Childhood Obesity and Islet Function

JOHAN STAAF
Abstract

The prevalence of childhood obesity and Type 2 Diabetes Mellitus (T2DM) has increased during recent decades. T2DM is accompanied with functional changes in the islets of Langerhans, which can be identified early in the pathogenesis. The aim of this thesis was to explore how metabolic changes caused by obesity early in life relate to islet function prior to overt T2DM.

To address this, Uppsala Longitudinal Study of Childhood Obesity (ULSCO) was established (paper I). Initially, the association between palmitate and insulin secretion was investigated using a translational approach with obese and lean normoglycemic juveniles and isolated human islets (paper II). Secondly, dynamics of islet-hormones insulin and glucagon, and gut-hormones glucagon like-peptide 1 (GLP-1) and glicentin (paper III) and magnetic resonance imaging of pancreatic fat fraction (PFF) (paper IV) were studied in association to glucose tolerance and beta-cell function. Finally, a novel method of analysing shape features of oral glucose tolerance test (OGTT) curves was introduced and evaluated (paper V).

Obese subjects had high prevalence of prediabetes and metabolic syndrome (MetS) (paper I). In obese pre-pubertal children with elevated palmitate levels, hyperinsulinemia was observed (paper II). In contrast, obese pubertal adolescents with similar palmitate levels showed moderate insulin levels during OGTT with delayed first phase insulin response. To explore mechanisms for these variations, isolated human islets were exposed to palmitate for different time periods in vitro. After 2 days accentuated insulin response was observed. Impaired beta-cell function and apoptosis were evident after 7 days, however. Hyperglucagonemia and disturbed GLP-1 and glicentin levels were associated with obesity and glycaemic status, with fasting glicentin being predictive of prediabetes (paper III). Furthermore, PFF was increased in obese subjects and associated to MetS and visceral adipose tissue, but not to beta-cell function (paper IV). OGTT curves were converted into geometric centres, centroids, which correlated with differences in glucose tolerance (paper V).

In conclusion, the islet function in obese children was associated with elevated levels of palmitate, but not pancreatic fat. Fasting palmitate and glicentin levels, as well as centroid analyses of OGTT curves, could potentially identify obese children at risk of prediabetes and subsequent T2DM.

Keywords: type 2 diabetes mellitus, Uppsala Longitudinal Study of Childhood Obesity, palmitate, glucose-stimulated insulin secretion, hyperinsulinemia, hyperglucagonemia, GLP-1, glicentin, magnetic resonance imaging, metabolic syndrome, oral glucose tolerance test, centroid

Johan Staaf, Department of Medical Cell Biology, Box 571, Uppsala University, SE-75123 Uppsala, Sweden. Department of Women's and Children's Health, Akademiska sjukhuset, Uppsala University, SE-75185 Uppsala, Sweden.

© Johan Staaf 2017

ISSN 1651-6206
ISBN 978-91-554-9801-6
urn:nbn:se:uu:diva-313310 (http://urn.kb.se/resolve?urn=nbn:se:uu:diva-313310)
To Emmelie, my wife
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
</thead>
<tbody>
<tr>
<td>AUC</td>
<td>Area Under the Curve</td>
</tr>
<tr>
<td>BMI</td>
<td>Body Mass Index</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
</tr>
<tr>
<td>DPP-IV</td>
<td>Dipeptidyl Peptidase-IV</td>
</tr>
<tr>
<td>FFA</td>
<td>Free Fatty Acid</td>
</tr>
<tr>
<td>FFAR-1</td>
<td>Free Fatty Acid Receptor-1</td>
</tr>
<tr>
<td>GI</td>
<td>Gastrointestinal</td>
</tr>
<tr>
<td>GLP-1</td>
<td>Glucagon Like Peptide-1</td>
</tr>
<tr>
<td>GSIS</td>
<td>Glucose Stimulated Insulin Secretion</td>
</tr>
<tr>
<td>HOMA</td>
<td>Homeostatic model assessment</td>
</tr>
<tr>
<td>IFG</td>
<td>Impaired Fasting Glucose</td>
</tr>
<tr>
<td>IG1</td>
<td>Insulinogenic Index</td>
</tr>
<tr>
<td>IGT</td>
<td>Impaired Glucose Tolerance</td>
</tr>
<tr>
<td>ISSI-2</td>
<td>Insulin Secretion-Sensitivity Index-2</td>
</tr>
<tr>
<td>KRBH</td>
<td>Krebs-Ringer Bicarbonate HEPES</td>
</tr>
<tr>
<td>LFF</td>
<td>Liver Fat Fraction</td>
</tr>
<tr>
<td>MRI</td>
<td>Magnetic Resonance Imaging</td>
</tr>
<tr>
<td>NGT</td>
<td>Normal Glucose Tolerance</td>
</tr>
<tr>
<td>OGTT</td>
<td>Oral Glucose Tolerance Test</td>
</tr>
<tr>
<td>PFF</td>
<td>Pancreatic Fat Fraction</td>
</tr>
<tr>
<td>SAT</td>
<td>Subcutaneous Adipose Tissue</td>
</tr>
<tr>
<td>T2DM</td>
<td>Type 2 Diabetes Mellitus</td>
</tr>
<tr>
<td>ULSCO</td>
<td>Uppsala Longitudinal Study of Childhood Obesity</td>
</tr>
<tr>
<td>VAT</td>
<td>Visceral Adipose Tissue</td>
</tr>
</tbody>
</table>
Table of contents

1. Introduction .......................................................................................................................... 11

2. Background .......................................................................................................................... 13
   2.1 Childhood obesity ............................................................................................................. 13
   2.2 Adipose tissue ................................................................................................................. 14
   2.3 Free fatty acids .............................................................................................................. 14
   2.4 The islet of Langerhans ................................................................................................. 15
   2.5 Pediatric Type 2 Diabetes Mellitus ................................................................................. 16
   2.6 Risk factors of T2DM in childhood obesity ................................................................. 17
   2.7 Incretins .......................................................................................................................... 18
   2.8 Shape analysis of OGTT curves ..................................................................................... 18
   2.9 Treatment strategies for childhood obesity ................................................................. 19

3. Aims ..................................................................................................................................... 20
   3.1 General aim ..................................................................................................................... 20
   3.2 Aims of each paper ......................................................................................................... 20

4. Materials and Methods ...................................................................................................... 21
   4.1 Design and setting of the study ....................................................................................... 21
   4.2 Ethical considerations ..................................................................................................... 21
   4.3 Anthropometric and blood pressure measurements ..................................................... 22
   4.4 Definitions of obesity, T2DM and MetS ......................................................................... 22
   4.5 Fasting blood sampling ................................................................................................. 22
   4.6 Oral glucose tolerance test ............................................................................................. 22
   4.7 Analytical procedures of blood samples ....................................................................... 23
   4.8 Calculations of beta-cell function and insulin resistance ............................................. 23
   4.9 Shape analysis using centroids ..................................................................................... 23
   4.10 Magnetic resonance imaging ....................................................................................... 24
   4.11 Culture of human islet .................................................................................................. 24
   4.12 Glucose-stimulated insulin secretion (GSIS) ............................................................... 24
   4.13 Islet insulin content and apoptosis measurements ...................................................... 24
   4.14 Statistical methods and analyses .................................................................................. 25

5. Results and Discussion ...................................................................................................... 26
   5.1 The ULSCO cohort .......................................................................................................... 26
   5.2 Palmitate and insulin secretion ....................................................................................... 26
   5.3 Islets function in obese children .................................................................................... 28
List of Papers

The thesis is based on the following papers, which are referred to in the text by their Roman numerals.

I Anders Forslund, **Johan Staaf**, Joel Kullberg, Iris Ciba, Marie Dahlbom, Peter Bergsten. Uppsala Longitudinal Study of Childhood Obesity – protocol description, Reproduced with permission from *Pediatrics*, Vol. 133, Page(s) 386-93, Copyright © 2014 by the AAP


V **Johan Staaf***, Joel Kullberg*, Hannes Manell, Daniel Weghuber, Håkan Ahlström, Peter Bergsten†, Anders Forslund†. Centroid analysis of OGTT-data for type 2 diabetes risk assessment (*manuscript*)

* First authors contributed equally. † Last authors contributed equally

Reprints were made with permission from the respective publishers.
1. Introduction

Obesity is defined as a state of excess body fat and has been recognized as a medical disorder already in ancient Greece (1, 2). More than 2 billion people are characterized as overweight (body mass index (BMI) between 25 kg/m² and 30 kg/m²) or obese (BMI above 30 kg/m²), and the latter state contributes to approximately 3.5 million deaths annually (3). The prevalence of obesity has increased during past decades, both among adults (3, 4) and children (3, 5). Childhood obesity has contributed to rise in juvenile type 2 diabetes mellitus (T2DM) (6, 7) and the combination, sometimes called “diabetes” (8, 9), constitutes a serious health issue in this century (10, 11) because of its association to diabetes-associated complications (12) and premature mortality (13).

Diabetes mellitus, a non-communicable disease that implicates dysregulation of blood glucose, has been known during millennia (14, 15). T2DM used to afflict predominantly elderly, but is currently increasing in prevalence among youths (7, 16). Children with obesity and T2DM display altered function of insulin-producing beta-cells in combination with insulin resistance (17, 18) and already prior to overt T2DM the postprandial glucose response is disturbed (19). Furthermore, subjects with obesity have elevated levels of free fatty acids (FFAs) (20, 21), which have been connected with adverse effects on beta-cells (22-24). This contributes to elevated glucose levels (25) and has been linked with increased amount of visceral fat (26). Moreover, altered secretion of proglucagon-derived hormones, including glucagon from pancreatic alpha-cells and glucagon like peptide-1 (GLP-1) from intestinal L-cells are involved in the pathophysiology of T2DM in adults (27, 28).

Fat accumulation in pancreas in augmented in obese children (29). To what extent this affects the islets of Langerhans and, in particular, changes in beta-cell function, has been disputed (30, 31). To determine the function of pancreatic islets and evaluate the body’s ability to maintain glucose homeostasis, an oral glucose tolerance test (OGTT) is commonly conducted and the shape of the curves generated have been used to predict glucose intolerance and T2DM (32).

This thesis aims at increasing the etiology of childhood obesity. More specifically, hormones secreted by islets of Langerhans will be investigated in
relation to childhood obesity. In addition, different factors that potentially affect the function of the islets will be explored.
2. Background

2.1 Childhood obesity

Approximately 10% of all children worldwide were in the beginning of the 21st century overweight or obese (33). Currently more than 40 million children below the age of 5 are characterized as obese (34) and in Sweden almost 3% of children in primary school have obesity (35). The prevalence in several other European countries is higher (36) which has contributed to the phrase “obesity epidemic” (37). Indeed, the amount of children and adolescents with obesity has increased rapidly and the trend is expected to continue, which is alarming due to onset of obesity-related complications early in life (38). Furthermore, obesity in childhood is often carried over to adulthood (39) with negative health consequences the afflicted individual and society as a whole (40-42).

Obesity in the pediatric population is defined as BMI, adjusted to age and gender, of more than 30 kg/m² (43) or above 2 BMI-standard deviations (BMI-sds) (44). Since late 1970s the World Health Organization (WHO) has recognized obesity as a disease (34). Development of obesity is caused by an interaction between genetic, epigenetic and environmental factors (45, 46), where increased consumption of high calorie foods is a fundamental aspect (47). Moreover, a sedentary life-style with a low degree of physical activity has been connected with increase risk of weight gain early in life (48). In addition socioeconomic aspects as well as the parental BMI and education level are known risk factors (35). Around 5% of obese subjects have known genetic mutations, which directly contribute to obesity (49).

A substantial number of subjects with obesity suffers from the metabolic syndrome (MetS) (50) and obesity is also associated with several other metabolic aberrations and ectopic fat deposition (29, 51, 52). Furthermore, the risk of early onset of T2DM is relatively higher in adolescent with obesity (7, 53). Obese subjects are also at increased risk of developing cardiovascular disease (CVD) early in life (54), caused primarily by a shift in the lipid profile (55). In addition, the risk of prediabetes and T2DM increases with degree of obesity (56). Severe obesity in childhood has been linked to both impaired glucose tolerance and insulin resistance, which are precursors of overt T2DM (57).
2.2 Adipose tissue

In subjects with obesity, the adipose tissue is expanded and contain more and larger adipocytes compared with in non-obese subjects (58). The adipose tissue is recognized not merely as a storage site of triacylglycerols (TAG), but also as a metabolic and endocrine active tissue, releasing numerous adipokines and cytokines into the blood circulation (59). As the BMI of a subject increases, the subcutaneous adipose tissue (SAT) expands. Fat is also deposited in the visceral adipose tissue (VAT) compartment as well as within intra-abdominal organs such as the liver and pancreas (60, 61). Adipocyte dysfunction, in particular in the visceral compartment, contributes to a state of dyslipidaemia with elevation of circulating FFAs as well as TAG and attenuated levels of high density lipoproteins (HDL), with the latter two included as factors of the MetS (26).

In order to examine the volume of various adipose tissue compartments, as well as the presence of ectopic fat infiltrating the organs, magnetic resonance imaging (MRI) is currently the most precise technique (62). Furthermore, MRI uses no ionizing radiation, making it appropriate for pediatric research. Using MRI, an accurate estimate of VAT can be obtained, which is expanded already in overweight individuals (63). An increase in VAT has been associated with elevated postprandial levels of FFAs (26) as well as insulin resistance in adults (64). In children, the ratio between VAT and SAT is found to be elevated in subjects with prediabetes (65), suggesting that the composition of intra-abdominal and subcutaneous fat compartments play a role in T2DM pathogenesis (66).

In adults, pancreatic fat fraction (PFF) has been in some studies been related to negative effects on beta-cell function (67) and insulin resistance (68) but not in other studies (31, 69, 70). Similarly, in overweight or obese children pancreatic fat has been associated with beta-cell dysfunction and insulin resistance in some studies (30, 60). However, other studies find no associations (71). In adult subjects with T2DM, PFF has been shown to be elevated while the total size of the pancreas is reduced by about a third compared with non-diabetic subjects (72).

2.3 Free fatty acids

During fasting, FFAs are at maximum levels and function as a major source of energy for most tissue in the body (24). In the post-prandial state FFA levels decline mainly due to the increase in insulin, which suppresses release of FFAs from the adipose tissue (24). Chronically elevated FFA levels have harmful effects on various cell types, including the insulin-producing beta-
cells. This is commonly described as lipotoxicity (22). Prolonged exposure to high FFA levels causes a reduction in insulin secretion and increase in oxidative stress within beta-cells (73). Obesity is accompanied by an increase in circulating levels of FFAs, demonstrated in both adults (20) and children (21). Moreover, in T2DM adult subjects, FFAs are roughly 30% higher during an OGTT compared with non-diabetic subjects, most likely due to de novo synthesis or reduced extraction of lipids from the blood stream (74).

Total FFAs consist of a spectrum of saturated and unsaturated FFAs, which all have different biological effects on beta-cell function (75). For instance, some unsaturated acids have been shown to be positive for beta-cells by preserving beta-cell viability and secretory function (76). Palmitate (C16:0) is a saturated fatty acid and one of the most abundant in circulation (77). It has been shown in vitro that long-term exposure to palmitate has negative affects on insulin secretion and beta-cell mass by increased generation of ceramide (78). Interestingly, the unsaturated FFA oleate (C18:1) can reduce the negative effects of palmitate (78, 79) and up-regulate fatty acid oxidation (80). This has also been demonstrated in muscle cells, where palmitate in vitro caused increased intracellular ceramide, decreased levels of phosphorylated Akt and insulin resistance; processes which were restored by oleate (81). Palmitate has also been connected with ER-stress (82), disturbed insulin processing and intracellular protein trafficking (83, 84) as well as alternations in the calcium homeostasis (85) and insulin secretion (86). Furthermore it has been linked to inflammation by activation of toll-like receptor (87) and elevation of cytokine levels in mice exposed to the fatty acid (88). Palmitate’s relationship to insulin secretion and risk of T2DM in children and adolescents with obesity is still unknown, however.

2.4 The islet of Langerhans

Pancreatic islets of Langerhans are highly vascularized clusters of multiple endocrine and non-endocrine cells (89), with the main task of maintaining blood glucose homeostasis. Approximately 1 million islets are situated within the pancreas in healthy individuals (90). Each islet contains a few thousand cells, with the main cell-type being insulin-producing beta-cells, which in humans makes up approximately 70% of the islet mass (91). Beta-cells can sense elevated blood glucose levels and respond by releasing insulin into the circulation. Hyperinsulinemia refers to elevated levels of circulating insulin and is often observed before overt T2DM and has also been suggested to play a role in early stages of childhood obesity (92, 93). In obese non-diabetic adults beta-cell mass is often expanded (94). Moreover, the blood flow is reduced and intrapancreatic fat deposition augmented, thus altering
the beta-cell milieu (95). Once diabetes is manifest the beta-cell mass tend to decline, but the structure of the islets remain intact (94, 96, 97). One study demonstrated more than 60% decrease in beta-cell mass in obese subjects with T2DM in comparison with non-diabetic obese subjects (94).

The second most common cell-type within the islet is the alpha-cell, which secrete glucagon in response to low glucose levels (98). Glucagon acts primarily on the liver, generating glucose from stored glycogen through the process of glycogenolysis (98). In adults, elevated glucagon levels have been implicated in the pathogenesis of T2DM, by contributing to elevate blood glucose (27). Within the islet somatostatin-secreting delta-cells, ghrelin-secreting epsilon-cells and pancreatic polypeptide-secreting PP-cells can also be found (91). This thesis will, however, focus on changes in function of only alpha- and beta-cells in obese children and adolescents.

2.5 Pediatric Type 2 Diabetes Mellitus

The global T2DM epidemic is connected with increased prevalence of obesity (37, 99) and is predicted to continue with an estimated half a billion people affected by 2030 (45). In recent decades there has been an unparalleled increase in the number of adolescents, in particular subjects with obesity, who develop the disease (6, 7). Indeed, a close biological and pathophysiological link between T2DM and obesity exist (53, 100). T2DM implicates an inability to maintain glucose levels within a normal range at fasting and after meals, which has been attributed to both impaired beta-cell function and insulin resistance (17, 101, 102). T2DM is a chronic disease and the consequences and complications are numerous including retinopathy and potential blindness, nephropathy and subsequent renal failure and increased risk of CVD (46) due to both micro- and macro-vascular complications (100).

Obese children and adolescents display various degrees of impaired beta-cell function and insulin resistance (18, 65). Early in the diabetes pathogenesis, a state of prediabetes often occurs, which is defined as impaired fasting glucose (IFG) and/or impaired glucose tolerance (IGT) (17, 103). Children with obesity and/or a family history of diabetes have increased prevalence of prediabetes (65, 104, 105). It is important to identify subjects with prediabetes because of increased incidence of cardiovascular complications (103) ectopic fat deposition (106) and risk of conversion to overt T2DM (103, 107).

The transition from normal glucose tolerance (NGT) to T2DM can occur swiftly and is often preceded by a decline in first phase insulin response and increased secretion of proinsulin from beta-cells (108). In beta-cells, proin-
Insulin is cleaved to insulin and c-peptide within secretory granules and released by exocytosis in response to glucose (109). However, as glycaemic control begins to deteriorate, the fasting and postprandial proinsulin levels increase (110), suggesting dysfunction of the hormone biosynthetic machinery within beta-cells (111). Indeed, in adults elevated proinsulin levels have been shown to predict future onset of T2DM (112).

2.6 Risk factors of T2DM in childhood obesity

There are several factors that contribute to beta-cell dysfunction, including lipotoxicity, inflammation, amyloid formation, insulin resistance, altered incretin and adipokine levels (101, 113-115). A majority of these factors are also attributes of obesity, which is why obesity is so closely related to T2DM (116). Other risk factors for developing T2DM include family history of diabetes, genetic predisposition, ethnicity and puberty (16, 117, 118). For instance, African-American children show relatively higher levels of circulating insulin and lower insulin sensitivity than other ethnic groups (119). Interestingly, increased levels of melatonin have recently been linked to T2DM risk (118), potentially implicating sleeping habits in the pathogenesis. During puberty the insulin demand is higher due to a temporary increase in insulin resistance (120), which post-puberty normalizes (121). Hence, puberty is a period of increased risk of T2DM for obese adolescents (7).

MetS is prevalent among obese children in Europe. It is a condition defined by several metabolic aberrations and is associated with central adiposity (122). Subjects with the MetS have an increased risk of developing T2DM (123). Altered lipid profile in combination with inflammation and insulin resistance is considered to link obesity and T2DM (124). As mentioned, high BMI is considered a strong risk factor of T2DM (125), where the distribution of adipose tissue within the body plays an important role (126). The expanded volume of abdominal fat, especially in the visceral compartment, has also been associated with T2DM risk (126). Expanded VAT volume is linked to insulin resistance, another major risk factor of impaired glucose tolerance and subsequent T2DM (64). In a study conducted on elementary school children, around 40% of children below the age of 10 years old had known risk factors of T2DM (117), which highlights the importance of screening and the need of novel, clinically useful markers and intervention strategies for T2DM.
2.7 Incretins

Obesity and T2DM are connected by changes in pancreatic hormone levels as well as incretins, which are hormones secreted from gut hormonal cells in response to meals. In obese adults, increased fasting and postprandial glucagon levels are present but reduced levels of GLP-1 (27). Postprandial levels of GLP-1 are changed in obese children. Whereas some report increased levels (127), others found decreased GLP-1 response in obese subjects (128). More research is warranted on this topic, in particular since obese children have successfully been treated with GLP-1 therapy in a few research studies (129).

L-cells are gut hormonal cells located primarily in the distal ileum and colon and release GLP-1 into the bloodstream when sensing fatty acids, amino acids and carbohydrates in the intestinal lumen (130). Active GLP-1 has a half-life in plasma of less than two minutes, since it is rapidly being degraded by the enzyme dipeptidyl peptidase IV (DPP-IV). The hormone acts as a glucose-dependent potentiator of insulin secretion (130). In addition, GLP-1 also preserves pancreatic islets viability, secretory capacity and has in an animal study been shown to normalize elevated insulin levels (131).

The role of glicentin in childhood obesity is unknown and generally little is known about the physiological function of the hormone. However, glicentin has in a few animal studies been connected with gastric motility, regulation of gastric acid and insulinotropic effects on beta-cell secretion (132-134), which mimics some of the effects of GLP-1 (135, 136). Both glicentin and GLP-1 are products of the pro-hormone pro-glucagon.

2.8 Shape analysis of OGTT curves

A common method of diagnosing T2DM and identify subjects with prediabetes is by performing an OGTT and analyse glucose at fasting and after 2 hours (12). However, the glycaemic regulation during the entire OGTT has proven to be of physiological importance (137). By measuring insulin and glucose concentrations at intermediate time points, a postprandial response curve is obtained. From the insulin and glucose concentration measurements during the OGTT, estimates of beta-cell function and insulin sensitivity can be derived (138). The shape of the OGTT curve has been subject to further analysis and curves are often categorised as monophasic or biphasic, consisting of one or two peaks respectively (32). Interestingly, this has been related to known risk markers of T2DM, such as FFA levels, beta-cell function and insulin sensitivity independent of fasting and 2-hour glucose levels in children (32). Glucose curve shape features have also been demonstrated to con-
tain information predictive of prediabetes in children (19) and the shape of the glucose curve has been shown to be predictive of T2DM in adults (139).

There are also some studies investigating the pattern of the insulin curve with regard to diabetes risk and they found that a delay first insulin response was related to increased risk of future diabetes in adults (140). How the insulin curve relates to diabetes risk in children is still not explored, however. In addition, several attempts have been made to create indices by mathematical modelling of OGTT curves and relate these indices to beta-cell function, insulin sensitivity and glucose tolerance (141-144).

2.9 Treatment strategies for childhood obesity

The basis of obesity treatment is lifestyle intervention with diet and increased physical activity. However, results from such interventions are inadequate and often show no effect. Novel strategies, including pharmaceutical therapies, are desperately needed. In adults, metformin is often used to increase insulin sensitivity and reduce weight gain (18, 53, 145). Its use in children is limited, however. In recent years GLP-1 analogues, which are used in adults with obesity and T2DM (146, 147), have been used on juveniles with obesity with subsequent weight reduction (129). GLP-1 contributes to weight control and prevents glucose dysregulation by potentiating insulin secretion from beta-cells, inhibiting glucagon release from alpha-cells, slowing down gastric emptying and promoting satiety mediated by changes in leptin and peptide YY levels (148, 149).

The current most efficient treatment of obesity in adults is bariatric surgery, which surgically alters the anatomy of the gastrointestinal tract and causes rise in postprandial GLP-1 levels (136). Bariatric surgery has also been tested in adolescents with severe obesity (150). However, the permanent change in anatomy as well as the risks associated with operation and lack of long-term follow-up data are aspects that need careful consideration and makes the approach less attractive in young obese patients (151).
3. Aims

3.1 General aim
To investigate changes in islet function in obese children and adolescents and to explore mechanism and prognostic markers and tools of obesity-related T2DM.

3.2 Aims of each paper
Paper I: Establish and maintain a pediatric cohort of both non-obese and obese subjects

Paper II: Investigate the role of palmitate and insulin hypersecretion for developing obesity and obesity-related T2DM using both the cohort and isolated islets of Langerhans.

Paper III: Explore the role of glucagon, GLP-1 and glicentin in children with obesity and various degrees of glucose intolerance, including children with T2DM.

Paper IV: Examine the role of pancreatic fat in obese children and relate it to risk factors of T2DM.

Paper V: Develop a method to analyse the shape of glucose and insulin curves obtained during OGTT and relate it to glucose tolerance.
4. Materials and Methods

4.1 Design and setting of the study

Uppsala Longitudinal Study of Childhood Obesity (ULSCO) is a cohort designed to study pediatric obesity and obesity-related co-morbidities (papers I). The cohort includes obese children and adolescents, males and females below the age of 18 years old, referred to the Pediatric Obesity Clinic at Uppsala University Children’s Hospital. In addition, lean subjects are recruited as controls.

Enrolled children are carefully examined and blood samples are obtained at annual visits. In order to successfully study development of pediatric prediabetes and T2DM and other obesity-related comorbidities the ULSCO cohort was designed with detailed assessments and a longitudinal study setting. Storage of biological samples in a biobank enables current and future studies to be conducted on the cohort.

In paper IV, which was a multicentre study, pediatric cohorts in Uppsala (ULSCO) and Salzburg were combined within the European project “Beta-cell function in juvenile type 2 diabetes and obesity” (Beta-JUDO).

The in vitro part of this thesis (paper II) was conducted using isolated human islets from deceased adult donors. Islets were kept in culture and exposed to palmitate for various time periods. After culture, cell function and viability were examined.

4.2 Ethical considerations

Research involving children and adolescents raises many ethical concerns (152). The studies involving the ULSCO cohort were approved by the regional ethics committee in Uppsala (2010/036 and 2012/318). Written and informed consent was obtained from study participants and legal guardians. Participation in the cohort was voluntary and could be terminated at any time by study participant or legal guardian.
Ethical approval for working with human islets *in vitro* was granted by the regional ethics committee in Uppsala (2010/006).

4.3 Anthropometric and blood pressure measurements

Calibrated scales and stadiometers were used to measure weight and height of study subjects. BMI was obtained by dividing the weight with the height squared (kg/m$^2$). Subsequently, Microsoft Excel add-in LMS Growth using WHO growth curves was utilized to estimate BMI-SDS (153). Aneroid sphygmomanometer was used to determine blood pressure.

4.4 Definitions of obesity, T2DM and MetS

Obesity was defined as BMI-SDS $\geq$ 2.0 and severe obesity BMI-SDS $\geq$ 3.0 (44, 153). T2DM was defined according to WHO (154) while prediabetes was defined as IFG and/or IGT according to American Diabetes Association (155) and MetS for children and adolescents according to the International Diabetes Federation (156).

4.5 Fasting blood sampling

Blood sampling at fasting was conducted with a stationary intravenous catheter. Some samples were transported immediately to the Academic Laboratory, Uppsala University Hospital for analysis in accordance with local procedures. Other samples were immediately centrifuged at 2500 g for 10 minutes in 4°C temperature, aliquoted in vials and stored in a -80°C freezer.

4.6 Oral glucose tolerance test

OGTT was performed to evaluate glycaemic status, estimate beta-cell function and insulin resistance (157) and analyse the shape of the glucose and insulin curves (158). At fasting, subjects were asked to drink 3 dL glucose-solution of 1.75 grams glucose/kg bodyweight (maximum of 75 grams). Blood samples were obtained at time points -5 (fasting), 5, 10, 15, 30, 60, 90 and 120 minutes.
4.7 Analytical procedures of blood samples

The following analyses were conducted at the Academic Laboratory, Uppsala University Hospital: glucose (Abbott Architect, Abbott Diagnostics, Lake Forest, Ill), insulin (Cobas E602, Roche Diagnostics, Indianapolis, IN), follicle-stimulating hormone, and luteinizing hormone (Cobas E601, Roche Diagnostics, Indianapolis, IN), TAG (Architect c800 instrument, Abbott Diagnostics, Solna, Sweden) and HDL (Architect c800 instrument, Abbott Diagnostics, Solna, Sweden).

Insulin, proinsulin, C-peptide, glucagon and glicentin analyses were conducted at the Department of Medical Cell Biology, Uppsala University and FFAs at the Department of Analytical Chemistry, Uppsala University. For the hormones ELISA-kits were used (Mercodia AB, Uppsala, Sweden). Active GLP-1 was assayed with an electrochemiluminescent enzyme-linked assay (Mesoscale Discoveries), measuring 7–36-amide GLP-1. Fasting levels of FFAs, including palmitate, were measured in plasma by gas-chromatography mass spectrometry (77).

4.8 Calculations of beta-cell function and insulin resistance

Beta-cell function and insulin resistance were calculated using several indices, based on both fasting and OGTT values. The trapezoid rule was used to calculate area under the curve (AUC). At fasting, beta-cell function was calculated by homeostatic model assessment (HOMA)-beta and proinsulin to insulin ratio, while insulin resistance was assessed by HOMA-IR and 1/fasting insulin (108, 159). OGTT data were used to calculate post-prandial beta-cell function; including Insulinogenic Index (IGI), oral disposition index (IGI * Matsuda index) and insulin secretion-sensitivity index-2 (ISSI-2) ([AUCinsulin/AUCglucose] * Matsuda index) (30, 108, 160, 161). Insulin sensitivity during OGTT was calculated using the Matsuda index (162). Stimulation index represents the initial hormonal response during OGTT and was derived by dividing the 30 min value by the baseline value.

4.9 Shape analysis using centroids

The geometric centre of the curve defined the centroid. In the OGTT glucose and insulin curves the centroid consisted of both x- and y-components, where the x-component corresponded to time and the y-component to glucose or insulin concentration. Details on the equation used to calculate the centroid are outlined in paper V.
4.10 Magnetic resonance imaging

Pancreatic fat fraction (PFF) as well as liver fat fraction (LFF), SAT and VAT was quantified by MRI using 1.5T MRI scanners from Philips Medical System (Best, The Netherlands). The examination was performed in a supine position using water-fat imaging techniques. A surface coil was added to the MRI machine when pancreas scans were conducted. To quantify pancreas fat, the image reconstruction of water-fat signals was conducted by a whole-image optimization approach (163, 164). Manual segmentation was carried out by a qualified operator using software ImageJ version 1.42q. Details on the analyses of other fat compartments are presented in paper IV.

4.11 Culture of human islet

Human islets were cultured at 37°C, 5% CO₂ in CMRL 1066 medium (Invitrogen, Paisley, UK) including 10% fetal bovine serum (Invitrogen), 1% Penicillin-Streptomycin and 1% Glutamine (Invitrogen). Palmitate 0.5 mM (Sigma Aldrich, St. Louis, MO) and 0.5% bovine serum albumin (BSA) (Roche Diagnostics, Mannheim, Germany) was added to the medium. Durations of the treatment were 0 (control), 0.25, 2, 4 or 7 days.

4.12 Glucose-stimulated insulin secretion (GSIS)

After culture, the insulin secreting capability of the islets were assessed by perifusion. Initially Krebs-Ringer Bicarbonate HEPES (KRBH) buffer containing 2 mM glucose was used for 60 min and samples collected during the final 20 min. The glucose concentration was then raised to 20 mM for an additional 20 min and samples collected at specific time intervals. Quantification of insulin was conducted by ELISA. Total protein in islets was measured using the Lowry method.

4.13 Islet insulin content and apoptosis measurements

Human islets were lysed using lysis-buffer containing 1% Triton and 0.1% protease inhibitory cocktail in a phosphate buffer solution. Insulin content was measured by ELISA. Apoptosis was assessed by measuring cleaved caspase 3 (normalized to actin) by western blot.
4.14 Statistical methods and analyses

Papers I-V: GraphPad Prism 6.0c (GraphPad Software Inc, La Jolla, CA) was used for statistical analyses. In papers IV-V, Statistica 12 (Statsoft Inc. Tulsa, OK) was also used. Significance was considered at p<0.05.

Paper II: To compare groups ANOVA was applied with post hoc t-tests (Mann-Whitney’s or Bonferroni’s tests). Normal distribution of data was analyzed using D'Agostino & Pearson omnibus normality test.

Paper III: One-way ANOVA with post hoc Fischer’s LSD test was applied to compare groups. Logistic regression analysis was conducted to analyze proglucagon-derived peptides ability to predict prediabetes. ROC-curve analyses were conducted and cut-off points were determined by the Youden’s index.

Paper IV: Spearman’s rank test (correlations) and Mann-Whitney U-test (group differences) were used. Data that showed normal distribution were analyzed using Student’s t-test (group differences). Adjusted regression models were used, including age and body composition as parameters. Two models were applied: Model A contained the factors age and BMI-SDS, while model B consisted of VAT, SAT, and LFF. VAT was controlled for when analyzing the association between PFF and MetS, by analyses of covariance.

Paper V: To evaluate the centroid against 2-hour glucose; simple regression and multiple regression analyses were conducted. For group analyses Kruskal-Wallis test with Dunn’s multiple comparison test were applied.
5. Results and Discussion

In this thesis, the islet function with regard to beta- and alpha-cell secretory patterns in childhood obesity was investigated. In addition, several factors such as palmitate, ectopic fat deposition and incretins levels were examined regarding their potential influence on islet function in obese children, and in some cases also in isolated islets of Langerhans. Finally, a novel approach of analysing OGTT curves was introduced, compared with current methods of curve analyses and related to glycaemic status.

5.1 The ULSCO cohort

The first paper of this thesis describes the ULSCO cohort, which is a pediatric cohort including mainly obese subjects. Included in the cohort are also subjects with severe and morbid obesity, who are at high risk of developing obesity-related complications including T2DM (56) and CVD (165). Early onset of these complications constitutes a problem of major health concern (126). Indeed, in the ULSCO cohort more than 40% of obese subjects have prediabetes (paper I), which is comparable to the prevalence reported in another Swedish cohort of severely obese children (105). Furthermore, in ULSCO around 5% of subjects are diagnosed with T2DM and well above 30% fulfil the criteria of MetS, making ULSCO suitable to study early events in the pathogenesis of T2DM. The detailed characterization of study subjects, with various analyses of blood samples obtained at fasting and during OGTT as well as MRI scans, enables detailed studies on changes in islet function, glucose metabolism and body composition prior to onset of T2DM.

5.2 Palmitate and insulin secretion

Higher fasting levels of circulating palmitate were observed in obese subjects compared with lean subjects (paper II). During the fasting state, plasma levels of FFAs are mainly determined by the lipolytic rate of adipose tissue, converting stored intracellular TAGs to glycerol and FFAs, which are subsequently released into the circulation (166). Previously, total FFAs have been found to be elevated in children with obesity (21). Total FFAs have also
been connected both with insulin resistance (24, 167) and hyperinsulinemia (21, 168). The present thesis resolved total FFA levels into specific FFA species. Palmitate was in focus due to its known connection with beta-cell function and viability (78, 83, 86, 169-171), and investigated both in vivo and in vitro. We found that palmitate is elevated in children with obesity. Moreover, elevated fasting levels of palmitate relate to first phase insulin secretion, but in different ways in children and adolescents with obesity. In pre-pubertal children with high palmitate levels, hyperinsulinemia was observed during OGTT, with an accentuated first phase insulin response, which was significantly higher than in both lean subjects and obese children with low palmitate levels (paper II). The rate of insulin secretion was highest 5-10 minutes after the glucose load. Since levels of total FFAs decrease during an OGTT due to an increase in insulin (24), the effect of palmitate on beta-cells can be expected to be less during the second half of the OGTT. Therefore, we focused on associations between fasting levels of palmitate, fasting insulin and first phase insulin response. Indeed, obese children with high fasting palmitate had significantly higher fasting insulin, insulinogenic index and area under the insulin curve compared with obese children with low palmitate, suggesting that an initial heightened response of beta-cells to glucose correlate with elevated circulating palmitate levels. After one hour, there was no detectable difference between the groups (paper II). In animal studies, diet-induced elevation of FFA levels has previously been connected with hyperinsulinemia and accentuated first phase insulin secretion, which was attenuated independent of variations in insulin resistance when FFA levels were normalized (168).

In obese pubertal adolescents with high palmitate levels, insulin levels were not elevated during OGTT compared with lean controls and were considerably lower than in obese children with high palmitate. Puberty induces insulin resistance (172) and would arguably result in an elevation of circulating insulin to counteract this rise, in particular in adolescents with obesity. Interestingly, in adolescents with elevated palmitate the insulin response curve was delayed, with peak after 1 hour of the OGTT. A delayed insulin peak has been associated with deterioration in glucose tolerance and increased risk of diabetes in adults (140, 142). Furthermore, it has been demonstrated that elevation of FFA levels, caused by a continuous lipid infusion over a 4-day period, resulted in attenuated insulin release from beta-cells in non-diabetic subjects with a family history of diabetes (173). In subjects with no family history of diabetes, the same procedure resulted an augmented insulin secretion (173), suggesting that palmitate can have various effects on insulin secretion depending on genetic factors. The genetic background of the children was not assessed in our study, however. We conclude that the association between elevated palmitate levels and insulin secretion is different in children and adolescents with regard to first phase insulin secretion, even
though insulin resistance estimated by HOMA-IR was the same between groups.

To investigate mechanisms behind these variations in insulin levels during OGTT in the obese children and adolescents further, isolated human islets of Langerhans were cultured in presence of elevated palmitate concentration for different periods of time. Whereas a significant rise in GSIS was observed after 2 days of culture, secretory decline, loss of insulin content and apoptosis was evident after extended exposure to the fatty acid (paper II). The mechanism behind the augmented insulin response observed after 2 days is suggested to involve events related both to activation of FFAR-1 and initiating intracellular signalling pathways independent of FFAR-1 (86). The reduced insulin secretion observed after 7 days of culture is proposed to occur partly due to a decline in intracellular insulin content (174), which is in line with our results. This effect may be the result of an incapacity to maintain or increase the biosynthesis of insulin (175) and a defective processing and formation of cytosolic insulin granules (84). Similar results were observed in rat islets exposed to palmitate for extended time periods with reduction in insulin secretion, partially attributed to down-regulation of proteins involved in the exocytotic machinery (176).

Indeed, palmitate can have different cellular effects depending on duration of exposure, but also on the concentration of the fatty acid (177). In vivo, circulating palmitate concentration is the results of endogenous production and exogenous intake. Dietary habits have been shown to influence the composition of circulating FFAs (178). Nevertheless, our results indicate that high fasting palmitate levels could initially cause insulin hypersecretion that potentially would lead to a subsequent decline in beta-cell function, where at least the latter has been implicated in the T2DM pathogenesis in adolescents (108). Longitudinal in vivo studies are, however, needed to investigate this hypothesis further.

5.3 Islets function in obese children

Within the islet of Langerhans, beta- and alpha-cells are the most common cell-types (91). The architecture of the islet renders that cells are in close proximity to capillaries and paracrine communication between beta- and alpha-cells can readily occur (97). Childhood obesity is associated with altered secretion of both these cell-types (17, 179). Comparing obese and lean subjects in the ULSCO cohort, insulin levels at fasting were approximately 3.5-fold elevated (paper I). Moreover, post-prandial insulin levels measured during OGTT are substantially raised (Fig 1B). Interestingly, the glucose levels are the same during the initial 30 min between obese and non-obese
subjects (Fig 1A), but the first phase insulin secretion is accentuated (Fig 1B). Insulin levels and glycaemic status were associated in obese subjects and the highest fasting insulin levels were found in subjects with obesity and T2DM (paper III). C-peptide is co-secreted with insulin and is also elevated in both the fasting and postprandial state in pediatric obesity (paper IV, Fig 1C), providing further evidence that the hyperinsulinemia is a direct result of increased rate of secretion and not merely a reduction in clearance.

**Figure 1**

**A**

<table>
<thead>
<tr>
<th>Glucose (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
</tr>
<tr>
<td>0</td>
</tr>
</tbody>
</table>

**B**

<table>
<thead>
<tr>
<th>Insulin (pM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
</tr>
<tr>
<td>0</td>
</tr>
</tbody>
</table>

**C**

<table>
<thead>
<tr>
<th>C-peptide (pmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
</tr>
<tr>
<td>0</td>
</tr>
</tbody>
</table>

**D**

<table>
<thead>
<tr>
<th>Proinsulin (pmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
</tr>
<tr>
<td>0</td>
</tr>
</tbody>
</table>

**Figure 1:** Glucose (panel A), insulin (panel B), C-peptide (panel C) and proinsulin (panel D) measurements during OGTTs conducted in obese (full lines) and non-obese (dotted lines) children and adolescents.

Insulin acts as an anabolic hormone and has been connected with development of obesity (180, 181). Furthermore, hyperinsulinemia has also been connected with MetS in obese children (182). In animal models, it has been demonstrated that by genetically suppressing insulin levels in young mice, they are protected against onset of obesity when given a high-fat diet by upregulation of uncoupling proteins in white adipose tissue (181). These findings advocate that the anabolic properties of insulin do contribute to development and progression of obesity. Another study shows that overfeeding of pre-pubertal rats resulted in hyperinsulinemia, obesity and ectopic fat accumulation (183). The causality between insulin hypersecretion and insulin
resistance is debated. Traditionally, hypersecretion of insulin has been described as a result of insulin resistance. However, hyperinsulinemia has also been suggested as a cause of insulin resistance, demonstrated in animal studies (181, 184).

Since both beta-cell function and insulin resistance are cornerstones in T2DM pathogenesis, it is imperative to understand the intricate relationship. They are correlated by a hyperbolic curve, known as the disposition index, which implies that they affect each other and change simultaneously (185). Nevertheless, we find a huge variation in insulin secretion between subjects, given certain insulin sensitivity, suggesting that there are other factors contributing to insulin release from beta-cells than just insulin resistance. An example of such a factor could be circulating palmitate levels (paper II). These factors are important to identify, since chronically elevated insulin levels has been directly linked to increased risk of CVD, partially due to the effect of insulin on TAG formation (186). One strategy in preventing T2DM is by attenuating hyperinsulinemia and alleviate the secretory machinery in beta-cells, to offer them a chance of recovery (187). This has been shown to take place after bariatric surgery (188). Also pharmacological intervention has been effective in providing “beta-cell rest”, which can improve both the secretory pulsatility of insulin as well as the circulating proinsulin to insulin ratio (189).

The hyperinsulinemia during an OGTT can be further studied by using the centroid of the insulin curve. The centroid is the geometric centre of the insulin curve, represented by an insulin concentration plotted to the y-axis and a time point plotted to the x-axis (paper V). The centroid calculated based on insulin concentrations obtained during OGTT correlated closely to AUC of insulin ($R^2 = 0.99$). By combining insulin centroids of concentration and time, a closer association to 2h-glucose than traditional OGTT-derived estimates of beta-cell function was obtained. Indeed, hyperinsulinemia during OGTT was found to be connected with deterioration in glucose regulation (paper V) and is a part of the T2DM pathogenesis.

Evidence indicate that T2DM is not only preceded by elevation in insulin levels but also an increase in fasting glucagon levels with disturbed alpha-cell response to glucose (190). Glucagon acts as an inhibitor of hepatic uptake of glucose and simultaneously stimulates gluconeogenesis, thus causing elevation of blood glucose levels and has been suggested to play a role in the pathogenesis of T2DM (191). In adults with obesity and T2DM, elevated glucagon levels at fasting and during OGTT has previously been reported (27). Elevated levels of fasting and post-prandial glucagon, which are observed in this thesis, were seen in obese subjects with NGT (paper III). No further increase was seen in obese subjects with prediabetes. However, in
obese adolescents with T2DM both fasting and post-prandial glucagon levels were increased (paper III). These subjects also show the highest insulin levels, which make the hyperglucagonemia paradoxical since insulin is known to inhibit glucagon secretion through insulin receptor substrate-1 activation and downstream PI3-kinase pathway activation (192). Still, already in the state of prediabetes the alpha-cell resistance to insulin may be present (193), which could partially explain the combined hyperinsulinemia and hyperglucagonemia seen in obese children with T2DM (paper III).

Furthermore, the hyperglucagonemia observed in T2DM obese subjects may also be caused by impaired suppression of alpha-cell secretion by GLP-1 (194). Indeed, the postprandial GLP-1 response and GLP-1 to glucagon ratio is decreased in subjects with obesity and T2DM, which is in agreement with previous reported results in adults (195). Indeed, glucagon levels are important to monitor in obese children at risk of T2DM, since they are intricately part of the disease progression. Antagonising the glucagon receptor has even been proposed as a possible treatment strategy of both obesity and diabetes (148). However, currently no medication is available. In animal studies a knockout to the glucagon receptor improved glycaemic control (196), indicating the potential of a drug targeting this receptor.

In addition to elevated insulin levels, we also observed increased circulating proinsulin in children and adolescents with obesity, both at the fasting and post-prandial state (Fig 1D). Proinsulin is the precursor of insulin and has limited biological activity. Indeed, proinsulin has about 20 times lower affinity for the insulin receptor than insulin (197, 198). No difference in the fasting proinsulin to insulin ratio was observed in obese subjects compared with lean controls, however. This may indicate proportional hypersecretion of both forms of insulin in the obese children (paper IV). This is in agreement with previous reported results, which demonstrated that circulating levels of proinsulin are gradually increasing with BMI, but the proinsulin to insulin ratio remain unchanged in young obese children with NGT (199). In contrast, in overweight and obese children with impaired glucose regulation, both levels of proinsulin and the proinsulin to insulin ratio are increased during fasting and in the post-prandial state (110), suggesting a connection to glycaemic status. Indeed, in adults elevated proinsulin has been shown to be predictive of diabetes independently of insulin resistance and first phase insulin secretion (112), and suggested as potential biomarker of beta-cell dysfunction and insulin resistance (200).

In animal studies investigating the effect of obesity on beta-cell function, the intracellular synthesis of proinsulin is elevated (201). At the same time, impairment in proinsulin processing and trafficking is observed (187), resulting in the exocytosis of unprocessed insulin. Elevated proinsulin levels have also
been associated with MetS (202). In paper IV we observe a trend in elevation of fasting proinsulin in subjects with MetS (p=0.07), although no change in the proinsulin to insulin ratio could be detected between groups.

5.4 The entero-insular axis and the role of incretins

Altered levels of incretins, for instance GLP-1, play an imperative role in development of T2DM (127). GLP-1 is secreted from L-cells in the gastrointestinal (GI)-tract as a response to nutrients and has multiple effects on both the cells within the islets of Langerhans and the GI-tract; it potentiates insulin secretion in a glucose-dependent manner while it suppresses secretion of glucagon from alpha-cells; it decelerates gastric emptying and have direct effects on satiety in the hypothalamus (135, 194). We can report that fasting levels of active GLP-1 is higher in obese children with normal glucose tolerance than in lean controls (paper III). No difference in AUC of GLP-1 was found between the obese NGT and lean groups, however, which stand in contrast to Giannini et al. (127). The difference in results could be explained by the different characteristics of the study subjects. Another study measured lower fasting levels of GLP-1 in overweight and obese adolescents, however (203). Furthermore, the stimulation index of GLP-1 was lower in obese children indicating an attenuated response to nutrient intake, which is in agreement with previous reported results in obese girls (128). The lowest AUC of GLP-1 was found in obese subjects with T2DM (paper III).

The metabolic role of glicentin is under examination. The hormone has been shown to stimulate insulin secretion in canines (134). Fasting glicentin levels was previously reported to be similar between adults with diabetes and normal glucose levels (204). We found no difference in fasting glicentin or total glicentin (AUC of glicentin during OGTT) between lean controls and obese subjects with NGT. The stimulation index was reduced in the obese group, however. On the other hand, both fasting and total glicentin was reduced in prediabetic obese subjects compared to obese subjects with NGT. In fact, of all hormones studied, including fasting glucose, glicentin levels at fasting were the best predictor of IGT (paper III). These results must be validated in larger cohorts, but could potentially be used as a fasting biomarker of prediabetes in children and adolescents with obesity.

Lastly, in obese subjects with T2DM we observe a shift to more pancreatically and less intestinally cleaved proglucagon, which has previously been observed in adults (195). This shift is also observed between lean subjects and obese NGT, indicating that the shift is occurring before glucose regulation deteriorates and could potentially also be used as a biomarker of T2DM in pediatric obesity. The shift in proglucagon-derived peptides is of major
metabolic disadvantage and in the progression towards T2DM, it has been shown in an animal experiment that alpha-cell are capable of secreting GLP-1, possibly in an attempt to improve glucose homeostasis (195).

5.5 Ectopic fat and the role of PFF

The distribution of fat and the metabolic activity of adipocytes are factors that contribute to metabolic disturbances as well as risk of CVD (205). With MRI the distribution of adipose tissue can be quantified (62). The method is radiation free and can therefore by used in pediatric studies (29, 30, 60, 70, 206). We found that all fat compartments, SAT, VAT, LFF and PFF, were elevated in subjects with obesity compared with non-obese subjects and that obese subjects with MetS had the highest PFF (paper IV). It has previously been shown that PFF is associated with beta-cell function in adults (67, 207) and in children (30), which prompted us to investigate this further. We found that PFF correlated with indices of beta-cell function and insulin resistance using simple regression models (paper IV). However, when multiple regression models were applied (controlling for BMI and other adipose tissue compartment), no association between either beta-cell function or insulin resistance could be established, which is in contrast to some previous studies but in agreement with other reports (69, 70). However, FFA levels may mirror the ectopic fat deposition and the best estimators of PFF has previously been shown to be VAT and FFA (70). Furthermore, FFA transport has been connected to play a vital role in ectopic fat deposition (183). Interestingly, it has been demonstrated that the beta-cell metabolism is disturbed in individuals with obesity, with beta-cells utilizing more FFAs and less glucose as substrate (95).

In our study, PFF could be detected in almost all subjects, reaching as high as 15% of the pancreas volume in some obese subjects (paper IV). Wong et al found in a large study on healthy adult volunteers that around 16% had infiltration of fat in the pancreas and that this was associated with adiposity, hyperlipidemia and insulin resistance (68). Infiltration of fat in the pancreas has previously been shown to correlate with age, but not BMI or VAT in adult subjects with and without T2DM (31). Interestingly, we find a correlation with BMI-sds for the whole study population, but when examining only the obese sub-cohort separately, no correlation was detected indicating that once obesity is overt other factors than body weight determine PFF. A similar relationship was found between PFF and liver fat, which previously have been closely associated to each other (208).
5.6 Centroids used for OGTT shape analyses

When intermediate time-points are measured during an OGTT, estimates of beta-cell function and insulin resistance can be calculated as well as the shape of the curve analysed. Using the time points of an OGTT glucose homeostasis, beta-cell function, insulin resistance and risk of both T2DM and gestational diabetes, both in adults (139-143, 209) and in children and adolescents (19, 32, 158) have been projected. Most commonly the glucose values of the OGTT curve have been used (19, 32, 139, 143, 158, 209, 210), while some have analysed the insulin curve (140, 142) or both the glucose and insulin curves (141). Several studies compare monophasic and biphasic curves, and conclude that monophasic curves are connected with the subject having more serious metabolic problems (32, 141, 158, 209, 211). The information in the measurements obtained during an OGTT forming the curve shape have been investigated as a predictor of prediabetes in youths (19) and diabetes in adults (139, 140).

We have in this thesis described a novel method of analysing the shape of OGTT curves using centroids, i.e. the geometric centre of the area. Using centroids, all data are included and it assigns the OGTT a value that can be compared between subjects and within a subject over time (paper V). The centroid generated by using concentrations for both glucose and insulin increased as the glucose tolerance deteriorated. Also, centroids where time was considered for both glucose and insulin curves were higher in subjects with prediabetes compared with obese with NGT (paper V). This indicates that as glucose regulation deteriorates, there is a shift in both the concentration and timing of glucose and insulin response curves. Combining the centroids in multiple regression analyses, a close association to 2h-glucose was found ($R^2=0.81$). These association coefficients are substantially better than standard measures of beta-cell function and insulin resistance that also can be obtained from the OGTT curve. The centroid method transforms an OGTT curve into coordinates, which can objectively be used when comparing curves between subjects or within a subject after repeated OGTTs. Further longitudinal studies are warranted to explore the method further with regard to diabetes risk.

5.7 Study limitations

The studies of this thesis were cross-sectional in design, which limits the capability to examine outcomes. Moreover, ethnicity and family history of diabetes were not included in the analyses, which may influence the results. In the translational study (paper II) we used human islets from deceased adults, which could behave differently than the islets of Langerhans in obese
children and adolescents. Moreover, only fasting palmitate was analysed, i.e. not measured during the OGTT. Another issue in the study designs was the difficulty in recruiting lean controls and the low prevalence of obese subjects with T2DM in the cohort.

5.8 Interpretation and perspectives

Obesity in childhood is linked to alterations in hormonal and FFA levels, not in the least insulin and glucagon, which are primarily responsible for maintaining glucose homeostasis. In subjects with severe obesity alpha- and beta-cells are hypersecreting, in particular when the glucose tolerance begins to deteriorate. This is accompanied by decreased levels of incretins and elevation of ectopic fat deposition and circulating palmitate. All these changes occur already in young children with obesity and measures need to be undertaken to prevent these manifestations and treat the obese children.

5.9 Clinical applicability and possible implementations

It is important that efforts are done to prevent onset of T2DM in children, in particular considering the increased risk of mortality at earlier age. In the present thesis levels of insulin, proinsulin, C-peptide, palmitate, glucagon, GLP-1 and glicentin were measured and used in clinical evaluation of the obese children. Potentially, fasting glicentin concentrations could be used as an indicator of glucose intolerance indicating that a more thorough investigation of the obese patient should be conducted. The centroid method assigns the OGTT curve a coordinate, which correlates with glucose tolerance and can facilitate precise comparisons between and within individuals.
6. Conclusions

6.1 General conclusion

The function of the islet is changed in children with obesity, which may partially be caused by altered levels of circulating palmitate and incretins. Pancreatic fat is elevated in children with obesity, which does not affect beta-cell function. Since changes in islet function can be detected by OGTT, it is imperative to conduct this test to identify subjects at risk of developing prediabetes and T2DM.

6.2 Conclusions of each paper

Paper I: Systematically investigating obese children and adolescents, such as in the ULSCO cohort, makes studies possible to address metabolic disease in these patients and obtain greater knowledge concerning childhood obesity.

Paper II: High palmitate levels are related to time-dependent changes in beta-cell function, which may put obese children at increased risk of developing prediabetes and T2DM.

Paper III: High fasting glucagon and low GLP-1 and glicentin levels, which in part reflect altered alpha- and L-cell function, increase the risk of T2DM in obese adolescents.

Paper IV: Increased PFF in obese children is not related to altered beta-cell function, but instead to MetS and rise in VAT. Once obesity is overt, PFF does not correlate with BMI-sds or liver fat in the pediatric population.

Paper V: The centroid efficiently summarizes the information of OGTT curves and can be used to detect changes in glucose homeostasis between individuals and within individuals conducting multiple OGTTs.

För att kunna studera förändringar i kroppen och i de Langerhansska öarna som uppkommer vid fetma startades en kohort vid Akademiska Barnsjukhuset i Uppsala som heter Uppsala Longitudinal Study of Childhood Obesity (ULSCO). Kohorten består idag av hundratals individer under 18 år, som följs upp över tid med olika undersökningar. De flesta lider av svår fetma, men även normalviktiga barn och ungdomar är inkluderade som kontroller. Nästan alla forskningspersoner genomgår en oral glukosbelastning (OGTT), som mäter hur kroppen hanterar ett standardiserat intag av sockerlösning. Under belastningen mätas även andra ämnen, såsom insulin, proinsulin och glukagon, för att undersöka förändringar i dessa nivåer. Flertalet ungdomar har även utfört en magnetisk resonanstomografi (MRT) undersökning för att analysera hur olika fettväv är lagrad i organ påverkas vid fetma och hur detta relaterar till funktionen hos de insulinproducerande beta-cellerna samt sockerbalansen i kroppen. En betydande del av barn och ungdomarna med fetma, som ingår i ULSCO kohorten, har glukosintolerans och nästan en tredjedel lider av det metabola syndromet med bukfetma, påverkat blodtryck och blodsök samt förhöjda blodfetter. Kohorten utgör
därmed en viktig resurs för att kunna studera utveckling av typ 2 diabetes tidigt i livet.


The work leading up to the completion of this thesis has spanned over many years. The research has mainly been conducted at the departments of Medical Cell Biology and Women’s and Children’s Health, Uppsala University, Sweden with several local and international collaborations. Over the years, numerous people have supported and helped me in different ways. I want to extend my deepest appreciation to all co-authors, colleagues, friends and family that made this work possible.

Professor **Peter Bergsten**, my main supervisor. You are a true inspiration and the reason I became interested in research. It has been a privilege to work with you during almost a decade, initially as an undergraduate student and later as a PhD student. I am thankful for your positive attitude and for always taking the time to discuss both scientific matters as well as personal issues.

Associate professor **Anders Forslund**, my co-supervisor. Your enthusiasm and guidance has meant a lot to me. Despite a busy schedule at the clinic, you always have time to discuss research matters during lunch or after hours. Your support and encouragement have been greatly appreciated.

Associate professor, **Joel Kullberg**, my colleague and dear friend. Thank you for all your invaluable advice and unconditional support. Our friendship means a lot to me and I consider you a great role-model for all young scientists. From late evenings discussing science to many hours on the tennis court, I truly appreciate all of it (=100%)!

My deepest thanks to several senior professors at Uppsala University, in particular **Håkan Ahlström, Arne Andersson, Jonas Bergquist, Per-Ola Carlsson, Ulf Eriksson, Jan Gustafsson, Erik Gylfè, Peter Hansell, Bo Hellman, Leif Jansson, Stellan Sandler, Anders Tengholm, Michael Welsh, Nils Welsh and Gunilla Westermark** for all your support and guidance.

Current and former Head of Department of Medical Cell Biology, Professor **Nils Welsh** and Professor **Erik Gylfè**, respectively. Current and former
Head of Department of Women’s and Children’s Health, Professor Agneta Skoog Svanberg and Professor Jan Gustafsson, respectively.

Thanks to all current and former colleagues and co-workers at the department of Medical Cell Biology, in particular: Ernest Sargsyan, Azazul Islam Chowdhury, Hannes Manell, Hjalti Kristinsson, Levon Manukyan, Jing Cen, Hanna Nyblom, Kristoffer Thörn and Kumari Ubhayasekera (at the department of Clinical Chemistry, Uppsala University). Without your support and friendship in the lab, during seminars and journal clubs I wouldn’t be were I am today.

Also my deepest gratitude towards the research team at the department of Women’s and Children’s Health especially Marie Dahlbom, Iris Ciba, Malte Lindström, Simon Forslund and Jakob Forslund. It is because of you that the ULSCO cohort is what it is today.

Thanks to all colleagues and friends in the Beta-JUDO consortium for wonderful collaborations and for hosting us many times in your hometowns.

I am very appreciative of all close friends I have made during my time as a PhD student, for the informal discussion regarding science as well as all non-scientific discussions, dinners and activities, especially Erik Wallin Öhman, David Berglund, Fanny Fredriksson, Daniel Espes and Carl-Johan Drott.

I want to extend my appreciation and gratefulness to Uppsala University and to Uppsala Läkarförening for funding part of my time as a PhD student. I want to thank Uppsala University Hospital for giving me the opportunity to combine research and clinical work within a forskar-AT position. Also thanks for all the support I have received by my current employer, the Psychiatric clinic at Uppsala University Hospital, in particular from my mentor Sofia Christopoulou.

My father Bo Staaf and my mother Liselotte Staaf, thanks for your love, support and for always believing in me. My brothers Magnus Staaf and Henrik Staaf, you are amazing siblings and great fun to be with. Never stop reminiscing! Also thanks to the support of my sister in law Wioletta Staaf. My grandmothers, Inga-Lill Larsson and Marta Spetz, you are both true inspirations and thanks for your support and for showing such interest in my research.

Thanks to my father in law Christer Brandt and my mother in law Inger Brandt for all your support. Also my brothers in law Fredric Brandt and Alexander Brandt as well as Julia Främberg; you guys are the best!
My wife, **Emmelie Staaf**, you are the love of my life, my best friend and the mother of our son **Sigvard Staaf**. Thank you for all support and being so encouraging during this whole time when I insisted to combine research with both medical school and later clinical work. I love you and dedicate this thesis to you.

The work presented in this thesis was made possible because of the following funding sources:

- European Commission FP7-project Beta-JUDO (grant 279153)
- European Regional Development Fund
- Swedish Medical Research Council
- Swedish Diabetes Association
- Uppsala Regional Research Council
- Gillbergska Foundation
9. References


80. Thorn K, Bergsten P. Fatty acid-induced oxidation and triglyceride formation is higher in insulin-producing MIN6 cells exposed to oleate compared to palmitate. *J Cell Biochem* 2010;111:497-507.


123. Yki-Jarvinen H. Non-alcoholic fatty liver disease as a cause and a consequence of metabolic syndrome. *Lancet Diabetes Endocrinol* 2014.


A doctoral dissertation from the Faculty of Medicine, Uppsala University, is usually a summary of a number of papers. A few copies of the complete dissertation are kept at major Swedish research libraries, while the summary alone is distributed internationally through the series Digital Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine. (Prior to January, 2005, the series was published under the title “Comprehensive Summaries of Uppsala Dissertations from the Faculty of Medicine”.)