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METHODOLOGICAL AND CLINICAL STUDIES ON INSULIN RESISTANCE IN CHILDHOOD

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ABSTRACT

Insulin resistance is a condition in which adequate amounts of insulin fail to give an adequate response in target tissues. This thesis is based on five different studies, aiming to investigate different aspects of insulin resistance and the assessment methods thereof in children and in adults. The rationale for performing these studies is that insulin resistance is a key component of the metabolic syndrome and crucial in the development of type 2 diabetes. Concomitant with the increase in obesity worldwide, insulin resistance has become an important and increasingly more common pathological condition, which needs to be efficiently diagnosed and treated.

- In Study I we investigated proxy measures of insulin sensitivity assessment as compared to the reference standard Frequently Sampled Intravenous Glucose Tolerance Test (FSIVGTT) in obese children and adolescents. The Homeostasis model assessment (HOMA-IR), the Quantitative Insulin Sensitivity Check Index (QUICKI) and fasting insulin were compared to the Sensitivity index (Si) of the FSIVGTT with the sample stratified by sex, puberty status and median of Si. This study demonstrated that fasting indices have a low validity in identifying insulin resistance in this group, and we generally discourage the use of these methods.

- Study II was performed in the same obese pediatric population with the aim of showing that HOMA-IR and QUICKI are interchangeable. The numerous comparisons between these methods should thus be avoided. Also, the high physiologic fluctuations in insulin levels further undermine the robustness of these methods. Studies I-II provide evidence that fasting indices as simple screening tools for insulin resistance in children and adolescents should, if used at all, be interpreted with caution.

- The third and fourth studies were performed with the aim of developing and validating models for the kinetics of C-peptide and Nonesterified fatty acids (NEFAs) respectively during an intravenous glucose challenge. An insulin modified FSIVGTT was performed on healthy normal weight young adults, with sampling of C-peptide and NEFAs included at all sampling points. In Study III a model was developed which assesses first phase C-peptide secretion, indirectly also estimating insulin secretion. Assessing insulin secretion is useful in understanding diabetes development, in assessing progression to a diabetic state and in monitoring the effect of therapeutic regimens.

- In Study IV a novel NEFA model was validated on a subject level, with curves well fitting the diverse range of NEFA responses to a glucose challenge. Validation of the model using static parameters derived from the dynamic counterparts showed high correlation of the model's dynamic parameters to static parameters. NEFA levels are elevated in insulin resistance and affect glucose homeostasis on both the short and long term. This NEFA model may provide a complementary way of estimating insulin sensitivity, with focus on the lipotoxic aspect of diabetes development.

- Study V provides a clinical perspective on insulin sensitivity by examining metabolic features of a cohort of long-term cancer survivors treated with stem cell transplantation including total body irradiation, as compared to healthy controls matched for age and sex. The study shows an intact β-cell function, but decreased insulin sensitivity, after a median follow-up of 18 years. An adverse body composition with higher proportion fat mass than in controls was seen in cancer survivors. Lower levels of growth hormone, higher levels of leptin and lower levels of adiponectin were found, all of which may explain the adverse body composition and the reduced insulin sensitivity.

In summary, several aspects of insulin resistance and insulin secretion have been studied. Existing methods of insulin secretion and sensitivity assessment have been investigated and implemented, and new methods described. We hope this will contribute further knowledge on diabetes development and treatment strategies.

LIST OF PUBLICATIONS

This thesis is based on three publications and two submitted manuscripts, and will be referred to by their roman numerals (I-V).

I. Rossner SM, Neovius M, Montgomery SM, Marcus C, Norgren S. Alternative methods of insulin sensitivity assessment in obese children and adolescents. Diabetes Care. 2008 Apr;31(4):802-4.

II. Rossner SM, Neovius M, Mattsson A, Marcus C, Norgren S. HOMA-IR and QUICKI: decide on a general standard instead of making further comparisons. Acta Paediatr. 2010 Nov;99(11):1735-40.

III. Rossner SM, Boston R, Carlsson K, Ward GM, Gustafsson J, Norgren S. Modeling First Phase C-peptide Secretion During the Insulin Modified Frequently Sampled Intravenous Glucose Tolerance Test. *Submitted.*

IV. Rossner SM, Norgren S, Carlsson K, Neovius M, Gustafsson J, Boston R. A Model for Nonesterified Fatty Acid Kinetics During the Frequently Sampled Intravenous Glucose Tolerance Test. *Submitted.*

V. Frisk P, Rossner SM, Norgren S, Arvidson J, Gustafsson J. Glucose metabolism and body composition in young adults treated with TBI during childhood. Bone Marrow Transplant 2010 Dec 13. *Epub before print*.

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LIST OF ABBREVIATIONS

AIR	Acute insulin response
AUC	Area under the curve
BMI	Body mass index
BMI-SDS	Body mass index standard deviation score
CRP	C-reactive protein
DAG	Diacylglycerol
DEXA	Dual-energy x-ray absorptiometry
Di	Disposition index
FSIVGTT	Frequently Sampled Intravenous Glucose Tolerance Test
FSIVGTT-MMOD	Frequently Sampled Intravenous Glucose Tolerance Test analyzed by Minimal Model
GH	Growth hormone
GLUT	Glucose transporter
HOMA-IR	Homeostasis Model Assessment of Insulin Resistance
IGF1	Insulin growth factor 1
IGFBP	Insulin growth factor binding protein
IKKβ	Inhibitor of nuclear factor kappa B kinase β
IRS	Insulin receptor substrate
JNK	c-Jun amino terminal kinase
MINMOD	Minimal model
mTOR	Molecular target of rapamycin
NEFA	Nonesterified fatty acid
NF-κB	Nuclear factor-κB
OGTT	Oral glucose tolerance test
PDK	Phosphatidylinositol-dependent protein kinase
PI-3	Phosphatidylinositol 3
PIP_3	Phosphatidylinositol triphosphate
PKB	Protein kinase B
PKC	Protein kinase C
QUICKI	Quantitative Insulin Sensitivity Check Index
ROC	Receiver operating characteristic curve
SCT	Stem cell transplantation

Sg	Glucose effectiveness
Si	Sensitivity index
TBI	Total body irradiation
TNF-α	Tumor necrosis factor α

1 INTRODUCTION

The world wide obesity epidemic is closely followed by an increase in insulin resistance, which in combination with impaired insulin secretion ultimately leads to type 2 diabetes (1). Insulin resistance has been extensively researched as a crucial component of the so-called metabolic syndrome (2), involving obesity, dyslipidemia (high triglycerides and low high-density lipoprotein cholesterol), hypertension and microalbuminuria in the definition from the World Health Organization (3), and along with this an increased risk of cardiovascular disease (4, 5). It is important both from a health economic and health care perspective to identify insulin resistant individuals, and numerous methods of insulin sensitivity assessment have emerged. This thesis has insulin resistance as its unifying theme, yet the five included studies can be divided into three distinct groups (**Figure 1**). Each group contributes to the thesis work and education in research by involving different methodologies, study populations and clinical perspectives. As the title suggests, both clinical and methodological aspects are covered, however apart from the first two studies, the focus is not primarily on children but includes a more general perspective on insulin resistance.

Most insulin sensitivity assessment methods focus on the homeostasis of glucose and insulin. Gold standard methods exist, but the complicated and relatively invasive nature of these tests has lead to the development of simpler tests in the form of fasting indices. These crude fasting indices have been examined in many studies, yet there is no consensus on which method to use**.** Thus, **Study I** explores the validity of fasting indices as compared to a gold standard method in a population of obese children and adolescents. An extension of this is described in **Study II**, in which recommendations for further insulin sensitivity assessment are investigated by contrasting fasting indices and fasting insulin to the gold standard method.

The second group of studies differs from the others in its methodology, which is focused on the modeling of biological systems. It includes two modeling studies, in which the kinetics of C-peptide (**Study III**) and nonesterified fatty acids, NEFAs, (**Study IV**) respectively, are investigated. A C-peptide secretion model can contribute valuable knowledge on insulin secretion, which itself is difficult to measure. ß-cell impairment eventually leads to diminished insulin secretion and diabetes, which makes this a relevant issue to explore. Glucose and NEFAs are intrinsically linked together, and there is evidence that the pathogenesis of insulin resistance is linked to elevated NEFA levels. A model of glucose and NEFA kinetics contributes further insights into diabetes pathogenesis and may provide a tool complementary to traditional glucose/insulin models in assessing and understanding insulin resistance.

Insulin resistance is found not only in obesity but also in other medical states, one being in cancer survivors who have undergone stem cell transplantation including total body irradiation. With the advancements in cancer therapy, long-term cancer survivors constitute a growing population at risk of developing the metabolic syndrome or components thereof. **Study V**, the third subgroup of this thesis, constitutes a clinical application in which insulin sensitivity, insulin secretion and body composition in long-term survivors of childhood cancer are explored.

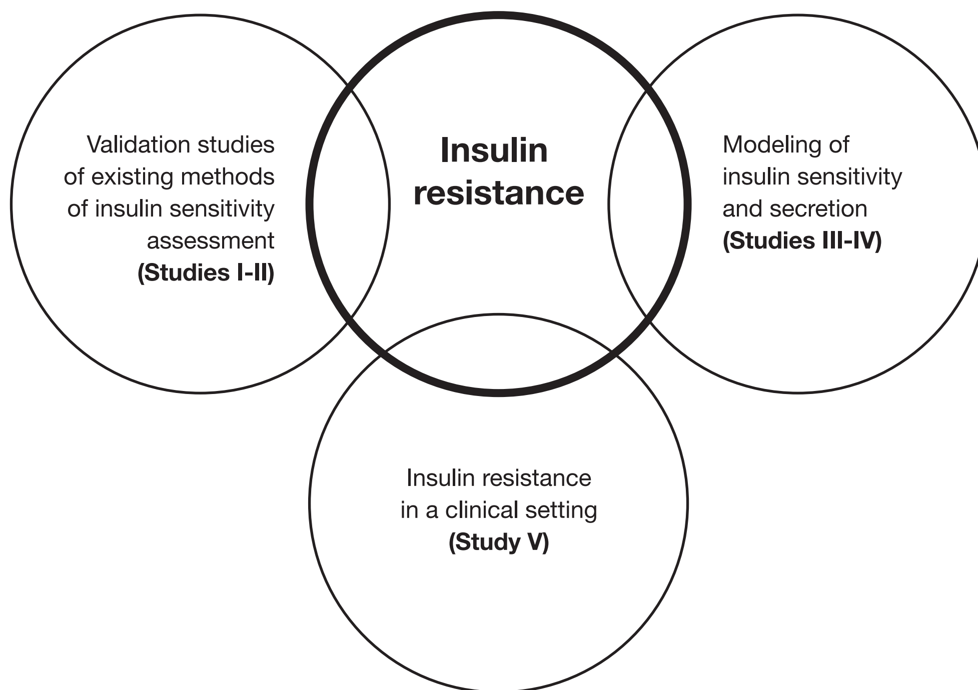

Figure 1. Overview of the five substudies included in the thesis, with insulin resistance as the unifying theme.

2 INSULIN

Insulin is a vital anabolic and regulatory hormone and is synthesized in the β-cells of the islets of Langerhans, which are scattered in groups throughout the pancreas. The human insulin gene is located on the short arm of chromosome 11. The insulin gene is transcribed and processed into messenger RNA, which is subsequently translated into preproinsulin. Within minutes, the preproinsulin molecule is cleaved from its signaling peptide to form proinsulin, which at this point is bound to the endoplasmic reticulum. After release from the endoplasmic reticulum, vesicles transport proinsulin to the Golgi apparatus, where it is sorted and packaged into secretory granulae. The proinsulin precursor molecule is then modified further by proteolytic enzymes, which cleave the center portion of the molecule from the C- and N-terminal ends, releasing C-peptide and insulin in equimolar amounts. The function of C-peptide seems to be to give the proinsulin the folding which is necessary for insulin to bind to its receptor. However studies also indicate that C-peptide has bioactive properties on its own and can have beneficial effects on endothelial function, blood flow and inflammation and thereby reduce diabetic complications in kidney and nervous tissue (6). The resulting secretory granulae thus contain an insulin polypeptide chain, consisting of 51 amino acids bound together in B- and A-chains by disulfide bonds and C-peptide. Insulin is stored as a hexamere, with six insulin molecules bound to a zink atom, while the active form of the hormone is a monomer. This storage form is effective and stable as long as pH levels are kept below neutrality. When stimulated, the β-cell releases insulin granulae by exocytosis, with granula membranes fusing with the plasma membrane of the β-cell thereby releasing its contents. The hexamere structure is dissolved by the higher pH value of the extracellular fluid and insulin is transported through the capillary wall and into the blood stream to exert its action.

2.1 INSULIN SIGNALING AND GLUCOSE TRANSPORT

The insulin signaling pathway, leading to glucose uptake primarily in skeletal muscle has been researched extensively. A better understanding of the signaling mechanisms may provide means to understand the pathogenesis of type 2 diabetes, as well as to identify possible novel treatment strategies. In insulin responsive tissues there are insulin receptors at the cell surface plasma membrane. These consist of two extracellular insulin-binding α-subunits and two β-subunits which span through the membrane. When insulin binds to the insulin receptor (IR), autophosphorylation on tyrosine residues occurs. This activates the tyrosine kinase of the β-subunits. Phosphorylated tyrosine residues on the activated insulin receptor protein serve as docking sites for the down-stream signaling molecules, the most extensively researched being the insulin receptor substrate (IRS) proteins, which are tyrosine phosphorylated by the β-subunits. The predominant IRS's in human skeletal muscle are IRS-1 and IRS-2, and these seem to have specific signaling roles. The tyrosine-phosphorylated IRS-1 mediates a cascade of signals to enzymes further downstream by binding to various other signaling proteins. Phosphatidylinositol 3 (PI-3) kinase (7) is an important step in this sequence. PI-3 kinase activates phosphatidylinositol-dependent protein kinase (PDK) and in turn the serine/threonine kinase Akt (also known as protein kinase B, PKB). Among other functions, Akt phosphorylates AS160 and these steps are necessary in promoting GLUT4 translocation (8). A simplified representation of the insulin signaling cascade is shown in **Figure 2**, ultimately leading to glucose transporter proteins translocating to the cell surface.

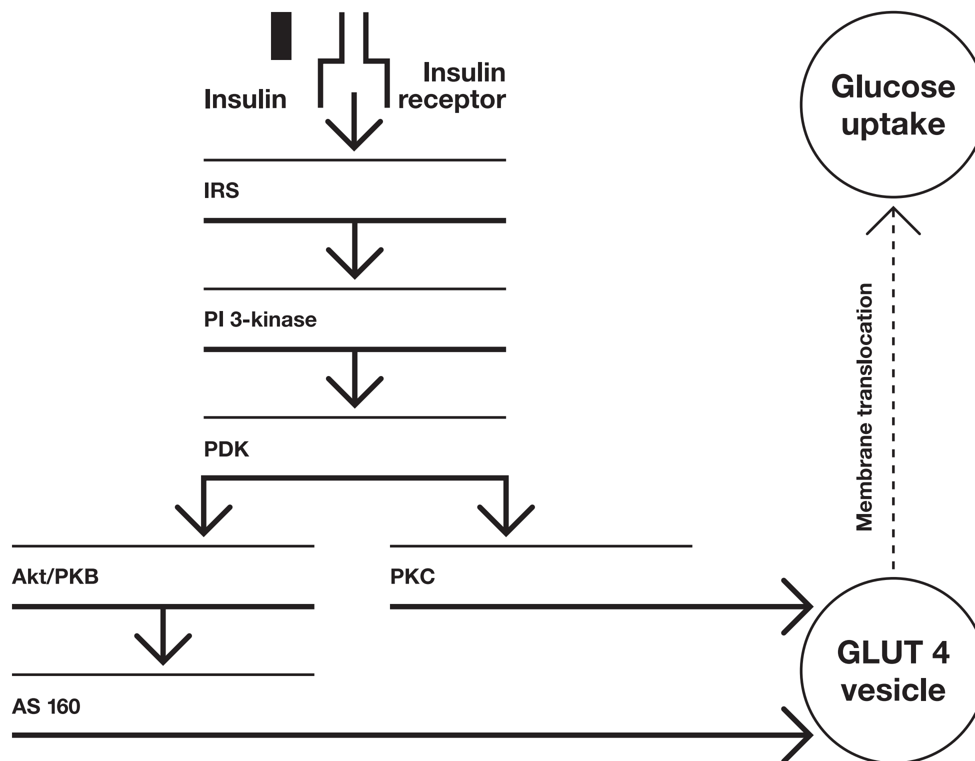

Figure 2. Schematic representation of insulin binding to its receptor, initiating a signaling cascade leading to glucose transporter (GLUT4) translocation and glucose uptake.

Glucose transport is the rate-limiting step for glucose metabolism. As glucose cannot pass the hydrophilic barrier of the cell membrane, access needs to be facilitated by the help of glucose transporters. The two main glucose transporter families in humans are sodium dependent transporters and facilitative glucose transporters (GLUT) (9, 10). Sodium dependent glucose co-transporters are involved primarily in glucose absorption in the gut and in the kidneys and will not be further discussed here. Several different GLUT family members have been identified, GLUT 4 being the most important form in skeletal muscle (11-13). In response to insulin or exercise intracellular vesicles containing GLUT4 fuse with the cell surface membrane in order to aid glucose transport into the cell. There are known impairments in GLUT4 action in insulin resistant skeletal muscle (14, 15). However, such impaired insulin-stimulated glucose transport into skeletal muscle seems not to be caused by a decrease in GLUT4 production predominantly, but rather by impairments in insulin signaling (8).

2.2 MOLECULAR MECHANISMS OF INSULIN RESISTANCE

Insulin stimulates the uptake of glucose in muscle and fat, as well as suppresses glucose production by the liver. When one or both of these functions of insulin action is impaired, insulin resistance at the skeletal muscle or fat level and/or hepatic level develops. Insulin resistance is defined as a state where normal levels of insulin fail to give an adequate response in tissues. However, besides its glucoregulatory role, insulin is also important in the metabolism of proteins and lipids, in ion fluxes, and as an anabolic hormone affecting cell growth and differentiation, inhibition of lipolysis, protein degradation and apoptosis. It seems that there is a selective insulin resistance affecting metabolic aspects of insulin action, but being distinctly different from

mitogenic aspects of insulin action (16). Various cellular and molecular defects underlying the pathology of insulin resistance have been identified (17), on the level of the insulin receptor, in signaling pathways and in glucose transport.

The IRS plays an important part in the insulin signaling cascade. The ability of the insulin receptor to phosphorylate tyrosine residues on the IRS-1 is reduced in type II diabetic muscle, which leads to diminished signaling in the entire pathway following the initial events (18). The exact molecular background to why the insulin receptor fails to tyrosine-phosphorylate the IRS molecule is not known. It has been shown, however, that if IRS is extensively phosphorylated on serine residues this will block access of tyrosine residues (19). Serine phosphorylation of IRS also reduces the ability of IRS proteins to attract PI3-kinase, which limits its activation (20-26), and causes an increased rate of degradation of IRS protein (27). Thus, serine phosphorylation interferes with the insulin signaling downstream and can occur in response to several different serine kinases and also other factors (**Table 1**). Further, insulin stimulated activation of phosphatidylinositol 3-kinase (PI3-kinase) is down-regulated in type 2 diabetics (18). The phosphorylation of other important signaling molecules, such as Akt/PKB and AS160 is also reduced in diabetic muscle (28). Since Akt/PKB regulates a number of metabolic processes a diminished activation will lead to changes in glycogen storage and protein metabolism.

A cellular nutrient sensor, mTOR (molecular target of rapamycin), has been identified as an important component in integrating cellular metabolism with growth factor signaling (29-32). mTOR phosphorylates and modulates the activities of p70 S6 kinase among others in response to insulin and amino acids. The activation of mTOR and p70SK1 kinase causes serine phosphorylation of IRS-1, with a concomitant decline in the IRS-1 associated PI 3-kinase activity. This has been suggested to negatively regulate insulin signaling and sensitivity (22, 33, 34). This is also thought to occur in response to nutrients and possibly hyperglycemia. In the overfed state, e.g. obesity and type 2 diabetes, hyperinsulinemia may hyperactivate the mTOR/p70 S6 pathway, causing serine phosphorylation of IRS-1, its degradation and further decline in PI 3 – kinase activity. It also appears that various serine kinases, such as c-Jun amino terminal kinase (JNK), stress activated protein kinases, tumor necrosis factor α (TNF-α) and protein kinase C (PKC), among others, can promote serine phosphorylation of IRS-1.

JNK has been shown to be activated by NEFAs, stress and inflammation (35-38), with a resulting diminished insulin signal along the metabolic pathway (39, 40). Also, activation of the pro-inflammatory kinase that phosphorylates the inhibitor of NF-κB, inhibitor kappa B kinase β (IKKβ), has been found to induce insulin resistance (41-43). Via a mechanism that involves impairment in the IRS-1/PI 3-kinase signaling pathway IKKβ has been implicated in the pathogenesis of insulin resistance.

TNF-α levels are increased in adipose tissue of obese, insulin resistant individuals and have thus been suggested to play a role in insulin resistance development in obesity (44-46). TNF-α has been shown to inhibit insulin signaling by inducing serine phosphorylation of IRS-1 (47), again leading to a decrease in IRS-1 associated PI 3-kinase activity. A further hypothesis is that mitochondrial dysfunction or reduced mitochondrial content with a concomitant decrease in mitochondrial NEFA oxidation will lead to accumulation of fatty acyl Coenzyme A and diacylglycerol, which may cause insulin resistance (48-50). Mitochondrial size and number are reduced in obesity and type 2 diabetes, and a reduced expression of a number of genes related to

mitochondrial growth and function have recently been identified in type 2 diabetics (51, 52). Another pro-inflammatory molecule, PKCθ has also been found to cause serine phosphorylation of IRS-1 (53, 54), supporting the role of serine kinases in the development and pathogenesis of insulin resistance.

A molecular mechanism which may potentially contribute to insulin resistance is an altered balance between the amounts of different PI 3-kinase subunits (55). These exist as heterodimers, with a regulatory subunit (p85) tightly associated with a catalytic subunit (p110). The main function of these kinases is to produce phosphatidylinositol triphosphate, PIP_3, which is a major signaling component of the cell. The regulatory subunit, p85, stabilizes the p110 subunit (56-58) and maintains it in a low activity state (59). In the normal state there is an excess of free p85 as compared to p110, and also a balance between the free p85 and the p85-p110 heterodimer, the latter being responsible for the PI 3-kinase activity. An imbalance in this system is thought to result in either increased or decreased PI 3-kinase activity. In this way excess p85 leads to increased insulin resistance. Causes of imbalance between PI 3-kinase subunits have been found to be induced by steroids (60), growth hormone (61), human placental growth hormone (61, 62), short-term overfeeding (63), obesity and diabetes (35).

Table 1. Causes of IRS-1 serine phosphorylation (16).

Mechanism	Activation
mTOR	P70S6 kinase-amino acids, hyperinsulinemia, nutrition
JNK	Stress, hyperlipidemia, inflammation
IKKβ	Inflammation
TNF-α	Obesity, inflammation
Mitochondrial dysfunction	
PKCθ	Hyperglycemia, diacylglycerol, inflammation

In summary, in spite of its importance as a health care problem, the molecular mechanisms of insulin resistance are as yet incompletely understood. Potential mechanisms have however been found, and a combination of these and other still unidentified factors may be necessary to induce clinically overt insulin resistance. Future studies are needed to explore this hypothesis.

3 ASSESSING INSULIN SENSITIVITY

In healthy normal individuals blood glucose levels are kept within a relatively tight range, which is maintained through periods of fasting as well as of feeding (**Figure 3**). This is achieved by a balance between glucose production and substrate control in the fasting state, and glucose absorption, production and uptake in the postprandial state. Insulin is a major player in this tightly controlled system by suppressing glucose production and stimulating glucose uptake. To assess insulin sensitivity numerous methods have been developed, most of which define insulin sensitivity on the basis of the ability of insulin to regulate blood glucose levels and glucose metabolism. Some of the most commonly used methods, namely the hyperinsulinemic euglycemic clamp, the Frequently Sampled Intravenous Glucose Tolerance Test (FSIVGTT), the HOMA-IR and QUICKI will be described here.

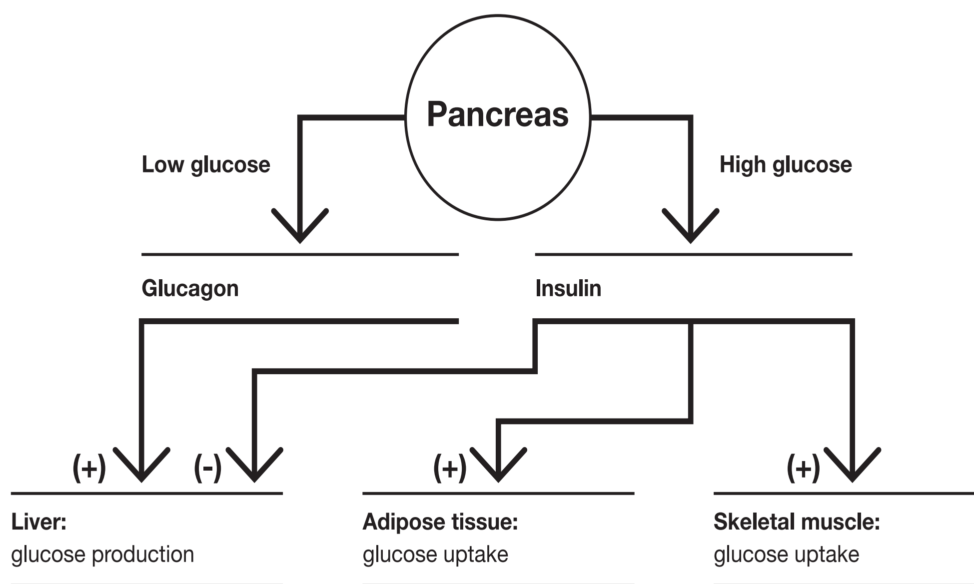

Figure 3. Glucose homeostasis is tightly regulated, with insulin stimulating the uptake of glucose primarily in skeletal muscle and adipose tissue, and inhibiting hepatic glucose production. This is counter-regulated by glucagon, which promotes glucose release.

3.1 HYPERINSULINEMIC EUGLYCEMIC CLAMP

The clamp method was developed by DeFronzo et al (64) and is accepted as a reference standard for the direct determination of insulin action. In the fasting state glucose production, mainly from the liver, equals glucose disposal in tissues. Insulin suppresses hepatic glucose production and stimulates glucose uptake, resulting in lower blood glucose. The clamp technique uses this situation by letting a fixed exogenous insulin administration induce hyperinsulinemia, which down-regulates hepatic glucose production. It then lets a variable exogenous glucose infusion counter the fall in blood glucose caused by the exogenous insulin infusion. Assuming that the hyperinsulinemic state is sufficient to suppress hepatic glucose production completely, the rate of glucose infusion necessary to maintain, “clamp”, a predetermined glucose level provides an index of the net effect of insulin on glucose production and utilization. A healthy

insulin sensitive subject will thus require higher glucose infusion rates as compared to an insulin resistant subject.

In practice, a peripheral catheter is inserted in each arm, one for sampling and one for infusion. Insulin is administered intravenously at a fixed infusion rate, and glucose is simultaneously administered at a variable rate aiming to maintain plasma glucose at a certain level. Glucose levels are tested every 5 to 10 minutes during the clamp in order to adjust the infusion rate accordingly.

The validity of the glucose clamp depends on achieving steady-state conditions, usually assumed to be attained at an arbitrary time point at the end of the clamp study, e.g. 120 minutes. The clamp method also assumes that hepatic glucose production is completely suppressed by the steady-state hyperinsulinemia. In normal insulin sensitive subjects this can be expected, however in insulin resistant subjects hepatic glucose production may not be completely suppressed. This can be overcome by choosing a higher insulin infusion rate. Alternatively, radiolabeled glucose tracers may be used in order to determine the origin of the plasma glucose levels. It must also be kept in mind that comparisons between different subjects are only valid if the same insulin infusion is used for all subjects, and that this infusion level is in a range where the glucose disposal rate can vary according to differences in insulin sensitivity. Thus the insulin infusion rate must be matched to the insulin sensitivity of the population being studied.

The main advantage of the clamp is that it estimates whole body glucose disposal at a given level of insulinemia under steady-state conditions. The approach is also straightforward, and the number of assumptions is limited. Limitations of the clamp however include the time consuming and labor intensive nature of the test, requiring skilled personnel to manage technical difficulties. This renders the clamp unsuitable for large epidemiological studies or routine clinical applications.

3.2 FREQUENTLY SAMPLED INTRAVENOUS GLUCOSE TOLERANCE TEST ANALYZED BY THE MINIMAL MODEL (FSIVGTT- MMOD)

The Frequently Sampled Intravenous Glucose Tolerance Test (65) (FSIVGTT) was developed by Bergman in 1979 and is a well validated method for insulin sensitivity assessment in adults as well as in children (64, 66-70). After an overnight fast, the subject receives a peripheral catheter in each arm. Baseline samples of insulin and glucose are taken, and at time 0 minutes glucose is infused in a given amount per kg body weight. Blood samples for determination of insulin and glucose are taken every minute during the first minutes of the test after which sampling is gradually spaced out following a standard protocol of 180 minutes. In the so-called modified protocol, at time 20 minutes a given amount of insulin per kg body weight is injected as an intravenous bolus dose (schematic representation shown in **Figure 4**). The insulin infusion is recommended in subjects whose endogenous insulin response may be insufficient to deal with the glucose load in such a way that the method can discriminate between the high glucose values and calculate a sensitivity index. An FSIVGTT glucose curve is represented in **Figure 5**, showing the typical glucose and insulin responses during the challenge.

The most established method for analysis of FSIVGTT data is to use minimal model assessment (FSIVGTT-MMOD) (71, 72), in which insulin and glucose data are entered into the so called MINMOD computer program to generate an insulin

sensitivity index, Si. The minimal model is defined by two coupled differential equations and includes four model parameters. The first equation describes plasma glucose dynamics in a single compartment, and the second equation describes insulin dynamics in a “remote compartment”. The structure of this model allows the MINMOD program to calculate model parameters which determine best fit to glucose disappearance during the FSIVGTT. The Si is derived from two of these model parameters and is defined as the fractional glucose disappearance per insulin concentration unit.

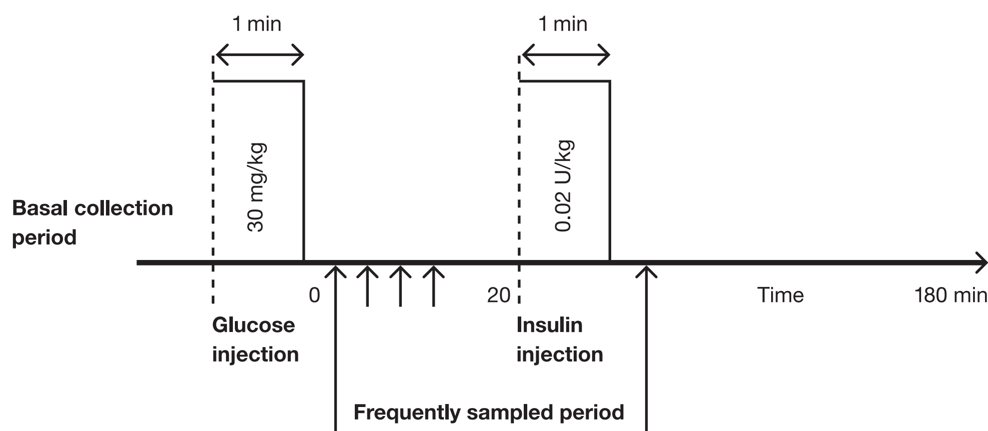

Figure 4. The FSIVGTT method shown schematically. Following a basal collection period, an intravenous bolus dose of glucose is given, and frequent sampling of insulin and glucose samples begins. After 20 minutes, an intravenous insulin bolus dose is given. Sampling is spaced out during the total glucose challenge of 180 minutes. Not drawn to scale.

In addition to Si, the FSIVGTT-MMOD can be used to estimate glucose effectiveness, termed Sg. This is a measure of glucose ability *per se* to enhance its own disposal and inhibit hepatic glucose production in the absence of an incremental insulin effect, i.e. at basal levels. Also, the Acute Insulin Response, AIR, may be determined which is a measurement of the insulin response (calculated as the area under the curve) during the first ten minutes of the test.

Advantages of the FSVIGTT-MMOD include being easier to perform than the glucose clamp method, steady-state conditions are not required, and the protocol is simply implemented without constant adjustments of intravenous infusions. Coefficients of variation for Si are comparable to insulin sensitivity estimates using the glucose clamp (73, 74), and reasonable correlations have been found between the reference glucose clamp and minimal model analysis of the FSIVGTT in healthy subjects, with weaker correlations in insulin resistant populations (69, 75, 76). Although sometimes used in relatively large studies, the FSVIGTT is still a costly and relatively complicated procedure. Another limitation may be the fact that the model relies on a simplification of the physiology of glucose homeostasis, such as grouping together the effects of insulin to promote peripheral glucose utilization and suppress hepatic glucose production. As insulin sensitivity/resistance varies, the relative contribution of hepatic glucose production to Si may vary to a significant extent (77). Also, due to the dynamic nature of the minimal model, estimates of Si are less reliable in individuals with impaired insulin secretion and/or significant insulin resistance. In these subjects, Sg may also be overestimated to accurately predict the disappearance of glucose during the FSIVGTT (78-80). Further, FSIVGTT-MMOD analysis may produce nonsensical

negative or zero Si values in subjects who have a profound insulin resistance and minimal insulin secretory capacity (76, 79, 81).

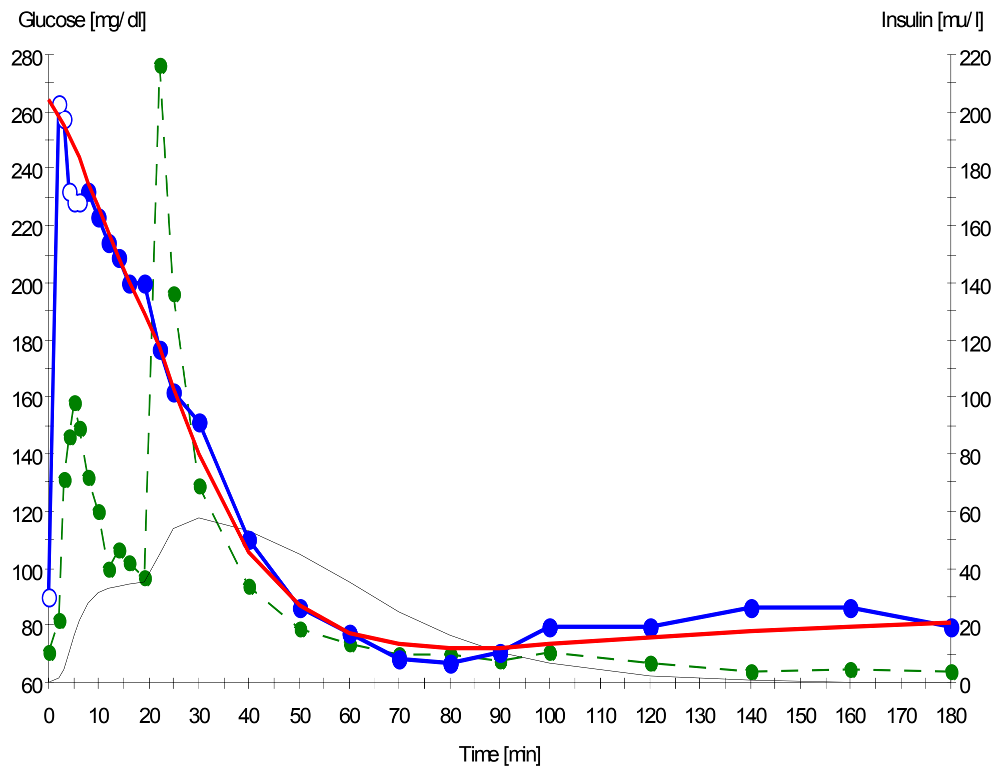

Figure 5. FSIVGTT curve from the MINMOD program showing the rapid increase in glucose after the bolus injection, followed by a first, endogenous insulin peak, and after 20 minutes a second insulin peak after the exogenous injection. Glucose levels gradually decline and may even descend below baseline levels.

3.3 SIMPLE FASTING INDICES FOR INSULIN SENSITIVITY ASSESSMENT

Fasting indices are derived from fasting steady-state conditions. In principle, after an overnight fast a single blood sample is taken for determination of fasting glucose and insulin. Alternatively, three samples at five-minute intervals may be taken and a mean calculated. In the healthy state, fasting represents a basal steady state where glucose is homeostatically maintained within a normal range, so that insulin levels are held within a narrow range and hepatic glucose production is constant. This means that basal insulin secretion rate by the pancreatic β-cells determines a relatively constant insulinemic level that will vary in accordance with insulin sensitivity/resistance such that hepatic glucose production matches whole body glucose disposal under fasting conditions. Fasting indices rely on the fact that subjects are strictly fasting and in basal steady-state conditions in regards to glycemia, insulinemia and hepatic glucose production. Fasting indices reflect primarily hepatic insulin sensitivity, but assuming that hepatic and skeletal muscle insulin sensitivity are proportional to each other this may provide an estimation of muscle insulin sensitivity as well. An obvious advantage of fasting indices is the inexpensive, simple set-up, which has made them extensively used in large epidemiological studies and in clinical practice. Limitations are explored in Studies I-II.

3.3.1 HOMA-IR

The Homeostasis model assessment, HOMA-IR, is derived from a mathematical assessment of the interaction between β-cell function and insulin resistance and was developed by Matthews et al in 1985 (82). It was developed as a computer-based model in which an array of fasting plasma insulin and glucose concentrations representative of varying degrees of β-cell deficiency and insulin resistance were plotted. The resulting array estimates insulin resistance and β-cell function on the basis of fasting insulin and glucose drawn from a given subject. The model assumes a feedback loop between the liver and β-cell, so that glucose concentrations are regulated by insulin-dependent hepatic glucose production, whereas insulin levels depend on the pancreatic β-cell response to glucose concentrations. Thus, impaired β-cell function reflects a diminished response of the β-cell to glucose-stimulated insulin secretion, and insulin resistance is reflected by a diminished suppressive effect of insulin on hepatic glucose production. Since the method was first published it has been widely used and is simplified to: HOMA-IR=(fasting plasma insulin x fasting plasma glucose)/22.5. The denominator 22.5 is a normalizing factor, i.e. the product of normal fasting plasma insulin of 5 μU/ml and a normal fasting plasma glucose of 4.5 mmol/l= 22.5, so that an individual with "normal" insulin sensitivity has a HOMA-IR=1. There is also a newer, updated model, however the original model is the most widely used due to its simplicity. HOMA-IR data are typically not normally distributed and need to be logarithmically transformed when used. HOMA-IR has been used in >500 publications, and is recommended in large cohort studies, cross-sectional epidemiology studies and physiologic studies in the normal population (83). HOMA-IR is the most widely used surrogate measure in children, however the accuracy of HOMA-IR as well as of fasting insulin is low (84) and it is thus not recommended for screening purposes (66).

3.3.2 QUICKI

The Quantitative Insulin Sensitivity Check Index, QUICKI, was developed in 2000 (81) with the aim of finding a new, simple test which could replace the clamp and FSIVGTT in large studies and in clinical practice. It is an empirically derived mathematical transformation of fasting glucose and insulin concentrations. Clamp and FSIVGTT studies were performed on 28 non-obese, 13 obese and 15 diabetic subjects. From these data a sensitivity analysis was carried out, and a novel index was determined which could be calculated using a single sample of fasting insulin and fasting glucose. A log transformation was included since the linear correlation to clamp data was improved when the skewed fasting insulin levels were log transformed. This correlation is however not maintained in diabetic subjects whose fasting hyperglycemia and impaired β-cell function is insufficient to maintain euglycemia. To account for this, log (fasting glucose) to log (fasting insulin) was incorporated into the formula. The reciprocal of this sum further transforms the data, generating an index which has a positive correlation with the clamp: 1/[log (fasting plasma insulin) + log (fasting plasma glucose). The correlation of QUICKI to clamp results was reported to be high and it was concluded that it was a valid proxy measure of insulin sensitivity. As is the case for HOMA-IR, the advantage of using QUICKI lies in the simple and inexpensive procedure, however in children the use of fasting indices, including QUICKI, is not recommended (66).

4 OBESITY AND METABOLIC ABNORMALITIES IN CHILDREN

Overweight and obesity are increasing health problems in children and adolescents worldwide. Based on measured height and weight in 1.6 million adolescent men in Sweden between 1969 and 2005, overweight tripled from 7.1% to 20.5%, obesity quintupled from 0.9% to 5.1% and morbid obesity increased ten-fold from 0.1% to 1.3% (85, 86). Also, the morbidly obese (BMI≥35) as share of the obese group increased from 13% to 25% during the same period (86). In Sweden, data from a nationally representative survey performed in 2008 in 7-9 year-old children show a 17% prevalence of overweight with a corresponding figure of 3% for obesity (87). Although there have been implications that this trend is levelling off (88), this is still highly alarming. The most established definition of overweight and obesity is the Body mass index (BMI), calculated as the weight in kilograms divided by the height in meters squared (kg/m^2). Cut-offs for adults have been defined by the World Health Organization to define overweight (≥25 kg/m^2) and obesity (≥30 kg/m^2) respectively (89), whereas in children and adolescents gender and age specific cut-offs have been defined by the International Obesity Task Force as to relate childhood BMI to the adulthood counterpart (90).

The complication of the metabolic syndrome has been increasingly recognized in children and adolescents, and has been reported to be as high as 50% in certain obese populations (91, 92). Until recently there has been no unified definition of the metabolic syndrome in the pediatric population, but the International Diabetes Federation (IDF) has now released such guidelines (93). A further study has suggested new criteria with age- and sex-specific cut-offs (94), and it is widely recognized that a consistent definition must be used. A recent study investigating the prevalence of the metabolic syndrome in European youth found this condition in 0.2% of 10-year old children and 1.4% of 15-year old children (95) using the IDF criteria. Although this is a low prevalence, a large proportion of the children (15-20%) had ≥2 risk factors or were centrally obese.

In certain pediatric populations a very high prevalence of glucose metabolic disturbances has been documented, yet in Sweden the number of children with type 2 diabetes is still low. However, in a yet unpublished Swedish study, impaired fasting glucose was found in 52.5% and impaired glucose tolerance in 20.6% of the obese children studied (n=139, personal communication Anna Mattsson). No cases of silent diabetes were found. Alarmingly, even in the youngest children (<10 years of age) impaired fasting glucose was found in 66.7% and impaired glucose tolerance in 14.3%. The prevalence of severe obesity with a BMI SDS score ≥2 and two or more criteria of the metabolic syndrome was found in 45%. As childhood metabolic syndrome often accompanies the growth transition with a three-fold increased risk of the metabolic syndrome in adulthood (96), this is especially concerning. Many of these children will thus be exposed to the adverse effects of obesity for a prolonged period of time, where puberty constitutes a particular risk period where physiological changes further decrease insulin sensitivity. Not only obese children are at risk for metabolic disturbances, but also children born small for gestational age, girls with polycystic ovary syndrome and children having undergone oncologic treatments among others. Early detection and treatment is thus crucial in order to reduce future morbidity and mortality.

5 NONESTERIFIED FATTY ACIDS AND INSULIN RESISTANCE

Fat storage in the form of triglycerides in adipose tissue constitutes a major fuel reserve in the body. Breakdown of triglycerides into glycerol and nonesterified fatty acids (NEFAs) liberates energy substrates as well as mediates other important pathways, such as influencing insulin action and glucose metabolism, acting as transcription factors and regulating insulin production. The breakdown of triglycerides, termed lipolysis, is carried out in a step-wise manner in which diglycerides, monoglycerides and lastly glycerol and NEFAs are produced. One molecule of triglyceride results in one molecule of glycerol and three molecules of NEFA, which are then oxidized to yield energy in the form of ATP. Some NEFAs are re-esterified into new triglycerides within the adipocyte. Hormone sensitive lipase, which catalyzes the first step of the lipolysis process, is regarded as the rate-limiting step and is regulated hormonally. The hormonal regulation of lipolysis in the short term is by catecholamines, insulin and natriuretic peptides, whereas in the long term growth hormone has a regulatory role. Lipolysis is however also regulated by a number of different factors besides hormones: paracrine factors (cytokines, adenosine, prostaglandins), age, gender, nutrition, physical activity, adipose region and genetics (97).

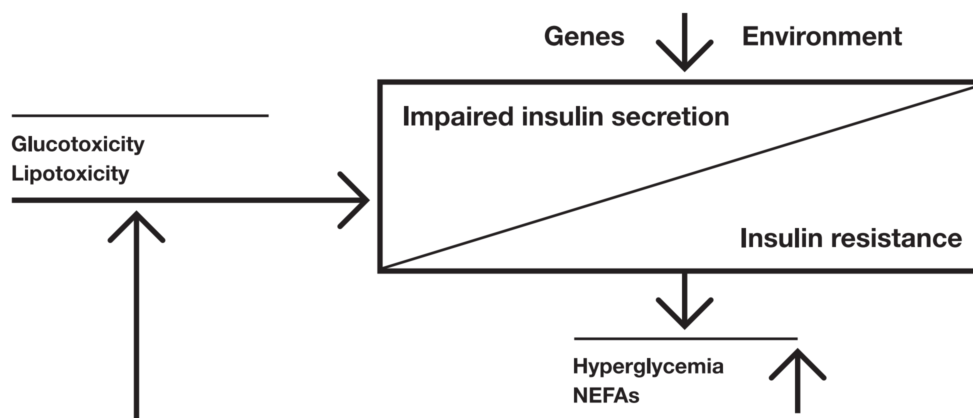

Figure 6. Genetic and environmental factors are involved in the pathogenesis of type II diabetes, a condition which is caused by a combination of insulin resistance and inadequate insulin secretion. The raised levels of glucose and NEFAs seen in this condition give rise to gluco- and lipotoxic effects on tissues, leading to further deterioration of insulin sensitivity and insulin secretory capacity.

Historically, type 2 diabetes has been considered to revolve around a glucose-insulin axis. It has however become apparent that NEFAs also play a very important role in the development of insulin resistance and thus eventually type 2 diabetes (**Figure 6**). As described above, NEFAs are released from adipocytes during periods of low energy intake. However, if plasma NEFA levels are elevated for more than a few hours, insulin resistance will develop (98). This may have desirable physiological effects such as preserving carbohydrate for use by vital tissues such as the brain, or during pregnancy when maternal relative insulin resistance preserves glucose for the growing fetus. However, in abundance of energy, the NEFA induced insulin resistance becomes counterproductive and has pathological consequences. The exact mechanism by which NEFAs induce insulin resistance in skeletal muscle is not known. The Randle cycle (99), first described in 1963, hypothesized that glucose and NEFA levels have an

inverse reciprocal relationship, so that glucose uptake is reduced when tissue energy needs are being met by NEFA oxidation and vice versa (**Figure 7**). The oxidation of NEFAs was thought to result in reduced glucose oxidation and increased levels of intracellular citrate, which would decrease glycolysis and glucose uptake. The Randle cycle has only been partially confirmed by in vivo and in vitro studies (98, 100-102), however, and other mechanism have been sought.

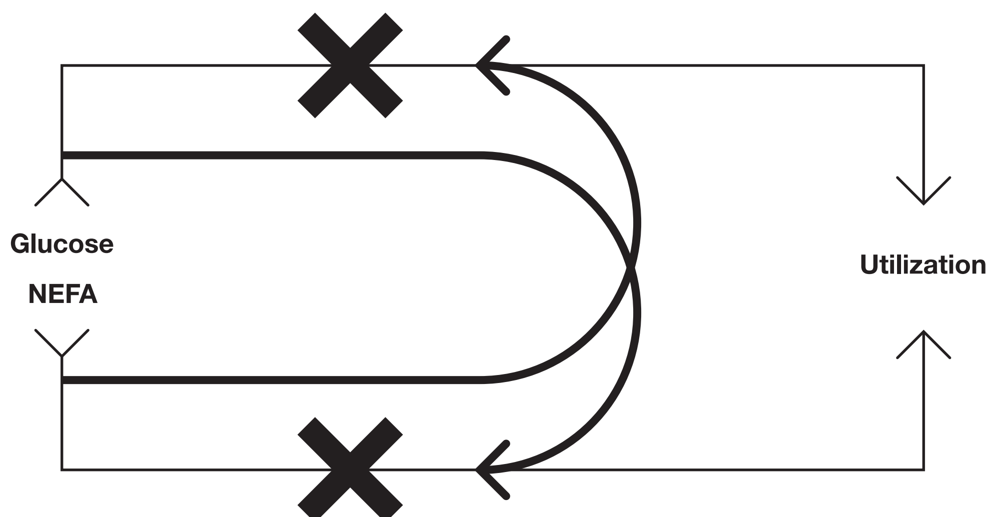

Figure 7. Schematic representation of how glucose and NEFAs interact in skeletal muscle, such that raised NEFA levels leads to the preferential oxidation of this substrate instead of glucose, and vice versa.

A further hypothesis is that NEFAs induce insulin resistance in human skeletal muscle at the level of insulin-stimulated glucose transport or phosphorylation by impairing the insulin-signaling pathway (103-105). The accumulation of metabolites such as diacylglycerol (DAG), an intermediate of triglyceride metabolism, is thought to play an important role. DAG activates protein kinase C (PKC) (106), which phosphorylates serine and threonine residues on the insulin receptor (107, 108) and IRS-1 (108, 109), and this may interfere with insulin signaling. The activation of the nuclear factor (NF)-κB pathway has also been shown to be affected by increasing DAG levels intracellularly (110). This pathway is linked to NEFA-induced insulin resistance in rodents (41, 111), and also plays a role in the development of coronary artery disease (112). NEFAs may also decrease insulin sensitivity by inducing oxidative stress (113), in which reactive oxygen species activate PKC and the NF-κB pathway, again leading to decreased insulin sensitivity (110, 114).

NEFAs also have direct effects on the liver. Insulin suppresses hepatic glucose production mainly by inhibiting glycogenolysis (115). NEFAs induce insulin resistance in the liver by inhibiting the suppression of glycogenolysis by insulin, leading to hepatic overproduction of glucose and thus hyperglycemia (116, 117). Insulin also stimulates NEFA uptake in the liver and production of intracellular triglycerides, and hepatic insulin resistance thus leads to elevated plasma NEFA levels. A general increase in visceral fat could also contribute to insulin resistance by an increased amount of inflammatory mediators being secreted from the adipocytes, such as tumor necrosis factor (TNF)-α (118), interleukin-6 (119), resistin (120), leptin (121) and adiponectin (122).

Besides having effects on insulin sensitivity, NEFAs also have effects on insulin secretion. Since NEFAs can cause insulin resistance in skeletal muscle as well as in the liver, it would be expected that all overweight or obese subjects, who to a great extent

have raised levels of NEFAs, would have elevated glucose levels. This is however not the case. The reason for this seems to be that NEFAs are potent insulin secretagogues and can compensate for the insulin resistance they produce in obese insulin-resistant subjects with intact β-cell function, since acutely raised NEFA levels stimulate insulin secretion (123), but also extended NEFA elevations potentiate glucose-stimulated insulin secretion in healthy subjects (124-126). In subjects who are genetically predisposed to type 2 diabetes, however, NEFAs are not fully able to compensatorily increase insulin secretion for the insulin resistance they induce. Thus, NEFAs play an important role both for the secretion and sensitivity of insulin.

6 WHAT IS MODELING?

Models are simplified representations of systems and can be used for the study of complex processes, which occur simultaneously (127). The different processes and their interactions can be described in mathematical terms by equations. The simultaneous solving of these equations becomes a representation of the behavior of the system, which can then be used to predict or describe the system.

The rationale for developing and using models is that it may be too costly, impossible or impractical to probe the real system. *Modeling* is the process of developing a model or set of equations to simultaneously represent the structure and behavior of a system. Modeling biological systems is based on observations of the system, for example to determine the kinetics of a certain drug after administration (**Figure 8**). As the structure of a biological system most often is unknown, a model developed to fit data from the system also becomes a hypothesis of how the system works, and the model will be refined and evolve as further studies provide more evidence on the underlying construction. There may be some limitations to modeling biological systems, such as incomplete data due to limitations on sampling sites, sampling times and number of studies that can be performed. Further, data may be imprecise and there may be constraints related to the biology of the system or to the experimental techniques.

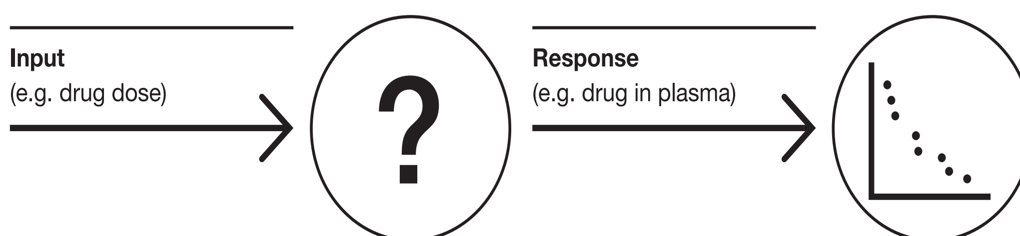

Figure 8. Modeling is based on using the observed response of a system to determine how the system works after a given input.

Within the frame of this thesis we have developed and validated two compartmental models. These assume that the compound or compounds of interest, in this case glucose, insulin, C-peptide and NEFAs, are distributed throughout the system in discrete entities, called compartments. A compartment is considered to contain kinetically indistinguishable material and may be defined in terms of a physical space (such as the plasma pool for example) or in conceptual terms (such as all particles that turn over at a specified rate).

6.1 BUILDING A MODEL

There are a number of steps involved in the development of a biological model. A schematic representation of the modeling process is shown in **Figure 9**, and the steps will be described in this section with the C-peptide model of Study III used as an example. As in research in general, the modeling process starts by identifying a problem or scientific question, which is then formulated to yield a hypothesis. In this case, the identification of a means of assessing insulin secretion by the use of C-peptide

is the problem we attempt to solve. We then define the system we are going to study, in this case the kinetics of C-peptide in a healthy normal weight population. We here define and set the boundaries for the investigation, such as the time (first twenty minutes of an FSIVGTT), the inputs and outputs (C-peptide concentrations and parameters). Also, we identify the purpose of the model, as this will form a basis of how the model should be developed. In the C-peptide model, the purpose is to calculate the secretion rate of C-peptide. When these initial conditions are set, the literature should be searched for existing models so that the model can be tested against new data, as opposed to developing a completely new model which may turn out to have only minor differences to older ones. In our case, the existing Boston C-peptide model was further elaborated for our purposes. In case a completely new model must be developed this can be done either by starting with the simplest possible model and adding features successively to fit the data, or by developing a model including all known information, then scaling it down stepwise to fit the data and lastly validate the model in another data set.

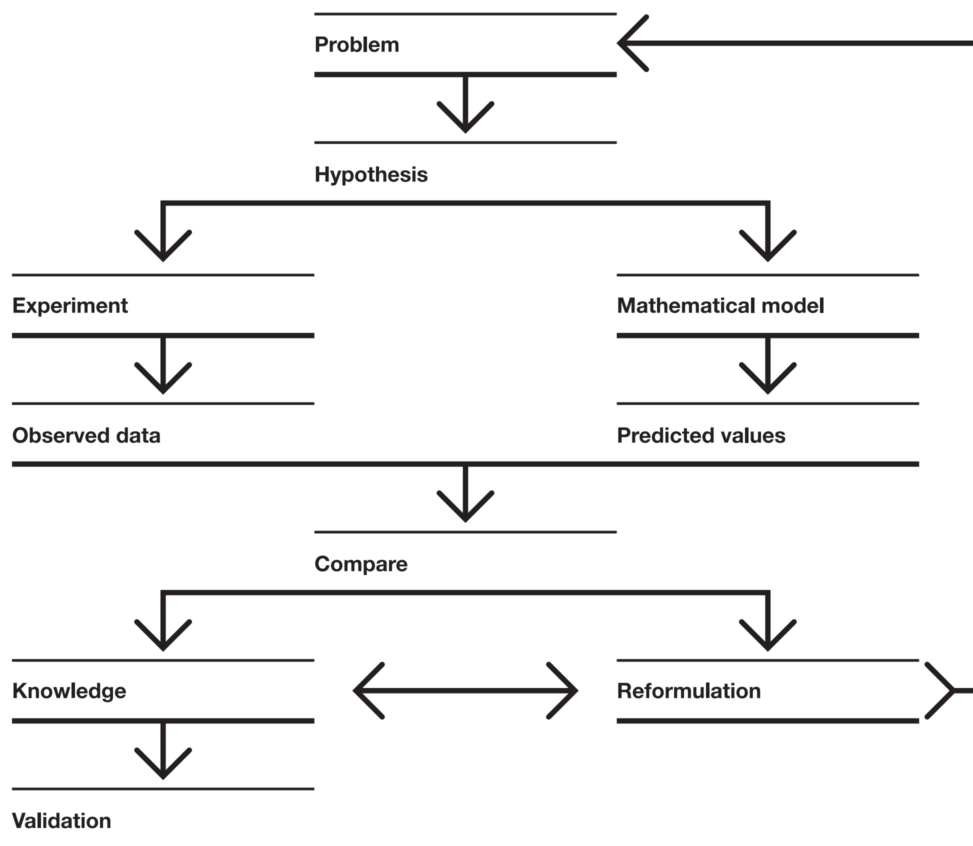

Figure 9. The modeling process starts with defining the actual problem to be solved. Following a number of steps either a new model is established, which contributes valuable knowledge and can be tested in other populations, or the model is rejected and must be reformulated and go through the modeling process once again.

In the next step, experimental data (C-peptide samples from our 15 study subjects) are to be compared to the model predictions. This is done by converting the model into a format which can be solved by a modeling computer package. In the present study, the so-called WinSAAM modeling program has been used. The following needs to be entered into the modeling program: model equations, parameters and their initial values, values for compartments at the start of the study, data, link between data and a

corresponding component of the model, possible changes in the experimental conditions of the study, conversion of units for observed data and calculated values and lastly functions. When the model has been set up and solved, comparison is made between observed data and model-calculated values. Initially, this can be done by graphically comparing the shape of the predicted curve to the observed data. Fitting data may be done manually by testing multiple values for a given parameter to see how this affects the model fit. This makes it easier to understand all parts of the curves and of the model. Once a model fit is obtained which seems close, a process of iteration is carried out which fits the data further. Refining the model may then have to be done to resolve possible errors and differences between observed and modeled data. When the fitting process is deemed successful, it needs to be critically evaluated with respect to how well the parameters are determined and the correlation between parameters. This can be done by looking at standard deviations of the parameters and making sure these are limited. When the model is finally consistent with the experimental data, it can be tested under different conditions as well as be published in order to be available for others to test and use.

6.2 WHY MODEL BIOLOGICAL SYSTEMS?

Modeling is increasingly being applied to the study of biological systems. The challenge in this approach is not to set up an arbitrary function which happens to fit the data, but to use modeling to understand the biological system being studied. Modeling can be used to determine the structure of a system, meaning how the different parts of the system relate to one another. This relationship may be between species of an ecological system, a nutrient in blood and some specific tissue, or a certain metabolite distributed throughout a cell. A model can be used to determine the relationship as well as the sequence of events which occur between various substances of interest. This information may in some systems be known already, whereas in others the structure can only be inferred from the data. Also, models can be used to determine parameters of interest, such as the size of a plasma pool, the clearance rate of a compound of interest, or transport rate of a substance between compartments. In this case it is important that the model is consistent with known biological information, so that parameters can be accurately assessed. Another purpose of a model may be to determine the interactions of parts of a system, for example linking the metabolism in one tissue with the metabolism of another, or to link the metabolism of a nutrient in one form with the metabolite in a second form.

When a model exists, it may be used to simulate and predict levels of a drug in the blood following various dosing regimens. The response of the system to the various inputs can be simulated on the short as well as long term. A useful application in this sense may be simulating likely scenarios before commencing complicated and expensive experimental data collections. This will not replace the actual experiment, but help avoid experiments with insufficient or inappropriate data, which in the end will not hold for testing the hypothesis. Another use for biological models may be to help identify sites of change in a given system when studied under different conditions. The varying conditions may result in large changes in the kinetic curves and in many pathways of the system, or it may only cause a subtle change in the data caused by a large change in a single parameter. In this case, models help to identify which parameters change between the conditions and the degree of change. Conditions may be a healthy vs. a diseased subject, a treated vs. untreated subject, or a normal vs. a high

intake of a specific nutrient. Lastly, models may provide a valuable tool for education purposes, such as demonstrating the properties of a system, teaching principles such as feedback loops or saturation kinetics and to test theories.

6.3 WHAT IS AN IDEAL MODEL?

As described, we create models to help examine our understanding of systems. Once a model has been created and seems to adapt to different circumstances of application, one needs to look at exactly how efficiently the model actually adapts to different settings and how the setting-specific index values sensibly characterize the situations. This involves investigating how well a model seems connected to the domains of its origin. On the one hand, parameters may be closely linked to the empirical processes visible in the data from which the model was developed. On the other hand, the parameters may be a property of the underlying hypothesis captured in the structure of the model, and possibly only loosely related to the original data. In the first case, where parameters are very tightly connected to the empirical setting, such models may serve little purpose beyond offering a summarization of the data. In the second case, where parameters are very loosely connected to the empirical setting, the parameters may describe how well the *model,* rather than the *biological system* studied, responds to different circumstances. Neither of these two alternative situations offers much understanding of the system. Instead, the ideal would be to see some relationship between the model parameters and their empirical counterparts, yet not an absolute concordance between them. Also, there should be some basis linking a model to other models in the same domain. In absence of this, one must wonder if model indices really bear any association to the physical system under investigation.

7 CLINICAL APPLICATION: LONG-TERM CANCER SURVIVORS

Insulin resistance is a known feature of obesity, type 2 diabetes, polycystic ovary syndrome among other clinical states. Beginning in the late 1980s, several studies have also shown an increased risk of the metabolic syndrome, with insulin resistance as a common component, in long-term survivors of first childhood cancer (128), and later also survivors of adult-onset cancer (129). With the increasing survival rates, this group constitutes an at-risk population of growing importance. Many studies have focused on one or more components of the metabolic syndrome, with few reports on the syndrome as a whole (130). Studies have found insulin resistance, hyperinsulinemia and impaired glucose tolerance after several childhood malignancies (128, 131-135), and patients treated with stem cell transplantation and total body irradiation seem to be at a heightened risk (136). Differences in cancer types and treatment strategies might lead to differences in etiology, however combining knowledge of the metabolic syndrome gained from studies of survivors of childhood and adult cancers could contribute valuable insight into the pathophysiology of the metabolic syndrome and intervention strategies thereof.

It is not clear what causes the metabolic derangements in cancer survivors. Hormonal changes which may contribute include damage to the hypothalamus-pituitary axis, leading to deficiencies in growth hormone, thyrotropin, gonadotropin and adrenocorticotropin and thus secondary deficiencies in thyroid hormones, sex hormones and adrenal hormones, all of which are associated with the metabolic syndrome or its components. The hypothalamus and pituitary are very sensitive to radiation, which is a common treatment in childhood cancer and may explain some of the disturbances. The specific effects of chemotherapy on the hypothalamus and pituitary have not been studied extensively, but there may be effects of chemotherapy contributing as well.

The development of the metabolic syndrome among cancer survivors has also been shown to be associated with signs of early atherosclerosis (137, 138), which may lead to an increased risk of cardiovascular disease. There is also a direct cardiovascular toxicity of some cancer treatments, which further elevates this risk. Further, survivors of some childhood cancers have been shown to have reduced energy expenditure during exercise and rest (139, 140), as well as decreased physical activity (141), which may contribute further to obesity and insulin resistance.

In conclusion, long-term survivors of childhood cancers constitute a group with an increased risk of the metabolic syndrome including insulin resistance, the exact background of which is not known. Currently, there are no specific guidelines for the treatment of these disturbances in cancer survivors and recommendations are thus the same as for the general population. The identification of a candidate etiological factor might however aid in developing more specific means of diagnosis and therapies and thus reduce complications. The fifth study of this thesis investigates insulin sensitivity and insulin secretion in long-term survivors of childhood hematologic malignancies with respect to body composition and hormonal disturbances in growth hormone and adipokines.

8 RATIONALE FOR PERFORMING STUDIES INCLUDED IN THIS THESIS

8.1 STUDY I

A number of methods of insulin sensitivity assessment have been developed, the most validated ones being relatively complicated, time- and labor consuming to perform, especially in children for whom a long fast, several hours of bed rest and multiple blood samples may be difficult. Establishing a robust yet simple test for insulin sensitivity assessment is attractive for both patients and caretakers. Less invasive, simple methods relying on only a single sample of fasting glucose and insulin have thus been developed (81, 82). Controversy however exists regarding the validity of these simple measures, and validation studies have been performed in various populations (see **Table 7**). Obese children and adolescents are an at-risk population for developing insulin resistance, and the validation of simpler fasting indices in this group is of great clinical interest. Study I was thus performed in order to investigate the validity of fasting insulin, HOMA-IR and QUICKI as proxy measures of insulin sensitivity as compared to the FSIVGTT-MMOD.

8.2 STUDY II

Since the development of fasting indices HOMA-IR and QUICKI, these two methods have been compared in a number of publications (see **Table 7**). However, where some have found these methods to be interchangeable, others have found one or the other method to perform better and have promoted the use of HOMA-IR before QUICKI or vice versa. Further, some authors find fasting insulin to be an equally good measure of insulin resistance as fasting indices in itself, whereas others state that calculating indices is superior to merely using fasting insulin. There have also been different views on the necessity of taking three fasting samples and using the mean for calculating indices as opposed to using a single sample. Study II was performed to test the hypothesis that fasting indices HOMA-IR and QUICKI are interchangeable. Further, fasting insulin was compared to fasting indices to establish if adding glucose to a formula adds any information. Lastly we investigated the quality of single sampling to using the mean of repeated samples.

8.3 STUDY III

Type II diabetes develops when the pancreatic β-cells fail to secrete enough insulin to compensate for the insulin resistance in peripheral tissues. Assessing insulin secretion is thus important in evaluating the progression from a pre-diabetic to a diabetic state. C-peptide and insulin are secreted from the pancreas in equimolar amounts, yet whereas insulin is extracted by the liver to some extent C-peptide does not undergo significant hepatic extraction. C-peptide can thus be used as an indirect marker of insulin secretion. Several C-peptide models have been developed using different mathematical approaches, a number of these using C-peptide secretion during the FSIVGTT. There are limitations to each of these previous studies, such as the complexity of the model or the need for additional experiments to supply C-peptide kinetics data (142). In study III a C-peptide model was developed for the indirect assessment of insulin secretion during the insulin modified FSIVGTT. The insulin modified protocol is preferable in settings where the subject's endogenous insulin production may be insufficient to

handle the glucose load (67, 143). In order to be clinically useful a model for this purpose should be user-friendly and easy to implement. Study III was thus performed to develop a model with the above characteristics, and to validate this model by comparison to previous work in this area.

8.4 STUDY IV

Levels of NEFA affect insulin sensitivity and elevated NEFA levels are often seen in pathological conditions such as diabetes, obesity, hypertension and coronary heart disease (144). Glucose, insulin and NEFA levels are interrelated and show a characteristic pattern during the FSIVGTT. A model of NEFA kinetics has the potential to make a contribution to the understanding of NEFA metabolism in different pathological states. A model has previously been reported for this purpose, however it has not been tested and validated on a subject level but only on mean data (145). There is a great variability among different subjects regarding the NEFA response, and for a model to be valid it should be able to fit this diversity of responses. Study IV thus provides a subject level validation of a newly developed NEFA model which can describe the major features of the NEFA response to an FSIVGTT.

8.5 STUDY V

As therapeutic strategies for childhood cancer have been successively improved, the population of long-term survivors of cancer has grown. This population is at risk for developing metabolic abnormalities, including obesity, insulin resistance, hypertension and dyslipidemia (136). The exact mechanism behind the adverse metabolic effects of cancer treatment is not known, but identifying the etiological background would make it possible to target interventions. Persons having undergone stem cell transplantation (SCT) seem to be at an increased risk of developing glucose metabolic derangements (131, 132, 146-154). In general, insulin resistance is closely linked to obesity, however most SCT survivors are normal weight when assessed by BMI (151, 155). The role of obesity in insulin resistance development is thus not clear in this group and other factors may be more important, or possibly BMI may not be an adequate measurement of obesity in this group (156). Study V was thus performed to investigate insulin sensitivity, insulin secretion and body composition in a cohort of long-term survivors of childhood cancer as compared to healthy matched controls.

9 OBJECTIVES

9.1 GENERAL OBJECTIVES

The overall aims of this thesis were

1. to examine existing methods of insulin sensitivity assessment,

2. to develop new methods for the assessment and further understanding of insulin secretion and sensitivity and

3. to apply these methods in a clinical setting.

9.2 SPECIFIC OBJECTIVES

Five specific questions have been addressed in this thesis:

1. What is the validity of HOMA-IR, QUICKI and fasting insulin as proxies for insulin sensitivity as measured by the sensitivity index of the FSIVGTT-MMOD?

2. Is there a rationale for further comparisons of fasting indices and fasting insulin as proxies for insulin sensitivity assessment?

3. Is it possible to develop an accurate yet simple model for insulin secretion assessment by the indirect use of C-peptide during the insulin modified FSIVGTT?

4. What is the subject level validity of a novel model of NEFA kinetics during the FSIVGTT?

5. What are the long-term consequences of stem cell transplantation including total body irradiation in terms of insulin sensitivity, insulin secretion and metabolic profile?

10 SUBJECTS AND STUDY DESIGN

10.1 STUDY I-II

Studies I-II included a population of 191 obese children and adolescents (109 females, 82 males) who were all investigated at the National Childhood Obesity Center at the Karolinska University Hospital in Huddinge, Stockholm, Sweden. These subjects were all referred to the center because of their obesity, and were investigated as in-patients for one week aiming to determine causes and complications to their obesity. During this week numerous assessments were performed including an FSIVGTT, body composition assessment using DEXA, blood sampling, and assessment of pubertal stage and presence of acanthosis nigricans. Heredity for obesity, diabetes mellitus and cardiovascular disease was documented. Clinical characteristics are shown in **Table 2**. The sample was stratified by sex, pubertal status and median of Si for the statistical analyses.

The Regional Ethics committee in Stockholm granted ethical approval for the studies, and parents and children gave informed consent.

Table 2. Clinical characteristics of the study population for studies I and II.

Variable	Males (n=82)	Females (n=109)	Total (n=191)
Age (years)	13.4±2.9	14.2±2.9	13.9±2.9
Weight (kg)	100.9±32.9	97.8±21.4	99.1±26.8
Height (cm)	164.2±15.7	161.3±10.9	164.0±13.2
BMI (kg/m^2)	36.5±6.7	37.3±5.7	36.9±6.2
BMI-SDS	6.4±1.8	5.9±1.4	6.1±1.6
Sensitivity index (10^{-4}min^{-1}/(µU/ml))	0.36±0.25 (0.30; 0.08-1.18)	0.39±0.27 (0.33; 0.08-1.65)	0.37±0.26 (0.32; 0.08-1.65)
HOMA-IR	3.6±2.4 (2.8; 1.2-14.4)	3.7±2.5 (3.3; 0.9-21.8)	3.6±2.4 (3.2; 0.9-21.8)
QUICKI	0.33±0.03 (0.34; 0.27-0.39)	0.33±0.02 (0.33; 0.26-0.41)	0.33±0.02 (0.33; 0.26-0.41)
Fasting insulin (pmol/l)	113.2±77.4 (87.0; 35.0-460.0)	112.8±56.7 (106.0; 26.0-458.0)	113.0±66.2 (97.0; 26.0-460.0)
Fasting glucose (mmol/l)	4.4± 0.4 (4.4; 3.5-5.6)	4.3±0.5 (4.2; 3.1-6.2)	4.3±0.5 (4.3; 3.1-6.2)

Results shown as mean±SD, (median; minimum-maximum)
BMI-SDS as described by Rolland Cachera et al (157)
HOMA-IR= Homeostasis model assessment, as described by Matthews et al (82)
QUICKI= Quantitative Insulin Sensitivity Check Index, as described by Katz et al (81)

10.2 STUDY III-IV

Studies III-IV included 15 healthy subjects (8 males, 7 females), 20-30 years of age who were all recruited by advertisement among medical students in the university hospital. Inclusion criteria were normal weight, healthy not taking any medication, no current pregnancy and no smoking. Clinical characteristics are shown in **Table 3**. Demographic data were collected and all subjects underwent an insulin modified

FSIVGTT and a hyperinsulinemic euglycemic clamp on two separate days. The FSIVGTT was complemented by C-peptide and NEFA samples at all sampling points.

The Regional Ethics committee in Stockholm granted ethical approval for the studies, and all subjects gave written informed consent.

Table 3. Clinical characteristics of the study population for studies III and IV.

Variable	Males (n=8)	Females (n=7)	Total (n=15)
Age (years)	24.1±2.4	23.6±2.3	23.9±2.3
Weight (kg)	72.9±8.4	55.7±6.5	64.9±11.5
Height (cm)	181.1±6.1	167.9±6.1	174.9±9.0
Body surface area (m^2)	1.9±0.1	1.6±0.1	1.8±0.2
BMI (kg/m^2)	22.2±1.8	19.8±1.7	21.0±2.1
Sensitivity index (mU/L^{-1} min^{-1})	6.6±2.0	10.2±2.6	8.3±2.9
Acute insulin response	313.7±67.3	308.7±163.5	311.3±117.2
Glucose effectiveness	0.03±0.01	0.07±0.12	0.05±0.09

Results shown as mean±SD

10.3 STUDY V

Long-term stem cell transplant survivors (defined here as alive 10 years after stem cell transplantation, SCT) treated with total body irradiation for acute lymphoblastic leukemia or lymphoblastic lymphoma were included in the study. These were recruited from those patients having undergone the pediatric SCT program at the University of Uppsala from October 1985 to June 1999. The cohort consisted of 18 long-term SCT survivors, 15 of which had been autografted and three of which had received an allogenic graft. Control subjects were selected by sending letters to 10 potential control subjects for each patient, randomly selected from a computerized registry of the population in Uppsala County. Controls were matched for age and sex. Of those who accepted by mail, the first one to reply to a phone call and deemed eligible for the study was chosen. Inclusion criteria were no known diseases, no medication other than contraceptives, no current pregnancy and no smoking. If none of the 10 controls accepted or were deemed eligible, a new set of 10 controls was selected and the process repeated. Clinical characteristics are shown in **Table 4**.

Patients and controls were investigated at the University Hospital in Uppsala. During two consecutive days and one night, subjects underwent an FSIVGTT, an OGTT, DEXA as well as blood sampling for insulin, c-peptide, IGF1, IGFBP3, CRP, leptin and adiponectin. A GH curve was obtained by sampling every 30 minutes during the night (12 h) with the maximum peak value of GH used as a measure of GH secretion capacity.

The Regional Ethical Review Board of Uppsala University granted ethical approval of the study, and all subjects gave written informed consent.

Table 4. Clinical characteristics of the study population in study V.

Variable	Patients median (interquartile range)	Controls median (interquartile range)	p
Fasting insulin (mU/L)	12.2 (7.3)	6.0 (2.8)	0.002
HOMA-IR	2.2 (1.7)	1.2 (0.5)	0.005
Sensitivity index (mU/L^{-1} min^{-1})	2.98 (1.99)	4.54 (2.02)	0.043
Acute insulin response	717 (754)	342 (258)	<0.001
BMI (kg/m^2)	21.6 (5.6)	24.5 (4.8)	0.093
Lean body mass (kg)	40.7 (12.5)	54.5 (18.6)	<0.001
%fat mass	34.9 (16.3)	24.3 (15.6)	0.011
GH_{max} (mU/L)	9 (12.9)	20.7 (16.3)	0.002
Leptin (µg/L)	18 (45.7)	4.8 (9.7)	<0.001

Results shown as median (interquartile range)
HOMA-IR= Homeostasis model assessment, as described by Matthews et al (82)
GH_{max}= Growth hormone maximum peak

11 MEASUREMENTS

11.1 FSIVGTT

The FSIVGTT was performed at 08:00 a.m. following an overnight fast. One peripheral intravenous catheter was inserted in each arm. Three fasting baseline samples for glucose and insulin were drawn at times -15, -10, and -5 minutes. At time 0 minutes, 0.3 g glucose per kg body weight was administered intravenously over one minute as 30% dextrose. At time 20 minutes, 0.02 U insulin per kg body weight was administered as an intravenous bolus dose. Blood samples for determination of glucose and insulin were drawn at times 0, 2, 3, 4, 5, 6, 8, 10, 12, 14, 16, 19, 22, 25, 30, 40, 50, 60, 70, 80, 90, 100, 120, 140, 160, and 180 minutes. MINMOD version 3.0 (Richard Bergman, 1994) was used to calculate the Si in Studies I-II and MINMOD Millennium (158) was used for studies III-V.

11.2 HYPERINSULINEMIC EUGLYCEMIC CLAMP

The hyperinsulinemic euglycemic clamp was performed after an overnight fast at the Karolinska University Hospital in Solna. A peripheral catheter was inserted in each arm, one for sampling and one for infusion. Insulin was administered intravenously at a fixed infusion rate of 1.0 mU/kg/min. Glucose (200 mg/ml) was simultaneously administered at a variable rate aiming to maintain plasma glucose at 5.0 mmol/L. Insulin and glucose testing was performed every 10 minutes during the 120 minute clamp.

11.3 MODELING PROCEDURE

After data collection, modeling was performed using the WinSAAM program (can be downloaded for free from http://www.winsaam.com). In Study III, a C-peptide model was created investigating the C-peptide secretion during the first 22 minutes of the FSIVGTT. The modeling technique is based on numerical deconvolution as outlined by Cutler (145, 159, 160) and employs a 2-compartment model to describe C-peptide disposition (161-164). This approach assumes that pancreatic C-peptide secretion during the FSIVGTT is described by two simultaneous processes: a constant basal C-peptide secretion and a first-phase C-peptide secretion described by a Gaussian function (142). The Gaussian function was chosen as it has been experimentally verified in vivo as describing the secretion profile and the rate of insulin secretion (142). The parameters of the model are the following: P_1 (picomoles per liter per minute) represents the height of the first-phase secretory pulse, P_2 (per minute^2) is related to the inverse of the pulse width at half-peak height, P_3 (minutes) represents the time of the peak of the first-phase C-peptide secretion rate, P_6 (picomoles per liter) is the initial C-peptide concentration and P_7 (picomoles per liter per minute) is a Gaussian function representing a first-phase C-peptide secretion pulse.

Results were validated by extrapolating the model to the first 22 minutes of the C-peptide response from the 8 subjects in the original description of the model, who had all undergone a full-length standard FSVIGTT. This was done by removing the second phase from the C-peptide secretion model and exploring the adequacy of a secretion sub-model comprising a first phase Gaussian pulse secretion identical to the subject's phase in the full (240 minute) study and a constant secretion sub-model. Only the

baseline secretion was allowed to adjust to accommodate the potential intrusion of the second (omitted) secretion phase. The allowance of baseline secretion adjustment could thus accommodate a possible overlap of the phases. The results of the current study were then re-fitted using just the first 22 minutes of the C-peptide data in order to show that valid and plausible parameters in agreement with previously reported data could be obtained.

In Study IV, a recently published NEFA model (145) was validated on a subject level by testing the existing model on the 15 study subjects' individual NEFA profiles. The general response of NEFAs to a glucose challenge is shown in **Figure 10**. The most important parameters of the model are S_{FFA} (μmol/l/min) and K_{FFA} (%/min). S_{FFA} is a parameter describing the rate of provision of NEFA to the plasma pool. After an overnight fast, the assumption is that intestinal absorption of NEFAs is negligible. S_{FFA} thus primarily represents the rate of lipolysis of adipose tissue and S_{FFA} is the potential maximum rate of lipolysis. K_{FFA} describes the rate at which NEFAs leave the plasma pool, and again, since patients are fasted, K_{FFA} primarily represents oxidation of fatty acids. To test the plausibility of the model, static parameters were developed from the dynamic curves using statistical software. The static counterpart of K_{FFA}, termed kffa, was determined from the slope of ln(NEFA) during the 10 minutes preceding the nadir of NEFA levels (shown in **Figure 10**). The static counterpart of S_{FFA}, termed sffa, was determined by multiplying basal NEFA level, kffa and the linear NEFA incline following the nadir (shown in **Figure 10**). The relationship between the dynamic parameters and their static counterparts as well as MINMOD indices were then investigated using regression analysis.

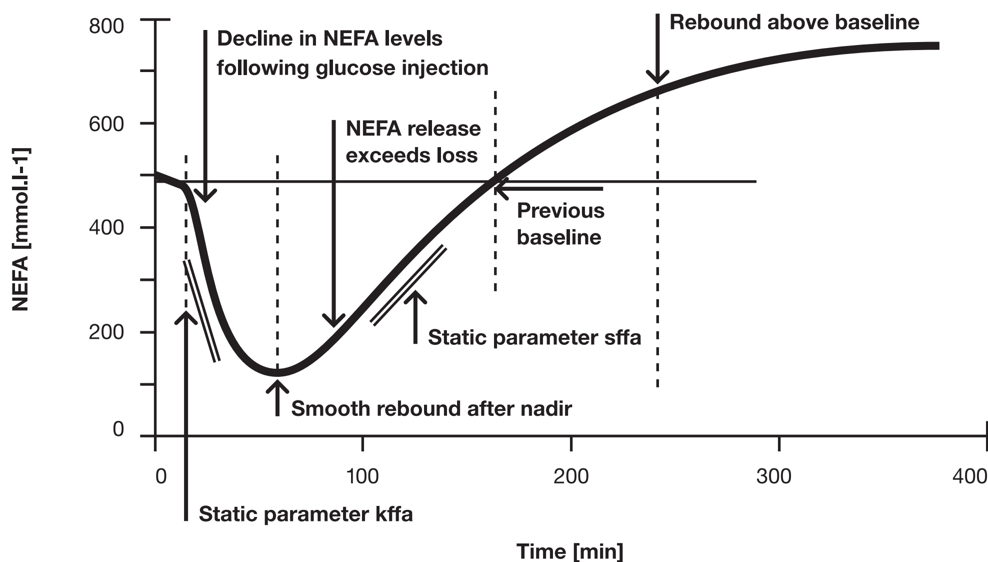

Figure 10. Typical NEFA curve during an intravenous glucose challenge. As glucose is injected, NEFA levels decline to a nadir, then slowly return to baseline and may also rebound above baseline levels. The derivation of the static parameters kffa and sffa also shown in the graph.

11.4 BIOCHEMISTRY

Samples from studies I-IV were analyzed at the accredited laboratory of the Karolinska University Hospital in Huddinge. For studies I-II, fasting insulin was analyzed using radioimmunoassay Insulin RIA 100 (Pharmacia Diagnostics AB, Uppsala, Sweden). For studies III-IV insulin was analyzed with electrochemiluminescense immunoassay,

ECLIA at Modular E (Elecsys, Roche Diagnostics, Scandinavia AB). The proinsulin cross reactivity with insulin ECLIA is 0.05 % according to the manufacturer. C-peptide was analyzed with electrochemiluminescense immunoassay, ECLIA (Elecsys, Roche Diagnostics, Scandinavia AB). Glucose was analyzed with the hexokinase enzymatic method Glukos HK (Roche) using a Roche Modular P-instrument. NEFAs were analyzed at the Centre for Inherited Metabolic Diseases at the Karolinska University Hospital in Huddinge with a Waco kit called NEFA-HR(2) on a Thermo T20xti. This is an enzymatic method with spectrophotometric detection using oleic acid as standard.

For study V CRP, leptin, adiponectin and GH were analyzed at the accredited laboratory at the Uppsala Academic Hospital. CRP was analyzed with an antigen-antibody complex method with spectrophotometric detection using the CRP Vario kit on an Architect instrument (Abbott). Leptin and adiponectin were analyzed using Human Leptin ELISA kit (LINCO) and Human Adiponectin ELISA kit (LINCO), respectively on a Wallac 1420 Multilabel Counter Viktor^2 (Perkin Elmer). GH was analyzed with an electrochemiluminescense immunoassay method using a kit from Siemens MSD AB on a DCP Immulite 2500 instrument. IGF1 and IGFBP3 were analyzed at the Karolinska University Hospital using an Immulite 2000 assay (Siemens), which is a chemiluminescent immunometric assay.

11.5 SEXUAL MATURATION, ANTHROPOMETRY AND BODY COMPOSITION MEASUREMENTS

Sexual maturation in Studies I-II was determined by a pediatrician according to Tanner criteria (165, 166). Genital development in boys and breast development in girls were used for classification into pubertal groups. In Study V, having reached final height was regarded as a sign of completed puberty.

In all studies, height and weight were measured in the morning. BMI was calculated as kg/m^2. In Studies III-IV, body surface area was calculated as described by Du Bois (167). In Study I-II, subjects underwent DEXA assessment at the Karolinska University Hospital in Huddinge using the equipment from Lunar Radiation, Madison, WI, USA. In Study V, DEXA was performed at the University Hospital in Uppsala using Lunar Radiation, Madison, WI, USA.

12 STATISTICAL ANALYSIS

Studies I-II and V were analyzed with the help of SPSS (version 14.0, SPSS inc., Chicago, IL, USA), studies I-II also with STATA (version 9.0, College Station, TX, USA) and Microsoft Excel including the application Analyse-It. Studies III-IV were analyzed using STATA (version 9.0, College Station, TX, USA). The statistical methods that have been used in this thesis are presented in **Table 5**.

Table 5. Statistical methods used in each substudy respectively.

Statistical method	Study I	Study II	Study III	Study IV	Study V
Descriptive statistics	x	x	x	x	x
Linear regression	x	x	x	x	x
Pearson's correlation coefficient	x	x			
Spearmans's correlation coefficient			x	x	x
ROC analysis		x			
Bland-Altman		x			
Wilcoxon signed rank test					x
Mann Whitney U test					x
Fisher's exact test					x
McNemar test					x

13 RESULTS AND DISCUSSION

In this thesis several aspects of insulin resistance have been explored, covering both clinical (studies I-II and V) and methodological aspects (studies III-IV). Existing and novel ways of insulin sensitivity assessment and insulin secretion have been investigated, and a clinical application in an at-risk population described.

Studies I-V are reprinted at the end of this thesis. In the following section the main findings of the studies will be discussed including strengths, limitations and future directions.

13.1 STUDIES I-II: USING FASTING INDICES IN INSULIN SENSITIVITY ASSESSMENT

Although it is clear that insulin resistance is highly related to obesity and cardiometabolic risk in children (168), it is not clearly established how insulin resistance in childhood should be assessed, in which clinical disorders it is present and whether it can be prevented and treated (66). Although relatively extensively used and validated in adults, the validity of HOMA-IR and QUICKI has been subject to debate in children and adolescents. Further, there is controversy regarding which of these indices best reflects the insulin sensitivity in a subject, and whether fasting insulin in itself is just as good a measure as fasting indices. These issues constitute the background of Studies I-II.

The major conclusion of Study I was that fasting indices should not be used in obese children and adolescents, or at least be used with caution. We base this on the poor validity of fasting indices in subgroups of our study. We found sex dependent explanatory power of Si, with generally higher explanatory power in males than in females. Also, the validity varied with pubertal stage with highest correlations in pubertal groups in both sexes (**Table 6**). Further, the validity was influenced by the degree of insulin sensitivity in females: while variations in HOMA-IR explained 33.7% of the variation in Si for subjects with high Si (insulin sensitive subjects) ($p<0.001$), the corresponding number for subjects with low Si (insulin resistant subjects) was only 3.2% ($p=0.197$). Fasting insulin explained 14.1% of the variation in Si in female subjects with high Si, ($p<0.004$), with corresponding number for subjects with low Si being 0.3% ($p=0.715$). In males, no such heterogeneity was detected. Thus, sex, pubertal stage, degree of insulin resistance and possibly other factors not accounted for in this study contribute to varying validity and appear to render fasting indices non-suitable for general use.

Table 6. Partial correlation coefficients of ln Si to ln HOMA-IR and ln fasting insulin respectively, adjusted for age and BMI.

	Ln HOMA-IR		Ln fasting insulin	
	Females r	Males r	Females r	Males r
Prepubertal	0.16 p=0.839 n=6	-0.43 p=0.028 n=28	-0.61 p=0.393 n=6	-0.38 p=0.053 n=28
Pubertal	-0.57 p=0.004 n=26	-0.78 p<0.001 n=28	-0.41 p=0.049 n=26	-0.71 p<0.001 n=28
Postpubertal	-0.53 p<0.001 n=77	-0.57 p=0.004 n=26	-0.37 p=0.002 n=77	-0.48 p=0.019 n=26
Total	-0.53 p<0.001 n=109	-0.67 p<0.001 n=82	-0.37 p<0.001 n=109	-0.61 p<0.001 n=82

The main conclusion of Study II was that focus should be directed at identifying and deciding on a general standard for insulin sensitivity assessment in clinical work and research practice. Numerous publications have been presented contrasting HOMA-IR and QUICKI as well as various other fasting indices, and an overview of some of these publications is presented in **Table 7**. Such comparisons yield no further information, since HOMA-IR and QUICKI show identical diagnostic accuracy as predictors of the FSIVGTT sensitivity index. This is illustrated by the ROC curves below, in which the QUICKI and HOMA-IR curves are superimposed (**Figure 11**). Insulin had a non-significantly lower area under the curve (AUC) than fasting indices, indicating that fasting indices offer no advantage over fasting insulin alone as proxies for insulin resistance in euglycemic children. A further conclusion was that due to large intra-individual physiologic variations in fasting insulin, HOMA-IR/QUICKI results will differ within each individual when measured at different times. This, in combination with the fact that insulin assays are not standardized and that testing of aliquots of a common sample analyzed in different laboratories shows disparate results (169) further discourages the use of this measure. Even in the presence of a general insulin assay standard, separate standards would need to be developed based on gender, ethnicity and pubertal stage (170-172). Thus, there is ample support for the general conclusion of Study I and II, discouraging the use of fasting indices as well as fasting insulin as proxies for insulin sensitivity in obese children and adolescents.

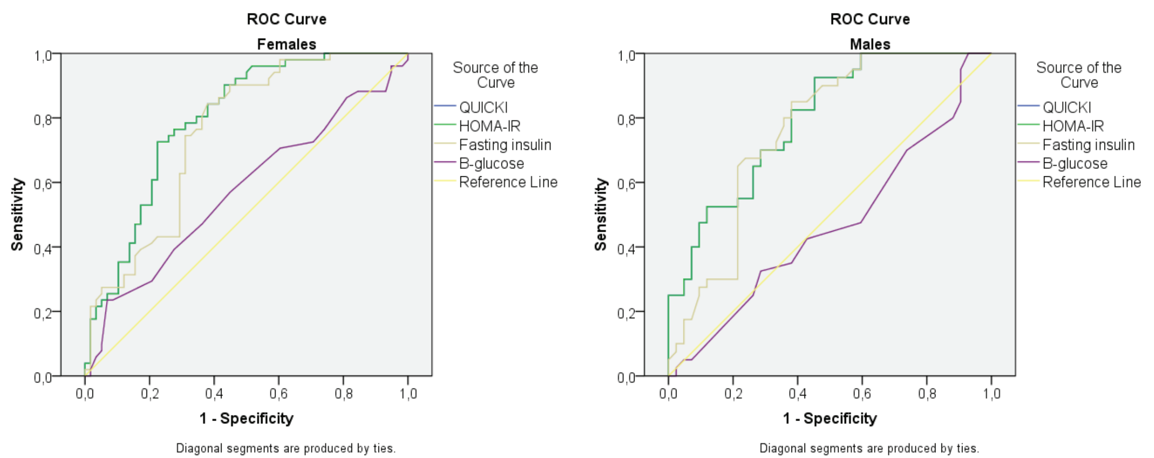

Figure 11. ROC curves illustrating the diagnostic accuracy of HOMA-IR, QUICKI, fasting insulin and fasting glucose in indentifying insulin resistance, here defined as below the population median of Si as determined by the FSIVGTT.

Reasons for the confusion regarding the similarity between HOMA-IR and QUICKI in children are not clear. When looking at the HOMA-IR and QUICKI formulas and transforming them, the relationship becomes: QUICKI = 1/[log(HOMA-IR) + log(22.5)]. From our point of view, this mathematical relationship makes it obvious that these two fasting indices should be highly related, and as has been pointed out, QUICKI is the log transformation of HOMA-IR and should be regarded as such (83). As for the equal validity of fasting indices and fasting insulin, even obese children have a relatively narrow range of glucose levels (92, 173) whereas insulin levels may vary considerably (174), making the contribution of adding glucose to the formulas insignificant in comparison to the effect of insulin levels.

An expert conference recently used evidence-based methods to investigate the state of the art related to insulin resistance in children (66). As for methods of screening criteria and methodology, the conference found no justification for screening children for insulin resistance, including obese children. As stated previously, invasive complicated methods are clearly not suitable. Fasting insulin and also fasting indices were found to be unreliable measures of insulin sensitivity. The fact that testing of insulin in different laboratories yields disparate results strengthens this conclusion. This is thus in agreement with the findings of Studies I-II. Merely the presence of obesity constitutes a risk for insulin resistance and should lead to intervention.

Table 7. Numerous publications in various study populations have been performed, with non-consistent conclusions regarding the superiority of one specific fasting index.

Study	n	Indices	Clinical characteristics	Statistical test	Conclusion
Sarafidis (175)	78	HOMA-IR, QUICKI, 1/ HOMA-IR index, McAuley index, clamp	Hypertensive diabetic adults	Regression analysis	HOMA-IR>QUICKI, but QUICKI better reproducibility
Keskin (176)	57	HOMA-IR, QUICKI, FGIR, OGTT	Obese children & adolescents	ROC	HOMA-IR>QUICKI
Cutfield (177)	79	HOMA-IR, QUICKI, FSIVGTT	Children (twins, SGA, premature, normal)	Spearman	HOMA-IR & f-insulin > QUICKI
Brady (178)	27	HOMA-IR QUICKI, revised QUICKI, FIRI, f-insulin, FGIR, Bennetts index, FSIVGTT	Healthy males	Pearson	Revised QUICKI>HOMA-IR, HOMA-IR & QUICKI equal
Rabasa-Lhoret (179)	148	HOMA-IR, QUICKI, revised QUICKI, new revised QUICKI, clamp	Normal, obese, PCOS, IGT, type II diabetic adults	Spearman	QUICKI> HOMA-IR
Uwaifo (180)	31	HOMA-IR, QUICKI, FGIR, Belfiore-FFA, c-peptide-to-insulin ratio, clamp	Lean & overweight children	Spearman	QUICKI> HOMA-IR
Brandou (181)	66	HOMA-IR, QUICKI, 40/insulin ratio index, FSIVGTT	Lean & obese children	Least square fitting	Equal result
Conwell (182)	18	HOMA-IR, QUICKI, FGIR, f-insulin, FSIVGTT	Obese children & adolescents	Spearman	Equal result
Diamanti-Kandarakis (183)	59	HOMA-IR, QUICKI, clamp	PCOS women	Pearson	Equal result
Gungor (184)	156	HOMA-IR, QUICKI, FGIR, glucose-to-insulin ratio, clamp	Healthy children & adolescents, PCOS, IGT	Spearman	Equal result
Henderson (185)	20	HOMA-IR, QUICKI, f-insulin, clamp, FSIVGTT	Healthy children	Spearman	Equal result
Ruige (186)	638	HOMA-IR, QUICKI, f-insulin, FGIR, glucose-to-insulin ratio, Belfiore GLY, revised QUICKI, McAuley index, Belfiore FFA, clamp	Lean, overweight, obese adults	ROC	Equal result
Schwartz (174)	323	HOMA-IR, QUICKI, FGIR, insulin-triglycerides, clamp	Healthy adolescents	ROC	Equal result
Yokoyama (187)	140	1/HOMA-IR, QUICKI, clamp	Type II diabetic & healthy adults	Regression analysis	Equal result

Belfiore GLY= 2/[(insulin * glucose) + 1]
Belfiore FFA= 2/[(insulin * NEFA) + 1]
Bennetts index= 1/log(glucose) * log (insulin)
FGIR= fasting glucose to insulin ratio
FIRI= (fasting insulin * fasting glucose)/25
HOMA-IR= Homeostasis model assessment
IGT= impaired glucose tolerance
ISA-FFA= antilipolytic insulin sensitivity index
McAuley index= $e^{[2.63-0.28 \ln(\text{insulin}) - 0.3 \ln(\text{triglycerides})]}$
Revised QUICKI= 1/log(glucose) + log (insulin)+log(NEFA)
QUICKI= Quantitative Insulin Sensitivity Check Index
PCOS= polycystic ovary syndrome

13.1.1 Strengths and limitations

A strength of studies I-II is the inclusion of a large number of very obese children and adolescents, all of which have undergone an FSIVGTT. However, the very wide age range introduces a limitation since subjects are at different stages of puberty, which is known to affect insulin sensitivity. We have tried to overcome this by stratification into pubertal subgroups.

Study II states that numerous comparisons between fasting indices have been made, and that this is unnecessary. Yet the study itself again does this comparison, which may seem like a contradiction. However, the aim is not the actual comparison of the methods, but rather the proof that the methods are interchangeable, as this has been debated in a number of studies with no real consensus. Also, there is no cut-off level of Si or other measure to define insulin resistance, and we have thus chosen the arbitrary level of the Si median in stratifying groups. However, most of these very obese children and adolescents are most likely insulin resistant to some extent, making a stratification into subgroups of "insulin-resistant" and "insulin-sensitive" misleading. Again, the aim of the second study is not to identify insulin resistance *per se* but to limit further comparisons of HOMA-IR and QUICKI, and this relationship remains the same no matter which cut-off level is chosen.

13.1.2 Future directions

The study sample in Studies I-II is of considerable interest for research as it contains a large fraction of Sweden's most overweight and obese children and adolescents. Data have been assembled from 1996-2002. The participants of this study are thus now (in 2011) adolescents, young adults and possibly even in their 30s. Childhood obesity and metabolic risk factors track into adulthood (96, 188, 189), and it is likely that a large proportion of these participants remain overweight or obese. As a future study, it would be interesting to investigate the development of insulin resistance, diabetes and cardiovascular risk in this sample of individuals with long-standing metabolic derangements from childhood onwards and see to what degree fasting indices, fasting insulin and Si in childhood and adolescence correlates to later metabolic disease. Possibly, these measures could add information to risk assessment in insulin resistance and obesity in the long-term. Further, future research in this area should aim at standardizing insulin measurements and at identifying strong surrogate markers of insulin resistance (66).

13.2 STUDIES III AND IV: USING MODELS TO ASSESS INSULIN SECRETION AND INSULIN RESISTANCE

The ability of the pancreatic β-cell to secrete insulin in response to glucose during an FSIVGTT is an important determinant of the glucose tolerance of an individual. Reliable methods for insulin secretion assessment are thus needed to complete the picture provided by MINMOD indices such as insulin sensitivity and glucose effectiveness. The discovery that C-peptide and insulin are secreted in equimolar amounts has lead to the development of several models which through the indirect use of C-peptide can describe pancreatic insulin secretion (190). Insulin undergoes extraction when passing the liver whereas C-peptide does not undergo this extraction to

any larger extent, which means measuring peripheral serum insulin will not reflect the actual insulin amount released by the pancreas but also hepatic extraction. C-peptide can thus be used as a marker of insulin secretion. Study III presents a novel, user-friendly method which describes C-peptide basal and peak secretion during the first phase of an insulin modified FSIVGTT in 15 healthy normal weight subjects. The model provided valid results for all 15 subjects, revealing an array of C-peptide secretion responses. Validation was carried out by extrapolating the model to the results of 8 subject from a previous full-length standard FSIVGTT study, which showed a very good fit. Further, the model parameters were in line with previously estimated parameters, strengthening the plausibility of the model. The main conclusion of Study III was thus that it is possible to accurately yet relatively simply assess first phase insulin secretion indirectly using C-peptide data from the first phase of a glucose challenge. This applies for the insulin modified as well as for the standard FSIVGTT protocol.

Elevated NEFAs are typically present in insulin resistance and in many pathological conditions such as diabetes, obesity, hypertension and coronary heart disease. Study IV aims to elucidate the dynamics of NEFA metabolism, in order to understand the contribution of NEFAs in the etiology of these conditions. The model was recently presented (145), using mean group data from previous experiments (191, 192). Study IV provides a subject level validation of the model, which is important since NEFA responses to an FSIVGTT show a diversity of patterns. The model was well able to fit all 15 individual profiles, demonstrating the robustness. To test the plausibility of the model, a statistical program was used to derive static counterparts from the dynamic parameters at certain intervals of the NEFA profile. The correlation between static and dynamic parameters was tested, as well as the correlations of these two groups of parameters to MINMOD indices. The major findings were that (1) the model derived parameters were in agreement with static parameters derived from the NEFA concentration curves, (2) the dynamic parameters reflecting the provision and removal of NEFA to and from the plasma compartment were closely correlated and (3) the static parameters, derived form dynamic counterparts, were correlated to dynamic parameters of glucose and insulin kinetics derived using the MINMOD.

A further finding was that at baseline the provision of NEFA to the plasma pool (reflecting predominantly lipolysis during fasting) was poorly related to the NEFA plasma concentration, indicating that plasma NEFA level *per se* is a poor estimate of the rate of lipolysis, even after an overnight fast. It also implies that the NEFA supply and demand system is not necessarily at steady state at baseline, which this model takes into account.

13.2.1 Strengths and limitations

The models were developed using the insulin modified FSIVGTT, meaning that an insulin infusion was given after 20 minutes. The purpose of the insulin modified test is to separate the glucose and insulin peaks, specifically in subjects whose endogenous insulin secretion is inadequate to respond to the glucose challenge of the FSIVGTT, e.g. diabetics or insulin resistant subjects. Using the insulin modified protocol may thus be regarded as a strength as it improves the precision of the estimation of the Si (193). However, it may also be argued that the insulin injection introduces a disturbance when estimating C-peptide and insulin secretion. We have overcome this potential distortion by using the first 22 minutes of the FSIVGTT, preceding the effects of the insulin injection, in the C-peptide model.

As for limitations, the sample size was quite small, with only 15 subjects. However, for method development purposes this is sufficient and well in line with similar previous studies (191, 192, 194, 195). Further, the study population is very homogenous in regards to age, body composition and insulin sensitivity. It may thus be argued that these models cannot be extrapolated to populations with glucose intolerance of some degree, or in subjects with a wider range of BMI. However, the models have now been developed and tested in this sample, and future studies are needed for validation in other more heterogeneous populations.

In the NEFA model we assume that the glucose load and the insulin response it elicits completely shuts down lipolysis. However, lipolysis is most likely not absolutely down regulated, and a constant infusion of stable labeled glycerol isotope would have been necessary to measure this exactly, as glycerol (in contrast to NEFAs to some extent) is not re-esterified in adipose tissue and thus provides a measure of lipolysis.

13.2.2 Future directions

Further validation studies in more heterogeneous populations are needed for both the C-peptide and the NEFA model. Combining the NEFA model with stable labeled glycerol isotope may be done in the future as to investigate the extent to which lipolysis is in actual fact shut down.

13.3 STUDY V: SURVIVING CANCER- AWAITING METABOLIC DISTURBANCES?

The focus of Study V is the long-term risk for survivors of stem cell transplantation treatment including total body irradiation following hematologic malignancies in childhood. This group has been reported to be at an increased risk of developing insulin resistance, hyperinsulinemia and impaired glucose tolerance. The prevalence of obesity also seems increased in this group. Growth hormone (GH) deficiency has been suggested to explain some of these metabolic abnormalities, with a prevalence of GH deficiency in adult survivors of childhood cancer ranging between 35% and 91% (136).

The results from our study (**Table 8**) indicate that even after a follow-up of as long as 18 years, β-cell function is still intact. However, insulin sensitivity is lower as compared to controls, and there is significantly higher fasting insulin, C-peptide and HOMA-IR in patients. The compensatory insulin secretion maintains normoglycemia to the same extent in patients as in controls, with non-significant differences in the prevalence of impaired glucose tolerance in patients and controls. Although there was no significant difference in BMI in patients versus controls, percentage fat mass was higher in patients and lean body mass lower. This indicates that BMI is not a suitable means of assessing body composition in this group and that future studies should include valid measurements of whole body and visceral obesity.

Growth hormone peak (GH_{max}) was significantly lower in the patients, however there were no differences in serum levels of IGF1 and IGFBP3. The levels of CRP and leptin were significantly higher in patients, whereas adiponectin levels were lower. Leptin and adiponectin data remained significant after adjustment for fat mass. Further, a HOMA-IR >2.86 (196) was associated with BMI ≥25 (5/8 vs 0/10, $p=0.007$) and central obesity (6/8 vs 1/10, $p=0.013$). Si had an inverse correlation to percentage fat mass ($r=-0.52$,

p=0.032) but not to waist circumference. GH_{max} correlated inversely with percentage fat mass (r=-0.63, p=0-009) but not with Si. GH_{max} correlated negatively (r=-0.62, p=0.008) and fat mass positively (r=0.51, p=0.037) with time since SCT.

Table 8. Main findings of Study V.

Variable	Patients median (interquartile range)	Controls median (interquartile range)	p
Fasting insulin (mU/L)	12.2 (7.3)	6.0 (2.8)	0.002
HOMA-IR	2.2 (1.7)	1.2 (0.5)	0.005
Sensitivity index ($mU/L^{-1} min^{-1}$)	2.98 (1.99)	4.54 (2.02)	0.043
Acute insulin response	717 (754)	342 (258)	<0.001
BMI (kg/m^2)	21.6 (5.6)	24.5 (4.8)	0.093
Waist circumference (cm)	82 (19)	87 (16)	0.69
Lean body mass (kg)	40.7 (12.5)	54.5 (18.6)	<0.001
%fat mass	34.9 (16.3)	24.3 (15.6)	0.011
GH_{max} (mU/L)	9 (12.9)	20.7 (16.3)	0.002
Leptin (µg/L)	18 (45.7)	4.8 (9.7)	<0.001
Adiponectin (mg/L)	4.9 (2.1)	7.5 (4.0)	0.008

Results shown as median (interquartile range)
HOMA-IR= Homeostasis model assessment, as described by Matthews et al (82)
GH_{max}= Growth hormone maximum peak

The past four decades have shown a dramatic increase in cancer survival in children and adolescents. Cancer in children is luckily rare, with an age-standardized incidence of 140 cases per million children aged 0-14 years in Sweden (197, 198). In the Nordic countries an 80% 5-year survival (199) has been attained, however the survival figures vary with the specific cancer diagnosis and the clinical presentation within each diagnosis. In the age group of 25-35 years, 1 in 700 is a childhood cancer survivor (200). In Europe the number of survivors is expected to be 300,000-500,000 individuals, and in Sweden the corresponding figure is 6,000-7,000. The advances in diagnosis and treatment leading to this increased survival rate are of course applauded, however there is also evidence that some cancer survivors may pay a high price in terms of late term complications. Individuals having undergone stem cell transplantation have been investigated in regards to how the transplantation has affected health, life situation and health-related quality of life (201, 202). Adult SCT survivors reported lower quality of life than the norm, and had significantly more psychiatric problems, cognitive difficulties and depressions; and the percentages being unemployed or preterm retired were higher. Besides these there is a number of other

complications after SCT in childhood, such as second malignancies, cardiac problems, pulmonary complications, impaired fertility, osteoporosis, obesity, dyslipidemia, hypertension, impaired glucose tolerance and insulin resistance (136).

13.3.1 Strengths and limitations

A major strength of Study V is that it constitutes the longest follow-up of SCT survivors to date, at least to our knowledge. A control group is included, which has undergone the same investigations as the patient group. Also, FSIVGTT is used for insulin sensitivity assessment and DEXA for body composition assessment, both of which are valid tests.

Growth hormone levels were significantly lower in SCT survivors compared to controls, and it is thus proposed that GH deficiency may be an underlying cause of the adverse body composition of survivors. However, in obesity and overweight, GH levels may be low in endocrinologically healthy individuals. Thus, whether patients display a real GH deficiency or whether the low GH levels are caused by their adiposity is not clear. In line with this argumentation is the fact that there were no differences in IGF1 and IGFBP3, which may be better markers for GH secretion. This may possibly undermine the conclusion that a real GH deficiency exists. To be able to draw a valid conclusion a more robust method of GH assessment would have to be used, such as an insulin tolerance test. However, GH assessment in this group is not straightforward. For example, a recent study has shown that cranially irradiated adult cancer survivors may have normal spontaneous GH secretion, but discordant peak GH responses to stimulation test (203). Further, it has been shown that IGF1 and IGFBP3 values may be normal or high despite low GH peaks in subjects treated with total body irradiation, suggesting a resistance to IGF1 caused by irradiation effects on bone (204). In summary, despite the adiposity and the normal IGF1 and IGFBP3 levels, the low GH levels in patients may still reflect a real deficiency. Also, we have not explored the level of physical activity, and this may confound our results as it has been shown that survivors of childhood cancer have a decreased level of physical activity (141). However, the differences in leptin and adiponectin cannot be explained by inactivity only, so there must be metabolic abnormalities existing in these individuals.

Further limitations of this study include a possible selection bias in that 7 of 25 eligible survivors declined participation, and the fact that there was a disproportionally large number of males among decliners. Also, the control subjects were matched for age and sex, but not for BMI and visceral adiposity. In retrospect, matching could have been done for this aspect as well. The control group was surprisingly overweight, with 44% having a BMI ≥25 kg/m^2. This is not representative of the normal population of this age group, in which <30% would be expected to be overweight (205). However, the results of the study would be strengthened in the case of a thinner control group, so we still believe our results to be valid.

13.3.2 Future directions

There are risk-based screening recommendations for long-term complications such as the metabolic syndrome in children (206). Currently, however, there are no available guidelines for screening for the metabolic syndrome in adult survivors of childhood cancer, and treatment recommendations are basically those which apply to “regular” metabolic syndrome patients. However, further knowledge on the etiology of the

metabolic syndrome in cancer survivors may lead to more specific therapies and prevention measures. Future studies thus need to identify the pathophysiological background and which interventions are best suited for these survivors, as well as to implement long-term follow up screening programs for early intervention. In line with this, we plan a further study with a similar but more extensive set-up, in which among other things lipid profile, adipokines, NEFAs, sex hormones, FSH, LH, thyroid profile, cytokines and level of physical activity using accelerometer and diary, will be explored.

14 CONCLUSIONS

Following the discussion and taking into account the limitations stated, we have conducted five studies with results covering a wide range of issues regarding assessment of insulin sensitivity, development of novel models of insulin secretion and sensitivity assessment as well as a clinical perspective:

(I) We have shown that fasting indices HOMA-IR, QUICKI and fasting insulin are relatively poor methods for assessing insulin sensitivity in obese children and adolescents. The validity of these methods varies by sex, by pubertal stage and in females by degree of insulin sensitivity. The use of fasting indices and fasting insulin for insulin sensitivity assessment in this group should thus be limited. Resources should be targeted at early intervention in obese children and adolescents, regardless of insulin sensitivity measurements.

(II) We have documented that HOMA-IR and QUICKI have equal diagnostic accuracy in detecting insulin resistance as measured by the FSIVGTT, and fasting insulin has non-significantly lower accuracy in itself. The variation in insulin levels when tested at different times further strengthens the conclusion that the use of simple surrogate indices is limited. Conducting and reporting further comparisons should be avoided.

(III) We have developed a novel method for the indirect assessment of insulin secretion by modeling C-peptide during the first phase of an insulin modified FSIVGTT. The assessment of insulin secretion is valuable for the understanding, diagnosis, treatment and follow-up of diabetes, which potentially makes this model a useful tool. Also, it can be used during the insulin modified FSIVGTT, which is preferred in settings with low insulin sensitivity or low endogenous insulin secretion.

(IV) We have performed a subject level validation of a novel model for NEFA kinetics following an FSIVGTT. With a lipotoxic view of diabetes development, complementary to the glucotoxic view, parameters of NEFA kinetics add information on the dynamics of insulin action.

(V) Lastly, we have shown that long-term survivors of stem cell transplantation and total body irradiation have intact β-cell function even after a follow-up of 18 years. Their insulin sensitivity is however lower than that of healthy controls, and their proportional fat mass is higher. Abnormalities in the secretion of or the sensitivity to leptin and adiponectin may play a role in the metabolic alterations of SCT survivors, as well as deficiency in GH secretion.

SAMMANFATTNING PÅ SVENSKA

Insulinresistens är ett tillstånd där vävnaders känslighet för insulin är nedsatt, så att en egentligen adekvat insöndring av insulin inte räcker till för att ge önskad effekt i målvävnader. Denna avhandling baseras på fem studier, vars mål är att undersöka olika aspekter av insulinresistens samt metoder för dess mätning. Insulinresistens är en viktig komponent av det metabola syndromet och kan ses som ett förstadium till typ 2 diabetes. I takt med att förekomsten av övervikt och fetma ökat har insulinresistens och dess komplikationer följt i samma spår. Insulinresistens har således blivit ett allt vanligare tillstånd, som kräver effektiv diagnos och behandling.

- En rad olika metoder för mätning av insulinkänslighet har utvecklats. En referensmetod är en form av intravenös sockerbelastning, (FSIVGTT= Frequently Sampled Intravenous Glucose Tolerance Test) och innebär att en sockerlösning samt en insulindos ges direkt i blodet till en fastande individ, varefter blodsocker och insulinvärden följs under 180 minuter. Då denna metod är komplicerad, dyr och relativt krävande för såväl patient som personal har det utvecklats en rad enklare metoder. Två av dessa kallas HOMA-IR och QUICKI, och baseras på ett enda blodprov med bestämning av fasteblodsocker samt fasteinsulin. **Studie I** och **II** jämför dessa enklare metoder med referensmetoden i en grupp feta barn och ungdomar. Slutsatsen är att dessa enklare metoder inte ger ett tillförlitligt mått på insulinresistens i den aktuella patientgruppen, varför vi avråder från att använda dessa metoder. Den kroppsegna variationen av insulinnivåer bidrar till ytterligare osäkerhet, så att dessa mått, om de skall användas överhuvudtaget, skall tolkas med stor försiktighet. Vidare jämförelser mellan HOMA-IR och QUICKI är inte meningsfullt då metoderna har samma tillförlitlighet.

- I **Studie III** och **IV** undersöktes 15 friska normalviktiga försökspersoner med en intravenös sockerbelastning som kompletterades med mätning av C-peptid och fria fettsyror. C-peptid bildas då den spjälkas av från ett förstadium till insulinmolekylen. För varje molekyl insulin som insöndras från bukspottskörteln till blodbanan insöndras en molekyl C-peptid. Insulin bryts dock ned i levern i högre grad än C-peptid, varför exakta insulinnivåer är svåra att mäta medan C-peptid därmed kan utgöra ett indirekt mått på insulinsekretionen. I studie III skapades därför en matematisk modell för att med hjälp av C-peptidnivåer indirekt kunna uppskatta insulinsekretionen från bukspottskörteln. Att kunna mäta insulininsöndringen är viktigt för att förstå utvecklingen av diabetes och effekten av olika interventioner.

- Höga halter av fria fettsyror har visat sig bidra till utvecklingen av insulinresistens. I studie IV undersöktes en modell som beskriver hur fria fettsyror omsätts under en sockerbelastning, och hur detta kan ge mått på insulinkänsligheten. Modellen speglade väl fria fettsyrors omsättning under sockerbelastningen, samt gav rimliga mått på hur dessa bildas och förbrukas. Detta arbete syftar till att utveckla nya metoder som kan utöka förståelsen för fria fettsyrors roll i diabetesutveckling.

- Insulinresistens förekommer inte enbart vid fetma, utan även hos personer som i barnaåren genomgått behandling mot cancer. Med allt bättre överlevnad vid ett flertal cancerdiagnoser är detta en växande grupp. **Studie V** undersöker

insulinresistens, insulininsöndring samt kroppssammansättning hos en grupp som i barndomen genomgått stamcellstransplantation på grund av olika typer av blodcancer. Slutsatsen av denna studie är att de stamcellstransplanterade patienterna efter en genomsnittlig uppföljningstid av 18 år har en lägre insulinkänslighet, som dock kompenseras av en ökad insulininsöndring, jämfört med friska kontroller. Patienterna hade också en högre andel fettmassa jämfört med friska kontroller. Rubbningar i insöndringen av tillväxthormon samt fettvävshormonerna leptin och adiponektin kan bidra till denna ofördelaktiga kroppssammansättning och utvecklingen av insulinresistens.

Sammanfattningsvis har vi studerat flera aspekter av insulinresistens och insulininsöndring. Vi har visat att enkla, alternativa metoder för mätning av insulinresistens inte är tillförlitliga bland feta barn och ungdomar, samt att fokus framöver bör vara riktat mot utvecklingen av bättre, standardiserade metoder. Fortsatta jämförelser av befintliga metoder har begränsat värde. Vi har också utvecklat och validerat två nya matematiska metoder för mätningen av insulininsöndring samt insulinresistens. Målet är att dessa metoder ska utgöra komplement till befintliga tekniker och öka kunskapen kring diabetesutveckling. Slutligen har vi visat att stamcellstransplanterade långtidsöverlevare av cancer i barndomen har en ökad metabol risk, men efter lång uppföljning fortfarande intakt funktion av bukspottskörtelns insulininsöndring.

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