been unsuccessful [75]. However, as only 10-15% of the patients have a first-degree family member with diabetes [76] environmental factors are strongly suspected in the aetiology. In further support, genetic changes alone cannot explain the rapid increase of type 1 diabetes over the latest decades. These potential risk factors may influence the risk of type 1 diabetes by directly triggering islet autoimmunity or they may accelerate disease progression in individuals with an ongoing autoimmune process. The most established trigger of autoimmunity to date is maternal enterovirus infection during the prenatal period, or direct exposure to these viruses in childhood [74]. Respiratory infections have in contrast been associated with both increased [77] and reduced risk (The hygiene hypothesis) [78]. Additional potential risk factors include higher maternal age and caesarean delivery [79] as well as stressful life events [80], suggestively mediated by increased cortisol levels and lower resistance to infections [81]. Early life dietary factors, including cow's milk consumption, vitamin D (deficiency), low omega-3 fatty acids and dietary toxins (nitrate and nitrite) [74] have further been implicated to trigger autoimmunity and β-cell apoptosis, mainly in individuals with a genetic predisposition. Collectively, these potential triggers have put forward the gut microbiota in the pathogenesis of type 1 diabetes [82]. The rapid increase in incidence has also coincided with the childhood obesity epidemic [83], implying that this may be an important promoter [84]. For instance high birthweight (discussed in section 2.4.2) [85, 86] mediated by maternal diabetes/obesity, high body mass index (BMI) (further discussed in section 2.4.3) [87, 88] and fast growth during the first years in life [89] have all been associated with increased risk of type 1 diabetes.

2.3.2 Type 2 diabetes

A large body of research has successfully investigated the aetiology of type 2 diabetes and its prevention. Excessive weight has been identified as the major risk factor [11]. The hallmark of type 2 diabetes, insulin resistance, is partly viewed as an effect of general obesity, but it is hypothesized that abdominal body fat may be more important (further discussed in section 2.4.3) [90, 91]. Other risk factors are low physical activity [92], sedentary lifestyle and hours of TV-watching (independent of total activity) [93] and smoking [94]. Diet has further been identified to play a fundamental role in the aetiology with excess risk associated with a high-fat and low fibre diet, and consumption of red and processed meat and sugar sweetened beverages [95]. In contrast, moderate alcohol consumption [96], high coffee consumption [95] and foods associated with the Mediterranean diet, including fruit and vegetables and nuts, are associated with reduced risk [97]. High and low birthweight also appear important and both are associated with risk of type 2 diabetes (see section 2.4.2) [98-100]. Mechanistically, the majority of factors associated with type 2 diabetes are suggested to modify risk of disease by insulin sensitivity. Type 2 diabetes is known to be largely preventable with lifestyle interventions resulting in weight loss, including healthy diet and increased physical activity [4, 5].

2.3.3 LADA

LADA is suggested to be a hybrid of type 1 and type 2 diabetes and consequently, risk factors may promote LADA both by way of promoting insulin resistance and/or by affecting β-cell autoimmunity. So far, limited attempts have been made to investigate potential risk factors in relation to LADA. The main reason is that few studies include both lifestyle information, antibody measurements to distinguish LADA from type 2 diabetes patients, and a suitable (diabetes-free) comparison group [101]. However results from the Norwegian HUNT and the Swedish ESTRID studies (described in detail in the methods section) imply that the aetiology of LADA may include lifestyle factors previously linked to type 2 diabetes such as overweight and obesity [102], physical inactivity [102], high consumption of sweetened beverages [103], smoking [104], sleep disturbances and low psychosocial well-being [105] and moderate alcohol intake (protective) [106]. These findings support the notion that insulin resistance may be important in the development of LADA. LADA has also been associated with factors suggested to increase the risk of type 1 diabetes including high education [107] and coffee consumption [108]. Previous studies further indicate that risk factors may differ for more and less autoimmune individuals, e.g. alcohol has been associated with a reduced risk of more type 2-like LADA but not more autoimmune type 1-like patients [109]. Studies are scarce hence there is a need for replications and extensions of these findings. Furthermore, the risk of developing LADA as a result of lifestyle factors likely depends on genetic susceptibility but few studies have investigated this notion in relation to LADA [110, 111]. In this thesis, LADA risk will be studied in relation to family history of diabetes, low birthweight, and overweight/obesity including its interaction with genetic susceptibility. These factors have previously been associated with risk of type 1 and/or type 2 diabetes, as presented in the following section.

2.4 POTENTIAL RISK FACTORS STUDIED IN THE THESIS

2.4.1 Family history of diabetes

Family history covers both genetic and shared environmental factors and is a strong predictor of risk of diabetes [112].

2.4.1.1 Type 1 diabetes

Familial clustering is a strong indicator of future risk of type 1 diabetes, first-degree relatives with diabetes increase the risk 9-fold [113]. The association is stronger for paternal diabetes [114, 115] but diminishes with age of the offspring. The strong genetic component is further illustrated by the 6-10% lifetime risk in dizygotic twins compared to >50% lifetime concordance rate in monozygotic twins [52, 76]

2.4.1.2 Type 2 diabetes

Diabetes in first-degree relatives confers a 3-fold increased risk of type 2 diabetes [112] and it has been suggested that the risk is higher if the mother has the disease, rather than the father [63]. A strong familial effect is further supported by twin studies indicating a concordance rate of 20-30% in dizygotic and ~70% in monozygotic twin pairs [63]. The heritability estimates are stronger at younger age and in lean compared to obese individuals [116].

2.4.1.3 LADA

Reports on family history in LADA are scarce but cross-sectional studies indicate a high prevalence [117, 118]. A 4-fold increased risk in those with a family history of any type of diabetes was found in the HUNT study [119], whereas the risk was increased 2-fold in the Finnish Botnia study [120]. Because LADA is suggested to be hybrid form of diabetes [16], which is also supported by evidence from some genetic studies [70], family history of both type 1 and type 2 diabetes may potentially promote LADA. Findings of a few smaller studies support this hypothesis [121-124]. Furthermore, prospective data from the Botnia study found that a mix of both forms of diabetes in the family was associated with a 2-fold increased risk of non-insulin requiring diabetes [120]. However, the relative importance of family history of type 1 versus type 2 diabetes in the aetiology of LADA has not been explored. Other questions that remain to be investigated include the influence of female vs. male relatives, number and closeness of afflicted family members and underlying mechanisms, e.g. the influence of family history of diabetes on indicators of autoimmunity and insulin resistance.

2.4.2 Birthweight

The intrauterine environment has been suggested to be essential in predicting various metabolic disorders, including diabetes [125]. The association between parental environmental factors such as diet, body composition, smoking and stress during time of conception, adverse foetal developmental and later chronic disease of the offspring, support this notion [126]. Epigenetic and metabolic reprogramming in response to these in utero stressors provide a mechanistic link [127].

2.4.2.1 Type 1 diabetes

With regard to type 1 diabetes a modest link to high birthweight (macrosomia) has been documented in two meta-analyses [85, 86] and this association was replicated in a recent nationwide Swedish study [128]. It was suggested that this reflects overstimulation of the foetal pancreatic β-cells from maternal diabetes/obesity during pregnancy [129] which in turn, has been associated with higher birthweight and type 1 diabetes [128]. This is likely an effect from maternal hyperglycaemia stimulating foetal insulin secretion; one of the key determinants of foetal growth [130]. A similar association with birthweight has been documented in other autoimmune diseases, including rheumatoid arthritis [131, 132].

2.4.2.2 Type 2 diabetes

It is well documented that both high and low birthweight increase the risk of type 2 diabetes [98-100]. As similar to type 1 diabetes, high birthweight correlates with gestational diabetes and maternal adiposity [133]. This leads offspring to reduced β-cell volume and function and impaired insulin action [129] with long-term metabolic consequences such as increased risk of obesity and type 2 diabetes [134]. However, genetics [135] and shared life-style factors within families [126] have also been suggested to underlie the association. It is hypothesised that low birthweight is an indicator of poor nutrition of the foetus, leading to β-cell dysfunction, impaired mitochondrial oxidation in muscles and reduced insulin sensitivity [126]. The 'thrifty phenotype hypothesis' introduced by Hales and Barker in 1992 [136], suggests that low birthweight leads to metabolic disturbances and an energy saving phenotype that when met with rapid postnatal growth, increases the risk of later obesity, insulin resistance and type 2 diabetes. Several studies support this hypothesis in relation to the risk of type 2 diabetes [137]. In further support, low birthweight in combination with adult overweight has been shown to synergistically increase the risk of myocardial infarction [138] and metabolic syndrome [139]. Genetic factors may also mediate low birthweight and type 2 diabetes and genes inherited from the father are suggested to be most important [140].

2.4.2.3 LADA

One may hypothesise that low birthweight increases the risk of LADA by way of promoting insulin resistance and furthermore, that low birthweight may interact with adult overweight in the aetiology of LADA, similarly to what has been reported in type 2 diabetes. In this thesis, birthweight was investigated in relation to the risk of LADA for the first time.

2.4.3 Overweight and obesity

The global prevalence of overweight and obesity is 39% in the adult population, and the prevalence has tripled in children sine the seventies [13]. Excessive weight is the main cause of insulin resistance – among obese Caucasians 70% are presumed to be insulin resistant [91]. The detrimental effects of obesity are also genetically determined and differ over ethnic groups with Asians being insulin resistant at lower levels of obesity [90, 91].

2.4.3.1 Type 1 diabetes

Autoimmune diabetes has commonly been viewed as a non-obese form of diabetes. Nevertheless, according to several studies, high BMI [87, 88] and rapid growth during the first years in life [141, 142] are associated with an elevated risk of type 1 diabetes. The 'accelerator hypothesis' [143] proposes that adiposity increases insulin resistance and accelerates β-cell apoptosis in already at-risk individuals. It also proposes that insulin resistance is the common underlying feature of all types of diabetes. A role for excessive weight in the aetiology of type 1 diabetes is further strengthened by the parallel increase in childhood obesity and type 1 diabetes incidence [83, 84, 144]. Obesity may also affect the risk by promoting β-cell autoimmunity; pro-inflammatory adipokines, released from excessive fat tissue have been shown to be involved in many immune mediating processes [145].

2.4.3.2 Type 2 diabetes

Overweight and obesity are the single most important risk factors for type 2 diabetes [11]. A meta-analysis of prospective cohort studies found a 3-fold increased risk in overweight individuals, and a 7-fold increased risk in obese individuals [146]. The pathway between excessive weight and insulin resistance has been linked to a cross-talk between adipose tissue, skeletal muscle and liver [91] where the ability to store excessive fat subcutaneously is instrumental. The failure to expand adipocytes and adipose tissue in response to surplus calories will lead to accumulation of fat in ectopic depots such as liver, skeletal muscle and intra-abdominal (visceral fat) depots which is highly associated with insulin resistance in these organs. Abdominal fat has therefore been suggested to be far more detrimental than whole body obesity [90, 91]. Recent studies also suggest that functional changes in the gut microbiome (e.g. reduced diversity) may have a role in obesity and type 2 diabetes by way of reduced insulin sensitivity [147].

2.4.3.3 LADA

Results from cross-sectional studies suggest that individuals with LADA have higher BMI than patients with type 1 diabetes but are leaner than patients with type 2 diabetes [48, 117, 148-152]. The clinical phenotype within LADA also varies and patients with low GADA levels usually have higher BMI than those with high GADA levels. As demonstrated above, obesity has been linked both to insulin resistance as well as insulin secretion. Thus the heterogeneity of LADA suggests that the risk may differ depending on level of autoimmunity and that several pathways could be involved. Previous analyses, based on a subset of the HUNT study, suggest that overweight and obesity are as strong risk factors for LADA as for type 2 diabetes [102]. However this study was based on only 81 cases of LADA and replications and extensions of these findings are warranted. It is still unknown if the association also relates to more autoimmune forms of LADA, and if overweight interacts with family history or genetic susceptibility in relation to the risk of LADA.

2.4.4 Interaction between overweight, obesity and genes

A potential relationship with excessive weight and LADA may depend on genetic susceptibility. Few studies have investigated gene-overweight interaction in relation to autoimmune diabetes. However, a Swedish study found that type 1 diabetes in children with low-risk HLA DQ haplotypes was associated with higher prevalence of overweight/obesity in a synergistic manner [153]. Further support came from a study of multiple sclerosis in which the authors reported a striking additive interaction between obesity and the high risk HLA-DRB1*15 genotype [154]. For type 2 diabetes, large-scale studies have found interaction between a genetic risk score, BMI status [155] and waist circumference [156]. Studies of specific genotypes are scarce. Nevertheless, a Chinese study recently reported a strong interaction between *TCF7L2* risk genotype (rs290487) and BMI status /waist circumference in relation to type 2 diabetes risk [157]. The *FTO* gene is also interesting as the risk variants seem to modify eating behaviour and body composition [64], at the same time, the risk of type 2 diabetes attenuates with physical activity [158, 159]. Studies of family history further support a strong gene-overweight interaction in the aetiology of type 2 diabetes [160-162].

3 AIMS

The overarching aim of this thesis is to provide new information on the influence of birthweight, overweight and obesity, family history and genes and their interplay on the risk of LADA.

The specific aims were as follows:

I. To study the risk of LADA and type 2 diabetes in relation to birthweight, and potential interaction between low birthweight and adult overweight (Paper I).

II. To study the role of family history of type 1 and type 2 diabetes in relation to the risk of LADA and type 2 diabetes (Paper II).

III. To study the impact of overweight and obesity on the risk of LADA and type 2 diabetes and to assess potential interaction between overweight and family history of diabetes (Paper III).

IV. To study the risk of LADA and type 2 diabetes related to the interaction of overweight and genetic susceptibility as indicated by high-risk genotypes of HLA DR-DQ, *TCF7L2* and *FTO* (Paper IV).

4 MATERIALS AND METHODS

This thesis is based on data from two large Scandinavian studies: The Swedish ESTRID Study (Epidemiological study of risk factors for LADA and type 2 diabetes); https://ki.se/imm/estrid (paper I-IV) and the Norwegian HUNT Study (Nord-Trøndelag Health Study): https://www.ntnu.edu/hunt (paper III -IV). Together these two data materials comprise the two largest population-based studies, to date, investigating environmental risk factors for LADA.

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

4.1.1 Study design

ESTRID is an ongoing population-based case-control study, initiated in 2010, aiming at clarifying how lifestyle factors, alone and in combination with genes, affect the development of LADA compared with type 2 diabetes. ESTRID (Figure 4.1) continuously recruit all incident cases of LADA and a random sample of incident cases of type 2 diabetes (4 per LADA case) from two diabetes registers: ANDIS (All New Diabetics in Scania; http://andis.ludc.med.lu.se), and ANDiU (All New Diabetics in Uppsala; http://www.andiu.se). The aim of these registers is to characterize all new cases of diabetes diagnosed at health centres and hospitals in Scania and Uppsala based on detailed clinical and genetic information. Diabetes-free population-based controls (six per every one case of LADA), ≥35 years of age to match the age criteria of the LADA patients, are enrolled by random sampling from the Swedish national population register using incidence density methodology [163].

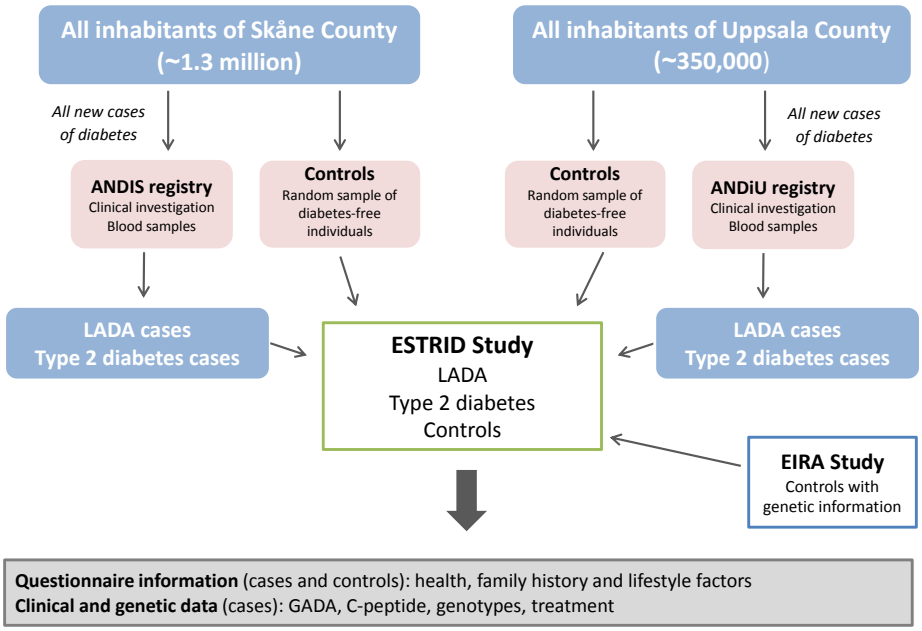

Figure 4.1 Flow chart of the ESTRID Study.

Since controls in ESTRID do not provide blood samples, for the BMI-gene interaction analysis in study IV, we used genetic controls from the EIRA (Epidemiological Investigation of Rheumatoid Arthritis; https://www.eirasweden.se) study (Figure 4.1) [164]. EIRA is based on the same case-control methodology as ESTRID and participants answer a very similar questionnaire. These control subjects, aged ≥35 years and free of diabetes and rheumatoid arthritis, are randomly selected from the Swedish population register and matched to the cases in ESTRID by age and sex.

4.1.2 Study population

Eligible in paper I were all participants included in ESTRID between September 2010 and 1 July 2014 with self-reported information on birthweight (45.0% of the total study population could recall this information) and complete information on all confounders (age, sex, BMI and family history of diabetes [FHD]); incident cases of LADA; n=134 and type 2 diabetes; n=350, and diabetes-free controls; n=603.

In paper II the analytical sample consisted of individuals recruited until 31 July 2015 with complete information on FHD and confounders (age, sex, BMI, smoking and education); incident cases of LADA; n=378 and type 2 diabetes; n=1 199, and diabetes-free controls; n=1 484 (99.5% of the total sample).

The study population in paper III was based on data from ESTRID gathered until July 2016 and included all incident cases of LADA (n=425) and type 2 diabetes (n=1,420), and diabetes-free controls (n=1,704) with complete information on BMI and confounders (age, sex, FHD, smoking and physical activity), comprising 98.2% of the total study sample.

Eligible in paper IV were all cases of LADA (n=394) and type 2 diabetes (n=1,290) included in ESTRID between September 2010 and July 2017 and all controls (n=2,656) from the EIRA study enrolled between 1996 and 2014 (free of diabetes and rheumatoid arthritis), with complete information on BMI and confounders (age, sex, smoking and physical activity) and with information on at least one of the genotypes of interest (HLA DR-DQ, *TCF7L2*-rs7903146) or *FTO*-rs9939609). Comprising 62% of the total sample.

4.1.3 Biochemical and clinical information

Fasting blood samples from time of diagnosis were collected for all cases and analysed at the university laboratory of Skåne and Uppsala. Classification of diabetes subtype was based on age at onset, glutamic acid decarboxylase autoantibodies (GADA) and fasting C-peptide. GADA was analysed with ELISA (enzyme-linked immunosorbent assay; RSR Limited, Cardiff, UK) and at the cut-off level of 10.7 IU/ml, specificity and sensitivity of the assay were 98% and 84% respectively [165], values above 250 IU/ml were censored at 250 IU/ml. C-peptide level was analysed using the Cobas e 601 analyser (Roche Diagnostics, Mannheim, Germany) or IMMULITE 2000 (Siemens Healthcare Diagnostics Product Ldt., Llanberies, UK). Fasting blood glucose and C-peptide were used to calculate Homeostatic model assessment (HOMA) indices of insulin resistance, insulin sensitivity and β-cell function with

the HOMA2 calculator (Oxford Centre for Diabetes, Endocrinology & Metabolism. Diabetes Trial Unit).

4.1.4 Classification of diabetes

Patients were diagnosed within the health care system of each county according to national standard. LADA was classed as age ≥35 years at diabetes onset, GADA positivity (≥10 IU/ml), and C-peptide level above the lower boundary of the normal range; ≥0.2 nmol/l (IMMULITE) or ≥0.3 nmol/l (Cobas). Type 2 diabetes were defined as age ≥35 years at diagnosis, GADA negativity (<10 IU/ml) and C-peptide >0.6 nmol/l (IMMULITE) or >0.72 nmol/l (Cobas).

4.1.5 Questionnaire information

The questionnaire in ESTRID contains a variety of items on general health, heredity and lifestyle including queries on leisure time physical activity, smoking, alcohol and education. The survey is sent out in parallel to cases and their matched controls. Cases receive the questionnaire as soon as possible after diagnosis (median 5 months) and are specifically asked to provide information on lifestyle habits as they were before diagnosis.

Birthweight (Paper I)

Self-reported birthweight was collected from the questionnaire and categorized according to low (<3 kg), normal (≥3 to <4 kg) and high (≥4 kg) weight. By linkage to the Swedish medical birth register, we were able to validate the self-reported information for those born in 1973 and onwards ($r= 0.86$, $p<0.0001$).

Family history of diabetes (Paper II)

Family members' diabetes status was inquired by questions on diabetes in first-degree relatives (mother, father, sisters, brothers, sons and daughters) and maternal and paternal grandparents. Information on age at onset and insulin treatment was available and relatives <40 years at onset and with insulin treatment were classed as having type 1 diabetes or else as having type 2 diabetes. There was an additional question on total number of other second-degree relatives (aunts, uncles, grandchildren, nephews, nieces) and first-degree cousins with diabetes.

Body mass index (Paper III, IV)

BMI was calculated using self-reported information on current weight and height as kg/m^2. The patients were asked to report weight before onset of diabetes and this information showed high correlation with clinically gathered information from time of diagnosis ($r=0.92$, $p<0.0001$). BMI was categorised according to WHO's definition as normal weight; <25 kg/m^2, overweight; 25–29.9 kg/m^2 and obese; ≥30 kg/m^2.

4.1.6 Genetic information

For cases (Paper II, III and IV), genotyping was performed using blood DNA samples analysed with iPLEX Gold technology (Sequenom Laboratories, San Diego, CA, USA). Infinium CoreExome v1.1 (Illumina, San Diego, CA, USA) was used for imputation of missing genotypes based on the Haplotype Reference Consortium (http://www.haplotype-reference-consortium.org/; version r1.1 2016) reference panel. All analyses were computed at the Lund University Diabetes Centre.

For controls (Paper IV), blood DNA samples from the EIRA biobank were genotyped for HLA variants using an Infinium Illumina 300K immunochip custom array (Illumina, San Diego, CA, USA) while genotyping of *TCF7L2* and *FTO* was based on GWAS data using the Illumina Global Screening array.

Single nucleotide polymorphisms (SNPs) were used to identify carriers of high-risk variants of *TCF7L2* (rs7903146 T-allele), *FTO* (rs9939609 A-allele) and HLA DRB1 and DQB1. For the HLA genotypes, three SNPs in the major histocompatibility complex (MHC) class II region (rs3104413, rs2854275, rs9273363) were combined to classify individuals as carriers of high (DR4/4, DR3/3, DR3/4, DR3/4-DQ8, DR4/4-DQ8, DR4/X-DQ8) or low/intermediate (DR4/X, DR3/X, DRX/X, DR4-DQ7) risk variants. This method has been validated with an overall accuracy of 99.3% [166].

4.2 THE HUNT STUDY (PAPER III, IV)

4.2.1 Study design

The HUNT Study (Figure 4.2) is a longitudinal study initiated with the overall aim of serving as a solid foundation for a large number of health related research topics [167]. The study comprises three separate health surveys conducted during the period 1984-2008: HUNT1 (1984-86), HUNT2 (1995-97) and HUNT3 (2006-08) to which the complete population aged ≥20 years, living in the Norwegian County of Nord Trøndelag was invited to participate. The surveys included questionnaires on lifestyle and health related matters and a clinical examination which included anthropometrical measurements and blood sampling. The overall response rate in HUNT is high but declined over time with a 90.3%, 71.3% and 54% response rate in the HUNT1, HUNT2 and HUNT3 surveys respectively [167]. Among those still alive, 78% attended both in the HUNT1 and HUNT2 surveys while the proportion of re-attenders between HUNT2 and HUNT3 was 70%.

4.2.2 Study population

Eligible for Paper III were all individuals attending at least two surveys, who were free of diabetes at baseline (HUNT1 or HUNT2), with complete baseline information on BMI, age, sex, FHD, physical activity and smoking (n=56,549). During 1,012,957 person-years of

follow-up (1984–2008) 147 incident cases of LADA and 2002 cases of type 2 diabetes were identified. This information was available for 89% of the responders.

We also formed a sub-cohort of individuals participating in the HUNT2 and HUNT3 surveys with complete information on waist and hip circumference (only available from baseline at HUNT2) and covariates. During 463,670 person-years of follow-up, 51 cases of LADA and 1,038 cases of type 2 diabetes were identified.

Paper IV comprised 48,599 individuals who participated in at least two surveys, were free of diabetes at baseline and with complete baseline information on BMI, age, sex, physical activity and smoking and with data on at least one of the genotypes we were interested to study (HLA, *TCF7L2* or *FTO*). This included incident cases of LADA (n=131) and type 2 diabetes (n=1,901) and 886,120 person-years of follow-up (79% of the responders).

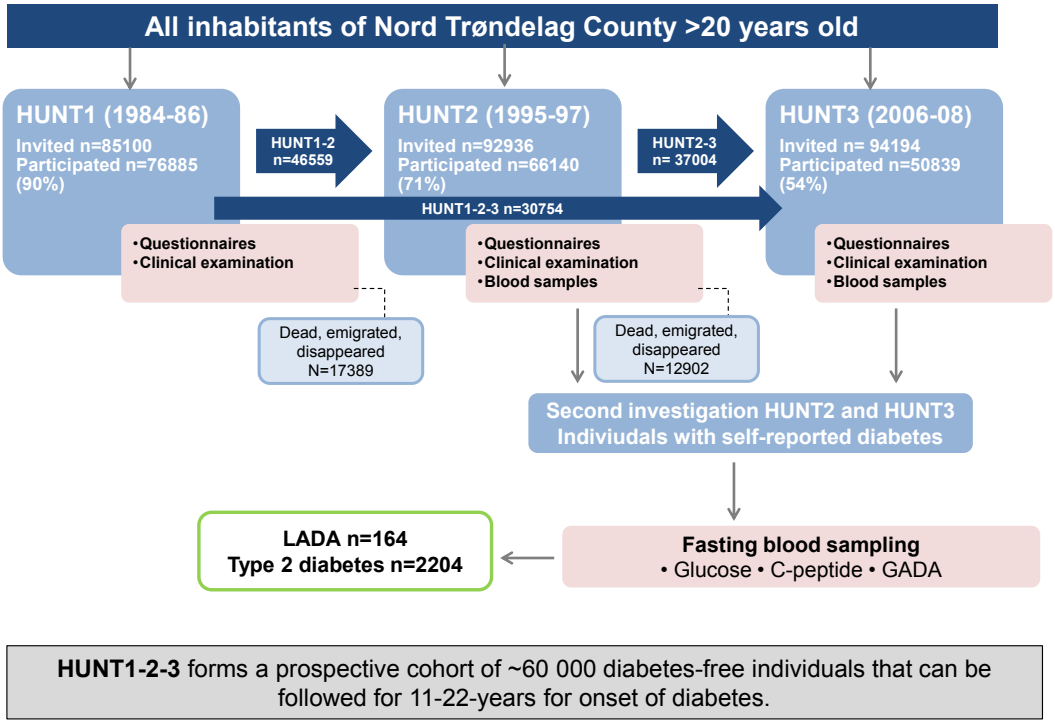

Figure 4.2 Flow chart of the HUNT Study. Adapted from Krokstad et. al [167].

4.2.3 Biochemical analyses

At follow-up (HUNT2 or HUNT3) incident cases of diabetes were identified by self-report. This information showed very high concordance with information from medical records (>95%) [168]. Participants reporting diabetes were invited to a follow-up investigation during which trained nurses probed for age at onset and diabetes treatment. Fasting blood samples were also collected (median 4 years after diagnosis) and serum samples were analysed for glucose, C-peptide and GADA titres. For those not attending the follow-up examination, GADA was measured in non-fasting serum available from the HUNT biobank [67]. Glucose was measured with a HemoCue monitor at Levanger Hospital, Levanger, Norway. C-peptide

was analysed with radioimmunoassay (Diagnostic Systems Laboratories, Webster, TX, USA) and GADA with immunoprecipitation radioligand assay by translation labelled ^{3}H-GAD65 (NovoNordisk, Bagsværd, Denmark) at Aker Hospital, Oslo, Norway. GADA was reported as an index value relative to standard serum. The specificity (1.00) and sensitivity (0.64) of the GADA method was tested in the Islet Antibody Standardization Program 2003 workshop and the cut-off antibody index ≥0.08 for positivity (equivalent to ≥43 IU/ml according to the WHO standard) was chosen to attain the highest possible specificity with reasonable sensitivity. HOMA indices were calculated as in ESTRID, with the HOMA2 calculator (Oxford Centre for Diabetes, Endocrinology & Metabolism. Diabetes Trial Unit).

4.2.4 Classification of diabetes

LADA was defined as age at onset ≥35 years and with GADA positivity (≥43 IU/ml) but because treatment information was missing for 12% of the antibody positive cases we did not use the insulin criteria. By this definition we have unavoidably included also type 1 diabetes patients with adult onset in our LADA group. However 81.7% of those with this information reported that they were without insulin treatment the year after diagnosis, indicating that the proportion of misclassified type 1 diabetes patients is small. In addition, in both paper III and IV we performed sensitivity analyses with a stricter LADA definition (i.e. no insulin within one year of diagnosis) and found similar results.

4.2.5 Questionnaire information

At baseline, participants answered self-administrated questionnaires including items on smoking status, alcohol intake, education, leisure time physical activity and FHD. Information on diabetes in first-degree relatives was available from HUNT1 (siblings), HUNT2 and HUNT3 (parents, siblings, children) and those with self-reported FHD in any of the three surveys were considered exposed. A detailed description of procedures and methods is found at: http://www.ntnu.edu/hunt.

4.2.6 Anthropometrical information

Participants' body measurements were obtained by trained nurses at the baseline clinical examination (HUNT1 or/and HUNT2).

Body mass index (Paper III, IV)

BMI (kg/m^2) was calculated from weight (rounded to nearest half kilo) and height (in whole centimetres) with participants wearing light clothing and no shoes and categorized as in ESTRID (WHO standard).

Waist-to-hip and waist-to-height ratio (Paper III)

Waist and hip circumference and height were used to calculate waist-to-hip ratio (WHR) and waist-to-height ratio (WHtR), two measures of abdominal obesity that has been suggested to best predict risk of diabetes [169]. Waist circumference was measured at the height of the

umbilicus and hip circumference at the thickest part of the hip. The measures were dichotomized according to previously used risk levels: WHR <0.85 vs. ≥0.85 (women) and <0.90 vs. ≥0.90 (men) and WHtR <0.50 vs. ≥0.50 [169].

4.2.7 Genetic information

DNA samples were available from the HUNT biobank and genotyped for SNPs associated with HLA (rs2854275, rs9273363, rs9272346), *TCF7L2* (rs7903146) and *FTO* (rs9939609) with HumanCoreExome arrays (Illumina, San Diego, CA, USA https://www.ntnu.no/hunt/gwas-data). Imputation, was performed with Minimac3 (v2.0.1, http://genome.sph.umich.edu/wiki/Minimac3) from a customized Haplotype Reference consortium release 1.1 (HRC v1.1). All analyses were computed at the NTNU Genomic Core Facility in Trondheim. The HLA SNPs were chosen based on previous literature [18, 68, 166] and participants were categorized as having HLA high-risk genotype if carrying at least one of the risk variants; rs2854275 (TT/TG), rs9273363 (AA) or rs9272346 (AA).

4.3 STATISTICAL ANALYSES

Conditional logistic regression analysis (Paper I, II, III, IV)

Data from ESTRID was analysed with classical case-control methodology, where the proportion of exposed cases is compared with proportion of exposed controls. This was done by calculating odds ratios (OR) and 95% confidence intervals (CI) of LADA and type 2 diabetes associated with birthweight, FHD, BMI and genotypes of interest using conditional logistic regression analysis (matched on date of inclusion and county). By applying incidence density sampling [163] i.e. by enrolment of incident cases of diabetes and controls randomly sampled from the source population at risk (eligible to become a case during the remainder of the study time) at the same time as the cases, the OR yields a direct estimate of the incidence rate ratio (IRR). In paper I, we performed both matched and unmatched analyses but chose to report ORs from the unmatched model as there was no appreciable difference between the analyses. In study IV, cases and genetic controls from EIRA were post-matched based on age (in 5 year strata) and sex.

Restricted cubic spline regression analysis (Paper III)

Restricted cubic spline models were applied in paper III (ESTRID data) in order to assess BMI (per unit increase) in relation to the risk of LADA and type 2 diabetes without making any assumption about linearity i.e. by fitting a smooth line which is allowed to vary over the whole range of BMI [170].

Linear regression (Paper III)

For the ESTRID data in paper III we also used linear regression models to estimate the association between BMI and degree of insulin resistance (logHOMA-IR) and levels of logGADA (applied with logarithmic transformation due to a non-linear association and differences in variance).

Population attributable risk (Paper III)

In Paper III, to assess what proportion of cases of LADA and type 2 diabetes that can be attributed to overweight and obesity we calculated population attributable risk (PAR) by applying the formula:

$$p(1 - [1/RR])$$

Where *p* is the prevalence (%) of the risk factor of interest among cases and RR is the adjusted effect estimate (OR or HR) [171].

Cox proportional hazards regression analysis (Paper III, IV)

In the prospective data from HUNT, we modelled Cox proportional hazards regression with age as underlying time-scale to estimate hazard ratios (HR) with 95% CI of LADA and type 2 diabetes associated with overweight, obesity and genes. For individuals participating at all three surveys (with baseline measurements from both HUNT1 and HUNT2), time-dependent variables were used such that exposure and covariate information were updated the second time of participation. Person-years of follow-up were accumulated from age at baseline (HUNT1 [1984] or HUNT2 [1995]) until age at onset of diabetes, death or age at follow-up (in HUNT2 [1997] or HUNT3 [2008]), whichever came first.

Interaction (Paper I, III, IV)

In this thesis we are referring to interaction on an additive scale i.e. interaction is present if the net effect of two independent risk factors exceeds the risk of each factor assessed independently (super additive association). In study I, interaction was calculated with relative excess risk due to interaction (RERI) with 95% CI for those with joint exposures (low birthweight and overweight).Whereas attributable proportion (AP) due to interaction with 95% CI was reported for doubly exposed individuals in study III (overweight and FHD) and in study IV (high-risk genotype and overweight) according to the following formulas:

$$RERI = RR^{11} - RR^{10} - RR^{01} + RR^{00}$$

$$AP = RERI \; / \; RR^{11}$$

Where RR is relative risk associated with the outcome when both (RR^{11}), either (RR^{10} or RR^{01}), or none (RR^{00}) of the exposures are present. Positive interaction is said to be present if RERI or AP > 0.

Meta-analysis (Paper IV)

In paper IV, RRs and APs from the separate BMI-gene analyses in ESTRID and HUNT were pooled to reduce random variation. The pooling was performed with EpiNet meta-analysis tool (freely available from www.epinet.se) using the inverse variance method and a fixed model approach [172].

Stratified analysis (Paper II, III, IV)

We also performed stratified analyses were we divided the LADA cases by median GADA level, referred to in the thesis as $LADA^{low}$ (<median) and $LADA^{high}$ (≥median). The median GADA-level was 177.5 IU/ml in paper II, 196.0 IU/ml (ESTRID) and 134.4 IU/ml (HUNT) in paper III and 206 IU/ml (ESTRID) and 151 IU/ml (HUNT) in paper IV.

Stata Statistical Software 14 (StataCorp, College Station, TX, USA) was used for calculating splines. All other analyses were calculated with Statistical Analysis Software (SAS) 9.4 (SAS Institute, Cary, NC, USA).

4.4 ETHICAL CONSIDERATIONS

Informed consent was obtained from all participants in ESTRID, EIRA and HUNT and the studies have been approved by ethical committees: ESTRID by the Ethical review board in Stockholm, including an extended approval for inclusion of EIRA controls, and HUNT, by the Norwegian Data Inspectorate and Regional Committee for Medical Research Ethics.

5 MAIN RESULTS

5.1 POPULATION CHARACTERISTICS

In both cohorts (Table 5.1), comparing individuals with LADA and type 2 diabetes, the former group had inferior insulin secretion (C-peptide), were more often treated with insulin and had greater proportion of HLA high-risk variants. In ESTRID, the LADA patients were also younger, leaner (BMI) and less insulin resistant (HOMA-IR) than patients with type 2 diabetes. Differences between patient groups in HUNT were smaller i.e. degree of insulin resistance and mean BMI were similar although LADA patients had less abdominal obesity (mean WHR).

5.2 BIRTHWEIGHT, LADA AND TYPE 2 DIABETES (PAPER I)

In paper I, we explored the association between birthweight, LADA and type 2 diabetes. We found that low birthweight was inversely associated with both subtypes of diabetes: OR increased with 52% per every kg reduction in birthweight (OR 1.52, 95% CI 1.12-2.08) for LADA and with 58% for type 2 diabetes (OR 1.58, 95% CI 1.23-2.04). Among participants weighing <3 kg compared with ≥4 kg at birth, OR was estimated at 2.38 (95% CI 1.23-4.60) for LADA and at 2.37 (95% CI 1.37-4.10) for type 2 diabetes. There seemed to be little impact from FHD on these risk-estimates, OR only changed marginally after adjustment (See paper I, table 2 for details). The highest risk was found in those exposed to both low birthweight and overweight; for LADA the OR was estimated at 3.26 (95% CI 1.69-6.29) and for type 2 diabetes at 39.93 (95% CI 19.27-82.71) when comparing to those with birthweights ≥3 kg and with normal weight. No interaction was found for LADA (Figure 5.1), however for type 2 diabetes there was a 25-fold excess risk attributed to the joint effect of low birthweight and overweight (Figure 5.1).

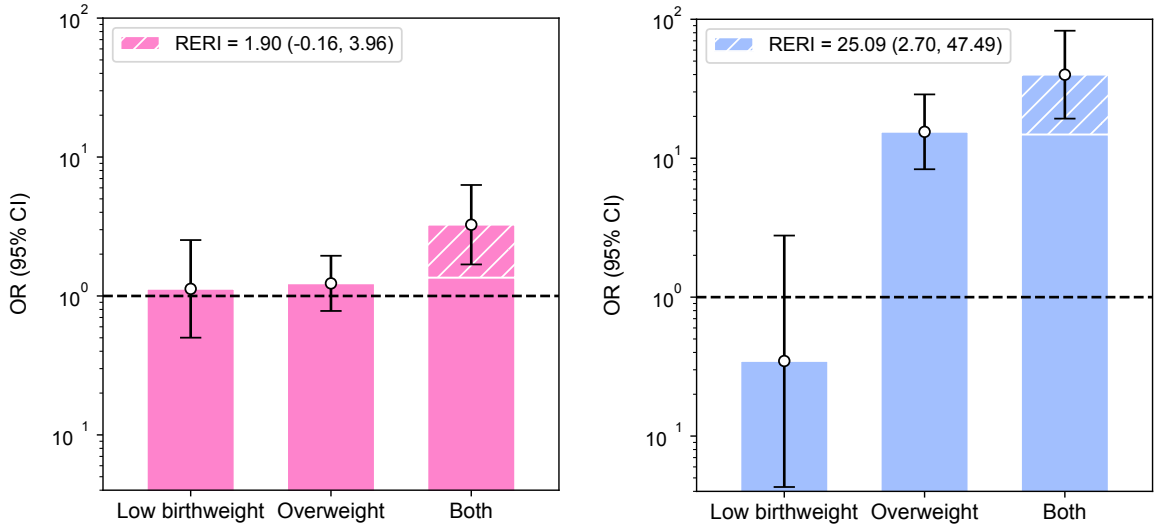

Figure 5.1 Combinations of low birthweight (< 3kg) and overweight (BMI ≥25) for LADA (left, pink bars) and type 2 diabetes (right, blue bars). Reference is birthweight (≥ 3 kg) and normal weight (BMI <25). The hatched area represents the relative excess risk due to interaction (RERI) for those with both risk factors. Models were adjusted for age, sex and FHD.

Table 5.1 Characteristics of the study participants from ESTRID and HUNT (included in paper III in the thesis) and genetic controls from the EIRA study (included in paper IV in the thesis).

	HUNT				ESTRID				
Characteristics	**No diabetes**	**Type 2 diabetes**	**LADA**	***P*** [a]	**Controls**	**Type 2 diabetes**	**LADA**	***P*** [a]	***Genetic controls***
No. of individuals	54,440	2,002	147		1,704	1,420	425		2,656
Women, %	53.2	47.3	51.7	*0.3028*	51.9	39.2	45.7	*0.0181*	*72.4*
Mean age (years) at diagnosis, (SD) [b]	–	60.9 (10.9)	59.9 (11.1)	*0.3039*	58.4 (13.5)	63.2 (10.3)	59.0 (12.3)	*<.0001*	56.6 (9.4)
Mean age (years) at baseline (HUNT), (SD)	48.3 (15.8)	54.8 (11.0)	54.4 (11.2)	*0.6928*	–	–	–	–	–
Mean BMI (kg/m^2), (SD)	25.5 (3.8)	29.8 (4.5)	29.2 (4.9)	*0.1704*	25.9 (4.2)	31.2 (5.4)	28.1 (5.3)	*<.0001*	25.5 (4.1)
Mean waist-to-hip ratio, (SD) [c]	0.84 (0.08)	0.90 (0.07)	0.87 (0.07)	*0.0260*	–	–	–	–	–
Any first-degree FHD, %	24.2	57.1	48.3	*0.0379*	24.4	49.8	45.2	*0.0951*	–
FHD-T2D, %	–	–	–	–	22.65	47.61	36.71	*<.0001*	–
FHD-T1D, %	–	–	–	–	2.58	5.00	11.29	*<.0001*	–
With insulin treatment, %	–	3.4	17.3	*<.0001*	–	5.9	41.2	*<.0001*	–
Median C-peptide (nmol/l), (IQR)	–	0.86 (0.60)	0.57 (0.78)	*<.0001*	–	1.20 (0.65)	0.69 (0.67)	*<.0001*	–
Median GADA, (IU/ml), (IQR)	–	–	134.4 (521.4)	–	–	–	196.0 (224.0)	–	–
Median HOMA-IR, (IQR)	–	2.20 (1.60)	2.10 (1.70)	*0.1119*	–	3.50 (2.20)	2.70 (2.60)	*<.0001*	–
Median HOMA-β, (IQR)	–	64.5 (49.2)	59.0 (50.8)	*0.4109*	–	68.1 (49.8)	37.8 (53.6)	*<.0001*	–
HLA high-risk genotype, % [d]	42.5	40.7	60.3	*<0.0001*	–	31.4	61.2	*<.0001*	*33.0*
***TCF7L2*-rs7903146, %** [e]	44.2	54.9	44.3	*0.0186*	–	52.1	51.8	*0.7795*	*45.8*
***FTO*-rs9939609, %** [f]	65.9	69.2	76.3	*0.0847*	–	67.5	66.3	*0.5156*	*64.3*

a) P for difference between LADA and type 2 diabetes. b) Age at inclusion for control participants. c) Information only available from baseline at HUNT2 (1995–1997). d) HLA high-risk: DR4/4, DR3/3, DR3/4, DR3/4-DQ8, DR4/4-DQ8 or DR4/X-DQ8. e) Proportion with the TT/TC genotype. f) Proportion with the AA/AT genotype.

5.3 FAMILY HISTORY OF DIABETES, LADA AND TYPE 2 DIABETES (PAPER II)

In study II we explored LADA and type 2 diabetes in relation to FHD and found that first-degree FHD more than doubled the risk of LADA (OR 2.22, 95% CI 1.73–2.85) and type 2 diabetes 2.66 (95% CI 2.17–3.28) (Figure 5.2) when compared to those without FHD. We additionally found that the association with LADA was much stronger had the relative type 1 diabetes with OR elevated 6-fold (OR 5.75; 95% CI 3.23-10.25). Still, LADA was clearly associated also with FHD-T2D (OR 1.89; 95% CI 1.45-2.47). Type 2 diabetes was foremost associated with FHD-T2D (Figure 5.2). For LADA, FHD-T1D vs. FHD-T2D was associated with a phenotype that were closer to that of type 1 diabetes such that GADA titres were higher (250 vs. 104 IU/mL*),*while C-peptide levels (0.65 vs. 0.91 nmol/L) and prevalence of HLA low-risk genotypes (5.0% vs. 28.6%) were lower.

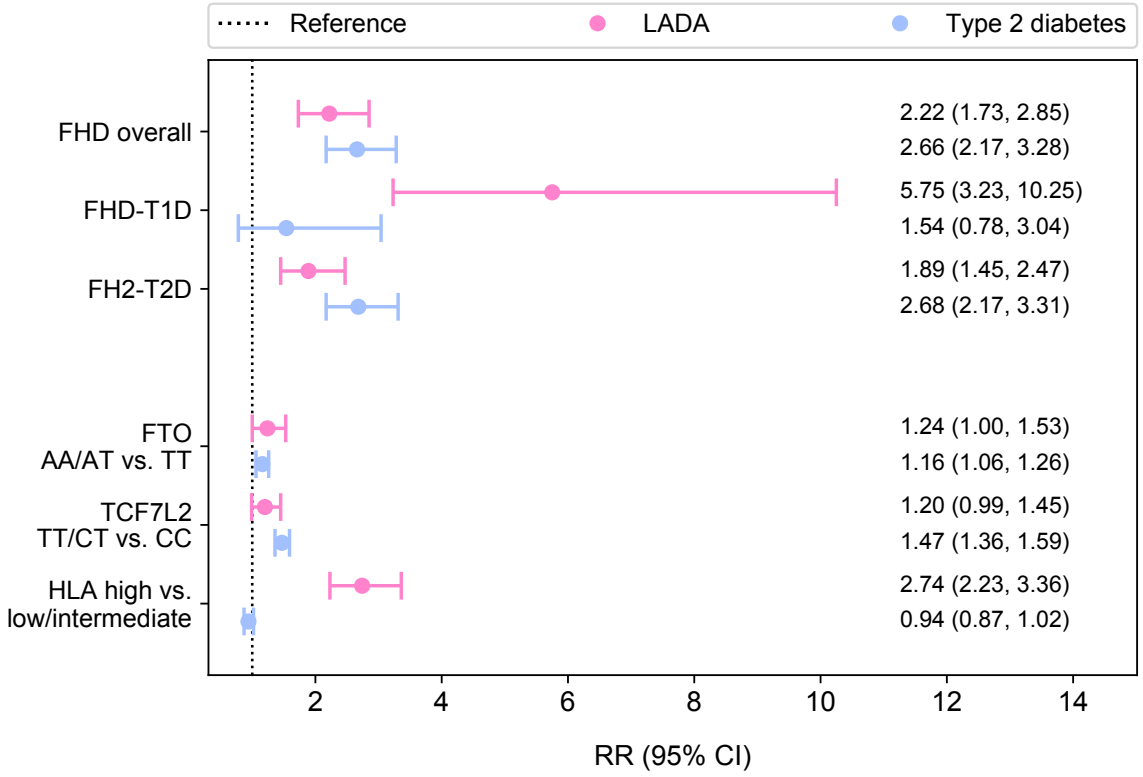

Figure 5.2 RR and 95% CI for LADA and type 2 diabetes in relation to FHD (reference; no FHD), using data from ESTRID, and genotypes of HLA, *TCF7L2* and *FTO,* using pooled data from ESTRID and HUNT. Analyses of FHD were adjusted for age, sex, BMI, education and smoking. In addition, FHD–T1D was adjusted for FHD–T2D and vice versa. Analyses of genotypes were adjusted for age and sex.

5.4 OVERWEIGHT AND OBESITY IN RELATION TO LADA AND TYPE 2 DIABETES (PAPER III)

Results from ESTRID indicated that obesity, compared with normal weight, was associated with a 3-fold increased risk of LADA (OR 2.93, 95% CI, 2.17-3.97) and a 19-fold increased risk of type 2 diabetes (OR 18.88 (95% CI 14.29-24.94). The relationship with LADA was seen foremost in LADA^{low} (OR 4.25; 95% CI 2.76-6.52) yet, present in LADA^{high} (OR 2.14; 95% CI 1.42-3.24). Data from HUNT confirmed these results suggesting even stronger associations (Figure 5.3). Data from HUNT2 further indicated that abdominal obesity (WHR and WHtR) augments the risk of LADA 2-3 fold. Corresponding estimates for type 2 diabetes are found in figure 5.3. The non-linear association between BMI and diabetes, presented with splines, can be found in paper III.

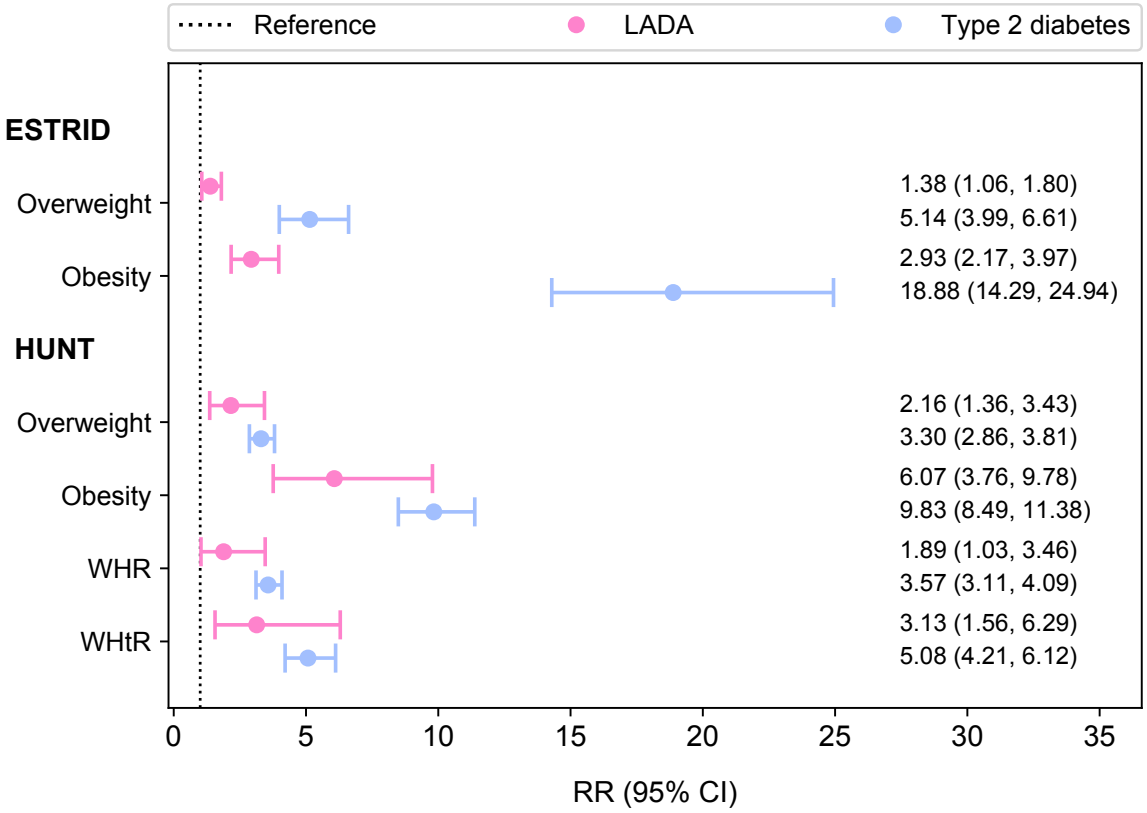

Figure 5.3 ORs (ESTRID) and HRs (HUNT) with 95% CI of LADA and type 2 diabetes in relation to overweight (BMI 25–29.9) and obesity (BMI ≥30) vs. normal weight (BMI <25) and HRs for LADA and type 2 diabetes in relation to WHR (<0.85 vs. ≥0.85 [women] and <0.90 vs. ≥0.90 [men]) and WHtR (<0.50 vs. ≥0.50) using data from HUNT2. All models were adjusted for age, sex, FHD, smoking and physical activity.

The combination of BMI ≥25 and FHD conferred the highest risk of LADA and type 2 diabetes (Figure 5.4). No interaction was observed for LADA in ESTRID (AP=0.06; 95% CI −0.25-0.37) however, results from HUNT indicated that 37% (AP=0.37, 95% CI 0.10-0.64) of the LADA cases can be attributed to the interaction between overweight and FHD. For type 2 diabetes, interaction was seen irrespective of data (Figure 5.4).

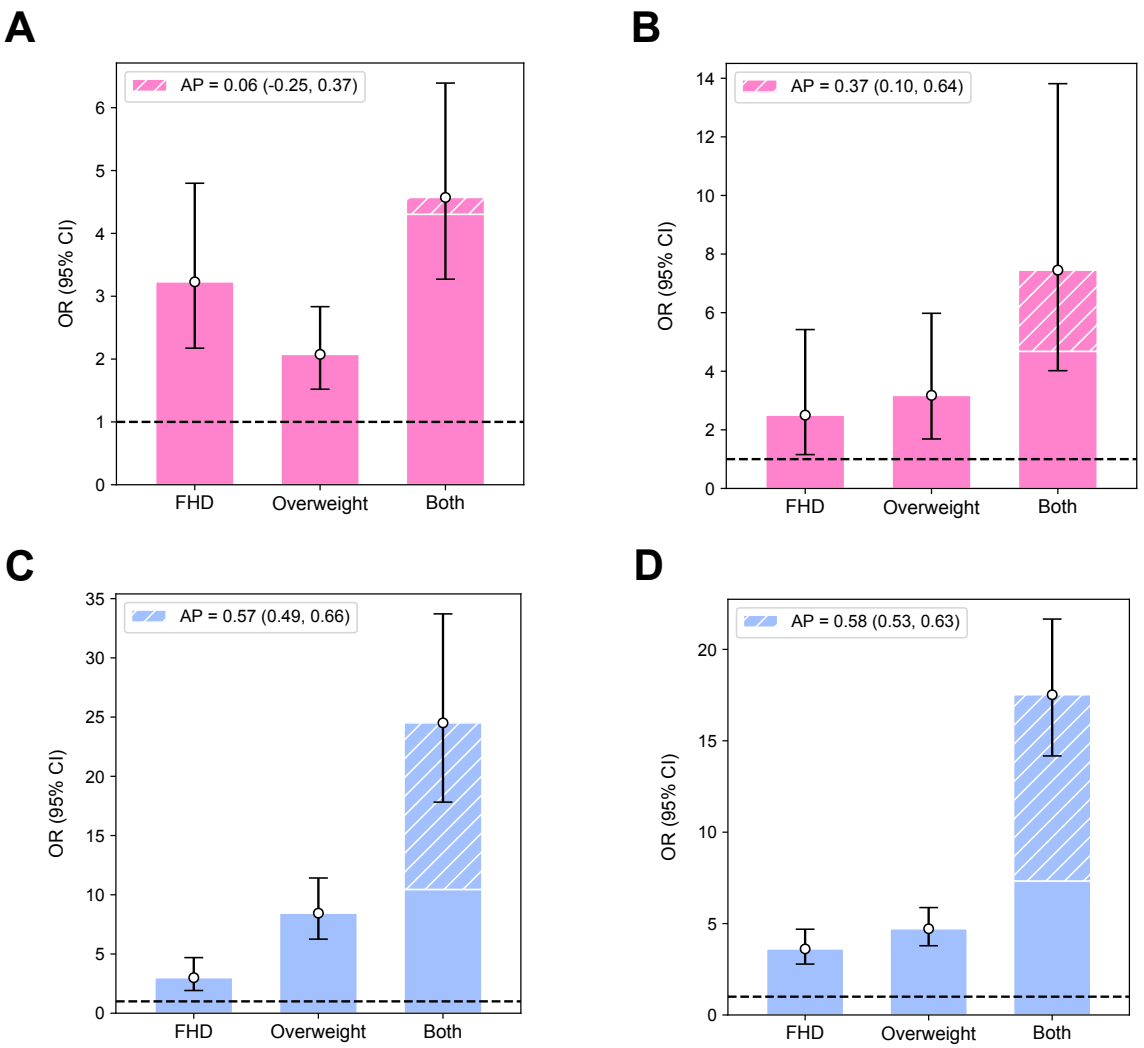

Figure 5.4 Combinations of overweight/obesity (BMI ≥25) and FHD in relation to the risk of LADA in ESTRID (**A**), LADA in HUNT (**B**), type 2 diabetes in ESTRID (**C**) and type 2 diabetes in HUNT (**D**). Reference is normal weight (BMI <25) and no FHD. The hatched area represents the attributable risk due to interaction (AP). Models were adjusted for age, sex, smoking and physical activity.

Obese vs. normal weight individuals with LADA displayed a more type 2-like phenotype indicated by greater levels of C-peptide and lower proportion of insulin treatment. In both studies, BMI was also positively associated with insulin resistance (HOMA-IR). Contrary, BMI was inversely associated with GADA in ESTRID but this association was weak in HUNT (details can be found in paper III). Moreover, calculation of PAR suggested that 31-56% of all LADA cases and 70-82% of all type 2 diabetes cases can be attributed to overweight/obesity.

5.5 BMI-GENE INTERACTION, LADA AND TYPE 2 DIABETES (PAPER IV)

In the fourth study, we pooled risk estimates from ESTRID and HUNT and continued to explore the interaction between BMI and heredity that was indicated in paper III, here focusing on high-risk genotypes of HLA, *TCF7L2* and *FTO*. Study-specific risk estimates can be found in paper IV. We first studied the association between diabetes and each variant separately; HLA high-risk genotypes were strongly associated with LADA in both cohorts

(RR_{pooled} 2.74; 95% CI 2.23-3.36) whereas *TCF7L2* was only associated with LADA in ESTRID and *FTO* only with LADA in HUNT (Figure 5.2). However in the pooled analysis, an overall association was seen between $LADA^{low}$ and high-risk genotypes of both *TCF7L2* and *FTO* (results for the stratified LADA analyses are found in the supplementary material of paper IV).

Moreover, the combination of overweight and each of the high-risk variants conferred an increased risk of LADA with RR_{pooled} of 7.59 (95% CI 5.27-10.93) for HLA, 2.65 (95% CI 1.97-3.56) for *TCF7L2* and 2.21 (95% CI 1.60-3.07) for *FTO* (Figure 5.6). We also found interaction between overweight and HLA (AP 0.29; 95% CI 0.10-0.47) *TCF7L2* (0.31; 95% CI 0.09-0.52) and *FTO* (AP 0.38, 95% CI 0.15-0.61). Using the Swedish data, the strongest risk was observed in overweight individuals with the DR4/4 genotype (RR 26.76, 95% CI 15.42-46.43, AP 0.58, 95% CI 0.32-0.83. These results indicate that about 29-38% of the doubled exposed LADA cases may be prevented by maintaining a healthy weight. Type 2 diabetes was associated with both *TCF7L2* and *FTO* but not with HLA (Figure 5.2). There was a significant interaction between overweight and *TCF7L2* (AP 0.26; 95% CI 0.19-0.33), but not between overweight and high-risk genotypes of HLA or *FTO* (Figure 5.6).

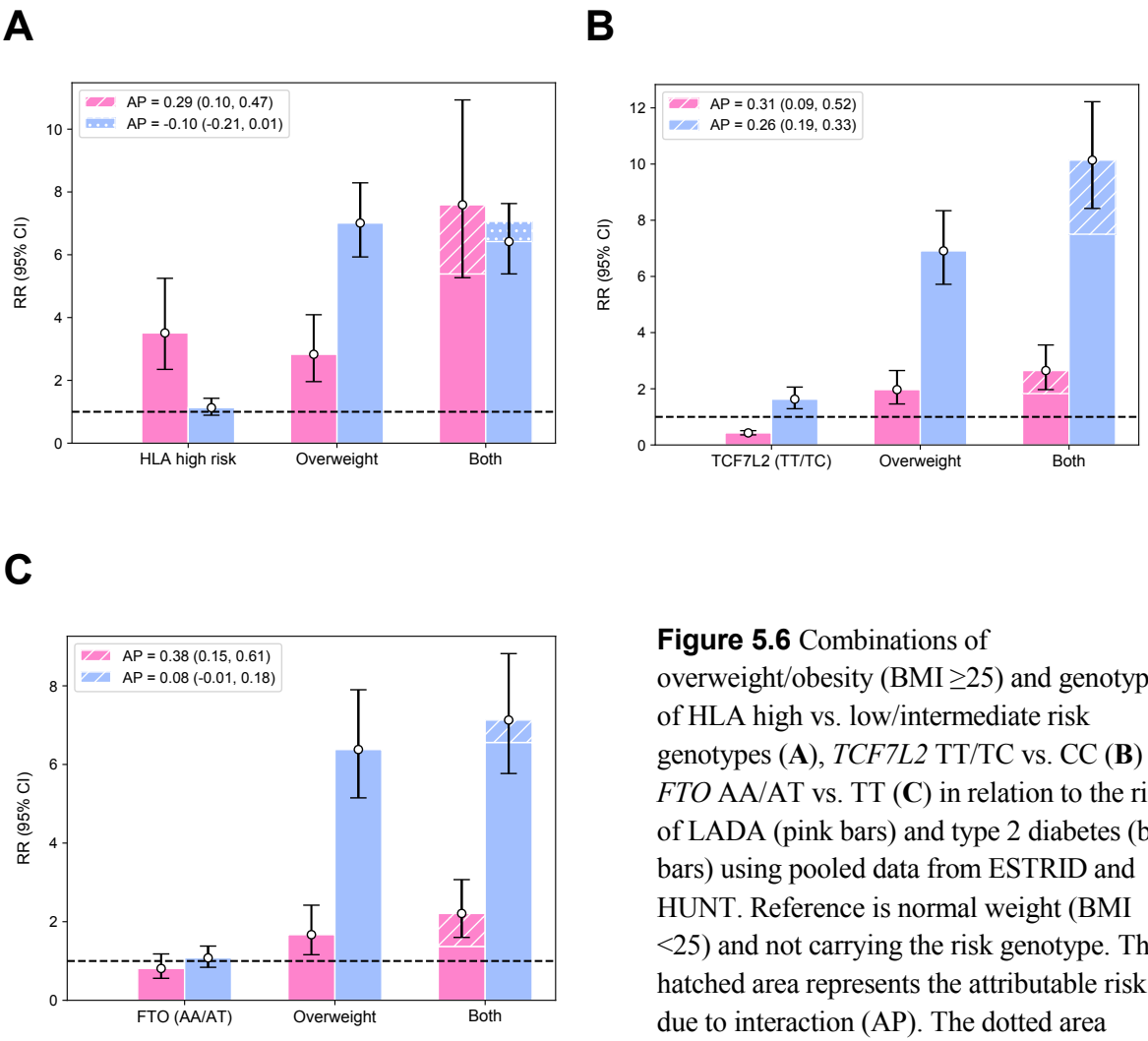

Figure 5.6 Combinations of overweight/obesity (BMI ≥25) and genotypes of HLA high vs. low/intermediate risk genotypes (**A**), *TCF7L2* TT/TC vs. CC (**B**) and *FTO* AA/AT vs. TT (**C**) in relation to the risk of LADA (pink bars) and type 2 diabetes (blue bars) using pooled data from ESTRID and HUNT. Reference is normal weight (BMI <25) and not carrying the risk genotype. The hatched area represents the attributable risk due to interaction (AP). The dotted area represents negative AP.

6 DISCUSSION

6.1 SUMMARY OF MAIN FINDINGS AND INTERPRETATIONS

The purpose of this thesis was to explore how FHD, low birthweight, overweight/obesity, and the potential interaction between these factors, influence the risk of LADA compared to type 2 diabetes. Regarding heritability, our findings indicate that FHD of type 1 diabetes confers the highest risk of LADA, but that the risk is also elevated for those with FHD-T2D. Likewise, there was a stronger link between LADA and HLA high-risk genotypes, associated with type 1 diabetes and autoimmunity, than between LADA and *TCF7L2* and *FTO* genotypes associated with type 2 diabetes. These findings support the notion that LADA is genetically closer to type 1 than type 2 diabetes [16]. We further observed that overweight/obesity increase the risk of LADA, and that the combination of overweight and FHD or HLA high-risk variants, in particularly the DR4/4 genotype, dramatically increased the risk of LADA. Additionally, there was interaction between overweight and risk variants of the *TCF7L2* and *FTO* genotypes in relation to LADA and with *TCF7L2* in relation to type 2 diabetes. Our findings further suggest that low birthweight is associated with increased risk of LADA as well as type 2 diabetes, particularly in combination with adult overweight. Overall, the results of this thesis suggest that LADA essentially is a consequence of genetic predisposition towards autoimmunity along with environmental exposures that promote insulin resistance.

6.2 HEREDITY

Using data from ESTRID, we first confirmed previous results showing that FHD is associated with increased risk of LADA [119, 173] and type 2 diabetes [112]. We further observed that the risk of LADA is considerably higher for individuals with first-degree FHD-T1D vs FHD-T2D, although the risk was clearly raised also for the latter group. This is concordant with cross-sectional studies indicating that the prevalence of FHD-T2D is as high in LADA as in type 2 diabetes, whereas FHD-T1D is much more prevalent in LADA [124]. Genetic analysis from paper IV, where we incorporated data from both ESTRID and HUNT, support this view; HLA high-risk genotypes were strongly associated with LADA in both studies. Likewise, the abundance of genetic studies during the last decades indicate that genetic susceptibility in LADA stems foremost from HLA genotypes associated with type 1 diabetes [69, 70].

Our results for the type 2 diabetes associated genes were contradictive and much weaker. A genetic overlap between LADA and type 2 diabetes is debated and while some find an association [66, 67, 72, 73], others do not [68, 69]. Stratification by median GADA titre however, revealed an overall association with both the *TCF7L2* and *FTO* risk genotypes in LADA with lower GADA level, which is in line with what has been reported by previous studies [67, 72]. It is likely that genes associated with type 2 diabetes are more important for less autoimmune, type 2-like LADA. This is further supported by a recent study that found that single GADA positive children with the *TCF7L2* genotype less often converted to

multiple antibody status than those without the risk genotype [174]. Mechanistically the *FTO* gene is associated with increased obesity, which may explain why the association with LADA was seen only in HUNT; these patients had both higher BMI and higher prevalence of the risk variant compared with LADA patients in ESTRID (paper IV, table 1). However, the majority of genes associated with type 2 diabetes susceptibility, including *TCF7L2*, primarily impact β-cell function and secretion [59, 61] rather than worsening peripheral insulin resistance. Similarly, in individuals with FHD-T2D, β-cell dysfunction is present long before the development of hyperglycaemia [175]. However, as the risk is modest it is not clear how much these risk variants *per se* actually contribute to the aetiology of LADA, even in case of mild autoimmunity.

Consistent with the above, the risk of type 2 diabetes was primarily associated with FHD–T2D and patients with FHD had lower levels of C-peptide than those without FHD [12,35]. We could further confirm that type 2 diabetes is associated with *TCF7L2* and *FTO* but not with HLA [16]. Notably, as heritability cannot completely be explained by genetic susceptibility [57], some of the association between LADA and FHD is likely a result of lifestyle factors shared within families, especially for individuals with FHD-T2D. Still, adjusting for BMI and physical activity had little impact on the association, which is in line with present and previous reports for type 2 diabetes [112]. This may implicate that epigenetic alterations, e.g. parental exposures at time of conception [126], underpin part of the association.

6.3 OVERWEIGHT AND OBESITY

Overweight/obesity was shown to be strongly associated with increased risk of LADA both in the Swedish case-control data and in the prospective data from Norway. The risk was less pronounced compared with type 2 diabetes which supports the belief that insulin resistance underlie the pathogenesis but is a less important factor for LADA than for type 2 diabetes [16]. In line with this, calculation of PAR suggested that a large part of the LADA patients may be prevented by keeping a normal weight but that this proportion is smaller than for type 2 diabetes. The association between LADA and obesity was strongest in less autoimmune LADA, and these patients displayed a more type 2-like phenotype with less HLA high-risk genotypes and better β-cell function. Notably, we did observe an increased risk also in the most autoimmune LADA group. In keeping with our findings an elevated risk has been seen both in children [87] and adults [88] with type 1 diabetes. Pathologically, our results favour insulin resistance as the primary pathway; there was a positive association between BMI and HOMA-IR indices. In contrast, an inverse relationship was observed with level of GADA which has been reported previously both for LADA [71] and for type 1 diabetes [176]. As insulin resistance amplify the need for insulin [2], it is conceivable that β-cells in people with autoimmunity and an already impaired insulin release will not be able to compensate for the increased requirement following obesity. This is compatible with the accelerator hypothesis which proposes that insulin resistance exhaust the β-cells and further, that insulin resistance is the common denominator for type 2 diabetes and autoimmune diabetes [143]. However, this

does not rule out a possible link between obesity and autoimmunity in LADA; it has been suggested that obesity induced inflammation mediates β-cell stress and increases antigen presentation, which may evoke an autoimmune reaction in individuals with a genetic predisposition [177, 178].

We further investigated potential interaction between overweight, FHD and genes in relation to diabetes. Indeed the strongest risk of both LADA and type 2 diabetes was seen in those exposed jointly to FHD and overweight. For LADA, the combined effect of overweight and HLA high-risk genotypes conferred the by far highest risk. In addition, a dramatic interaction between HLA high-risk genes and overweight was observed which was most evident for those with the DR4/4 variant. Previous studies of type 1 diabetes in children [153, 179] and of multiple sclerosis [154] support our findings of a synergistic effect between BMI and HLA risk-genotypes. Additionally, there was interaction between overweight and *TCF7L2* and *FTO* in relation to LADA. The interaction with *TCF7L2* was also confirmed in relation to type 2 diabetes. This in line with earlier studies of type 2 diabetes [155, 156], reporting interaction between overweight and a genetic risk score. Moreover, a strong interaction between *TCF7L2* and obesity was recently reported in a Chinese population [157].

It is plausible that the double burden of carrying both HLA genes, associated with autoimmunity, and obesity, which besides leading to reduced insulin sensitivity puts an extra load on the β-cells, further intensifies the risk of LADA. Similarly, in individuals carrying the *TCF7L2* genotype and a genetically induced insulin deficiency (which alone only marginally increases the risk), the risk of LADA exacerbates when combined with insulin resistance. The observed interaction between overweight and *FTO* in relation to LADA indicated that the risk associated with *FTO* is only elevated when combined with overweight. This is in keeping with previous reports suggesting that the risk allele increase the risk of obesity [64], while in those who stay physically active, the risk attenuates [158]. There was no interaction between *FTO* and overweight for type 2 diabetes which may be due to overweight being a much more prominent risk factor for type 2 diabetes than for LADA even without genetic risk.

From the above, it can be hypothesised that genes and environment follow a reciprocal relationship in the aetiology of LADA such that individuals with low (HLA) genetic susceptibility demand more lifestyle factors promoting insulin resistance for manifest disease. In the presence of a high genetic load, insulin resistance plays less of a critical role. However, when these factors are present together the risk rises “through the roof”. Interaction is also useful from a public health perspective, i.e. by identifying subgroups of individuals with additional risk, preventive measures can be undertaken more efficiently. Importantly, our findings suggest that LADA, in line with type 2 diabetes, may be prevented with weight management. It is noteworthy that this seems to apply also to individuals with genetic susceptibility.

6.4 BIRTHWEIGHT

Using data from ESTRID, we found an inverse relationship between birthweight and LADA such that low but not high birthweight was associated with excess risk. This is the first study investigating birthweight in relation to LADA, but the result fits with a large amount of type 2 diabetes studies [98]. Our results for type 2 diabetes were also in line with these earlier findings. The study was hampered by the small number of participants and it is possible that also high birthweight, mediated by maternal diabetes/obesity during pregnancy, would confer risk of LADA in a larger sample. Especially since high birthweight has been associated with both type 1 [85, 86, 128] and type 2 diabetes [99, 100]. Supporting this notion, the association with low birthweight was strengthened after excluding those reporting maternal diabetes. However, adjusting for FHD did not impact the results, neither for LADA nor for type 2 diabetes. This implies that the association between LADA and low birthweight is related to the in utero environment rather than being genetically determined. A considerable body of evidence supports this possibility [126], e.g. maternal malnutrition during pregnancy or paternal metabolic disease before conception and during spermatogenesis may adversely impact the metabolic health of offspring in later life [180]. The combination of low birthweight and adult overweight further increased the risk of LADA and type 2 diabetes, which particularly favours the thrifty phenotype hypothesis [136]. Clearly, the findings for LADA need to be extended and replicated and even though the topic of in utero environment has been investigated intensely, the field of epigenetics is still in its infancy and many more questions need to be addressed before drawing any conclusions.

6.5 METHODOLOGICAL CONSIDERATIONS

6.5.1 Random error

The presence of random variation should be kept in mind when interpreting data and in general, small numbers increases the risk of imprecise results. Confidence intervals in stratified analyses were occasionally wide and overlapping. Thus, interpretation of risk magnitudes and results from subgroup analyses should be made with some caution. This is foremost a concern in paper I in which half of the study population was lost due to participants not recalling their birthweight. The results were based on cases and controls recruited between 2010 and 2014. When re-analysing the data with cases recruited until 2017, the same result is obtained. It is of course still important to replicate the findings in other materials.

6.5.2 Systematic errors

6.5.2.1 Selection bias

Selection bias occurs if participation in a study is related, in a non-random way, to the exposure and disease under study. In a case-control study, the key feature of the controls is to mirror exposure frequency in the study base. If this is violated, selection bias is introduced which could either over- or underestimate the effect estimates. Controls were recruited continuously and randomly (incidence density sampling) from the same population that produced the cases. The method has a high validity [163] but a spurious association may arise if participating controls differed from those who did not. For instance, individuals with higher education are generally overrepresented among responders. Higher education is in turn associated with a healthier lifestyle, why it can be expected that participants tend to be leaner and more active compared to the general population. Response rate among controls in ESTRID is 62%, to estimate if this is a representative sample of the study base we compared our controls to the general population in Skåne with regard to level of education and found that it was similar (http://www.statistikdatabasen.scb.se). Moreover, the proportion of obesity among our controls and the general population of Skåne (16-84 years) is the same (15%). However, it should be noted that this estimate is based on data from the national public health survey with only 42% response rate (https://www.folkhalsomyndigheten.se). The genetic controls from EIRA were slightly leaner (12% were obese). This could have led us to overestimate the results in paper IV. Still, the overall association with overweight and obesity is in line with results from paper III where we used the controls recruited within ESTRID, suggesting that this did not impact the results to any large extent.

The case response rate in ESTRID was high (83% for LADA and 79% for type 2 diabetes). Even so, an additional concern is whether cases recruited from the ANDIS and ANDiU registers in Skåne and Uppsala differ from those not covered by these registers. Still, the absolute majority of the patients in ESTRID were recruited from ANDIS, which at present covers >90% all eligible patients in Skåne County [18]. Out of these ~95% of the LADA

cases are invited to participate in ESTRID. Furthermore, there is nothing to suggest that health care centres not covered by these registers differ systematically from those included.

Internal non-response may also be a problem if cases and controls differ in that respect. Especially in paper I were only 45% of the participants knew their birthweight. However, there is no reason to suspect that birthweight would be linked to non-response. Importantly, response rates were similar among cases and controls. We also called all participants with missing information and could confirm that these people did not know their birthweight. In addition, when we compare register-based data of the controls with and without self-report (n = 81), we found that those without this information have slightly higher mean birthweight, (3.55 kg vs. 3.43 kg). If anything, this suggests that we have underestimated the association between low birthweight and diabetes. Notably, our results for type 2 diabetes were in line with a large number of register-based studies [98].

In HUNT, response rate dropped during the course of the study, from 91% in the first survey to 54% in the last one. Thus loss to follow-up may be a problem and may affect the association with BMI if dropping out is linked to both exposure and disease (e.g. if obese individuals with diabetes are less likely to attend the follow-up study or died before follow-up). In such case, the association would most likely be underestimated. In support of low selection bias, validation studies where re-attenders were compared to those who did not attend the follow-up showed that the groups were similar with regard to most health characteristics. However, older participants who discontinued their participation had a higher rate of co-morbidities compared to participants in the same age [167, 181].

6.5.2.2 Misclassification of exposure

The self-reported exposure information is problematic. In ESTRID, were we used a retrospective approach, this may lead to a differential exposure misclassification if cases remember and report birthweight, FHD and BMI exposures differently than controls. This could either under- or overestimate our associations. By using incident cases approached in close proximity to diagnosis and by emphasizing the importance of reporting lifestyle according to the year prior to disease onset, the likelihood of response bias should be reduced. Still, the self-reported BMI information in ESTRID is a limitation. As pointed out by Rothman and others [182, 183] the most likely scenario would be that responders underreport their current weight (especially if obese) and over report height. The implication is that we will underestimate the association between overweight/obesity and diabetes, as long as cases and controls do not differ in their degree of underreporting. If cases underreport more, e.g. due to the fact that they lost weight following diagnoses and report accordingly, this would lead us to underestimate the association with BMI further. In this context, it is also noteworthy that among cases, self-reported BMI information (weight and height) was highly correlated (r=0.92, $p < 0.0001$) with BMI calculated at diagnosis (information available from ANDIS). Importantly we could confirm our results using objectively obtained information from the HUNT study.

Likewise, for individuals born after 1973, the self-reported birthweight information was validated against register data with a very good result; r=0.86 (*p <0.0001*). We were not able to validate the self-reported information for older participants, whom at the same time may have reported less accurate information. To handle this we performed separate analysis for individuals aged ≥60 years. These estimations only marginally changed the results, indicating a non-biased result. In support are other studies demonstrating a high validity of birthweight information also for older individuals [98, 140, 184].

With regard to the self-reported information on FHD, it is possible that cases are more aware of relatives' diabetes status which would lead us to overestimate the association with diabetes. For the same reason, relatives may be more prone to seek medical care if they experience diabetes like symptoms. However, cases are approached close to diagnosis which reduces the time window for raised awareness among the relatives. Also relatives' age at onset and insulin treatment, used to determine FHD-T1D vs FHD-T2D, was based on self-reported information from the participants. Our rather crude classification may have led relatives with autoimmune diabetes and onset after 40 years of age to be classified as having type 2 diabetes. This could explain part of the association between LADA and FHD-T2D. However, using a more stringent classification of FHD-T2D (≥40 years and no insulin treatment) did not change the association.

6.5.2.3 Misclassification of disease

Patients in ESTRID were diagnosed within the health care system. The self-reported diabetes in HUNT could potentially be a problem. However validation studies, comparing questionnaire data with medical records of the patients showed that the methods were highly correlated [168]. Nevertheless, individuals with diabetes can sometimes be asymptomatic for many years before diagnosis. Consequently, it is possible that we have cases of undiagnosed diabetes among the controls/individuals not reporting diabetes. If the likelihood of being diagnosed is related to exposure i.e. birthweight, FHD and BMI, this may bias (overestimate) the relative risk estimates in present thesis. On the other hand, the rate of undiagnosed diabetes in HUNT is reportedly low (0.3%) [168]. In ESTRID, one can assume that controls with undiagnosed diabetes would be more similar to the cases in which case, we would have underestimated the associations.

The possibility of false positive LADA patients is a concern, in particularly in LADA patients with low GADA levels. To separate LADA patients from type 2 diabetes we used GADA, which has been shown to be the antibody that best predicts autoimmunity and progression to disease [25]. In ESTRID the method has high specificity: 98%. This implies that 2% of type 2 diabetes cases may be misclassified as cases of LADA. Simulations where we attempted to remove a proportion of obese subjects, hypothesized to be cases of type 2 diabetes, indicate that this potential misclassification is not enough to remove the risk of LADA associated with excessive weight. Furthermore, the fact that we found an association with obesity and with FHD-T2D regardless of GADA level clearly speaks against a large impact from false positive LADA patients (i.e. type 2 diabetes patients misclassified as LADA). In addition the

association with BMI and LADA was even stronger in HUNT were the specificity of the GADA assay was 100%. Most importantly, in both studies there was a clear association between HLA high-risk genotypes and LADA with low GADA which is in contrast to what we see for type 2 diabetes, supporting the autoimmune nature of this group. Further support, a previous study of HUNT indicate a real impact of even low and transient levels of GADA; these low GADA individuals displayed lower fasting C-peptide levels [185] than antibody-negative individuals who were classified as type 2 diabetes. Similarly, in paper III we found a higher proportion of insulin treatment among $LADA^{low}$ than in type 2 diabetes patients.

The sensitivity of the assays (84% in ESTRID and 64% in HUNT) implies that we have false negative LADA patients among individuals with type 2 diabetes which may have diluted the associations for type 2 diabetes. Moreover, even though GAD is the most common antibody in LADA, the use of only one autoimmune marker means that some LADA individuals may have been classified as autoantibody negative, i.e. as having type 2 diabetes. In a previous study based on HUNT, 99% of LADA cases were positive for GADA and less than 1% was exclusively positive for IA-2A or ZnT8A [185]. However, in Action LADA [25], 10% of the LADA cases were GADA negative. If we assume that 10% of all LADA cases were positive only for these other autoantibodies and thus classified as having type 2 diabetes (by multiplying the number of LADA patients by a factor 1.105) this implies that 14 of the type 2 diabetes patients in HUNT and 42 of the type 2 diabetes patients in ESTRID (numbers from paper IV) could be misclassified LADA cases. Also related to false negatives; because GADA can disappear after long duration of disease [185] it is possible that LADA patients in HUNT, for whom GADA titres were measured several years after diagnosis, appeared GADA negative and were consequently included in the type 2 diabetes group. However, GADA has been shown to be more stable in LADA compared with type 1 diabetes [16]. Most importantly, the lack of association between type 2 diabetes and HLA risk genotypes indicates that the proportion of misclassified LADA is most likely small.

6.5.2.4 Confounding

A strength of this thesis was that we had the possibility to adjust for a large number of potential confounders including physical activity, smoking, alcohol and education. Residual confounding or unmeasured confounding should still be kept in mind when interpreting the results. For instance in paper I, it would have been interesting to adjust for gestational diabetes as this likely diluted our findings for low birthweight. Parental exposures during childhood would also be interesting considering both the association with birthweight and with FHD, especially for the association with FHD-T2D.

6.5.3 Generalisability

Our findings are based on two large population-based studies: The Swedish study aimed at covering all incident cases of diabetes in two counties in Sweden together with a random sample from the general population. The Norwegian study covered one whole county in Norway which has been shown to be a fairly good representative of the rest of Norway [186].

Thus we have no reason to believe that these findings could not be generalised to other Nordic countries with similar rates of LADA and exposures under study. With regard to the rest of Europe, and other parts of the world where LADA is less prevalent, differences in the genetic set-up and pattern of additional risk factors could limit generalisability. Of note, estimation of PAR is based on the estimated effect sizes as well as prevalence of overweight in the population. In a population with different levels of BMI, the proportion of diabetes cases attributed to these risk factors would be different.

7 CONCLUSIONS

The aim of this thesis was to add new knowledge to the aetiology of LADA, by exploring the impact of birthweight, overweight and obesity, FHD and genes and their interaction on the risk of LADA. Our results suggest that FHD is a strong risk factor. The risk is foremost associated with FHD of type 1 diabetes and HLA genotypes promoting autoimmunity, but is also associated with FHD of type 2 diabetes. Possibly also with genes promoting type 2 diabetes and β-cell deficiency in a non-autoimmune way. Moreover, the results indicate that overweight and obesity are important risk factors in relation to LADA, probably mediated by insulin resistance. The risk was especially high in combination with FHD and a considerable interaction was observed in individuals with HLA high-risk genotypes. However, interaction was also seen in those with risk variants of *TCF7L2* and *FTO*. This indicates that excessive weight is a particularly detrimental risk factor for LADA in individuals with genetic susceptibility. The results further imply that low birthweight may be as strong risk factor for LADA as for type 2 diabetes. The association appeared to be independent of FHD, which support the hypothesis that the intrauterine environment rather than genetic factors explain the association. The combination of low birthweight and adult overweight seems to be an especially important risk factor for LADA which supports the thrifty phenotype hypothesis.

Taken together, the results from this thesis support that factors linked to insulin resistance and type 2 diabetes also constitute risk factors for LADA, while genetic susceptibility is linked foremost to genes associated with autoimmunity and type 1 diabetes. These findings contribute to the limited but gradually increasing number of studies indicating that lifestyle plays an important role in the aetiology of LADA. Notably, under the assumption of causality, our results suggest that a large proportion of the LADA cases are attributable to excessive weight and may therefore be preventable in a similar way as for type 2 diabetes. The results also indicate that lifestyle interventions may be especially beneficial among people with HLA high-risk genotypes. Still, these findings are suggestive, and to verify and extend these results to other settings is of importance. To evaluate the real preventive potential of LADA, intervention studies will be crucial.

8 FUTURE PERSPECTIVES

Risk factors for LADA are still largely an unexplored area. The ESTRID and HUNT data materials, which this thesis is based on, provide great opportunities to continue exploring potential environmental risk factors that could alter the risk of LADA. Such factors include physical inactivity, a large number of dietary factors and many others.

However, considering that research in this area primarily is based on two Scandinavian studies, new research initiatives involving other populations exhibiting other characteristics, ethnic backgrounds and genetic set ups, are clearly warranted.

Further examination of overweight interactions and type 1 diabetes genes outside the HLA complex, e.g. *INS* and *PTPN22* and *SH2B3*, should be considered. Additionally, environmental risk factors other than excessive weight would be interesting to explore in a gene-environmental setting, some potential candidates being birthweight, physical activity, diet, smoking and alcohol consumption.

Importantly, the best treatment regimen for LADA is still not established and few studies have explored long term consequences such as cardiovascular disease. Hence, more remains to be investigated in terms of prognosis of LADA.

Lastly, intervention studies are a necessity to be able to evaluate to what extent LADA actually can be prevented by lifestyle modification. High-risk individuals, e.g. persons with HLA high-risk genotypes and antibody positivity, could be a good target group for such intervention.

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