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ROLE OF CIRCULATING LEVELS OF PALMITATE AND
PALMITOLEATE IN DEVELOPMENT OF BETA CELL
DYSFUNCTION AND INSULIN RESISTANCE IN
PEDIATRIC OBESITY

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Abstract

Obesity and T2DM show a significant relationship and are affecting increasing number of individuals. Obesity ($\text{BMI} > 30 \text{ kg/m}^2$) has been portrayed as a pandemic of the 21st century with a prevalence of more than 1 billion people. Juvenile obesity has increased considerably in the last decades and is linked to alternation in insulin levels. In these individuals early manifestations of beta cell dysfunction can be studied. We hypothesized that hypersecretion of insulin is predictive of future beta cell failure and precedes insulin resistance. Furthermore elevated levels of palmitate have adverse effects on beta cells, whereas high levels of palmitoleate have cytoprotective characteristics and promote insulin sensitivity. Using GC-MS, circulating levels of NEFAs were measured in 55 obese non-diabetic children. Fasting insulin, glucose and additional metabolic parameters were obtained. In addition, OGTT was conducted and allowed determinations of beta cell function and insulin sensitivity. To further examine palmitate's effects on beta cells, GSIS on human islet was measured after exposure to 0.5 mM palmitate for 0 (control), 1, 24 and 48 hours. Islet apoptosis was also measured after exposure to the fatty acid. In individuals with high palmitate levels, hypersecretion of basal and stimulated insulin levels was observed in the youngest subjects. Stimulated hypersecretion showed a significant decline with age, indicating a loss of beta cell mass and/or secretory function caused by ER-stress and oxidative stress. In vitro increased apoptosis was observed in islets after 48 hours culture with palmitate levels greater or equal to 0.5 mM. Islets displayed hypersecretion of basal and glucose stimulated insulin secretion after 1 hour, which was further accentuated after 24 hours. After 48 hours decreasing levels of insulin secretion were observed, however. Circulating palmitoleate levels correlated with high insulin sensitivity and low basal insulin levels, promoting metabolic deceleration and beta cell recovery. In conclusion, hypersecretion of insulin promotes anabolism, trapping young obese individuals in a vicious cycle, surging towards T2DM.

Abbreviations

ACC = Acetyl CoA Carboxylase
ATP = Adenosine Tri Phosphate
BMI = Body Mass Index
BSA = Bovine Albumin Serum
CPT 1 = Carnitine Palmitoyl Transferase 1
CRP = C - reactive protein
EDTA = EthyleneDiamineTetraacetic acid
ER = Endoplasmatic Reticulum
FABP = Fatty Acid Binding Protein
FAS = Fatty acid synthesis
FFA = Free Fatty Acids
FSH = Follicle Stimulating Hormone
GC-MS = Gas Chromatography-Mass Spectrometry
GLUT = Glucose Transporter
GnRH = Gonadotropin Releasing Hormone
GPR = G-Protein Receptor
GSIS = Glucose Stimulated Insulin Secretion
HDL = High Density Lipoprotein
IFG = Impaired Fasting Glucose
IGF-1 = Insulin Growth Factor-1
IGT = Impaired Glucose Tolerance
LDL = Low Density Lipoprotein
LH = Lutein Hormone
mM = mille Molar
mU/L = micro Units / Liter
MUFA = Mono Unsaturated Fatty Acids
NAD = Nicotinamide Adenine Dinucleotide
NEFA = Non Esterified Fatty Acids
NF- κ B = Nuclear Factor – κ B
OGTT = Oral Glucose Tolerance Test
PDH = Pyruvate Dehydrogenase
PERK = Pancreatic Endoplasmatic Reticulum Kinase
PFK = Phosphofructokinase
PPAR = Peroxisome Proliferator-Activated Receptor
SCD 1 = Stearoyl CoA Desaturase 1
SNP = Single Nucleotide Polymorphism
T2DM = Type 2 Diabetes Mellitus
TAG = Triglyceride
TNF- α = Tumor Necrosis Factor- α
UPR = Unfolded Protein Response
VLDL = Very Low Density Lipoprotein

1. Introduction

Obesity and T2DM – a “joint pandemic” of the 21st century

Obesity and type 2 diabetes mellitus (T2DM) is afflicting an increasing number of individuals on a global scale [1-3]. Obesity has been referred to as a pandemic of 21st century [4], not only affecting citizens of western countries, but also many people in countries in Africa and Asia [5]. It is estimated that more than 1 billion people suffer from obesity [4] and the prevalence is increasing rapidly [4-5]. During the first decade of the third millennium, the prevalence of obesity in Europe increased by 10% and is consuming a large proportion of healthcare resources [5]. Obesity has a complex etiology and affects individuals of all ages [4]. In recent decades a dramatic rise in childhood obesity has been observed [5]. In 2003, 22 million children worldwide, 5 years old or younger, were reported obese or overweight [4]. Numerous reports demonstrate a rising prevalence of paediatric obesity in Sweden the past decades [6-7]. However, recent data indicate that the trend in Sweden is levelling off [8]. Obese children have an augmented risk of future morbidity [4, 6] and obesity in itself can predict premature mortality and potentially counteract the current rise in life expectancy [9]. A strong relationship between obesity and prevalence of T2DM, not long ago considered a disease of the older generation, has been established [2]. WHO estimated that around 170 million people worldwide suffered from diabetes mellitus at the turn of the century and this number is expected to more than double by 2030 [10]. Up to 85% of children, who are diagnosed with T2DM, are obese or overweight [11]. Type 1 diabetes mellitus has been the most common type of diabetes observed in paediatric patients. With the growing obesity pandemic, T2DM is expected to surpass T1DM in children [2]. T2DM is the result of beta cell dysfunction and insulin resistance [12-15]. The exact mechanisms linking obesity with T2DM are not fully understood, however. In this study we propose that hyperinsulinemia, in essence hypersecretion of insulin by the beta cell, is particularly relevant for understanding early manifestation of T2DM in obese individuals. Also, we propose that early events of beta cell failure can be investigated by studying obese children.

Defining childhood obesity and the relevance of puberty

The general method of defining obesity is by using body mass index (BMI) [16]. In adults, the BMI is simply calculated by dividing the weight (kilograms) by the height (meters) squared, thus obtaining the unit kg/m^2 . According to international standards, overweight is defined as $\text{BMI} > 25\text{kg/m}^2$ and obesity as $\text{BMI} > 30\text{kg/m}^2$, respectively. Cut-off points are related to increased health risks. When applying the BMI formula on children and adolescents, adjustments have to be made with regard to age and sex (ISO-BMI) [16], since children undergo rapid growth and development, especially during puberty. During puberty a decrease in insulin sensitivity of approximately 30% has been observed in both lean and obese adolescents [17]. The mechanisms behind this are not clear, though an appealing theory is that insulin sensitivity is reduced as a consequence of a transitory rise of growth hormone (GH) during puberty. GH has been shown to affect lipolysis and the concentration of free non esterified fatty acids (NEFA) in the bloodstream [17], which could potentially fuel insulin resistance. Interestingly, the compensatory increase in insulin secretion by the beta cells was lower than expected, signifying an attempt of protecting and preserving beta cell function [17]. In isolated beta cells, GH has been shown to cause a rise in insulin secretion [18], enhancing anabolic pathways and promoting insulin resistance.

Cellular mechanisms of beta cell dysfunction

T2DM is a metabolic disease with four main characteristics: beta cell dysfunction accompanied or not with inadequate insulin action leading to hyperglycemia and obesity [19]. Hyperinsulinemia, caused by beta cell dysfunction, has been proposed as a potential primary cause of early progression towards T2DM [15]. T2DM is a disease that slowly develops over an extended time period. This is due to the fact that beta cells possess a great reserve capacity, which enables insulin output to be increased dramatically in times of nutrient excess [20]. It has been demonstrated both *in vitro* and *in vivo* that glucose potentiates beta cell proliferation [20]. This is achieved by increase in beta cell mass, mitoses of existing beta cells and *de novo* generation of beta cells from progenitor cell within the pancreas [20]. At the time of diagnosis of T2DM, a 25-50% reduction in beta cell-mass can be observed, suggesting initial compensation for the decline in number of beta cells by hypersecretion of insulin from remaining beta cells [20].

Beta cell dysfunction arises from environmental and genetic factors [21]. Environmental factors are primarily chronically elevated levels of glucose and lipids, which lead to harmful effects including endoplasmic reticulum (ER) and oxidative stress. This stress is caused by an increased metabolic demand, in terms of insulin secretion and biosynthesis, on beta cells (Fig 1.1) [22].

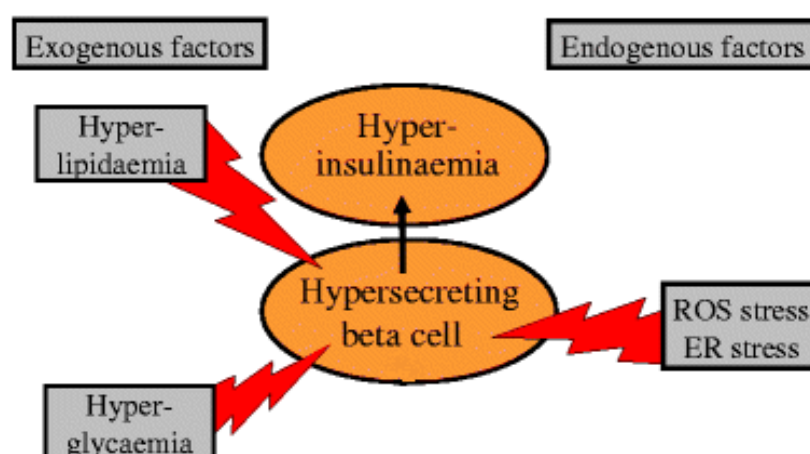


Figure 1.1 Hypersecretion of insulin is dependent on exogenous factor (hyperlipidaemia and hyperglycaemia) and endogenous factors (oxidative stress and ER-stress). Adapted from Rustenbeck et al [22]

Hypersecreting beta cells are showing different manifestations of intracellular stress (Fig 1.1), which elicit defense mechanisms including the unfolded protein response (UPR) [23]. Over time, the growing secretory demand on beta cells in obese individuals will promote apoptosis, with a subsequent loss of total beta cell mass, however [22]. Loss in beta cell mass is a sign of incipient T2DM. The exact pathway between hypersecretion of insulin and T2DM is still to be defined. Possible explanations could be cellular fatigue or inability to handle oxidative and ER-stress [15, 24]. ER-stress occurs when the ER is burdened by excess protein synthesis [15]. Secretory cells, beta cells included, have a very capable ER and are therefore particularly sensitive to ER-stress [15]. Some actually refers to diabetes as “*misfolded protein disease*” [25], demonstrating the important role of the ER in beta cell function

[15]. Beta cells have a lot of ER transducer proteins, which main function is to intervene in case of ER-stress [15]. Pancreatic-ER kinase (PERK), which is a part of the UPR, senses protein folding status of the ER and can trigger “rescue cascades” involving phosphorylation of eIF2 α [25]. This will temporarily halt protein synthesis, in order to deal with misfolded proteins within the ER, thus reducing insulin secretion. *In vitro* studies have demonstrated the role of ER-stress in beta cell dysfunction, where a knockout of PERK lead to beta cell apoptosis and elevated glucose levels [26].

High glucose levels cause increased glycolytic flux, which results in generation of reactive oxygen species (ROS) within mitochondria, generating oxidative stress [15]. Oxidative stress causes intracellular damage, affecting multiple part of the cell including the DNA. Ca²⁺ levels are disturbed, which compromise the entire secretory machinery, and eventually the beta cell will undergo apoptosis [15]. *In vitro* studies show increased oxidative stress in beta cells cultured in a glucolipotoxic milieu [15]. Patients with T2DM show increased levels of oxidative stress markers as well as markers for ER-stress [15], indicating its significance in pathogenesis and pathology of T2DM.

Glucose and lipid metabolism

Elevated levels of glucose and fatty acids will fuel oxidative pathways. An intricate relationship between glucose oxidation and fat oxidation exists within cells, however [27]. In 1963, Philip Randle proposed a theory linking metabolism of glucose and fatty acids in the ‘*Glucose-fatty acid cycle*’ [28]. He acknowledged that relationship of glucose- and fatty acid metabolism is reciprocal, hence elevated influx of fatty acids would lead to increased ratios of acetyl CoA/CoA and NADH/NAD⁺ in the mitochondria. This would result in decreased activity of pyruvate dehydrogenase (PDH) and elevated levels of citrate in the Krebs cycle [29]. High levels of citrate inhibit phosphofructokinase (PFK), a key regulatory enzyme in the glycolytic pathway. By inhibition of PFK, fructose-6-phosphate will accumulate within the cell, which will inhibit hexokinase and lead to reduced levels of glucose flux [29].

The main function of beta cells is the biosynthesis and secretion of insulin, which is tightly regulated by ambient glucose levels. High levels of glucose will cause increased glucose oxidation and lead to elevated levels of malonyl-CoA. Malonyl-CoA is a key building block in fatty acid biosynthesis (Fig 1.2). In addition, malonyl-CoA inhibits β -oxidation by suppressing the rate limiting enzyme carnitine palmitoyltransferase 1 (CPT1), thus preventing NEFAs from being transported into the mitochondria and undergo β -oxidation (Fig 1.2) [27]. Regulation and activity of CPT 1 play essential roles in maintenance of viability and secretory function in beta cells. Elevated levels of NEFAs, in particular palmitic acid (palmitate; C16:0), in combination with impaired CPT1 activity lead to intracellular accumulation and synthesis of lipids, which eventually precipitate beta cell dysfunction and apoptosis [27]. In contrast, it has been shown that via activation of GPR40 and subsequent peroxisome proliferator-activated receptor (PPAR)-signaling, NEFA actually stimulate CPT1 gene expression [3]. This would result in an increased β -oxidation, thus reduced intracellular levels of lipids.

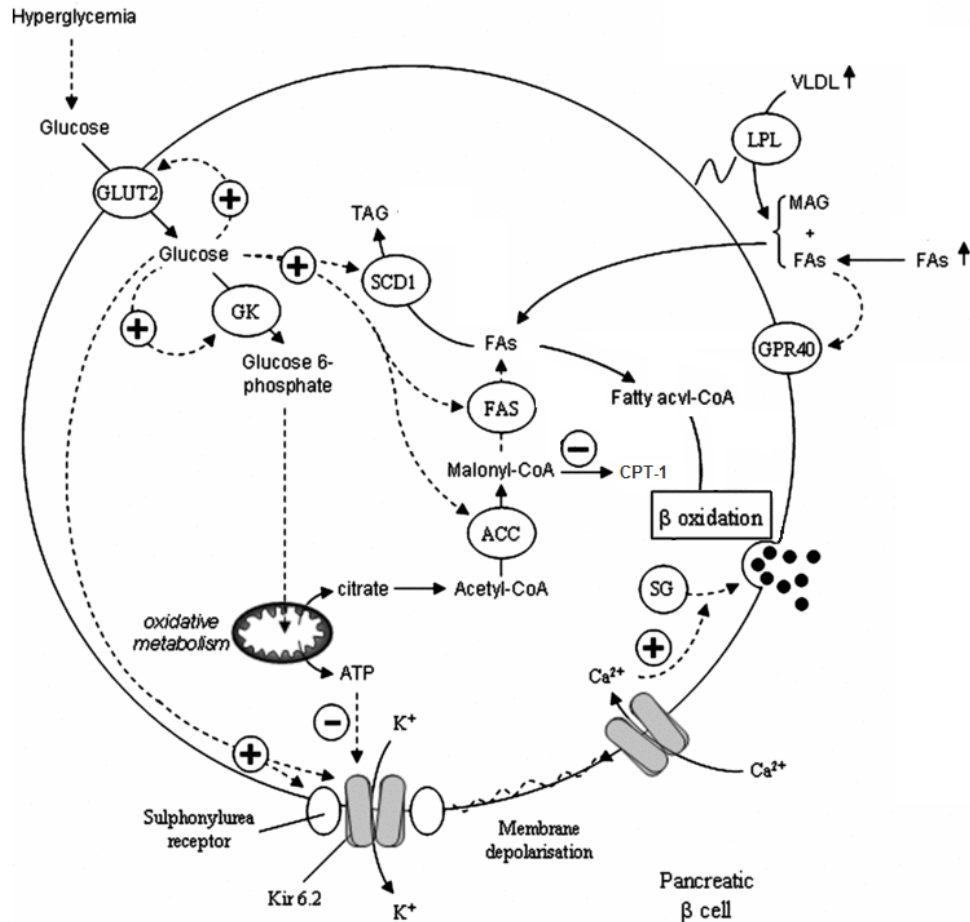


Figure 1.2 Increase in glucose oxidation causes depolarization of beta cells and insulin release but also lipid accumulation. High glucolytic flux will stimulate acetyl-CoA carboxylase (ACC), which will biosynthesize malonyl-CoA from acetyl-CoA. Malonyl-CoA promotes lipid biosynthesis and TAG formation and inhibits CPT 1, the rate limiting enzyme in β -oxidation. This will have adverse effects on the beta cells by accumulation of intracellular lipids, a concept known as lipotoxicity. Adapted from Sugden, Holness [30].

Consequences of insulin hypersecretion

A prominent feature in obese children is elevated level of fasting insulin [31], which promotes accumulation of glucose and lipids and consequently further development of obesity [32]. Chronically elevated basal insulin levels have negative effects on glucose stimulated insulin secretion (GSIS), where reduction in the number of insulin vesicles present for instant release has been proposed as one mechanism [33]. Furthermore, chronically elevated basal insulin levels will lead to desensitization and internalization of peripheral insulin receptors [33], which will be compensated for by increased insulin release and further rise in basal insulin levels. An interesting theory is that high basal insulin levels also desensitize insulin receptors on beta cells, resulting in attenuated glucose sensitivity and impaired feedback regulation, which will result in insufficient first phase insulin secretion [33].

Factors that determine if an individual will hyper- or hyposecrete insulin at a given insulin sensitivity are not fully understood [33]. Many potential mechanisms have been suggested, including serum NEFA composition and concentration [34], insulin clearance rate [35], secretory signaling pathways [36], insulin receptor expression on beta cells [37] and alternations parasympathetic activity directed towards the pancreas [38]. Genetics has been shown to play a crucial role in

predisposing dysinsulinemia; the gene coding for the pancreatic beta cell glucose carrier, GLUT2, has been identified as a strong candidate. GLUT2 is involved in regulating stimulated insulin secretion and a mutation in this gene trigger onset of diabetes mellitus [33]. The majority of known single nucleotide polymorphisms (SNP) linked to obesity and T2DM are connected with beta cell function [21].

Role of non esterified fatty acids on beta cell function

Long-term exposure of beta cell to NEFAs, such as palmitate, causes beta cell apoptosis [39-40]. We wanted to examine the effects of palmitate in obese children and on islets *in vitro*. The term lipotoxicity is commonly used when addressing lipid's toxic effect on a cellular level. The loss of secretory function and viability of beta cells, when exposed to NEFAs for an extensive period of time, plays an important role in the development of T2DM [40]. However, the effects NEFAs have on beta cells depend on length and degree of saturation of the NEFA. Differences in beta cell response to long chain saturated or mono unsaturated fatty acids have been documented, indicating that they work through different intracellular mechanisms [40]. Cells treated with high levels of palmitate display morphological alterations in membrane structures within the cell, most likely the ER, manifestations of ER-stress, loss of viability and apoptosis [24, 41]. Moreover, experiments on isolated islets show that prolonged (24 hours) levels of elevated palmitate induce rise in basal insulin secretion [42], which is detrimental to the metabolic homeostasis. On the other hand, when exposing beta cells *in vitro* to mono-unsaturated fatty acids like palmitoleic acid (palmitoleate; C16:1), much less destructive effects on the cells are observed [40]. Thus, palmitoleate appears to mediate positive effects, or at least counteract the negative apoptotic effects of palmitate, when the two NEFAs are administered together [40]. Moreover, palmitoleate averts steatosis of the liver in mice, possibly by lowering the levels of lipogenic enzymes in the liver [43-44]. This endocrine communication between adipose tissue, myocytes and hepatocytes reflects the general action of a hormone. Indeed, palmitoleate has been referred to as a lipid hormone or 'lipokine' [43].

Role of non esterified fatty acids on insulin resistance

In the present study we set out to examine the effects of palmitate and palmitoleate on insulin resistance in obese individuals. Elevated levels of circulating NEFAs have been associated with development and progression of insulin resistance and impaired glucose tolerance although the exact mechanisms are poorly apprehended [29]. Possible explanations are effects exerted on the cellular plasma membrane and, perhaps more importantly, NEFAs' capability to operate as signalling molecules within cells [45]. It has been shown that palmitate aggravate glucose homeostasis by causing insulin resistance [29]. In contrast, palmitoleate has been linked to improved insulin sensitivity *in vitro* [29] and in adult humans, independent of age, sex and adiposity [43-44]. The function of glucose transporter (GLUT) 4 is dependent on insulin and is found in the plasma membrane of adipocytes, hepatocytes and skeletal muscle cells. Both palmitate and palmitoleate appears to have direct effects on insulin signalling and insulin dependent transport of glucose by GLUT4 [29]. It has been shown that palmitoleate's effect is partly mediated by recruitment of GLUT4 to the plasma membrane and retention of GLUT4, thus preventing down regulation of the important glucose carrier by internalization [29]. Fatty acid binding protein (FABP) 4 and 5, lipid chaperons in control of lipid traffic within cells [43], have both been found to repress palmitoleate synthesis [44]. The actions of FABP incorporate

lipolysis and initiation of inflammatory pathways in adipocytes, mostly located around the central viscera [44]. Data from animal models show that mice with a knockout of aP2, the gene that codes for FABP, do not develop insulin resistance when they become obese. Moreover, the expression of tumour necrosis factor- α (TNF α), a cytokine released from adipose tissue and related to obesity-induced insulin resistance, is impeded in the absence of FABP [45].

Hypotheses

The aim of the present study was to elucidate the roles of palmitate and palmitoleate in the development of beta cell dysfunction and insulin resistance observed in young obese individuals. NEFA-related mechanisms of beta cell dysfunction were also addressed in isolated islets of Langerhans, obtained from deceased non diabetic donors.

We hypothesized that:

- (1) Elevated levels of palmitate in obese individuals precipitate beta cell dysfunction, displayed by early hypersecretion of insulin followed by beta cell failure.
- (2) High levels of palmitoleate counteract hypersecretion of insulin and improve insulin sensitivity, hence attenuating the detrimental anabolic and metabolic drive in obese children.

2. Material and methods

Study population

Study subjects consisted of 55 children suffering from obesity (ISO-BMI > 30 kg/m²), who had been referred to the overweight clinic at the Academic Children's Hospital in Uppsala. The individuals were part of the larger cohort Uppsala Longitudinal Study of Childhood Obesity (ULSCO) for which ethical approval was obtained 2010. Inclusion criteria for the study population were age under 18 and ISO-BMI > 30 kg/m².

Exclusion criteria were manifest type 1 or type 2 diabetes mellitus. Parents had together with the child filled in an extensive anamnesis form, covering physical exercise, eating habits, heredity etc. Presently, ULSCO is expanding, taking a leading part in a large European collaboration between pediatric, biomedical and industrial partners in Uppsala, Sweden; Cambridge and Manchester, UK; Leipzig and Mainz, Germany; Vienna, Austria; Geneva, Switzerland and Espoo; Finland. This joint effort is called Beta-JUDO (Beta cell function in JUvenile Diabetes and Obesity) and aims at clarifying the role of early insulin hypersecretion for the development of juvenile obesity and diabetes.

Sampling

Blood sampling was conducted following a 12-hour overnight fast. A standard winged infusion set with a "butterfly needle" was used. Before the venipuncture, patients were electively treated with local anesthesia by applying an EMLA-plaster one hour beforehand. For measurements of clinically relevant parameters, blood was collected in test tubes and sent for analysis at the Academic Hospital laboratory. After these samples had been collected, blood was obtained using an EDTA test tube for analysis of NEFAs. The test tubes were subsequently centrifuged; plasma was thereafter carefully collected using a syringe, aliquoted, coded and stored in -70 °C. Additional parameters measured and utilized in this study include insulin, glucose, HbA1c, C-peptide, cholesterol, triglycerides (TAG), low density lipoprotein (LDL), high density lipoprotein (HDL), C-reactive protein (CRP), albumin, follicle stimulating hormone (FSH), luteinizing hormone (LH), testosterone, estrogen and insulin growth factor-1 (IGF-1).

Measurements of body mass index (BMI) and body composition

Weight and length were measured in order to calculate BMI. Body composition was measured by multiple techniques including bioelectrical impedance, caliper and BodPod. These results were used to estimate total body fat percentage.

Oral glucose tolerance test (OGTT)

A standard 75g-OGTT was conducted during the morning. Baseline blood samples were drawn from a vein catheter. The patients were instructed to drink a 75g glucose solution. Blood samples were collected at the following time points: 30, 60, 90 and 120 min. All blood samples were analyzed with regard to insulin and glucose. Hemolysis in blood sample generated a false low value and such samples were discarded. To include a patient's OGTT result in the study a maximum of one discarded sample was allowed. Moreover, the youngest subjects were not asked to perform OGTT. Thereby we were left with 33 subjects with OGTT measurements, which we could use for analysis. Fasting insulin and glucose values were obtained for all 55 subjects.

OGTT is a standardized test regularly used to estimate beta cell function and insulin resistance, since the dynamic changes in plasma insulin and plasma glucose over time reflect these two parameters well [46]. It is a useful diagnostic tool, when distinguishing between diabetes mellitus (DM), impaired glucose tolerance (IGT) and impaired fasting glucose (IFG) [10]. Patients with IFG have a baseline (fasting) glucose level of 6.1-6.9 mM. IGT is defined as a baseline glucose level of <7 mM and a glucose level at the 120 min time point of 7.8-11.1 mM. The diagnosis diabetes mellitus is given to patients with a baseline glucose level of >7 mM or a 120 min glucose level of >11.1mM (Table 2.1) [10, 31].

<i>Diagnosis</i>	<i>Criteria</i>
Diabetes Mellitus	Fasting plasma glucose ≥ 7.0 mM <i>or</i> 2h plasma glucose at a standard 75g-OGTT of ≥ 11.1 mM.
Impaired Glucose Tolerance	Fasting plasma glucose < 7.0 mM <i>and</i> 2h plasma glucose at a standard 75g-OGTT of ≥ 7.8 mM and < 11.1 mM.
Impaired Fasting Glucose	Fasting plasma glucose ≥ 6.1 mM and < 7.0 mM <i>and (if measured)</i> 2h plasma glucose at a standard 75g-OGTT of < 7,8 mM.

Table 2.1: Diagnostic cut-off points of diabetes mellitus, impaired glucose tolerance and impaired fasting glucose according to WHO [10].

Measurements of NEFAs

Chemicals

Standard samples of fatty acids and internal standard were obtained from Larodan Fine Chemicals AB (Malmö, Sweden). Dimethylamine and Deoxo-Fluoro reagent (bis(2-methoxyethyl)amino-sulfur trifluoride) were bought from Sigma-Aldrich (Stockholm, Sweden). Additional chemicals and solvents were bought from Merck Eurolab AB (Stockholm, Sweden), unless stated otherwise.

Extraction of lipids

A slightly modified version [47] of lipid extraction according to Dole [48] was used. Frozen plasma samples were placed in room temperature for 30 min to thaw. Plasma (300 μ L) together with 12.8 μ g of internal standard (C17:0) were thoroughly vortexed for 40 min with 3 mL of organic solvent mixture, consisting of iso-propanol, heptane and 1M HCl (vol. 40:10:1 mL). Thereafter, samples were incubated at room temperature for 10 min. H₂O (2 mL) was added to samples and plasma lipid were extracted into 4 mL of heptane. During extraction, lipids were protected against oxidation by supplying the organic solvent mixture with 0.05 mg/mL butylated hydroxytoluene (BHT). The heptane was dried under nitrogen and lipids were thereafter dissolved in 200 μ L of dichloromethane (CH₂Cl₂). All samples were stored at -20°C until further derivatization.

Derivatization of NEFA

Diisopropylethylamine (10 μ L) and dimethylamine (30 μ L) were added to lipids dissolved in CH_2Cl_2 (200 μ L). The mixture was put on ice for 15 minutes. Then 10 μ L bis(2-methoxyethyl)amino-sulfur trifluoride (DEOXO) was added and the test tubes were vortexed for 30 seconds. Test tubes were then kept at 0°C for 30 min. Samples were incubated in room temperature for 10 min. Water (2 mL) and 4 mL (2 \times 2) of heptane were added and mixed thoroughly. Samples were then centrifuged at 1000 g for 10 min in two steps. Finally, the upper heptane layer was collected and evaporated under nitrogen. Dried amide derivative was dissolved in 200 μ L of heptane prior to analysis by GC-MS.

Identification of NEFAs by GC-MS

Analysis of NEFA was performed using a 8000 Top Series gas chromatograph (GC) and a AS800 autosampler (CE Instrument, ThermoQuest Italia S.p.A., MI, Italy) coupled to Voyager mass spectrometer (MS) with Xcalibur version 1.2 software (Finnigan, ThermoQuest, Manchester, UK). Separation of NEFAs was conducted on a non-polar capillary column, DB-5MS (J&W Scientific, Folsom, CA, USA), 30 m \times 0.18 mm \times 0.18 μ m film thickness. As gas carrier, helium was used at an inlet pressure of 80 kPa. The volume of 1 μ L of each sample was injected into the GC at an injection temperature of 250°C. During the first minutes the oven temperature was 130°C, then raised to 270°C at a rate of 50°C/min for 10 min and finally the temperature was increased to 290°C at a rate of 1°C/min for another 10 min. The mass spectra were recorded at electron energy of 70 eV and the ion source temperature was 200°C. The spectra were scanned in the range 50-500 m/z. NEFAs identification was done by comparing mass spectra of standard samples of amides of fatty acids with our samples. Quantification of NEFAs was done by using heptanoic acid (C17:0) as internal standard, with a retention time of 19.55 min (Fig 2.1).

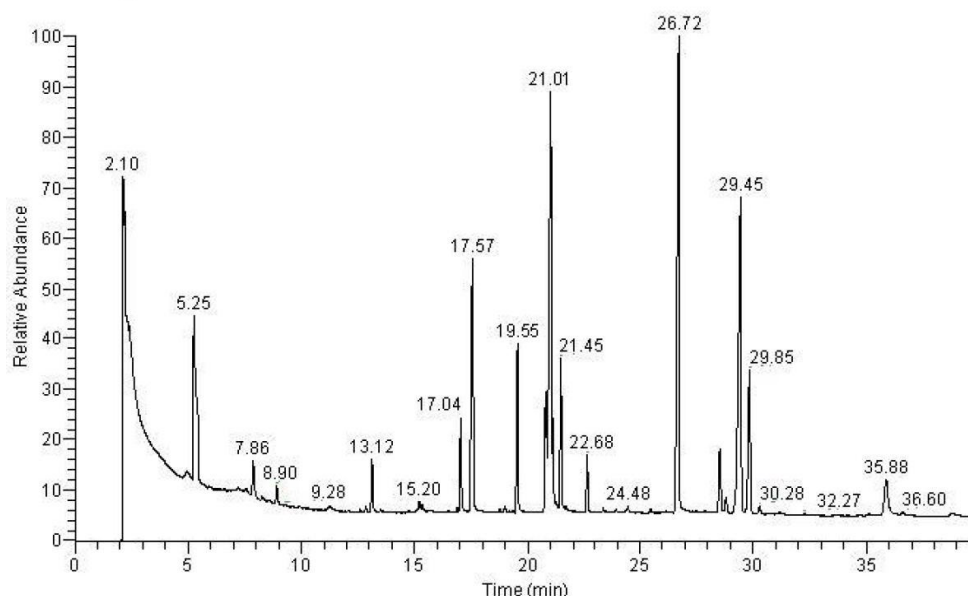


Figure 2.1: Chromatogram from plasma obtained from one study subject. Heptanoic acid was used as internal standard, with a retention time of 19.55 min. Palmitate can be identified as the peak with a retention time of 17.57 min. Palmitoleate has a slightly lower mass than palmitate and is represented by the peak to the left with a retention time of 17.04 min.

Measurements of insulin sensitivity

Insulin sensitivity is inversely proportional to insulin resistance and can be calculated in several different manners. Using the fasting values for insulin and glucose, the homeostatic model for assessment of insulin resistance (HOMA-IR) [49] was used to evaluate the peripheral insulin resistance at baseline in the patients. HOMA-IR is equivalent to $Insulin_0 (mU/L) \times Glucose_0 (mM) / 22,5$ [49]. Insulin sensitivity was also estimated using values from the OGTT. The insulin sensitivity index (ISI) proposed by Matsuda and DeFronzo was used; $10000/\sqrt{(Insulin_0 \times Glucose_0 \times Insulin_{mean} \times Glucose_{mean})}$ [50]. This index is referred to as ISI-composite.

Measurements of beta cell function

To evaluate pancreatic beta cell function, homeostatic model for assessment of β -cell function (HOMA- β) [49] and the Insulinogenic index was used. HOMA- β is calculated by $20 \times \text{fasting insulin} (mU/L) / \text{fasting glucose} (mM) - 3.5$ [49]. The Insulinogenic index is derived from the basal and 30 min values of an OGTT and is calculated by $Insulin_{30} - Insulin_0 / Glucose_{30} - Glucose_0$ [51].

In vitro experiments on human islets

Cell culture

Human islets, harvested from diseased non-diabetic donors, were cultured at 37°C with 5% CO₂ in CMRL 1066 medium (5.5 mM glucose), obtained from Sigma. Media was supplemented with PEST (Penicillin 100U/mL and Streptomycin 100µg/mL) and 2 mM glutamine. Ethical approval for the islets studies had been granted by the regional ethics committee in Uppsala.

Palmitate medium and islet treatment

Stock solution of palmitate medium (100 mM) was prepared by dissolving palmitate (Sigma) in 50% ethanol. Stock solution was then used to obtain a culture medium of 0.5 mM palmitate. BSA (0.5%) was added and medium placed in 37°C water bath for 30 min. In each well 5 islets were placed and cultured in the presence of 0.5mM palmitate for 0 (control), 1, 24 and 48 hours. Islets were also cultured for 48 hours with different concentration of palmitate, which were prepared from stock solution. Concentrations of palmitate used were 0 (control), 0.25, 0.5 and 1.0 mM.

Glucose-stimulated insulin secretion (GSIS)

After culture, GSIS [52] was performed on cells, cultured at 0.5 mM palmitate for different time periods. Prior to GSIS standard KRBH consisting of 130 mM NaCl, 4.8mM KCl, 1.2 mM MgSO₄, 1.2 mM KH₂PO₄, 2.5 mM CaCl₂, 10 mM HEPES, 5.0 mM NaHCO₃ and 0.1% BSA was prepared. The pH was adjusted to 7.4 by 1 M NaOH. Stock solution of glucose (200 mM) was prepared by dissolving glucose in KRBH. The glucose stock solution was then used to make KRBH with 2 and 20 mM glucose, respectively. Medium was removed and cells were washed once with 2 mM KRBH. Then 1 ml KRBH (2 mM) was added to each well, and islets were incubated (37°C and 5% CO₂) for 1 hour. Subsequently, 0.5 ml of either 2 or 20 mM KRBH was added to wells and an additional 30 min incubation followed. Islets were then simultaneously placed on ice, to prevent further insulin secretion. KRBH (200 µL)

was transferred from each well and stored at -20°C until insulin quantification by ELISA [53].

Measurements of insulin secretion by ELISA

In brief, samples from GSIS were diluted appropriately with buffer containing 0.1% BSA. Levels of insulin were measured by ELISA [53]. Initially, buffer consisting of 125 mM NaCl, 5.9 mM KCl, 1.2 mM MgCl₂, 1.28 mM CaCl₂ and 25 mM HEPES was prepared and adjusted to a pH of 7.4. Phosphate buffer, consisting of 10.5 mM Na₂HPO₄ and 3.5 mM NaH₂PO₄, was prepared and used to dilute insulin antibodies (20 mL:1 µL). Buffer containing insulin antibodies (100 µL) was added to a 96-well plate and left sealed at 4°C overnight. In addition, blocking buffer was prepared by dissolving casein (1%) in the same buffer. The solution was stirred gently overnight.

On the following day the plate was room tempered and washed twice with washing buffer. Then, 100 µL blocking buffer was added to each well and the plate was incubated for 2 hours at 37°C. Insulin standard was prepared and pre insulin-peroxidase made by dissolving 0.12 g NaCl and 1.18 g BSA in 20 mL phosphate buffer. The solution was sealed and left overnight. Following the 2 hour incubation, the plate was room tempered for 30 min and then washed twice with washing buffer. Thereafter, the plate was left upside-down at 4°C for 25 min. Insulin standards and samples were added to the wells and left at 4°C overnight.

On the final day, the plate was room tempered for 30 min and washed twice with washing buffer. Next, plate was left upside-down at 4°C for 25 min. Insulin peroxidase (IP) buffer was prepared by adding IP to buffer (10 µL:10 mL). The buffer was added to each well and the plate was sealed and incubated at 4°C for 4 hours. Potassium-citrate was room tempered in the dark. Following incubation, the plate was room tempered for 30 min and washed twice with washing buffer. Substrate solution was prepared by mixing 20 mL potassium-citrate with 1 mL TMB. Then, 100 µL of substrate solution was added to each well and the plate was incubated in room temperature in darkness for about 35 min. Finally, absorption was measured at 370 nm using a spectrophotometer. Insulin levels were normalized to total protein, which was measured in each well by DC protein assay (Bio-Rad).

Measurements of apoptosis

Cell death detection kit ELISA^{PLUS} (Roche Diagnostics) was used to measure apoptosis, following instructions of the manufacturer. Levels of apoptosis were normalized to DNA content, which was determined by measuring absorption (260 nm) of cells lysed in ddH₂O.

Statistical analysis and data presentation

Data are presented as means ± SEM. All analyses of statistics were conducted using GraphPad Prism 5. Linear regression was used to investigate correlations between data. Unpaired t-test was used to compare differences between groups. Statistical significant was considered when $P < 0.05$.

3. Results

Subjects characteristics

Individuals participating in the present study suffer from pediatric obesity and belong to the Uppsala Longitudinal Study of Childhood Obesity (ULSCO) cohort. The number of subjects was 55 with mean age of 12.4 ± 0.5 years and age distribution ranging from 3 to 17 years (Fig 3.1). Out of the 55 subjects 33 were males and 22 were females. The age of the males ranged from 8 to 17, while the age of the females ranged from 3 to 16.

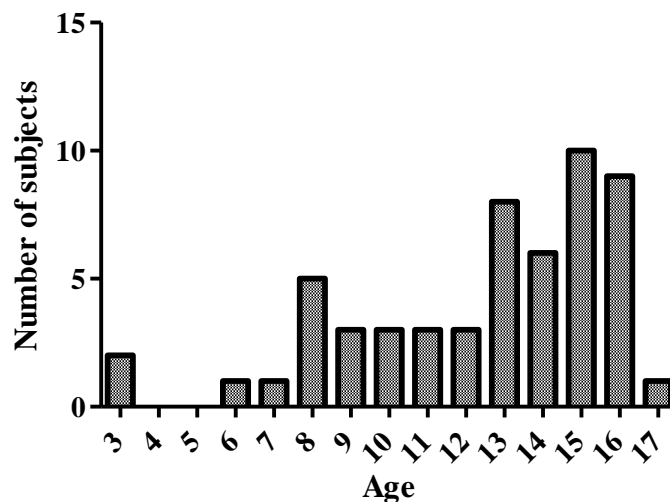


Figure 3.1 Age distribution in study population.
Results expressed as the number of individuals at different ages. Mean age was 12.4 ± 0.5 (N=55)

Sex hormonal alterations affect insulin sensitivity [12, 17]. Hence, subjects were also categorized based on whether or not they had reached puberty. This was conducted by measuring circulating levels of FSH and LH, which were obtained in approximately 90% of the subjects and are in combination used to assess the onset of puberty. Using reference interval for pre-puberty and puberty levels of FSH and LH, 21 children were categorized as pre-puberty, while 34 children were in puberty. In the pre-puberty group, there were 8 females and 13 males with a mean age of 9.3 ± 0.7 . In the puberty group there were 14 females and 20 males with a mean age of 14.4 ± 0.3 .

Anthropometric measurements including BMI, waist circumference and body fat percentage were obtained for each subject (Table 3.1). There were no significant differences in mean age, BMI or waist circumference between males and females. Females showed a significantly higher body fat percentage ($P < 0.0001$) in comparison with males, which was expected considering the difference in body composition between genders.

	All	Males	Females	Puberty	Pre-puberty
Number	55	33	22	34	21
Age	12.4±0.5	12.6±0.5	12.1±0.9	14.4±0.3	9.3±0.7
BMI (kg/m²)	34.3±0.8	33.3±0.9	35.8±1.5	36.5±0.9	30.7±1.1
Waist circumference (cm)	106.6±2.3	105.3±2.4	108.5±4.3	113.5±2.3	95.4±3.5
Body fat (%)	33.2±0.7	29.7±0.4	38.4±0.8*	33.7±1.0	32.2±0.9

Table 3.1 Study population characteristics. Results are presented as means ± SEM.

*P<0.0001 compared with males.

Fasting insulin and glucose levels in study subjects

Insulin values were measured in the young obese individuals, following an over-night fast. Levels were elevated almost 2.5-fold compared to reference values (<11 mU/L), but showed no gender- or puberty-related differences (Table 3.2). Hypersecretion of basal insulin was found in approximately 90% of the subjects. Large variability in insulin levels was observed with values ranging between 5.3 and 80 mU/L in the 55 subjects (Fig 3.2).

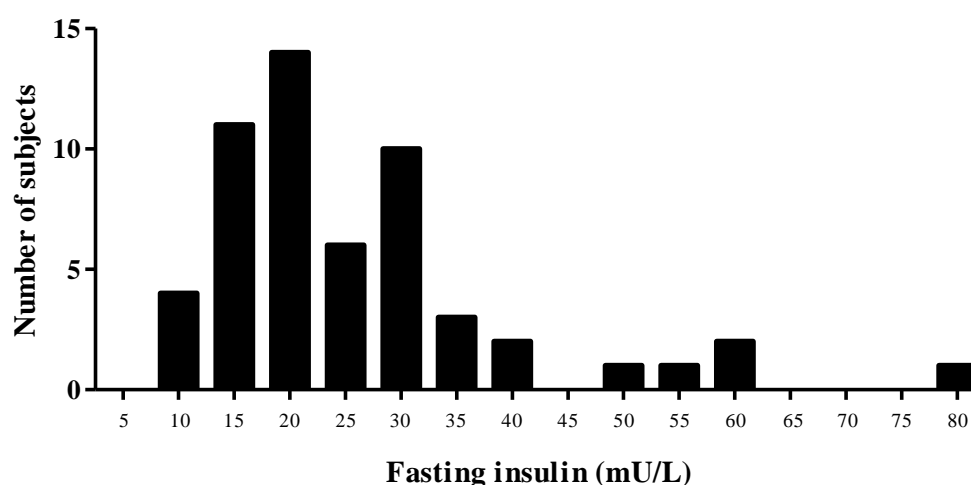


Figure 3.2 Fasting insulin levels in all subjects. Skewed distribution with a mean of 24.0±1.9. (N=55) Values on x-axis represent number of subjects with equal to or lower fasting insulin value.

The variable insulin levels were not correlated to variations in fasting glucose levels, which showed minor fluctuations. Moreover, all subjects had normal fasting glucose values (<6 mM) and normal HbA1c levels (4.0-5.9%), implicating that all study subjects were non-diabetic. However, two subjects displayed a disturbed glucose homeostasis during OGTT with a 120 min value between 7.8 and 11.1 mM. By definition they had impaired glucose tolerance and are at risk of progressing towards developing T2DM [31].

	All	Males	Females	Puberty	Pre-puberty
Basal insulin (mU/L)	24.0±1.9	24.4±2.6	23.3±2.9	24.6±2.3	22.9±3.4
Basal glucose (mM)	4.95±0.05	5.00±0.07	4.86±0.08	4.92±0.06	4.99±0.08
HbA1c (%)	4.47±0.04	4.47±0.05	4.49±0.07	4.42±0.05	4.55±0.07
C-peptide (nmol/L)	1.15±0.06	1.15±0.08	1.16±0.11	1.20±0.08	1.07±0.12

Table 3.2 Fasting insulin, glucose, HbA1c and C-peptide levels of study subjects. Basal insulin is increased almost 2.5-fold in comparison with reference value (11 mU/L). All other values are within reference range. Data are presented as means ± SEM.

Elevated insulin levels may be accounted for by reduced insulin clearance [54]. To address this possibility, C-peptide levels were measured (Table 3.2). C-peptide is formed by enzymatic cleavage of proinsulin and plays an important role in insulin synthesis. The peptide is then co-secreted with insulin [55]. A strong correlation between fasting insulin and C-peptide levels was observed (Fig 3.3) supporting the notion that insulin levels reflected variations in insulin secretion.

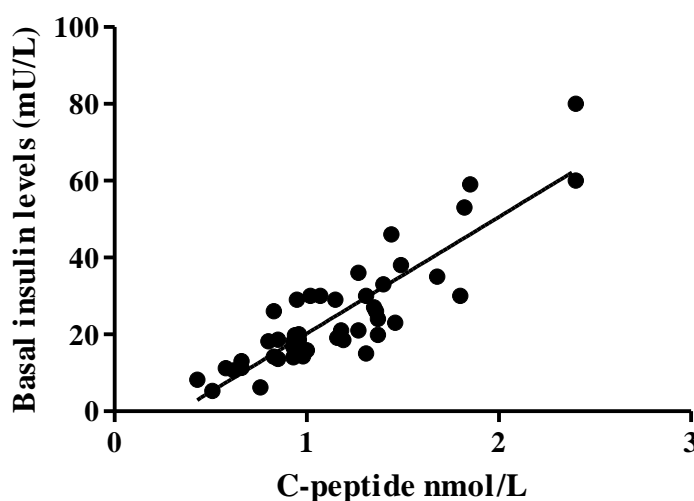


Figure 3.3 Correlation between basal insulin levels and C-peptide. Highly significant correlation ($P < 0.0001$) was obtained, indicating that levels of insulin represent secreted insulin. (N=46)

Insulin and glucose levels in study subjects after glucose challenge

In obese individuals, beta cell dysfunction is in addition to changes in fasting insulin levels, also manifested in altered insulin secretion upon a glucose challenge [31]. Whereas accentuated secretory response is evident in young obese individuals [31], decline in insulin response is observed later [51] precipitating development of T2DM. To address how insulin levels were altered in children with obesity, insulin responsiveness to elevated glucose levels was measured during an OGTT. The mean

glucose curve (Fig 3.4) was comparable with control individuals [31]. The corresponding insulin values were elevated at all time points (Fig 3.5), however, indicating hypersecretion of insulin.

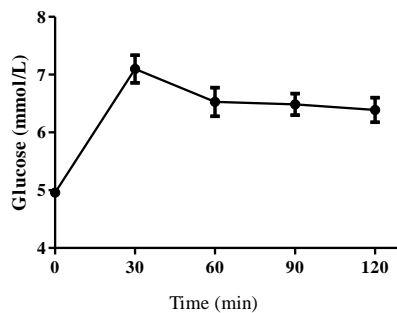


Figure 3.4 Glucose curve during OGTT. Curve based on means \pm SEM for study population that conducted OGTT (N=33)

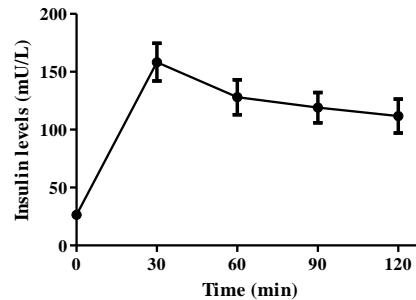


Figure 3.5 Insulin curve during OGTT. Curve based on means \pm SEM for study population that conducted OGTT (N=33). Insulin levels are above control at all time points.

Next, we investigated how insulin levels obtained at 30 min of OGTT varied with age. An age-dependent decline in the 30 min insulin value existed in the study subjects (Fig 3.6). Thus, young individuals both showed elevated basal and stimulated insulin levels (at the 30 min time point of the OGTT). Stimulated insulin levels were significantly lower in the group with individuals at age 15-16, comparing with the youngest age group, aged 8-9 (Fig 3.6).

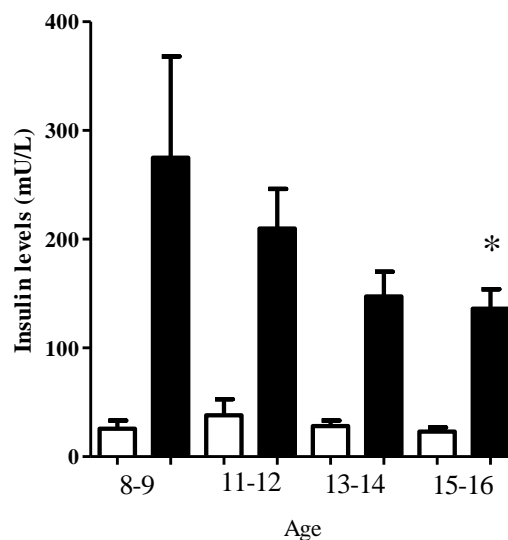


Figure 3.6 Study subjects arranged in groups according to age. White bars denote basal insulin levels and black bars stimulated insulin levels at 30 min during OGTT. N(8-9)=3; N(11-12)=4; N(13-14)=9; N(15-16)=14. The group 15-16 show a significantly lower stimulated insulin levels than group 8-9 * P<0.05

When the 30 min insulin value was plotted against the corresponding 30 min glucose value a significant correlation was obtained (Fig 3.7). The graph demonstrates that individuals with equal glucose levels had dissimilar insulin levels, however. Hence, additional factors than circulating glucose determine the 30 min insulin levels.

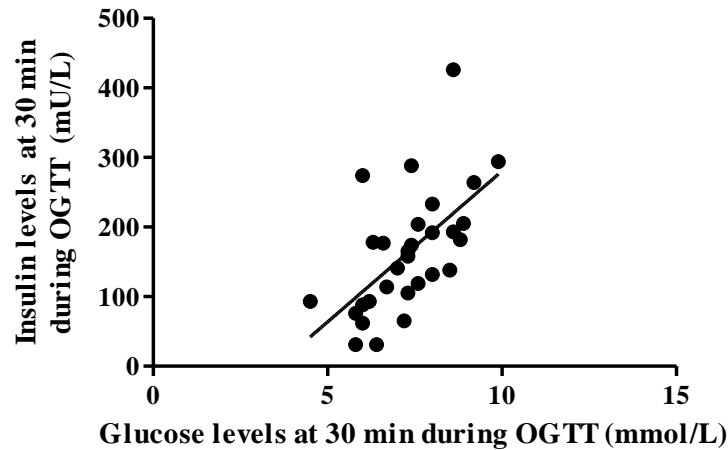


Figure 3.7 Insulin and glucose levels at 30 min during OGTT. Strong correlation was obtained ($P < 0.001$). However considerable variation observed within subject that have equal glucose levels, indicating that other factors contribute to variations insulin levels. (N=30)

When puberty was considered as such a factor influencing OGTT values, the pre-puberty group displayed a significantly higher ($P < 0.01$) insulin secretion at 30 min after ingesting the sugar load than the puberty group (Fig 3.8). The accentuated secretion of insulin observed in the pre-puberty group is most likely not explained by higher degree of insulin resistance since insulin resistance is elevated during puberty due to hormonal changes [17]. Instead, we investigated the role of free NEFA for the observed hypersecretion early in life and hyposecretion later in life in these young obese individuals.

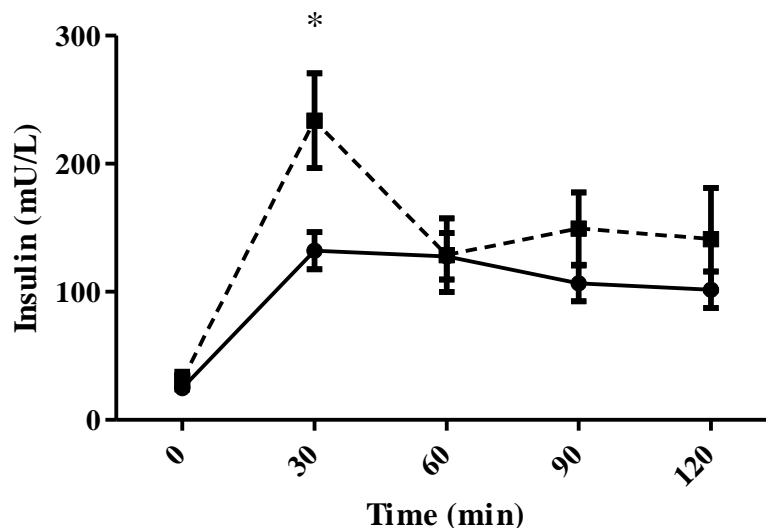


Figure 3.8 Insulin levels during OGTT Full line and round dots denotes puberty group (N=24), while dotted line and square dots denotes pre-puberty group (N=9). Data expressed as mean \pm SEM. * $P < 0.01$

Circulating palmitate and insulin levels

A rise in basal insulin levels is observed in islets exposed to elevated NEFAs during relatively extended time periods [42]. In particular, saturated fatty acids with long carbon chains have pronounced effects on insulin secretion [56]. Chronic elevation of lipids has been connected with fall in basal insulin secretion [42]. Also, the insulin secretory response to glucose is modulated, when circulating lipid levels are elevated. When the glucose challenge is performed following a short duration of elevated NEFAs, amplification of the secretory response is observed, where GPR40 signaling is a component part [3]. In contrast, reduction in the secretory response is observed if the glucose challenge is performed after prolonged, elevated levels of NEFAs, especially for saturated NEFA such as palmitate [24]. Furthermore, it is known that chronic levels of palmitate increase the proinsulin/insulin ratio within beta cells, indicating a secretory distress [57-58]. Although these short- and long-term effects of circulating fatty acids on glucose-induced alterations in circulating insulin levels are the results of many factors, the role of palmitate was addressed by investigating how fasting and 30 min following OGTT insulin levels in study subjects with low or high palmitate levels varied with age.

Palmitate is a prominent component of lipid emulsions provoking elevated insulin levels [36]. We hypothesized that levels of palmitate would be increased in young obese subjects showing rise in basal and stimulated insulin levels. Also, we proposed that a time-dependent reduction in insulin levels would be observed in individuals with long-standing elevated palmitate levels. The latter hypothesis is based on that prolonged periods of elevated levels of palmitate have been connected with impaired beta cell function [24]. Two assumptions were made. Firstly, the measured palmitate level for the individual is taken as representative of prevailing palmitate levels of that individual, in essence the palmitate concentration beta cells have been exposed to for a longer time period. Secondly, the age of the individual is an estimate for how long time the beta cells have been exposed to the measured palmitate level.

Levels of circulating palmitate were measured in the obese young individuals (Fig 3.9). A normal distribution was observed with a mean of 0.22 ± 0.01 . Similar to measurements in insulin levels, variability in palmitate levels was observed between the individuals ranging from 0.11 to 0.34 mM.

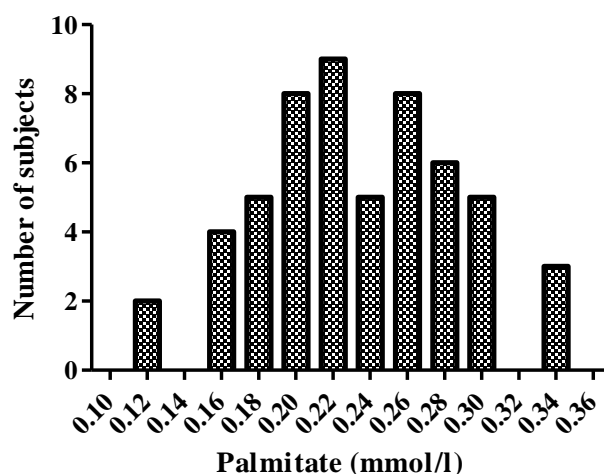


Figure 3.9 Distribution of palmitate in the study population.
Mean = 0.22 ± 0.01 mmol/L (N=55)

The study subjects were divided into two groups based on fasting palmitate levels. The divisor was the median value of 0.22 mM, dividing the individuals into two equally sized groups. Whereas individuals with low circulating palmitate levels had essentially age-independent 30 min insulin levels (Fig 3.10), age-dependent declining 30 min insulin levels were observed in individuals with high palmitate levels (Fig 3.11). Fasting insulin levels were normal in the youngest individuals with low palmitate, whereas levels were two-three fold elevated in the older individuals (Fig 3.10). In individuals with the higher palmitate levels an age-dependent decline in basal insulin levels was observed (Fig 3.11). Basal insulin levels were approximately three-fold higher in the youngest individuals with high palmitate compared to the youngest individuals with low palmitate levels. For stimulated insulin levels, a significant ($P<0.05$) decline was found in individuals with high levels of palmitate.

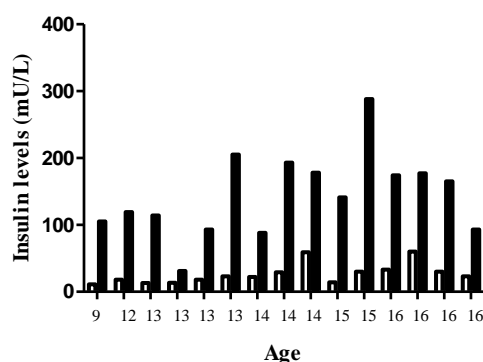


Figure 3.10 Individuals with low levels of palmitate (<0.22 mM) White bars represent basal insulin secretion. Black bars represent insulin secretion at 30 min of OGTT. (N=15) Subjects within the same age group are arranged according to individual levels of palmitate.

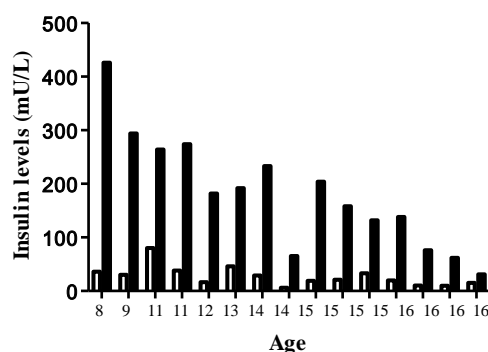


Figure 3.11 Individuals with high levels of palmitate (>0.22 mM) White bars represent basal insulin secretion. Black bars represent insulin secretion at 30 min of OGTT. (N=15) Subjects within the same age group are arranged according to individual levels of palmitate.

In addition, cholesterol, triglycerides, LDL, HDL, levels of additional NEFAs: palmitoleic, linoleic, oleic, stearic and docosahexaenoic acid, total NEFA and albumin were also determined (Table 3.3). Levels of NEFAs were also measured in 13 lean control individuals (data not shown). No significant differences in NEFAs levels between obese subjects and lean controls could be observed. Albumin was measured, because its ability to bind circulating NEFAs. No correlation was found between albumin levels and circulating NEFAs, suggesting that the pool of free NEFAs was not altered by albumin levels. No gender- or puberty-related changes in the lipid-related parameters were established.

	All	Males	Females	Puberty	Pre-puberty
Cholesterol (mM)	4.54±0.12	4.58±0.17	4.48±0.18	4.49±0.18	4.62±0.15
TAG (mM)	1.60±0.13	1.47±0.17	1.79±0.20	1.68±0.18	1.45±0.19
LDL [mM]	2.82±0.12	2.94±0.17	2.70±0.14	2.83±0.16	2.86±0.16
HDL [mM]	1.09±0.03	1.08±0.04	1.11±0.05	1.04±0.04	1.18±0.06
NEFA [mM]	0.772±0.026	0.776±0.033	0.765±0.042	0.761±0.033	0.789±0.042
16:1 <i>Palmitoleic acid</i>	0.047±0.003	0.045±0.03	0.050±0.005	0.048±0.004	0.046±0.03
16:0 <i>Palmitic acid</i>	0.229±0.007	0.234±0.08	0.222±0.013	0.223±0.009	0.240±0.012
18:2 <i>Linoleic acid</i>	0.077±0.004	0.078±0.06	0.076±0.06	0.075±0.004	0.081±0.008
18:1 <i>Oleic acid</i>	0.326±0.014	0.323±0.018	0.329±0.024	0.328±0.020	0.322±0.021
18:0 <i>Stearic acid</i>	0.075±0.003	0.079±0.004	0.068±0.05	0.069±0.003	0.084±0.005
22:6 <i>Docosahexaenoic acid</i>	0.017±0.001	0.016±0.001	0.019±0.002	0.018±0.001	0.016±0.002
Albumin [g/L]	40.3±0.4	40.6±0.5	39.9±0.7	40.7±0.5	39.7±0.8

Table 3:3 Lipid status at fasting measured in the young obese subjects. Data are presented as means ± SEM. No significant differences between males and females or the puberty and pre-puberty group were observed.

Palmitate-induced beta cell dysfunction

The function of the beta cell was further examined in isolated islets. Prolonged exposure (24 h) of islets to palmitate has been reported to be connected with elevated levels of insulin secretion at non-stimulatory glucose levels and reduction in glucose-stimulated insulin secretion [42]. In the obese young individuals in the present study elevated insulin levels at fasting and reduced insulin levels after OGTT were observed (Fig 10 and 11). However, the two secretory manifestations were typically not observed in the same individual. Instead elevated basal insulin levels in the present study were associated with normal or enhanced insulin levels after OGTT. Also, reduced stimulatory levels were observed in individuals with low fasting levels.

Manifestations of palmitate on beta cell function are time and concentration dependant. We therefore cultured human islets in the presence of palmitate for different time periods. After culture, GSIS was assessed by measuring insulin secretion at 2 and 20 mM glucose. Basal and GSIS was enhanced from islets cultured in the presence of palmitate for one hour (Fig 3.12). Elevation in basal and stimulated insulin secretion was also observed after 24 hours culture. After 48 hours culture basal and stimulated insulin secretion had declined, although they were still elevated compared to control islets. This partial normalization of the secretory pattern does not represent regain of function since enhanced apoptosis was observed in islets exposed to levels of 0.5 mM palmitate or above for 48 hours (Fig 3.13).

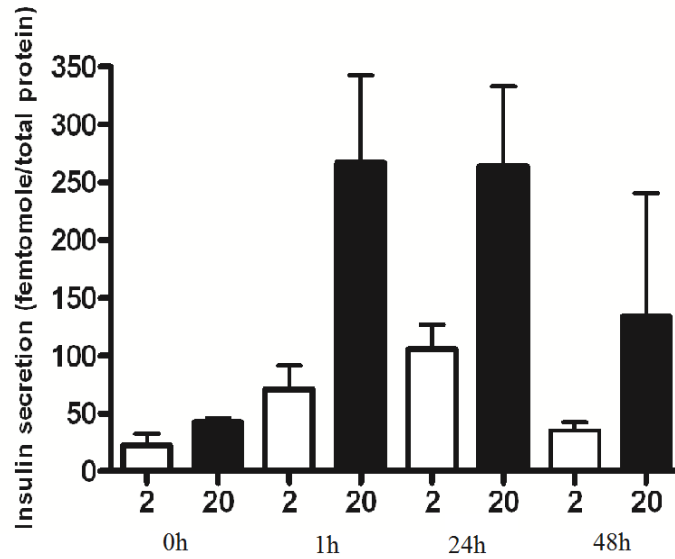


Figure 3.12 Glucose-stimulated insulin secretion from human islets exposed to 0.5 mM palmitate during different time periods. White bars represent basal insulin levels (2 mM glucose) and black bars insulin secretion after stimulation with 20 mM glucose. Results are means \pm SEM for 2 independent experiments.

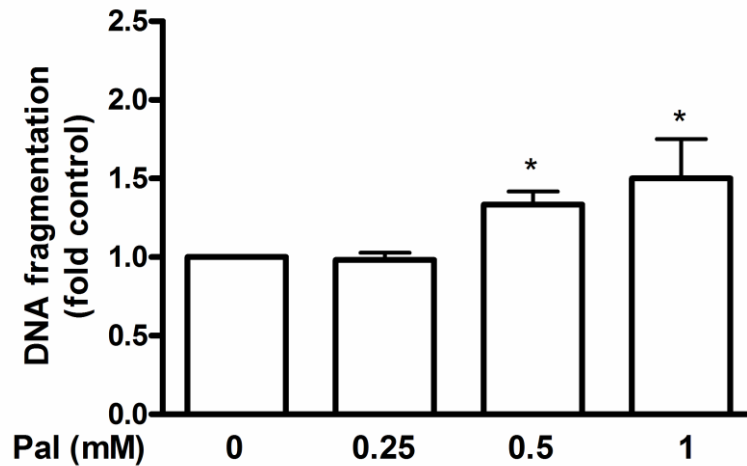


Figure 3.13: Apoptosis in human islets exposed to different concentrations of palmitate during 48 hours. Results are means \pm SEM of 5 independent experiments. * denotes $P < 0.05$ compared with control.

Fasting palmitoleate and insulin levels in study subjects

Palmitoleate, an omega-7 NEFA, is the mono-unsaturated counterpart of palmitate. Based on the documented effects of the mono-unsaturated fatty acid to counteract effects of palmitate in the beta cell [40], we hypothesized that palmitoleate would dose-dependently counteract fasting hyperinsulinemia. To examine if such a relationship existed fasting levels of palmitoleate were measured in the obese subjects. The measured palmitoleate levels in the fasting young obese individuals showed no gender- or puberty-related differences (Table 3.3) but varied substantially between individuals (Fig 3.14).

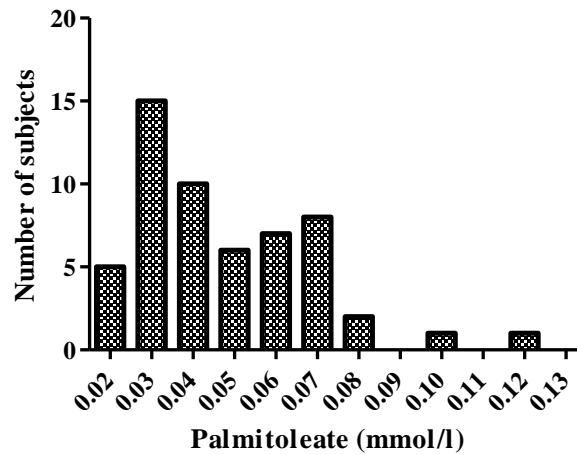


Figure 3.14. Distribution of palmitoleate in the study population.
Mean = 0.047 ± 0.003 mmol/L (N=55)

The study population was divided into 5 groups based on levels of palmitoleate, each group containing 11 individuals. When comparing the group with lowest levels of palmitoleate (0.025 mM) with the group with highest levels of palmitoleate (>0.065 mM), a significant attenuation in basal insulin levels was observed with rising palmitoleate (Fig 3.15).

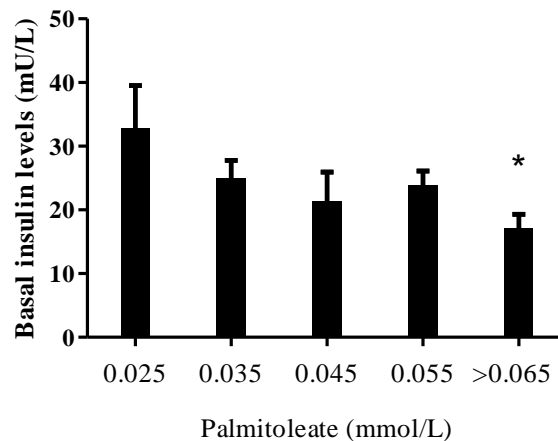


Figure 3.15 Correlation between basal insulin levels and palmitoleate. The study population (N=55) was divided into 5 groups, 11 individuals in each group. Numbers on X-axis represent approximate mean value of each group. Basal insulin levels was significantly lower with increasing palmitoleate ($P < 0.05$), comparing the group with lowest palmitoleate levels with the group with highest palmitoleate levels.

Beta cell function and insulin resistance

Fasting glucose and insulin values were used to estimate beta cell function and insulin sensitivity by the homeostatic model assessment (HOMA) [49]. Since no statistical difference existed between the puberty and pre-puberty groups with regard to fasting glucose and insulin (Table 3.2), all subjects were examined together. HOMA-IR is a validated method of examining insulin resistance, confirmed by the hyperinsulinemic-euglycemic clamp technique [59]. The mean values for HOMA beta cell function (HOMA- β) and for HOMA insulin resistance (HOMA-IR) were 349 ± 29 and $5.30 \pm$

0.44, respectively (Table 3.4). Normal values of HOMA-IR have been reported between 1.7 and 2.5 in healthy subjects [60], which demonstrates that the obese subjects in the study have a significantly elevated insulin resistance.

The OGTT values were used to calculate the Matsuda index (ISI-composite) (N=33) and the Insulinogenic index (N=30). The Matsuda index is a generally accepted method for calculating insulin sensitivity and the Insulinogenic index is useful for estimating early phase beta cell function (Table 3.4).

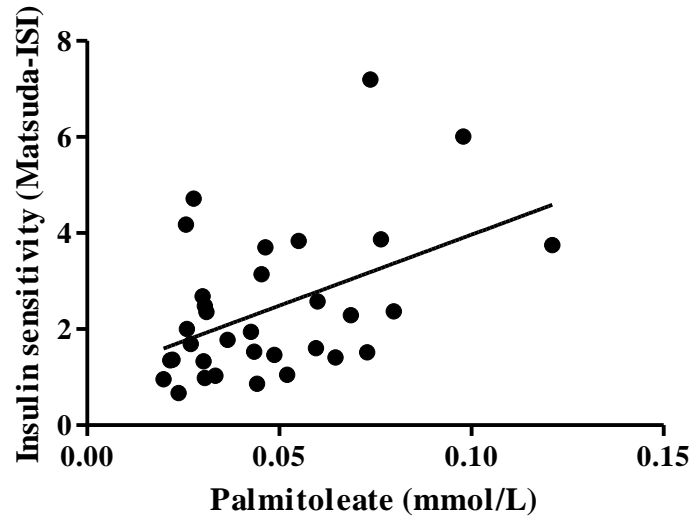
	Puberty	Pre-puberty	All
HOMA-β (%)	376 \pm 41 (N=34)	306 \pm 35 (N=21)	349 \pm 29 (N=55)
HOMA-IR	5.37 \pm 0.51 (N=34)	5.18 \pm 0.83 (N=21)	5.30 \pm 0.44 (N=55)
Insulinogenic index	58.7 \pm 7.0 (N=22)	83.1 \pm 31.3 (N=8)	65.2 \pm 9.7 (N=30)
ISI-composite	2.62 \pm 0.33(N=24)	1.86 \pm 0.37 (N=9)	2.42 \pm 0.27 (N=33)

Table 3.4 Beta cell function and insulin resistance of study subjects. Calculations of HOMA, Insulinogenic index and ISI-composite. Data are expressed as means \pm SEM.

HOMA- β is a measurement in percent of beta cell activity to maintain normal fasting glucose homeostasis. The subjects had a mean HOMA- β of 349%, indicating that the beta cells secrete substantial amounts of insulin at fasting to keep the blood glucose level within range. No correlation was found when the Insulinogenic index was plotted against HOMA-IR, which indicates that early phase insulin secretion is independent of basal insulin resistance. This confirms that there are multiple factors, which affect insulin secretion in addition to insulin resistance.

Palmitoleate and insulin sensitivity

Given the positive effect of palmitoleate to counteract negative effects of palmitate on the beta cell and the observed effect of the unsaturated fatty acid to lower fasting insulin levels, we proposed that palmitoleate would dose-dependently promote insulin sensitivity. Therefore, palmitoleate levels were plotted against ISI-composite. Significant correlation between circulating palmitoleate levels and insulin sensitivity was observed (Fig 3.16).



4. Discussion

The main finding and subsequent area of investigation in this study was the distinct basal hypersecretion of insulin in obese children. Basal insulin levels were measured with a mean of 24.0 mU/L, which was almost 2.5-fold above reference. A strong correlation between basal insulin and C-peptide was found, confirming that insulin levels do not reflect insulin recirculation, but are a direct result of beta cell secretion. Basal and glucose-stimulated insulin secretion (GSIS) levels were most accentuated in young individuals, with a following significant age-dependent decline in glucose stimulated insulin levels. Moreover, a steadily decreasing trend in basal insulin levels could be observed with age. Traditionally, insulin resistance has been described as the main reason behind insulin hypersecretion and subsequent beta cell failure, which eventually will precipitate T2DM. Reaven proposed in 1988 that elevated basal insulin levels merely are a reflection of insulin resistance [61], a concept which has been generally accepted over the years. It has been argued that elevated peripheral resistance of insulin acts as the main driving force behind hyperinsulinemia, with endocrine pancreas desperately attempting to compensate for the insulin desensitized target tissue [22]. Undeniably, a relationship between basal insulin levels and peripheral insulin sensitivity is present. However, only about 50% of variability in basal insulin levels between individuals could be explained by insulin sensitivity, suggesting that there must be other mechanisms present [33]. In this study insulin resistance (HOMA-IR) did not differ significantly between puberty and pre-puberty groups and did not correlate with the Insulinogenic index, hence other factors must contribute to the observed beta cell dysfunction.

Obese individuals have generally an increased nutrient load with elevated level of circulating NEFAs, which have been connected with beta cell dysfunction and secretory distress [52]. Chronically elevated levels of the saturated NEFA palmitate have adverse effects on the beta cell with repression of GSIS [3, 42]. In contrast, short term palmitate exposure accentuates both stimulated and basal insulin secretion in beta cells [3]. It has been shown that palmitate elevates intracellular levels of Ca^{2+} by closing of ATP dependent K^{+} channels, most likely caused by increased metabolism [57]. The NEFA receptor GPR 40 is involved in alternations in insulin secretion. Over expression of the receptor in a mouse model resulted in a diabetes phenotype, while a knockout of GPR 40 inhibited the onset of obesity-induced diabetes [3]. In the study, short (2 h) and long (48 h) term effects of palmitate on beta cells in GPR40 $+/+$ and GPR40 $-/-$ islets suggest that palmitate stimulate both basal insulin and GSIS in the short perspective via the receptor. In contrast, chronic (48 h) exposure to palmitate attenuates GSIS in GPR40 $+/+$ islets. This attenuation in GSIS was also seen in GPR40 $-/-$ islets not exposed to palmitate, indicating alternative roles of GPR40 in long term regulation of insulin secretion [3].

In the present study we demonstrate that isolated human islets exposed to 0.5 mM palmitate show early hypersecretion of insulin followed by a deterioration of beta cell function and apoptosis after 48 hours. Furthermore, we show that apoptosis of beta cells are dependent on the concentration of palmitate. In islets exposed to low levels of palmitate, no significant increase in beta cell apoptosis could be observed. However, palmitate levels of 0.5 mM or above caused a significant increase in apoptosis and a subsequent loss in beta cell mass. In the *in vivo* experiment, levels above 0.22 mM palmitate were considered “high level”. We showed that individuals with high palmitate display an age-dependent decline in GSIS and basal insulin levels. No such relationship was established in individuals with low palmitate levels. These

results suggest that decline in beta cell function is dependent on time and concentration of palmitate. In the *in vitro* study human islets were exposed to 0.5 mM palmitate, which was more than twice the concentration observed *in vivo* (0.22 mM). To be able to draw parallels between these two experiments, it must be remembered that only free circulating palmitate was measured *in vivo*, in essence palmitate unbound to albumin. Levels of albumin showed no significant variability between study subjects, hence did not affect levels of free circulating palmitate. *In vitro*, bovine serum albumin (BSA) was added to the medium, which binds a considerable portion of palmitate. Thus, free levels of palmitate may very well be lower *in vitro* than *in vivo*, legitimizing 0.22 mM palmitate as a “*high level*” of palmitate. Moreover, whereas human islets were subjected to controlled culture conditions, the patient situation has lots of confounding factors, conditions which must be taken into consideration when observing the data.

Palmitate induced time-dependent alternations in basal insulin levels in the isolated islets with initial elevated levels followed by lowered levels. Similar time-dependent effects of palmitate on basal insulin secretion have been observed previously [42]. Hence, beta cell function, in essence insulin secretion and biosynthesis, is negatively affected by palmitate in a time-dependent manner, initially promoting insulin secretion [42], but ultimately leading to beta cell failure [62]. In addition, initial elevation of GSIS was also observed in our study, which makes the model display similar insulin secretory characteristics as observed in the obese individuals. Interestingly, the negative effects on beta cells *in vitro* can be reversed, simply by removal of palmitate [42]. This indicates that lowering of circulating levels of palmitate *in vivo*, would have beneficial health effects especially for children with insulin hypersecretion.

We have described that the saturated NEFA palmitate has negative effects on beta cells. In contrast, elevated levels of the MUFA palmitoleate has been shown to have cytoprotective effects on beta cells [40] and promoting insulin sensitivity [44]. In the present study we show that increasing levels of palmitoleate correlate with lower basal insulin levels, hence promoting metabolic deceleration and normalization of basal insulin levels. *In vitro*, it has been demonstrated that palmitoleate counteracts the adverse effects of palmitate on beta cells, if they are administered together [40]. Although these palmitoleate-related cellular effects are not entirely clear, attenuation of palmitate-induced caspase 3/7 is one proposed mechanism [40]. Moreover, palmitate also affects insulin sensitivity, by compromising GLUT4 activity in muscle and adipose tissue [63]. In the present study, no correlation between palmitate and insulin sensitivity was found. Instead, palmitoleate was found to correlate strongly ($P < 0.01$) with insulin sensitivity in obese children. Same relationship has been established in adults [44]. The exact mechanisms of palmitoleate are still unclear. One proposed function is promoting GLUT 4 recruitment from intracellular vesicles and retention of GLUT4 at the plasma membrane hence accentuating insulin sensitivity [29]. As pointed out before, this could be the result of lowering insulin levels with following positive effects on insulin sensitive tissues.

Palmitoleate is biosynthesized from palmitate with the enzyme stearoyl CoA desaturase 1 (SCD1), which is a key step in optimizing insulin release [64]. SCD1 is involved in lipogenesis and it is believed that the activity of this enzyme is the main regulator of circulating levels of palmitoleate [44]. Treatment with rosiglitazone increased gene expression of SCD1 by almost 50% and boosted activity of the enzyme in adipose tissue, thereby sensitizing target tissue of insulin [64]. Interestingly, animal studies with SCD1 knockout mice show contradictory results

with increased insulin sensitivity and β -oxidation, thus making these animals resistant to obesity [65-66]. Humans and mice differ considerably in SCD1 gene expression and isoforms, making it difficult to draw parallels between the two species in this aspect, however [64]. Moreover, a knockdown of SCD1 has adverse effects on beta cells, by increased susceptibility to apoptosis and ER-stress induced by palmitate, since SCD1 is involved in regulation of intracellular palmitate levels in beta cells [66].

T2DM can be defined as inadequate insulin response to a rise in plasma glucose [19]. The disease is the consequence of two major mechanisms: beta cell dysfunction with impaired insulin secretion and insulin resistance in insulin sensitive tissue [15]. Basal level of insulin is used to calculate insulin resistance using different formulae and is used to estimate risk of T2DM [33]. Could elevated basal insulin levels alone be predictive of future T2DM, or even involved in the pathogenesis of this pandemic disease [33]? In the present study, we propose that hypersecretion of insulin is an early manifestation of beta cell dysfunction and an essential warning sign of subsequent progression towards T2DM, independent of insulin resistance. To fully evaluate this statement, further measurement with the hyperinsulinemic-euglycemic clamp must be performed. Development of T2DM in pediatric individuals increases the risk of progression towards vascular complication, affecting both macro- and microcirculation, relatively early in life [2]. None of the obese subjects in the current study had yet developed T2DM, which allowed us to study manifestations of early β -dysfunction and address new potential avenues of intervention.

A majority of the obese children are caught up in a self-sufficient vicious cycle (Fig 4.1). In obese individuals, beta cells are put under constant pressure of secreting insulin, which in the long run will cause ER-stress and oxidative stress [15, 39]. Insulin has anabolic effects in particular [17], promoting glucose and lipid uptake as well as glycogen storage and biosynthesis of fatty acids. This, in turn will lead to gain of adipose tissue and insulin resistance, which will contribute to even higher basal insulin levels and eventually beta cell failure. Imagine this vicious cycle located on a slippery slope, slowly revolving downhill with T2DM at the far end. Unless the cycle is broken by dietary intervention, exercise regimes or medication [67] the consequences on health will be devastating.

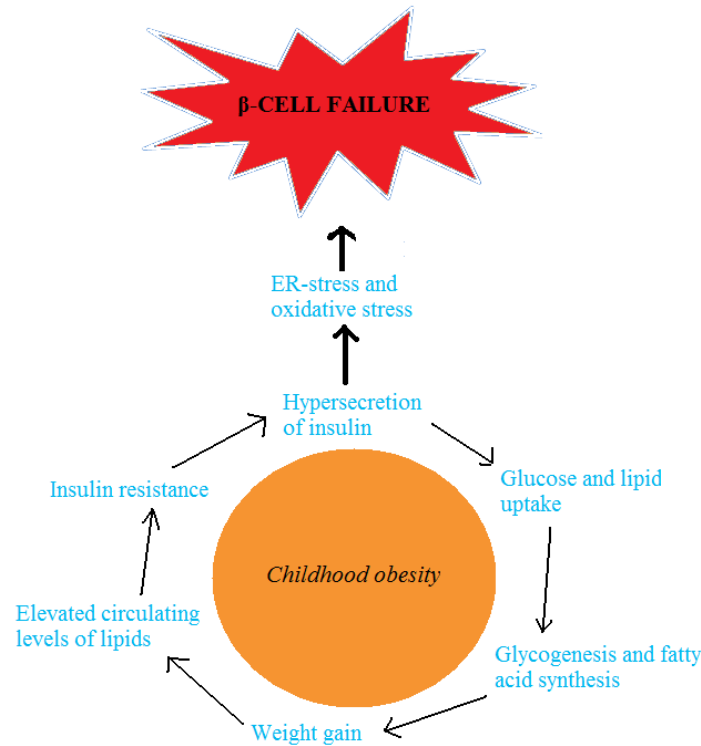


Figure 4.1: Vicious cycle hypothesis of childhood obesity with hypersecretion of insulin as the conductor and most important aim of intervention. Eventually the cycle will lead to beta cell failure.

Notably, treatment with insulinotropic drugs, such as sulfonylureas, is today the drug of choice in many countries, in an attempt to restore beta cell function. However, this will merely cause accentuated ER-stress and oxidative stress in beta cell already under increased stress and speed up the progression towards T2DM [15]. As an alternative, diazoxide treatment has been shown to improve viability and secretory function in vitro [52]. Diazoxide temporarily prevents insulin secretion by inhibition Ca^{2+} influx and exocytosis of insulin vesicles through hyperpolarisation of the cell by opening of ATP dependent K^{+} channels [52]. This provides beta cells with a chance of recovery and could very well be a potential future therapy.

The results of this study demonstrate that although average palmitate and palmitoleate levels are not different in obese compared to lean young individuals, variability in NEFA concentration is considerable between obese individuals. In fact the composition of NEFAs seems to be of great importance in development of hyperinsulinemia and insulin resistance [44-45]. A concentration- and time-dependent effect of palmitate on both basal and stimulated insulin levels was observed *in vivo*. These results were mimicked in isolated islets exposed to palmitate; hence islet can further be used to investigate the role of NEFAs in insulin hypersecretion. We conclude that hypersecretion of insulin is an early manifestation of beta cell dysfunction and an early warning sign of beta cell failure. A high circulating ratio of palmitate/palmitoleate affects both beta cell function and insulin sensitivity negatively. As a final point, metabolic deceleration with transient beta cell arrest, display positive effects by relieving ER-stress and restoring secretory function in beta cells [52]. Further research is urgent within this field, as we struggle with razing the “bridge of hyperinsulinemia” linking childhood obesity and T2DM.

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References

1. McCarthy, M.I., *Genomics, type 2 diabetes, and obesity*. N Engl J Med, 2010. **363**(24): p. 2339-50.
2. Alberti, G., et al., *Type 2 diabetes in the young: the evolving epidemic: the international diabetes federation consensus workshop*. Diabetes Care, 2004. **27**(7): p. 1798-811.
3. Steneberg, P., et al., *The FFA receptor GPR40 links hyperinsulinemia, hepatic steatosis, and impaired glucose homeostasis in mouse*. Cell Metab, 2005. **1**(4): p. 245-58.
4. Malecka-Tendera, E. and A. Mazur, *Childhood obesity: a pandemic of the twenty-first century*. Int J Obes (Lond), 2006. **30 Suppl 2**: p. S1-3.
5. Archenti, A. and L. Pasqualinotto, *Childhood obesity: the epidemic of the third millenium*. Acta Biomed, 2008. **79**(2): p. 151-5.
6. Ekblom, O.B., E.A. Bak, and B.T. Ekblom, *Trends in body mass in Swedish adolescents between 2001 and 2007*. Acta Paediatr, 2009. **98**(3): p. 519-22.
7. Holmback, U., et al., *Overweight more prevalent among children than among adolescents*. Acta Paediatr, 2007. **96**(4): p. 577-81.
8. Sjoberg, A., et al., *Recent anthropometric trends among Swedish school children: evidence for decreasing prevalence of overweight in girls*. Acta Paediatr, 2008. **97**(1): p. 118-23.
9. Franks, P.W., et al., *Childhood obesity, other cardiovascular risk factors, and premature death*. N Engl J Med, 2010. **362**(6): p. 485-93.
10. WHO. *Definition and diagnosis of Diabetes Mellitus and intermediate hyperglycemia*. 2006; Available from: http://whqlibdoc.who.int/publications/2006/9241594934_eng.pdf.
11. Matyka, K.A., *Type 2 diabetes in childhood: epidemiological and clinical aspects*. Br Med Bull, 2008. **86**: p. 59-75.
12. Abdul-Ghani, M.A., et al., *Risk of progression to type 2 diabetes based on relationship between postload plasma glucose and fasting plasma glucose*. Diabetes Care, 2006. **29**(7): p. 1613-8.
13. Carpentier, A.C., *Postprandial fatty acid metabolism in the development of lipotoxicity and type 2 diabetes*. Diabetes Metab, 2008. **34**(2): p. 97-107.
14. Lillioja, S., et al., *Insulin resistance and insulin secretory dysfunction as precursors of non-insulin-dependent diabetes mellitus. Prospective studies of Pima Indians*. N Engl J Med, 1993. **329**(27): p. 1988-92.
15. Aston-Mourney, K., et al., *Too much of a good thing: why it is bad to stimulate the beta cell to secrete insulin*. Diabetologia, 2008. **51**(4): p. 540-5.
16. Cole, T.J., et al., *Establishing a standard definition for child overweight and obesity worldwide: international survey*. BMJ, 2000. **320**(7244): p. 1240-3.
17. Goran, M.I., G.D. Ball, and M.L. Cruz, *Obesity and risk of type 2 diabetes and cardiovascular disease in children and adolescents*. J Clin Endocrinol Metab, 2003. **88**(4): p. 1417-27.
18. Sekine, N., et al., *Postreceptor signalling of growth hormone and prolactin and their effects in the differentiated insulin-secreting cell line, INS-1*. Endocrinology, 1996. **137**(5): p. 1841-50.
19. Weyer, C., et al., *The natural history of insulin secretory dysfunction and insulin resistance in the pathogenesis of type 2 diabetes mellitus*. J Clin Invest, 1999. **104**(6): p. 787-94.
20. Chang-Chen, K.J., R. Mullur, and E. Bernal-Mizrachi, *Beta-cell failure as a complication of diabetes*. Rev Endocr Metab Disord, 2008. **9**(4): p. 329-43.

21. Petrie, J.R., E.R. Pearson, and C. Sutherland, *Implications of genome wide association studies for the understanding of type 2 diabetes pathophysiology*. Biochem Pharmacol, 2010.
22. Rustenbeck, I., S. Baltrusch, and M. Tiedge, *Do insulinotropic glucose-lowering drugs do more harm than good? The hypersecretion hypothesis revisited*. Diabetologia, 2010. **53**(10): p. 2105-11.
23. Wu, J. and R.J. Kaufman, *From acute ER stress to physiological roles of the Unfolded Protein Response*. Cell Death Differ, 2006. **13**(3): p. 374-84.
24. Hoppa, M.B., et al., *Chronic palmitate exposure inhibits insulin secretion by dissociation of Ca(2+) channels from secretory granules*. Cell Metab, 2009. **10**(6): p. 455-65.
25. Wiseman, R.L. and W.E. Balch, *A new pharmacology--drugging stressed folding pathways*. Trends Mol Med, 2005. **11**(8): p. 347-50.
26. Harding, H.P., et al., *Diabetes mellitus and exocrine pancreatic dysfunction in perk-/- mice reveals a role for translational control in secretory cell survival*. Mol Cell, 2001. **7**(6): p. 1153-63.
27. Sol, E.M., et al., *Glucolipotoxicity in INS-1E cells is counteracted by carnitine palmitoyltransferase 1 over-expression*. Biochem Biophys Res Commun, 2008. **375**(4): p. 517-21.
28. Randle, P.J., *Regulatory interactions between lipids and carbohydrates: the glucose fatty acid cycle after 35 years*. Diabetes Metab Rev, 1998. **14**(4): p. 263-83.
29. Dimopoulos, N., et al., *Differential effects of palmitate and palmitoleate on insulin action and glucose utilization in rat L6 skeletal muscle cells*. Biochem J, 2006. **399**(3): p. 473-81.
30. Sugden, M.C. and M.J. Holness, *Role of nuclear receptors in the modulation of insulin secretion in lipid-induced insulin resistance*. Biochem Soc Trans, 2008. **36**(Pt 5): p. 891-900.
31. Brufani, C., et al., *Glucose tolerance status in 510 children and adolescents attending an obesity clinic in Central Italy*. Pediatr Diabetes, 2010. **11**(1): p. 47-54.
32. Odeleye, O.E., et al., *Fasting hyperinsulinemia is a predictor of increased body weight gain and obesity in Pima Indian children*. Diabetes, 1997. **46**(8): p. 1341-5.
33. Weyer, C., et al., *A high fasting plasma insulin concentration predicts type 2 diabetes independent of insulin resistance: evidence for a pathogenic role of relative hyperinsulinemia*. Diabetes, 2000. **49**(12): p. 2094-101.
34. Koyama, K., et al., *Tissue triglycerides, insulin resistance, and insulin production: implications for hyperinsulinemia of obesity*. Am J Physiol, 1997. **273**(4 Pt 1): p. E708-13.
35. Lillioja, S., et al., *Exaggerated early insulin release and insulin resistance in a diabetes-prone population: a metabolic comparison of Pima Indians and Caucasians*. J Clin Endocrinol Metab, 1991. **73**(4): p. 866-76.
36. Zhou, Y.P., et al., *Basal insulin hypersecretion in insulin-resistant Zucker diabetic and Zucker fatty rats: role of enhanced fuel metabolism*. Metabolism, 1999. **48**(7): p. 857-64.
37. Kulkarni, R.N., et al., *Tissue-specific knockout of the insulin receptor in pancreatic beta cells creates an insulin secretory defect similar to that in type 2 diabetes*. Cell, 1999. **96**(3): p. 329-39.

38. Weyer, C., et al., *Exaggerated pancreatic polypeptide secretion in Pima Indians: can an increased parasympathetic drive to the pancreas contribute to hyperinsulinemia, obesity, and diabetes in humans?* Metabolism, 2001. **50**(2): p. 223-30.
39. Kharroubi, I., et al., *Free fatty acids and cytokines induce pancreatic beta-cell apoptosis by different mechanisms: role of nuclear factor-kappaB and endoplasmic reticulum stress.* Endocrinology, 2004. **145**(11): p. 5087-96.
40. Morgan, N.G., et al., *The cytoprotective actions of long-chain mono-unsaturated fatty acids in pancreatic beta-cells.* Biochem Soc Trans, 2008. **36**(Pt 5): p. 905-8.
41. Diakogiannaki, E., H.J. Welters, and N.G. Morgan, *Differential regulation of the endoplasmic reticulum stress response in pancreatic beta-cells exposed to long-chain saturated and monounsaturated fatty acids.* J Endocrinol, 2008. **197**(3): p. 553-63.
42. Zhou, Y.P. and V.E. Grill, *Long-term exposure of rat pancreatic islets to fatty acids inhibits glucose-induced insulin secretion and biosynthesis through a glucose fatty acid cycle.* J Clin Invest, 1994. **93**(2): p. 870-6.
43. Cao, H., et al., *Identification of a lipokine, a lipid hormone linking adipose tissue to systemic metabolism.* Cell, 2008. **134**(6): p. 933-44.
44. Stefan, N., et al., *Circulating palmitoleate strongly and independently predicts insulin sensitivity in humans.* Diabetes Care, 2010. **33**(2): p. 405-7.
45. Fukuchi, S., et al., *Role of fatty acid composition in the development of metabolic disorders in sucrose-induced obese rats.* Exp Biol Med (Maywood), 2004. **229**(6): p. 486-93.
46. Stumvoll, M., et al., *Use of the oral glucose tolerance test to assess insulin release and insulin sensitivity.* Diabetes Care, 2000. **23**(3): p. 295-301.
47. Kangani, C.O., D.E. Kelley, and J.P. Delany, *New method for GC/FID and GC-C-IRMS analysis of plasma free fatty acid concentration and isotopic enrichment.* J Chromatogr B Analyt Technol Biomed Life Sci, 2008. **873**(1): p. 95-101.
48. Dole, V.P. and H. Meinertz, *Microdetermination of long-chain fatty acids in plasma and tissues.* J Biol Chem, 1960. **235**: p. 2595-9.
49. Matthews, D.R., et al., *Homeostasis model assessment: insulin resistance and beta-cell function from fasting plasma glucose and insulin concentrations in man.* Diabetologia, 1985. **28**(7): p. 412-9.
50. Matsuda, M. and R.A. DeFronzo, *Insulin sensitivity indices obtained from oral glucose tolerance testing: comparison with the euglycemic insulin clamp.* Diabetes Care, 1999. **22**(9): p. 1462-70.
51. Kuroe, A., et al., *Impaired beta-cell function and insulin sensitivity in Japanese subjects with normal glucose tolerance.* Diabetes Res Clin Pract, 2003. **59**(1): p. 71-7.
52. Sargsyan, E., et al., *Diazoxide-induced beta-cell rest reduces endoplasmic reticulum stress in lipotoxic beta-cells.* J Endocrinol, 2008. **199**(1): p. 41-50.
53. Bergsten, P. and B. Hellman, *Glucose-induced amplitude regulation of pulsatile insulin secretion from individual pancreatic islets.* Diabetes, 1993. **42**(5): p. 670-4.
54. Arslanian, S.A., et al., *Hyperinsulinemia in african-american children: decreased insulin clearance and increased insulin secretion and its relationship to insulin sensitivity.* Diabetes, 2002. **51**(10): p. 3014-9.

55. Chowta, M.N., et al., *Serum C peptide level and renal function in diabetes mellitus*. Indian J Nephrol, 2010. **20**(1): p. 25-8.
56. Stein, D.T., et al., *The insulinotropic potency of fatty acids is influenced profoundly by their chain length and degree of saturation*. J Clin Invest, 1997. **100**(2): p. 398-403.
57. Jeffrey, K.D., et al., *Carboxypeptidase E mediates palmitate-induced beta-cell ER stress and apoptosis*. Proc Natl Acad Sci U S A, 2008. **105**(24): p. 8452-7.
58. Bjorklund, A. and V. Grill, *Enhancing effects of long-term elevated glucose and palmitate on stored and secreted proinsulin-to-insulin ratios in human pancreatic islets*. Diabetes, 1999. **48**(7): p. 1409-14.
59. Bonora, E., et al., *Homeostasis model assessment closely mirrors the glucose clamp technique in the assessment of insulin sensitivity: studies in subjects with various degrees of glucose tolerance and insulin sensitivity*. Diabetes Care, 2000. **23**(1): p. 57-63.
60. Karne, R.J., H. Chen, and M.J. Quon, *Diagnosing insulin resistance by simple quantitative methods in subjects with normal glucose metabolism*. Diabetes Care, 2004. **27**(5): p. 1247-8; author reply 1249.
61. Reaven, G.M., *Banting Lecture 1988. Role of insulin resistance in human disease*. 1988. Nutrition, 1997. **13**(1): p. 65; discussion 64, 66.
62. Hovsepian, M., E. Sargsyan, and P. Bergsten, *Palmitate-induced changes in protein expression of insulin secreting INS-1E cells*. J Proteomics, 2010. **73**(6): p. 1148-55.
63. Tsuchiya, Y., et al., *Palmitate-induced down-regulation of sortilin and impaired GLUT4 trafficking in C2C12 myotubes*. J Biol Chem, 2010. **285**(45): p. 34371-81.
64. Riserus, U., et al., *Rosiglitazone increases indexes of stearoyl-CoA desaturase activity in humans: link to insulin sensitization and the role of dominant-negative mutation in peroxisome proliferator-activated receptor-gamma*. Diabetes, 2005. **54**(5): p. 1379-84.
65. Ntambi, J.M., et al., *Loss of stearoyl-CoA desaturase-1 function protects mice against adiposity*. Proc Natl Acad Sci U S A, 2002. **99**(17): p. 11482-6.
66. Thorn, K., M. Hovsepian, and P. Bergsten, *Reduced levels of SCD1 accentuate palmitate-induced stress in insulin-producing beta-cells*. Lipids Health Dis, 2010. **9**: p. 108.
67. Morrison, J.A., et al., *Determinants of persistent obesity and hyperinsulinemia in a biracial cohort: a 15-year prospective study of schoolgirls*. J Pediatr, 2010. **157**(4): p. 559-65.