Optimizing Nicotinic Acid Delivery for Durable Antilipolysis and Improved Metabolic Control

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Doctoral Thesis
Swedish University of Agricultural Sciences
Uppsala 2016

Acta Universitatis agriculturae Sueciae
2016:31

ISSN 1652-6880
ISBN (print version) 978-91-576-8564-3
ISBN (electronic version) 978-91-576-8565-0

Print: SLU Service/Repro, Uppsala 2016

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Abstract

Type 2 diabetes is a devastating disease affecting hundreds of millions worldwide. Lipid accumulation in peripheral non-adipose tissues is a major driver of insulin resistance, a central pathophysiological defect of this disease. Plasma free fatty acids (FFA), derived from adipose tissue lipolysis, are an important source of the intracellular lipid pools. Hence, adipose tissue antilipolysis may be an approach for reversing peripheral tissue lipid overload and the down-stream negative consequences, including insulin resistance.

Nicotinic acid (NiAc) is a potent inhibitor of adipose lipolysis, acutely reducing plasma FFA concentrations. However, a major FFA rebound occurs upon abrupt NiAc washout and sustained exposures are associated with tolerance development, with FFA returning to pre-dose levels. A key principle of this work was the use of precisely defined plasma NiAc exposure profiles, produced using a programmable, implantable minipump. Metabolic consequences of NiAc-induced FFA lowering were assessed in a translationally relevant preclinical model of the metabolic syndrome, the obese Zucker rat.

A feedback turnover model adequately described acute FFA responses to NiAc. This model aided in designing a gradual NiAc termination protocol which minimized FFA rebound. The strategy of around-the-clock exposure failed to deliver sustained FFA lowering, due to tolerance development. By contrast, an intermittent strategy succeeded in preserving acute FFA lowering and insulin sensitizing effects. A more complex model was required in order to capture the development of complete tolerance in response to sustained NiAc exposure. Further experiments revealed that NiAc timed to feeding decreased triglycerides in liver and heart and reduced plasma fructosamine. During an oral glucose tolerance test, plasma FFA levels were reduced with amelioration of hyperglycemia and hypertriglyceridemia. By contrast, NiAc timed to fasting did not reduce tissue lipids, ameliorate glucose intolerance or dyslipidemia.

In conclusion, the NiAc exposure profile has a major influence on metabolic control. A macro-pharmacologic approach succeed in identifying a rational NiAc delivery profile that suppressed rebound and tolerance and profoundly improved metabolic control in obese Zucker rats. The work shows the power of a multi-disciplinary drug discovery approach, using a comprehensive understanding of the relationship between pharmacokinetics and pharmacodynamics combined with knowledge of metabolic physiology.

Keywords: Antilipolysis, Niacin, Insulin resistance, Dyslipidemia, Feedback modeling

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Dedication

To my girls… Helene, Clara and Edith

Table of Contents

List of Publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I Ahlström, C., **T. Kroon**, L. A. Peletier, J. Gabrielsson (2013). Feedback modelling of non-esterified fatty acids in obese Zucker rats after nicotinic acid infusions. *J Pharmacokinet Pharmacodyn* 40(6), 623-638.

II **Kroon, T.**, A. Kjellstedt, P. Thalén, J. Gabrielsson, N. D. Oakes (2015). Dosing profile profoundly influences nicotinic acid's ability to improve metabolic control in rats. *J. Lipid Res.* 56(9), 1679-1690.

III **Kroon, T.**, T. Baccega, A. Olsén, J. Gabrielsson, N. D. Oakes. Nicotinic acid timed to feeding reverses tissue lipid accumulation and improves glucose control in obese Zucker rats. (*Submitted manuscript*).

IV Andersson, R., **T. Kroon**, J. Almquist, M. Jirstrand, N. D. Oakes, J. Gabrielsson. Third generation turnover model – Nicotinic acid-induced tolerance of insulin and free fatty acids. (*In manuscript*).

Papers I-II are reproduced with the permission of the publishers.

The contribution of Tobias Kroon (T.K.) to the papers included in this thesis was as follows:

I T.K. assisted during parts of the experimental *in vivo* work and assay analysis and contributed to writing the manuscript. TK handled the majority of the scientific journal correspondence.

II T.K. had a major role in the idea and hypothesis of this work. T.K. performed all experimental *in vivo* work with assistance from P.T. and A.K. T.K. performed all the assays, except for the mRNA analysis. T.K. summarized the data and wrote the manuscript together with N.D.O. and J.G. and handled the scientific journal correspondence.

III T.K. had a major role in the idea and hypothesis of this work. T.K. performed all experimental *in vivo* work with assistance from T.B. T.K. performed all the assays, except for the mRNA analysis, performed by A.O. T.K. summarized the data and wrote the manuscript together with N.D.O. and J.G. and handled the scientific journal correspondence.

IV T.K. contributed to the idea and hypothesis of this work. T.K. performed all experimental *in vivo* work and assay analysis and contributed to writing the manuscript together with R.A. and J.A.

Abbreviations

AC	Adenylate cyclase
ACC1	Acetyl-CoA carboxylase 1
AMP	Adenosine monophosphate
ApoB	Apolipoprotein B
ATGL	Adipocyte triglyceride lipase
ATP	Adenosine triphosphate
AUC	Area under the curve
cAMP	Cyclic adenosine monophosphate
CETP	Cholesteryl ester transport protein
ChREBP	Carbohydrate-responsive element-binding protein
CL	Plasma clearance ($L \cdot min^{-1} \cdot kg^{-1}$)
Cl_d	Intercompartmental distribution ($L \cdot min^{-1} \cdot kg^{-1}$)
C_p	Drug plasma concentration (*e.g.* µM)
DAG	Diacylglycerol
DNL	De novo lipogenesis
F, *R*	Plasma FFA response (mM)
F_0, R_0	Baseline FFA concentration (mM)
FAS	Fatty acid synthase
FFA	Fee fatty acids
GLUT4	Glucose transporter 4
GPCR	G-protein coupled receptor
GPR109A	G protein-coupled receptor 109A (nicotinic acid receptor)
HDL	High-density lipoprotein-cholesterol
HSL	Hormone sensitive lipase
I	Plasma insulin response (nM)
I_0	Baseline insulin concentration (nM)
IC_{50}	Potency (*e.g.* nM)
I_{max}	Efficacy
IRS-1	Insulin receptor substrate 1

k_a	First order absorption rate (min^{-1})
K_m	Michaelis-Menten constant (μM)
k_N	1st-order rate constant (min^{-1})
k_{out}	Fractional turnover rate FFA (min^{-1})
k_{tol}	Fractional turnover rate of moderator (min^{-1})
lbm	Lean body mass (kg)
LC-MS/MS	Liquid chromatography mass spectrometry
LDL	Low-density lipoprotein-cholesterol
M	Moderator compartment (*e.g.* mM)
N	Concentration in hypothetical NiAc action compartment
n, γ	Hill coefficient
NAFLD	Nonalcoholic fatty liver disease
NASH	Nonalcoholic steatohepatitis
NiAc	Nicotinic acid, niacin
nPKC	Novel protein kinase C
PDE3B	Phosphodiesterase-3B
PI3K	Phosphatidylinositol-3 kinase
PK/PD	Pharmacokinetics/pharmacodynamics
PKA	Protein kinase A
PKC	Protein kinase C
PPARγ	Peroxisome proliferator-activated receptor γ
R_{ss}	Response at steady state (*e.g.* mM)
RT-PCR	Reverse transcriptase polymerase chain reaction
SCD1	Stearoyl-CoA desaturase-1
sdVLDL	Small dense very low-density lipoprotein cholesterol
SEM	Standard error of the mean
S_I	Insulin sensitivity
S_N	NiAc sensitivity
SREBP-1c	Sterol regulatory element-binding protein-1c
Synth	Endogenous turnover rate of NiAc ($\mu mol \cdot kg^{-1} \cdot min^{-1}$)
$t_{1/2}$	Half-life (min)
T2D	Type 2 diabetes
TG	Triglycerides
V_c	Central volume ($L \cdot kg^{-1}$)
VLDL	Very low density lipoprotein cholesterol
V_{max}	Maximal velocity ($\mu mol \cdot min^{-1} \cdot kg^{-1}$)
VPC	Visual predictive check
V_t	Peripheral volume ($L \cdot kg^{-1}$)

1 Introduction

Disturbances in metabolism underlie several of the most debilitating and costly diseases of the industrialized world, type 2 diabetes (T2D) and cardiovascular diseases (Inzucchi *et al.,* 2012; Mozaffarian *et al.,* 2016). In health, metabolism is dynamic and modulated by multiple redundant systems (Frayn, 2009). In this context, the standard pharmacological approach of activating a target mechanism around-the-clock often fail because of time dependent loss of drug efficacy and rebound above pre-dose levels. This thesis explores the idea that a more comprehensive understanding of the relationship between pharmacokinetics (PK) and pharmacodynamics (PD) combined with knowledge of the physiologic regulation of metabolism (*i.e.* considering the effect of key biomarkers) can be used to mitigate these barriers enabling invention of new pharmacotherapies. This was done in the multi-disciplinary drug discovery environment at AstraZeneca R&D Gothenburg. The results in this thesis exemplify the value of comprehensive physiologic and PK/PD understanding by applying this approach to an ancient drug, nicotinic acid (NiAc). Thus, a well-defined exposure profile, including shape, extent and timing of drug intervention, was designed sequentially across several studies. This resulted in suppression of tolerance and rebound and most importantly, profound improvements of the metabolic profile of a preclinical disease-model.

1.1 Type 2 diabetes

During the past few decades there has been a global trend in lifestyle changes, dominated by an increased intake of high-fat/energy-dense foods, together with a sedentary lifestyle. These factors have caused the worldwide prevalence of obesity to escalate dramatically. In 2014, over 600 million people were obese, representing 13% of the global adult population (World Health Organization, Fact sheet N 311, 2015). Obesity is highly associated with T2D (Kahn *et al.*, 2006), a devastating disease currently affecting ~380 million people world-

wide, and predicted to increase to almost 600 million by 2035 (Guariguata *et al.*, 2014). T2D is associated with several risk factors, including increased risk of heart disease and stroke (Morrish *et al.*, 2001), kidney failure (Alwan, 2011) and an overall increased risk of premature mortality (Roglic & Unwin, 2010).

The major hallmark of the pathophysiology of T2D is insulin resistance, a situation where cells no longer respond appropriately to insulin. Although several mechanisms may trigger insulin resistance, it occurs primarily at the level of so-called insulin sensitive tissues such as liver, muscle and fat (Boucher *et al.*, 2014). Genetic predisposition may be an important component in the development of T2D (Beck-Nielsen & Groop, 1994). However, insulin resistance emerges primarily as a consequence of over nutrition coupled to a sedentary lifestyle, resulting in severely disrupted fuel metabolism (Reaven, 1995). Particularly pronounced is a perturbed lipid metabolism (McGarry, 1992), fueled by an imbalance between lipid supply and oxidation at the tissue level (Flatt, 1988; Frayn, 2002; Eckel *et al.*, 2005). This imbalance produces an elevated systemic FFA flux which results in peripheral lipid accumulation; *i.e.* lipid deposition in non-adipose tissues, such as, liver and skeletal muscle. Peripheral lipid accumulation has been shown to be a major driver of insulin resistance, nonalcoholic steatohepatitis (NASH) and dyslipidemia (Shulman, 2014; Lomonaco *et al.*, 2016; Krauss, 2004). New pharmacological therapies are needed to decrease tissue lipid loading and thereby reverse the negative metabolic consequences.

Insulin resistance is a prominent feature of both obesity and T2D (Kahn *et al.*, 2006; Guilherme *et al.*, 2008) and characterized by the failure of insulin to exert its normal functions. One important function is the insulin-induced fuel-switch in skeletal muscle (Fig. 1): from predominant FFA oxidation during fasting to glucose oxidation following meal ingestion. In obese and insulin resistant people this fuel-switching is impaired, resulting in a state of metabolic inflexibility (Kelley *et al.*, 1999).

Over time, insulin resistance results in a progressively worsening hyperinsulinemia, as the hypersecreting pancreatic β-cells tries to compensate for reduced insulin action in various tissues (Reaven, 1988). Control of fuel homeostasis is normally governed by insulin-stimulated glucose uptake into peripheral tissues and efficient suppression of lipolysis in adipose tissue (Frayn, 2009). Numerous metabolic abnormalities surface as a result of defective insulin action and secretion. This includes hyperglycemia, due to impaired insulin-stimulated glucose uptake into skeletal muscle (Kelley & Mandarino, 1990), paralleled by an elevated glucose production in the liver (DeFronzo *et al.*, 1989). Furthermore, insulin resistance gives rise to an abnormal lipid metabolism, resulting in elevated levels of circulating lipids

(Mooradian, 2009). These chronic increases in plasma glucose and lipid concentrations can further damage insulin action and secretion, exacerbating the insulin resistant state (Muoio & Newgard, 2008). Obese and insulin resistant humans can, however, remain in this β-cell compensatory state for long periods of time without developing diabetes. Nonetheless, at some point a subset of these individuals will transition from an insulin resistant state to overt T2D (Weyer *et al.*, 1999; Festa *et al.*, 2006), brought about by β-cell failure (Kahn, 1998).

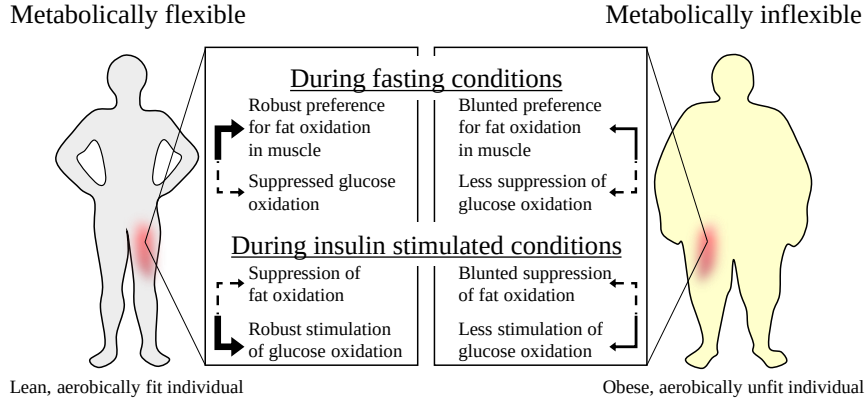

Figure 1. Metabolic flexibility. The ability of skeletal muscle to switch between oxidizing glucose or fat is heavily related to insulin sensitivity, fitness and obesity status. *During fasting conditions*, insulin levels are normally low and in a lean individual there is high dependence on fat oxidation. In an obese, sedentary individual, however, there is less dependence on fat and a greater reliance on glucose oxidation. *During insulin stimulated conditions*, in skeletal muscle of a lean individual, insulin strongly suppresses fat oxidation and induces a high reliance upon glucose oxidation, whereas in skeletal muscle of an obese, sedentary individual, there is less stimulation of glucose oxidation by insulin and blunted suppression of fat oxidation. (*Adapted from (Kelley et al., 1999)*).

Dyslipidemia is the primary cause for cardiovascular disease in T2D (Grundy *et al.*, 1999). A vast body of evidence suggests that insulin resistance can be ascribed a central role in diabetic dyslipidemia with an increased FFA flux as a major contributing factor (Taskinen, 2003; Krauss & Siri, 2004; Solano & Goldberg, 2005; Chahil & Ginsberg, 2006). Dyslipidemia is characterized by three cardinal features, namely elevated plasma concentrations of triglycerides (TGs), reduced high-density lipoprotein-cholesterol (HDL) and perhaps most importantly an increased amount of low-density lipoprotein cholesterol (LDL) and changes in its composition (Howard, 1999); often termed the atherogenic lipoprotein phenotype (Austin *et al.*, 1990). Hyperinsulinemia along with the elevated systemic FFA flux, promotes enhanced hepatic TG production, subsequently causing an increased secretion of apolipoprotein B (ApoB) and very-

low-density lipoprotein cholesterol (VLDL) (Frayn *et al.,* 2006). The ensuing TG and VLDL elevation worsens the lipoprotein phenotype. Specifically, HDL is reduced while small dense (sd)-LDL is increased (Mooradian, 2009). Circulating cholesteryl ester transfer protein (CETP) will, under these conditions, catalyze the transfer of cholesteryl ester from HDL into VLDL while TG moves in the opposite direction. This results in cholesterol-rich VLDL remnants and TG-enriched, but cholesterol-depleted HDL (Lewis & Rader, 2005; Mooradian *et al.*, 2008); both of which are atherogenic (Ginsberg, 2002). A similar mechanism account for the increase in sdLDL particles; due to elevated VLDL, CETP facilitates exchange between LDL and VLDL, producing TG-enriched but cholesterol-depleted LDL. The TG-enriched LDL particle is subsequently hydrolyzed by hepatic lipase or lipoprotein lipase (Packard, 2003), leaving behind a lipid-depleted pro-atherogenic sdLDL particle (Lamarche *et al.*, 1997).

Nonalcoholic fatty liver disease (NAFLD) is an increasingly recognized clinical condition in obesity and highly associated with insulin resistance and the pathogenesis of T2D (Dixon *et al.,* 2001; Bugianesi *et al.,* 2005). NAFLD describes a range of liver diseases but they all share a common pathophysiology of abnormal intracellular retention of lipids (steatosis) in the liver (Angulo, 2002). However, the degree may range from isolated steatosis to nonalcoholic steatohepatitis (NASH). Patients with NAFLD and T2D are at increased risk of developing more aggressive forms of liver disease, including NASH, advanced fibrosis, cirrhosis or liver cancer (Adams *et al.,* 2010; Cusi, 2009; Starley *et al.,* 2010; Bugianesi *et al.,* 2007). Although the exact mechanisms that triggering NASH progression are not fully understood, insulin resistance at the level of adipose tissue is highly associated with the degree of liver disease (Lomonaco *et al.,* 2016). Thus, lipotoxicity appears to be the likely culprit (Neuschwander-Tetri, 2010; Cusi, 2012; Lomonaco *et al.,* 2012).

1.2 Mechanisms of lipid-overload induced insulin resistance

The molecular mechanism by which lipids induce insulin resistance is not fully understood. However, a strong correlation exist between the intracellular lipid pool and insulin resistance (Krssak *et al.,* 1999; Perseghin *et al.,* 1999; Hwang *et al.,* 2007; Korenblat *et al.,* 2008; Magkos *et al.,* 2012). Circulating lipids, including plasma FFAs and TGs are important sources of this lipid pool (Frayn *et al.,* 2006). When insulin binds and activates the insulin receptor, signal transduction requires a coordinated relay of intracellular events. These events mostly involve phosphorylation or dephosphorylation of various proteins along the transduction pathway. In skeletal muscle, following activation of the

insulin receptor, the cascade initiates via phosphorylation of insulin receptor substrate 1 (IRS-1). When IRS-1 becomes phosphorylated on tyrosine residues it functions as a docking protein for other second messengers, *e.g.* phosphatidylinositol 3-kinase (PI3K). PI3K is activated upon binding to IRS-1 and subsequently, via several additional signaling intermediaries, promotes glucose transporter type 4 (GLUT4) to translocate to the plasma membrane. The fusion of GLUT4 with the plasma membrane ultimately results in glucose uptake into the skeletal muscle (Berg *et al.,* 2006; Youngren, 2007). In both rodent and human skeletal muscle, impaired insulin-stimulated phosphorylation of IRS-1 and PI3K activation, has been shown to occur following *i.v.* lipid infusions or high-fat diets. Diacylglycerol (DAG) activated protein kinase C, *i.e.* novel PKC (nPKC) was then found to cause a serine-threonine cascade, resulting in serine phosphorylation of IRS-1 which inhibits the insulin-stimulated tyrosine phosphorylation of IRS-1. The theta isoform (PKCθ) is thought to be involved in lipid-induced insulin resistance in skeletal muscle (Perseghin *et al.,* 1996; Schmitz-Peiffer *et al.,* 1997a; Schmitz-Peiffer *et al.,* 1997b; Griffin *et al.,* 1999; Dresner *et al.,* 1999; Yu *et al.,* 2002).

Insulin has profound effects on carbohydrate and lipid homeostasis in the liver. It promotes anabolic pathways such as stimulation of glycogen synthesis, de novo lipogenesis (DNL) and synthesis of lipoproteins, while suppressing gluconeogenesis, glycogen breakdown and VLDL secretion (Exton & Park, 1967; Scrutton & Utter, 1968; Madison, 1969; Sparks & Sparks, 1994; Hellerstein *et al.,* 1996). Many of the effects that insulin mediate in the liver are regulated via gene transcription; promoting expression of lipogenic and glycolytic genes while suppressing transcription of gluconeogenic genes (O'Brien & Granner, 1996). An important mechanism that interferes with insulin signaling in the liver also involves DAG. In this tissue DAG activates another nPKC, namely PKC epsilon (PKCε). Upon DAG-activation, PKCε binds to the insulin receptor tyrosine kinases, which inactivates the receptor. Thus, subsequent phosphorylation and activation of down-stream transduction mediators fails. Two major pathways inactivated via this mechanism are glycogen synthase activity and insulin-stimulated inhibition of gluconeogenesis (Samuel *et al.,* 2007; Hammond *et al.,* 2005; Nagle *et al.,* 2007).

Insulin promotes DNL in the liver by activating the transcription factor sterol regulatory element-binding protein-1c (SREBP-1c), which increases transcription of genes essential for hepatic lipid biosynthesis (Brown & Goldstein, 1997; Horton *et al.,* 2002). The newly formed TGs are then secreted into the circulation as VLDLs destined for storage in adipose tissue, or to be used as fuel in muscle. Whereas the pathway for gluconeogenesis in liver becomes insulin resistant, insulin sensitivity in the SREBP-1c pathway is

maintained (Shimomura *et al.*, 2000; Ferré & Foufelle, 2007). The insulin resistant status forces the insulin producing pancreatic β-cell to hyper-secrete insulin in a futile attempt to maintain metabolic homeostasis. Thus, hyperinsulinemia promotes an elevated activation of SREBP-1c and thereby an accelerated VLDL secretion and hepatic TG accumulation. FFA derived from this cycle contributes to an increased systemic FFA flux, worsening the insulin resistant state in muscle and adipose tissue. Consequently, insulin resistance severely disrupts metabolic regulation resulting in hyperglycemia, hyperinsulinemia, and hypertriglyceridemia (McGarry, 2002; Lann & LeRoith, 2007).

1.3 Adipose tissue lipolysis

The main role of adipose tissue is to store (as TG) and release (as FFA) energy. It is now recognized, however, that adipose tissue is more than just a lipid storage depot. In fact, several hormones and paracrine signaling molecules are secreted from adipocytes, establishing this tissue as an endocrine organ with an important role in metabolism (Frayn *et al.*, 2006; Kershaw & Flier, 2004).

During periods of food deprivation, the primary energy source comes from circulating plasma FFA, released from adipose tissue. The mechanism by which lipid-stores are mobilized from adipose tissue, *i.e.* lipolysis (Fig. 2), is mediated via cAMP-activated lipases, primarily hormone sensitive lipase (HSL) and adipocyte triglyceride lipase (ATGL). Upon their activation, TGs stored in intracellular lipid droplets are hydrolyzed into glycerol and FFA and subsequently released to the circulation (Sztalryd *et al.*, 2003; Zimmermann *et al.*, 2004). Hormones, *e.g.* catecholamines, can activate lipolysis in adipocytes in times of elevated energy demands, *e.g.* acute stress or prolonged intensive physical activity. Catecholamines interact with the β-adrenoceptor, a G-protein coupled receptor (GPCR or GPR), resulting in an increased adenylate cyclase (AC) activity and thus increased intracellular concentrations of cAMP; thereby activating the cAMP dependent protein kinase A (PKA). Subsequently, PKA phosphorylates, thus activate, HSL and ATGL (Collins & Surwit, 2001).

Insulin exerts a powerful inhibitory effect on adipose tissue lipolysis (Large *et al.*, 2004). During food deprivation, circulating insulin levels are low, with minimal insulin-induced inhibition of adipocyte FFA release. However, following meal ingestion and the subsequent secretion of insulin, a rapid and robust inhibition of lipolysis occur (Frayn, 2002). Upon binding of insulin to the insulin receptor, downstream enzymes are phosphorylated, ultimately activating phosphodiesterase-3B (PDE-3B). PDE-3B is an enzyme that breakdown cAMP into AMP, resulting in less activation of HSL (predominantly)

and thus inhibition of lipolysis (Loten & Sneyd, 1970; Degerman *et al.*, 1998; Wijkander *et al.*, 1998).

Several GPCRs are involved in regulating adipocyte lipolysis, *e.g.* GPR43, GPR81 and GPR109A (Ge *et al.*, 2008; Sakurai *et al.*, 2014; Offermanns, 2014), via modulation of cAMP. These receptors all have an inhibitory effect on lipolysis (*i.e.* antilipolysis) with metabolites stemming from tissue fuel oxidation as their natural endogenous ligands. Their physiological role is thought to involve regulation of lipid-stores under various extreme metabolic situations (Lafontan & Langin, 2009). For example, the ketone body β-hydroxybutyrate, a metabolite produced upon FFA oxidation, is the endogenous ligand for GPR109A (Taggart *et al.*, 2005). Thus, under prolonged periods of starvation, β-hydroxybutyrate negatively regulates its own production and may thereby function as a homeostatic feedback regulator to prevent ketoacidosis.

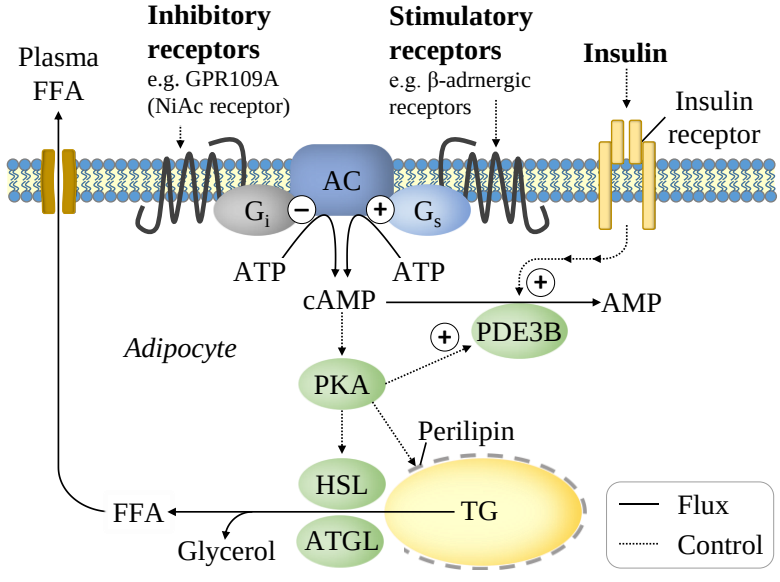

Figure 2. G-protein coupled receptor and insulin involvement in regulating adipose tissue lipolysis. Stimulatory effects from GPR109A agonism *vs.* inhibitory effects of β-adrenergic receptor agonism and insulin on adipocyte lipolysis: breakdown of TG to FFA and glycerol. Adenylate cyclase (AC); protein kinase A (PKA); hormone sensitive lipase (HSL); adipocyte triglyceride lipase (ATGL); G-protein coupled receptors (GPR); phosphodiesterase-3B (PDE3B).

1.4 Nicotinic acid-induced antilipolysis

Nicotinic acid (NiAc; or niacin) is an ancient drug with a clinical history of over six decades and was the first medication employed in clinical treatment of dyslipidemia. In 1955, Altschul and co-workers discovered that serum cholesterol levels were markedly reduced following large doses of NiAc (Altschul *et al.*, 1955). It is now recognized that NiAc has a pluripotent, dose-dependent effect on lipids and plasma lipoproteins, including decreased TG, LDL-cholesterol and lipoprotein(a) and increased HDL-cholesterol (Carlson, 2005).

At therapeutic concentrations, NiAc functions as a GPR109A agonist, potently inhibiting adipose tissue lipolysis resulting in a rapid reduction in plasma FFA concentrations (Tunaru *et al.*, 2003; Offermanns, 2006). Despite this acute effect, clinical dosing designed for dyslipidemia management has not been associated with improved glucose control. A possible explanation may be that prolonged NiAc exposure is associated with tolerance development, with return of FFA to pretreatment levels (Oh *et al.*, 2011). Furthermore, during rapid NiAc washout a major FFA rebound is seen, overshooting pretreatment levels (Ahlström *et al.*, 2011); a phenomenon also observed with oral dosing in humans (Carlson & Oro, 1962; Lauring *et al.*, 2012). This may be one reason for the apparent worsening of glycemic control with NiAc (Aye *et al.*, 2014; Blond *et al.*, 2014; Koh *et al.*, 2014; Hu *et al.*, 2015; Ooi *et al.*, 2015; El Khoury *et al.*, 2016; Goldie *et al.*, 2016). It should be remembered that the clinical NiAc dosing regimen has not been designed to lower FFA or reduce peripheral lipid accumulation, rather the goal has been to ameliorate dyslipidemia, via pathways possibly independent of the FFA lowering mechanism (Lauring *et al.*, 2012). Thus, a better understanding of NiAc's non-lipoprotein effects (*i.e.* FFA lowering) may help to define rational dosing strategies that lead to improvement in overall metabolic control via peripheral tissue lipid unloading.

Another important consideration may be the currently used extended release dosing regimen; once daily at bedtime to minimize flush (NIASPAN®, Prescribing Information). FFA increases naturally in the fasting state and it has recently been suggested that bedtime dosing might limit NiAc efficacy by triggering powerful counterregulatory mechanisms (Guyton *et al.*, 2015). It is not unreasonable that bedtime dosing might also be involved in the above mentioned glucose metabolic impairments. Furthermore, FFA lowering alone induces a major shift from whole body fat to carbohydrate oxidation, due to decreased substrate competition (Wang *et al.*, 2000). Therefore, NiAc-induced antilipolysis given at the right time (*i.e.* in conjunction with carbohydrate containing food) may be an approach for improving glucose control.

1.5 The obese Zucker rat – a model of metabolic disease

Experimental work in this thesis makes extensive use of the obese (fa/fa) Zucker rat. This is one of the most widely used animal models of the metabolic syndrome disease cluster: obesity, insulin resistance, moderate hyperglycemia, glucose intolerance, dyslipidemia, lipid intolerance and hepatic lipid accumulation (Rohner-Jeanrenaud *et al.,* 1986; Terrettaz *et al.,* 1986; Oakes ND *et al.,* 2005; Wallenius *et al.,* 2013). Not only does this model display key features of human disease but effects of antidiabetic and antidyslipidemic pharmacotherapies are predictive of responses in patients (Wallenius *et al.,* 2013; Fagerberg *et al.,* 2005), (Oakes ND *et al.,* 2005; Fagerberg *et al.,* 2007), (Chaput *et al.,* 2000; Abourbih *et al.,* 2009), (Balkan *et al.,* 1999; Iepsen *et al.,* 2015).

During cross-breeding of Sherman and Merck stock M rats a spontaneous fa-mutation was discovered (Zucker & Zucker, 1961). By ~5 weeks of age, animals with homozygous allele mutation (fa/fa) are noticeably obese and by 14 weeks, on *ad lib* standard rodent chow, body composition of the obese Zucker rat is >40% fat (Zucker & Antoniades, 1972). The fa/fa mutation results in a non-functioning leptin receptor (Phillips *et al.,* 1996). Leptin is a lipokine and thus secreted from adipose tissue, particularly in response to insulin. Although leptin has been shown to have widespread systemic effects, it is heavily involved in energy homeostasis. Following binding of leptin to its receptor in the hypothalamus it functions as a satiety factor, reducing the drive to eat (Ahima & Flier, 2000). Therefore, obese Zucker rats are hyper-phagic in nature with a disrupted normal nocturnal eating pattern resulting in an excessive energy intake causing overt obesity (Becker & Grinker, 1977).

1.6 Quantitative approaches

To aid the search for efficacious dosing regimens, quantitative models describing the dynamics of FFA in response to NiAc were employed. Quantitative pharmacology involves the temporal description of drug exposure and/or the drug-induced response (Holford, 1990; Levy, 1993; Mager *et al.,* 2003). Typically, modeling is a sequential endeavor starting with the temporal analysis of plasma drug concentration, followed by the drug-induced response modelled as a function of the plasma kinetic model, *i.e.* pharmacokinetic-pharmacodynamic (PK/PD) modeling (Gabrielsson & Weiner, 2006).

Tolerance models are needed in order to capture the phenomenon of decreased response following constant or repeated drug exposure. In biological systems, sustained drug exposures often disturb homeostasis, which may invoke counterregulatory mechanisms, opposing the drug-induced effect. For a simple turnover model, the response may be described by a single equation

where the rate of turnover depend on the level of response (Fig. 3A). As the response reaches some physiological limit, a decrease in response will occur (Yao *et al.*, 2006; Peletier & Gabrielsson, 2009). In the pool/precursor tolerance models the response (*R*) originates from a physiological pool (*P*). Build-up and loss of *P* is governed by its turnover rate (k_{in}) and fractional turnover rate (k_{tol}), respectively, whereas *R* is produced and lost by k_{tol} and its fractional turnover rate (k_{out}) respectively (Fig. 3B). Thus, the mass of the pool serves as input for the response compartment (*R*) and can either be stimulated or inhibited by a drug function. If a given fraction of the pool is pushed into the response compartment, a positive effect area (AUC_E) is produced. However, an equal fraction is now needed to refill the pool, in order to re-establish equilibrium. This refill produces a rebound area (AUC_R) and results in the intrinsic function that $AUC_E = AUC_R$, which in biological systems are rare (Licko & Ekblad, 1992; Bauer & Fung, 1993; Sharma *et al.*, 1998). A more flexible class of tolerance models are the moderator governed models. In these models, feedback is governed by an endogenous moderator (*M*), which counteracts changes in *R*. When drug-induced changes occur in *R*, simultaneous changes will also occur in *M*, which in turn affects the level of *R* negatively (Fig 3C). When the impact of *M* on *R* is slow, *i.e.* $k_{tol}<k_{out}$, the system has a tendency to overshoot, before settling at a pharmacodynamic steady state (R_{ss}). A rapid drug removal in these systems will in turn produce a rebound, due to the slow counteracting effects of *M*. In this system $AUC_E \neq AUC_R$ (Wakelkamp *et al.*, 1996; Zuideveld *et al.*, 2001; Bundgaard *et al.*, 2006; Gabrielsson & Peletier, 2007).

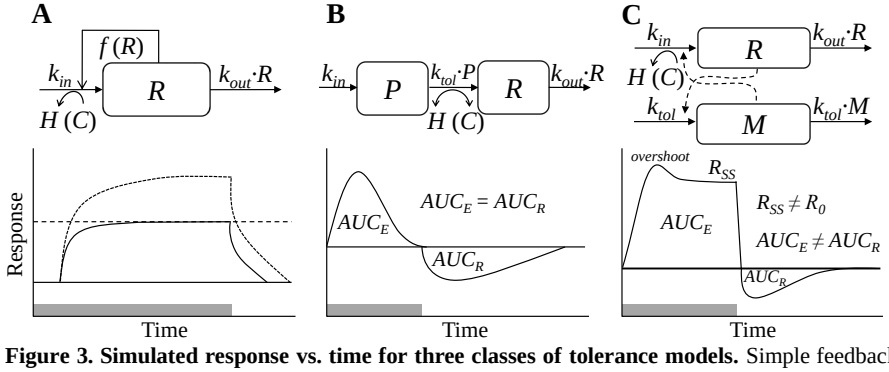

Figure 3. Simulated response vs. time for three classes of tolerance models. Simple feedback on turnover rate **A)**, pool/precursor model **B)** and negative feedback via moderator **C)**. The measured effect is represented by *R* while *P and M* represent the precursor of response and an endogenous moderator, respectively. The k_{out} and k_{tol} are the 1st-order rate constants of production and loss of *R* and *M*, and loss of *P*. A drug may inhibit or stimulate a system via a drug function, represented by *H(C)* and grey bars depict length of exposure. AUC_E and AUC_R represent the positive effect area and the rebound area, respectively. The dashed horizontal line in **A** represent a physiological limit while the dashed curve the response in a system without tolerance. (Adapted from (Gabrielsson & Weiner, 2006)).

1.7 Problem formulation

Available evidence suggest that a major driver of insulin resistance and metabolic disease is excessive accumulation of lipids in non-adipose tissues, including muscle and liver. An important source of these lipids is derived from circulating plasma FFA, released from adipose tissue via lipolysis. Therefore, a potential strategy for correcting metabolic disease is to suppress lipolysis and thereby reverse peripheral lipid accumulation. NiAc potently inhibits lipolysis acutely, however, sustained NiAc exposures result in tolerance development and major FFA rebound upon abrupt drug withdrawal. Novel pharmacological approaches are needed to define dosing strategies to mitigate these issues and durably maximize FFA lowering.

Rather than focusing on the molecular or physiological mechanisms underlying rebound and tolerance, a macro-pharmacologic approach has been used. A key principle has been the use of rational and well defined NiAc exposures. Thus, studies were performed at therapeutically relevant NiAc exposures *i.e.* at NiAc concentrations sufficient but not excessive for suppression of FFA. Tailor-made, well defined exposure profiles were produced with a low degree of invasiveness using a programmable, implantable mini-pump. The physico-chemical properties of NiAc were ideal for this purpose. Furthermore, the rapid plasma half-life of NiAc offers the freedom to realize virtually any desired exposure profile. The metabolic consequences of FFA lowering experiments were performed in a translationally relevant preclinical model, the obese Zucker rat.

2 Aims

The overall aim of this thesis was to explore the idea that a more comprehensive understanding of the relationship between pharmacokinetics and pharmacodynamics, combined with knowledge of metabolism, can be used to mitigate challenges in drug discovery and thereby enable increased translatability of preclinical findings and invention of new pharmacotherapies. Specifically, to find a NiAc delivery profile (shape and timing of exposure) that overcomes challenges such as tolerance and rebound in order to establish durable FFA lowering, sufficient to reverse peripheral lipid accumulation and improve insulin sensitivity in obese Zucker rats.

Specific hypotheses tested in Papers I-IV were:

I That a turnover model of NiAc-FFA captures experimental data from obese Zucker rats. Apply the model to explore future dosing regimen designs.

II That 1) continuous therapeutically relevant NiAc exposure delivers durable FFA lowering and 2) that intermittent (12 hr/day) NiAc exposure circumvents tolerance development and improves insulin sensitivity in conscious lean and obese rats.

III That: 1) gradual *vs.* abrupt NiAc withdrawal attenuates FFA rebound; 2) NiAc applied during feeding, but not fasting, improves metabolic control.

IV That a revised quantitative model, which alongside NiAc kinetics includes insulin dynamics and time-dependent tolerance development, improves model description of FFA dynamics.

3 Overview and progression of studies

The results described in this thesis originate from *in vivo* experiments in lean and obese rats. An important goal of this thesis was to optimize NiAc-induced antilipolytic treatment to better metabolic control. An overview and progression of studies is summarized in Table 1.

Table 1. *Thesis overview, including main problem formulation, hypotheses, key findings, conclusions and drug discovery (Dx) implications.*

	Paper I	**Paper II**	**Paper III**	**Paper IV**
		Optimizing nicotinic acid delivery to better metabolic control		
Problem	Quantitative tool for tolerance & rebound	Retaining drug efficacy upon repeated dosing	Address FFA rebound & further improve tolerance	Improved quantitative model
Hypotheses	Model captures response in obese rats	Intermittent dosing will -reduce tolerance -improve metabolic control	Exposure timed to feeding with gradual washout will -reduce rebound & tolerance -improve metabolic control	Inclusion of insulin, kinetics & tolerance improves description of FFA dynamics
Design	Feedback turnover model Obese rats Acute dosing	Acute & repeated dosing Continuous *vs.* intermittent Lean & obese rats	Repeated dosing Timed exposure food *vs.* fast Obese rats	Meta-analysis of Paper I-III
Key findings	Model captures obese data Marked & rapid tolerance	Intermittent dosing -reduced tolerance -retained drug efficacy -acutely better insulin sensitivity	Exposure timed to feeding -reduced rebound & tolerance -improved metabolic control	Model captures -repeated -intermittent -gradual washout Insulin-FFA potency
Conclusions	Tolerance may reduce efficacy chronically Next step Repeated dosing for chronic disease	Intermittent dosing retains efficacy Next step -Post-prandial dosing? -Rebound tackled by shape?	Timing & shape essential for improved metabolic control	Modeling requires drug & endogenous modulators Future encompasses timing & shape of exposure
Drug Dx	Importance of time-series Disease model	Continuous *vs.* intermittent drug exposures Programmable, implantable mini-pump	Time exposure to physiology Shape of exposure	Meta-analysis Rank candidates Predict designs

4 Materials and methods

4.1 Animals

All experimental procedures were approved by the local Ethics Committee for Animal Experimentation (Gothenburg region, Sweden). Male Sprague Dawley (lean) and obese (fa/fa) Zucker rats (obese) were purchased from Charles River Laboratories (USA). Animals were housed in an Association for Assessment and Accreditation of Laboratory Animal Care accredited facility with climate-controlled room temperature of 20-22°C and relative humidity of 40-60% with a 12 hr light-dark cycle (lights on at 06:00). For Paper III animals only, light-dark cycle was offset by 1 hr (lights on at 07:00). The animals were housed in groups of 5 and given free access to standard rodent chow (R70, Laktamin AB, Stockholm, Sweden) and regular tap water.

4.2 Nicotinic acid exposure selection and formulation

A key aspect throughout the study designs was to achieve therapeutically relevant plasma NiAc concentrations in the rat. Based on the acute relationship between plasma NiAc concentration and FFA lowering obtained in Paper I, therapeutic plasma NiAc concentrations of ~1 µM were established. For intravenous (*i.v.*) infusions, NiAc (pyridine-3-carboxylic acid, Sigma-Aldrich, St. Louis, MO, USA), was dissolved in sterile saline. For subcutaneous (*s.c.*) infusions, NiAc was dissolved in sterile water and adjusted to physiological pH using sodium hydroxide. The final concentrations of the *s.c.* NiAc dosing solutions ranged between ~0.35-1.0 M. Vehicle for control animals consisted of saline at equimolar concentrations. Freshly prepared formulations were loaded into the infusion pump (see below) via a 0.2 µm sterile filter (Acrodisc®, Pall Corporation, Ann Arbor, MI, USA) just before pump implantation.

4.3 Study Protocols and surgical preparations

Paper I

Acute NiAc-induced changes in plasma FFA concentrations were explored after different rates and durations of intravenous NiAc infusions. The exposure-response relationships were determined and quantified on the basis of a turnover model with feedback mechanisms, and used together with the parameter estimates to detect differences between lean and obese animals (Ahlström, 2011; Tapani *et al.*, 2014).

Surgical preparations: Surgery was performed under isoflurane anesthesia, with body temperature maintained at 37°C. Catheters were placed in the left carotid artery for blood sampling and right external jugular vein for NiAc/saline infusion. Catheters were exteriorized at the nape of the neck and sealed. After surgery, rats were housed individually and allowed a minimum of 5 days recovery before initiation of the experiments.

Experimental protocols: In the morning of the acute experiment, in over-night fasted rats, venous catheters were connected to an external infusion pump (CMA 100, Carnegie Medicin AB, Stockholm, Sweden). Lean and Obese animals were randomly allocated into 2 NiAc infusion groups: 20 $\mu mol \cdot kg^{-1}$ over 30 min or 51 $\mu mol \cdot kg^{-1}$ over 300 min. Following adaptation, basal blood samples were collected prior to NiAc administration. After this, multiple samples were drawn over 100 min in the 30 min infusion groups, and over 500 min in the 300 min infusion groups. Blood samples were briefly kept on ice until centrifuged and stored at -20°C pending plasma NiAc and FFA analysis.

Paper II

Paper II included three studies. In Study I, the ability of an intermittent (12 hr/day) *vs.* continuous (24 hr/day) NiAc exposure to suppress FFA levels were compared in lean and obese rats. In Study II whole body insulin sensitivity was assessed in obese rats following acute, continuous or intermittent NiAc exposure. In Study III, effects of alternating NiAc exposures on adipose tissue gene expression, and TG content in liver was assessed.

Surgical preparations: Surgery was performed under isoflurane anesthesia, with body temperature maintained at 37°C. For NiAc/saline administration, a programmable mini-pump (iPrecio® SMP200 Micro Infusion Pump, Primetech Corporation, Tokyo, Japan) was implanted subcutaneously, via a dorsal skin incision. To allow blood sampling from animals in Study I, a catheter was placed in the right jugular vein. Animals were then housed individually and allowed 3 days of recovery before start of the pre-programmed pump infusion.

Experimental protocols: Lean and obese animals were divided into 3 dose groups and NiAc was given acutely (NiAc naïve) or following 5 days with either continuous (Cont. NiAc) or intermittent (Inter. NiAc) administration. Each dose group was matched with corresponding saline infused controls. NiAc infusions were given subcutaneously at 170 $nmol \cdot min^{-1} \cdot kg^{-1}$. On the last treatment day, an overnight fast was initiated (food removed at 24:00) and animals entered into one out of the three terminal acute experiments: Study I, Study II or Study III.

Study I: Jugular catheters were connected to a swivel system and animals were left to adapt (3-4 hr) before blood sampling was initiated. Basal samples were taken in the 60 min period prior to NiAc/saline infusion (note that, in the Cont. NiAc groups, infusion pumps were on throughout this sampling period). Multiple blood samples were then collected under 8 hr, including a 5 hr NiAc/saline infusion period followed by a 3 hr washout period. Blood samples were briefly kept on ice until centrifugation and storage at -20ºC pending plasma analysis of NiAc, FFA, glucose and insulin.

Study II: Whole body insulin sensitivity was assessed in anesthetized obese Zucker rats using hyperinsulinemic-isoglycemic clamps (for acute surgery preparations, please see Paper I). Following stabilization, 2-3 basal blood samples were taken (with no NiAc/saline infusions in any group except Cont. NiAc). NiAc/saline was then *i.v.*-infused until end of experiment. After 60 min of NiAc/saline infusion, human insulin was infused at a constant rate based on estimated lean body mass, lbm (Oakes ND *et al.*, 2005) at 60 $pmol \cdot kg_{lbm}^{-1} \cdot min^{-1}$. Target plasma glucose level for the clamp was determined for each animal to be equal to its own basal level (isoglycemia). Plasma glucose was clamped with a variable rate *i.v.* infusion of 20% (w:v) glucose. Steady state, in both plasma glucose level and glucose infusion rate (GIR), was generally achieved within 90 min of clamp start. Blood samples were collected throughout, centrifuged immediately and stored at -20ºC pending analysis of plasma FFA and insulin concentrations.

Study III: After 5 days of continuous or intermittent NiAc/saline treatment, animals were anesthetized with isoflurane and tissues (liver and epididymal adipose tissue) were dissected, snap frozen in liquid nitrogen and stored at -80°C pending analysis of adipose tissue mRNA and liver TG content.

Paper III

Paper III included two studies. Study I was performed in anesthetized obese Zucker rats to address the issue of FFA rebound during NiAc withdrawal. Acute metabolic responses to either rapid (NiAc-Off) or gradual (NiAc-Stp-Dwn) NiAc withdrawal were assessed under basal fasting or glucose infused

conditions. The results of Study I were used to select the NiAc delivery profile deployed in Study II (protocols and results for Study I can be found in Paper III). In Study II, metabolic responses of fasting-period *vs.* feeding-period NiAc exposure was compared. Obese Zucker rats, with food freely available during nighttime only (to entrain defined periods of feeding and fasting in these hyper-phagic animals), were treated for 5 days with NiAc (NiAc Day *vs.* NiAc Night). Acute experiments were then performed in the conscious state and metabolic control was assessed using an oral glucose tolerance test (OGTT).

Surgical preparations: Surgery was performed under isoflurane anesthesia and body temperature maintained at 37°C. For NiAc/saline administration, a programmable mini-pump was used, as described above. A catheter was placed in the right jugular vein. Animals were housed individually until study completion, with a 3 day recovery before treatment start.

Experimental protocol: For the Study I experimental protocol the reader is referred to Paper III. *Study II:* During 5 days of treatment, food was freely available during the 12 hr dark period only (lights on at 07:00). A daily NiAc exposure profile, with gradual step-wise decline, was timed either to day (NiAc Day group) or night (NiAc Night group) including a 12 hr drug holiday period. Daily NiAc dosing profiles commenced at 06:00 for NiAc Day and at 18:00 for NiAc Night, given as an 8.5 hr constant infusion at 170 $nmol \cdot min^{-1} \cdot kg^{-1}$ followed by a 3.5 hr step-down protocol. All NiAc protocols were matched with saline infused controls. In the morning of day 5, the jugular catheter was connected to a swivel system. Following adaptation, blood sampling was initiated at 13:00. Corresponding to the timing of breaking the fast (19:00), animals received an oral glucose tolerance test (OGTT, 4.1 $g \cdot kg_{lbm}^{-1}$, 8.2 $mL \cdot kg_{lbm}^{-1}$). Blood samples were centrifuged and plasma stored at -80ºC pending analysis for NiAc, FFA, glucose, insulin, fructosamine and TG. Following the last blood sample, animals were anesthetized with isoflurane and tissues (liver, heart and epididymal adipose tissue) were dissected and snap frozen in liquid nitrogen and stored at -80°C pending TG and mRNA analysis.

Paper IV overview

A more physiologically based FFA model was developed. The FFA dynamics are driven by both NiAc pharmacokinetics and the endogenous insulin dynamics. A nonlinear mixed-effects approach was applied to capture both population and individual behaviors using pooled data from several studies.

Meta-analysis data: The developed models were challenged using data from Paper II (Study I) and Paper III (Study I, Glu- groups only). In addition, a group of lean animals were included, given 12 hr *s.c.* NiAc infusions followed by either abrupt or gradual switch-off (n=5/group, with corresponding saline

infused controls). A NiAc naïve group was also included (lean and obese, *n*=4-5/group, with corresponding controls) given a 1 hr constant *i.v.* NiAc infusion followed by either abrupt (Off 1 hr) or gradual switch-off (Stp-Dwn 1 hr). A summary of data included in meta-analysis is given in Table 2.

Table 2. *Paper IV study data (number of saline infused controls are given in parenthesis)*

State	Route	Pre-treat. (hr)	Acute inf. (hr)	Protocol/group	*n* Lean	*n* Obese
Conscious	*s.c.*	0	5	NiAc naïve	7 (2)	7 (5)
Conscious	*s.c.*	120	5	Cont. NiAc	6 (2)	8 (2)
Conscious	*s.c.*	120	5	Inter. NiAc	6 (2)	8 (3)
Anesthetized	*i.v.*	0	1	Off 1 hr	4 (3)	5 (3)
Anesthetized	*i.v.*	0	1 [+3.5 stp-dwn]	Stp-Dwn 1 hr	5 (2)	5 (2)
Anesthetized	*s.c.*	0	12	Off 12 hr	5 (2)	4 (2)
Anesthetized	*s.c.*	0	12 [+3.5 stp-dwn]	Stp-Dwn 12 hr	5 (3)	4 (3)

Subcutaneous (*s.c.*); intravenous (*i.v.*)

4.4 Analytical methods

Plasma concentrations of FFA (Wako Chemicals, Germany), glucose (Horiba ABX, France), TG (Roche Diagnostics, Germany), fructosamine (Horiba ABX, France), as well as liver and heart TG content, following isopropanol extraction (Horiba ABX, France) were determined by enzymatic colorimetric methods using an ABX Pentra 400 (Horiba ABX Diagnostics, Kyoto, Japan). Plasma glucose in Paper II was measured using a portable blood glucose monitoring device (ACCU-CHEK® Compact Plus, Roche Diagnostics Indianapolis, Indiana, USA). Obese rat plasma insulin was analyzed by radioimmunoassay (RI-13K Rat Insulin RIA kit, Merck Millipore, Germany while lean rat plasma insulin concentrations were determined using a colorimetric ELISA kit (Ultra Sensitive Rat Insulin ELISA Kit, Crystal Chem INC, Downers Grove, IL, USA). The ELISA was used for lean rats to minimize blood sample volume (only 5 µL plasma required *vs.* ~50 µL plasma for RIA). The RIA was used for the obese rats because high lipid levels in the plasma of these animals interfere with the ELISA but not the RIA measurement. Because of the hyperinsulinemia in the obese animals only 5 µL of plasma was required. For Lean rat plasma (with low lipid levels) the absolute insulin measurement are equivalent for the RIA and ELISA assays (in house analysis). For plasma samples collected during the glucose clamp study (Paper II, Study II), total (rat + human) insulin was determined using the rat RIA and human insulin was determined by a species specific RIA (human insulin specific RIA kit, Millipore). Plasma NiAc concentrations were analyzed using LC-MS/MS with a hydro-

philic interaction liquid chromatography (HILIC) approach, separated on a 50 x 2.1 mm Biobasic AX column, with 5 µm particles (Thermo Hypersil-Keystone, Runcorn, Cheshire, UK). Analytical assays are summarized in Table 3.

Area under the concentration-time curves (*AUC*) for plasma NiAc, FFA, insulin, glucose and TG were calculated by trapezoidal approximation, using GraphPad Prism 6.01 (GraphPad Software Inc., La Jolla, CA, USA). Lean body mass (lbm) was estimated from body weight as previously described (Oakes ND *et al.*, 2005).

RNA was extracted from liver and epididymal adipose tissue (EAT) and isolated according to the manufacturer's instructions, using RNeasy® Mini Kit (Qiagend AB, Solna, Sweden). cDNA was reverse transcribed using a high capacity cDNA reverse transcription kit (Applied Biosystems, Foster City, CA, USA). Differences in gene expression was determined using a QuantStudio™ 7 Flex Real-Time PCR System (Applied Biosystems). Please see Paper II and Paper III for primer sequences or TaqMan assay IDs.

Table 3. Summary of analytical methods used for plasma and tissue biomarkers

Biomarker	Assay principle	Manufacturer	LLOQ	Intra-assay (CV%)	Inter-assay (CV%)
FFA	Colorimetric method	Wako Chemicals	0.01 mM	≤1	≤5
Fructosamine	Colorimetric method	Horiba, ABX	13 µM	≤2	≤4
Glucose	Gluco-meter	Roche	0.6 mM	≤3	≤5
Glucose	Colorimetric method	Horiba, ABX	0.1 mM	≤1	≤2
Insulin (Lean)	ELISA	Crystal Chem	0.02 nM	≤10	≤10
Insulin (Obese)	RIA	Millipore	0.02 nM	≤5	≤10
NiAc	LC-MS/MS	Sciex API 5500	1-6 nM	≤10	≤10
Plasma TG	Colorimetric method	Roche/Hitachi	0.05 mM	≤1	≤2
Tissue TG	[a]Colorimetric method	Horiba, ABX	0.08 mM	≤3	≤2

Enzyme-linked immunosorbent assay (ELISA); Radioimmuno-assay (RIA); Lower limit of quantification (LLOQ); [a]Isopropanol extraction.

Statistics: In Paper II-III, statistical significance of post-hoc comparisons were evaluated based on 1-way ANOVA, adjusted for multiple comparisons, performed using GraphPad Prism 6.01 (GraphPad Software Inc., La Jolla, CA, USA). Comparisons between groups for repeatedly measured variables were based on AUC estimates. $P<0.05$ was considered statistically significant. Throughout, results are reported as mean ± standard error of the mean (SEM).

4.5 Numerical methods

Paper I

NiAc-induced inhibition of FFA release was described by $I(C_p)$ and given by

$$I(C_p) = 1 - \frac{I_{max} \cdot C_p^{\gamma}}{IC_{50}^{\gamma} + C_p^{\gamma}} \qquad (1)$$

where C_p, I_{max}, IC_{50} and γ denote the NiAc concentration in plasma, maximum drug-induced inhibitory effect, NiAc's potency on FFA release and the Hill coefficient, respectively.

The feedback of the Paper I model (Fig. 4A) is governed by a moderator (M), distributed over 8 transit compartments. M_1 inhibits the release of FFA while M_8 stimulates FFA plasma clearance. The moderator assumes to capture all endogenous FFA modulators, including insulin. The moderator is affected by FFA (R) via a 1st-order build-up of M ($k_{tol} \cdot R$), with a transit time of $1/k_{tol}$.

Following FFA lowering, production of moderator will be reduced with a subsequent decrease in M_1. With FFA formation inversely proportional to the moderator, raised to the power of p (M_1^p), FFA will increase when M_1 decreases. The signal transduction will eventually propagate through the moderator compartments, causing the level of moderator in M_8 to fall, reducing the loss of FFA. The concentrations of R and $M_{1\text{-}8}$ will eventually equilibrate. Lipoprotein lipase can hydrolyze TG into FFA in the capillaries, and thus not affected by NiAc. This was initially incorporated as a zero-order production term k_{cap} in the modeling of the obese rats. The FFA (R) dynamics are given by

$$\frac{dR}{dt} = k_{in} \cdot \frac{1}{M_1^p} \cdot I(C_p) + k_{cap} - k_{out} \cdot R \cdot M_8 \qquad (2)$$

where M_1 and M_8 are described above, k_{in} is the turnover rate, p the amplification factor, $I(C_p)$ the inhibitory drug mechanism function, k_{cap} the lipoprotein lipase dependent FFA formation, and k_{out} the fractional turnover rate of R. The turnover of the moderators are given by:

$$\begin{aligned} \frac{dM_1}{dt} &= k_{tol} \cdot (R - M_1) \\ \frac{dM_2}{dt} &= k_{tol} \cdot (M_1 - M_2) \\ &\vdots \\ \frac{dM_8}{dt} &= k_{tol} \cdot (M_7 - M_8) \end{aligned} \qquad (3)$$

A more in depth description of the model is available in Paper I.

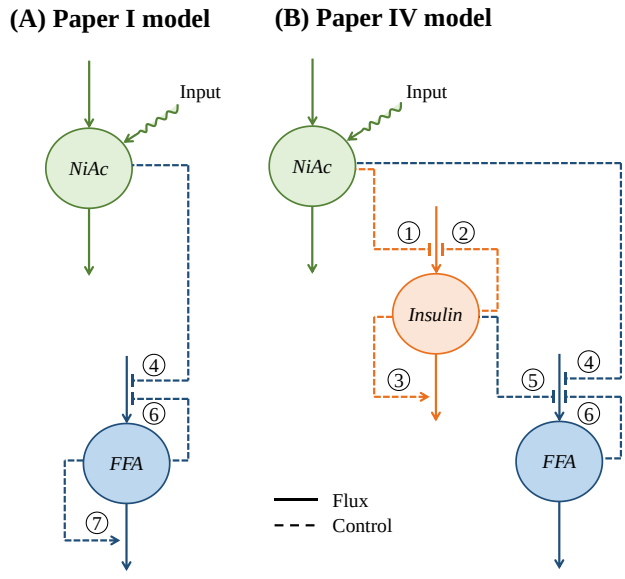

Figure 4. Diagrams of Paper I (**A**) and Paper IV (**B**) models. Solid and dashed lines represent flow and control, respectively. (**1**) NiAc inhibits insulin secretion. (**2**) Insulin have feedback mechanisms that inhibits its own turnover and (**3**) stimulates its own fractional turnover. Both (**4**) NiAc and (**5**) insulin inhibits FFA turnover. In Paper IV, (**6**) FFA have a single feedback mechanism, inhibiting its own turnover, while in the Paper I model, FFA were modelled using an (**7**) additional feedback mechanism which stimulates its fractional turnover.

Paper IV

The model developed in Paper IV is schematically described in Fig. 4B. For in depth description of the NiAc disposition model please see Paper IV.

Insulin turnover model

Insulin (I) dynamics were described by a feedback model and given by

$$\frac{dI}{dt} = k_{inI} \cdot H_{NI}(C_p) \cdot \frac{M_{0I}}{M_{1I}} - k_{outI} \cdot \frac{M_{2I}}{M_{0I}} \cdot I$$

$$\frac{dM_{1I}}{dt} = k_{tolI}(I - M_{1I}) \qquad (4)$$

$$\frac{dM_{2I}}{dt} = k_{tolI}(M_{1I} - M_{2I})$$

with initial condition $I(0)=I_0$ and $M_{1I}(0)=M_{2I}(0)=M_{0I}=I_0$. M_{1I} and M_{2I} is the first and second moderator, respectively. k_{inI} and k_{outI} represent insulin's turnover rate and fractional turnover rate, respectively, and k_{tolI} the moderators fractional turnover rate. NiAc's inhibitory function on insulin, $H_{NI}(C_p)$, is given by

$$H_{NI}(C_p) = 1 - E_{NI}(N_I)\frac{C_p^n}{IC_{50NI}^n + C_p^n} \quad (5)$$

where C_p is the plasma NiAc concentration , IC_{50NI} the drug potency, and n the Hill coefficient. $E_{NI}(N_I)$ represents the drug efficacy, which is dependent on the concentration in a hypothetical NiAc action compartment (N_I), given by

$$E_{NI}(N_I) = I_{maxNI}\left(1 - S_{NI}\frac{N_I}{N_{Iss}}\right) \quad (6)$$

where I_{maxNI} is the initial efficacy in a NiAc-naïve system, N_{Iss}=C_{pss} and thus the steady-state plasma NiAc concentration, and S_{NI} the long-term NiAc sensitivity on insulin. The dynamics of N_I, is given by

$$\frac{dN_I}{dt} = k_{NI}(C_p - N_I) \quad (7)$$

where k_{NI} is the 1st-order rate constant of the NiAc action compartment, with initial condition $N_I(0)$=$C_p(0)$. To capture individual variations, random effects were incorporated. To determine which parameters that were allowed to vary between individuals, an *a priori* sensitivity analysis was performed and the four parameters that had the highest impact on the system output were chosen. Residual variation was modelled using an additive model.

FFA turnover model

The FFA (F) turnover model is given by

$$\frac{dF}{dt} = k_{inF}\cdot H_{NF}(C_p)\cdot H_{IF}(I)\cdot\frac{M_{0F}}{M_F} - k_{outF}\cdot F \quad (8)$$

where k_{inF} and k_{outF} represent the turnover and fractional turnover of FFA, respectively. H_{NF} is the drug mechanism function representing NiAc's inhibitory effect on FFA, while H_{IF} represent insulins inhibitory effect on FFA and C_p the plasma NiAc concentration. M_F is the moderator compartment and given by

$$\frac{dM_F}{dt} = k_{tolF}(F - M_F) \quad (9)$$

with initial condition $M_F(0)$=M_{0F}=F_0. k_{tolF} represent the fractional turnover rate of the moderator compartment. H_{NF} is given by

$$H_{NF}(C_p) = 1 - E_{NF}(N_F)\frac{C_p^\gamma}{IC_{50NF}^\gamma + C_p^\gamma} \quad (10)$$

where IC_{50NF} is NiAc's potency on FFA lowering and γ is the Hill coefficient. NiAc's efficacy on FFA is flexible and able to change over time and given by

$$E_{NF}(N_F) = I_{maxNF} \cdot \left(1 - S_{NF} \frac{N_F}{N_{Fss}}\right) \quad (11)$$

where I_{maxNF} is the initial efficacy in a NiAc naïve system. S_{NF} represent the long-term NiAc sensitivity on FFA, N_F the concentration in a hypothetical NiAc action compartment acting on FFA and N_{Fss} the concentration in the action compartment at steady state. N_F is described by

$$\frac{dN_F}{dt} = k_{NF}(C_p - N_F) \quad (12)$$

with initial condition $N_F(0)=C_p(0)$. k_{NF} is the 1st-order rate constant of the NiAc action compartment acting on FFA. The insulin mechanism function H_{IF} is given by

$$H_{IF}(C_p) = 1 - E_{IF}(N_F)\frac{I}{IC_{50IF} + I} \quad (13)$$

where IC_{50IF} represent insulins potency on FFA release and E_{IF} its efficacy given by

$$E_{IF}(N_F) = I_{maxIF} \cdot \left(1 - S_{IF} \frac{N_F}{N_{Fss}}\right) \quad (14)$$

where I_{maxIF} represent the initial efficacy in a NiAc naïve system. S_{NF} represent the long-term insulin sensitivity on FFA.

Random effects were based on an *a priori* sensitivity analysis (as described for the insulin model) and residual variation was modelled using an additive model.

5 Results

5.1 Paper I

NiAc disposition: NiAc infusions at 170 nmol·min^{-1}·kg^{-1} resulted in plasma concentrations of ~1 µM in both lean and obese rats, with a tendency of slightly higher concentrations in obese *vs.* lean (Fig. 5A). NiAc disposition parameters are available and covered in detail in Paper I.

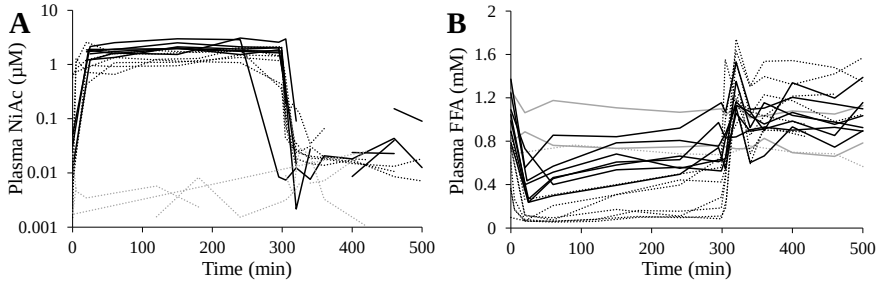

Figure 5. Plasma concentration-time profiles of NiAc (**A**) and FFA (**B**), in lean (dotted lines) and obese (solid lines) during and after 300 min infusion of 51 µmol·kg^{-1} NiAc (black) or saline (grey) administration.

NiAc-induced changes in FFA response: In Lean rats, plasma FFA decreased rapidly to a lower physiological limit of ~0.055 mM. In Obese, however, the FFA reduction was slower with no apparent plateau level, indicating a reduced NiAc efficacy in obese *vs.* lean. During ongoing NiAc infusion (0-300 min), pronounced tolerance development was observed (Fig. 5B). Following NiAc cessation, the return of FFA to baseline was slower in obese *vs.* lean rats, with FFA rebound being less pronounced.

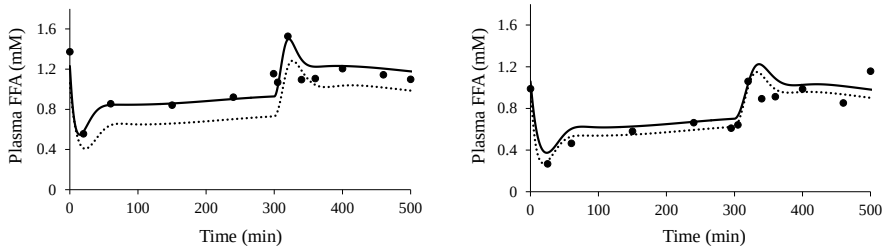

Figure 6. Typical model fits of plasma FFA concentration-time data in obese Zucker rats after 300 min infusion of 51 µmol·kg^{-1} NiAc. Black circles are observed data while solid and dotted lines depict individual and population fits, respectively.

Model prediction: Predicted and experimental data were consistent, and the model captured the elevated FFA baseline concentrations, slowly developing tolerance and the small oscillatory FFA rebound following 300 min infusions in obese rats. Representative observed and population predictions of changes in FFA concentrations in Obese are shown in Fig. 6.

Concentration-response relationship: The equilibrium NiAc concentration-FFA response relationships of lean and obese rats were simulated using the final parameter estimates (available in Paper I). It can be seen from Fig. 7 that the steady state FFA concentration (R_{SS}) was noticeably higher in obese *vs.* lean, over the entire range of simulated concentrations. There was a sigmoid relationship between the simulated steady state concentration of NiAc and FFA in lean, with decreasing FFA as NiAc concentrations increased from ~0.02 to 0.5 µM. In Obese, FFA concentrations decreased progressively with increasing NiAc concentrations, with no evident plateau. Thus for obese, the curve was more shallow and shifted upwards *vs.* lean.

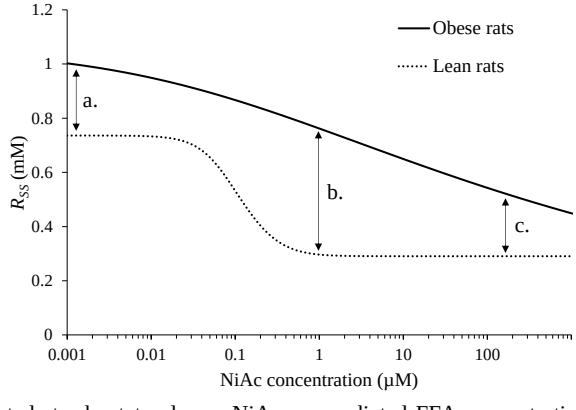

Figure 7. Simulated steady state plasma NiAc *vs.* predicted FFA concentrations at equilibrium (R_{SS}) for obese (solid line) and lean (dotted line) rats. Differences between lean and obese rats are given at (**a**) low, (**b**) therapeutic and (**c**) high NiAc concentrations.

5.2 Paper II

Nicotinic acid exposure: The target steady state plasma NiAc concentration of ~1 µM was successfully achieved in both lean and obese groups (Fig. 8).

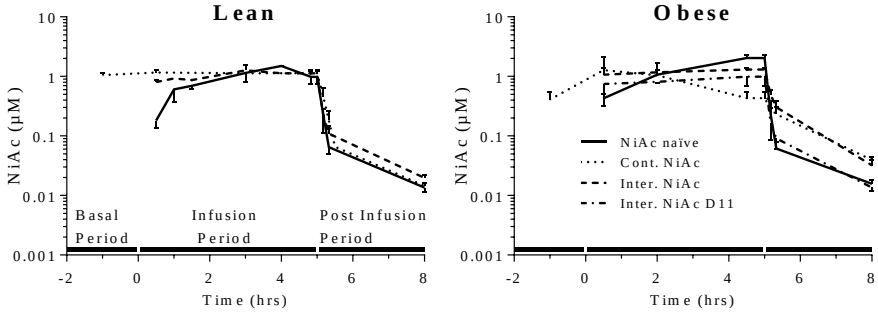

Figure 8. Plasma NiAc concentration in lean (left) and obese (right) with NiAc (170 nmol·min^{-1}·kg^{-1}) given acutely (NiAc naïve, *n*=7/group) or following 5 days continuous (Cont. NiAc, lean *n*=4, obese *n*=8) or intermittent (Inter. NiAc, lean *n*=4, obese *n*=9) or 11 days intermittent (Inter. NiAc D11, obese *n*=4) dosing. Data presented as mean ± SEM. Bottom black bars represent the Basal, Infusion and Post Infusion Periods, respectively.

Plasma FFA: In both lean and obese rats, NiAc-induced FFA lowering was completely lost in the Cont. NiAc group after 5 days of continuous, uninterrupted NiAc infusion (Fig. 9). In contrast, intermittent NiAc dosing succeeded in retaining significant FFA lowering during the Infusion Period *vs.* corresponding saline control (lean: P<0.05; obese: P<0.001, Paper II Fig. 4A). Unlike lean, in the obese Inter. NiAc group, there was some loss of the extent of FFA lowering *vs.* NiAc naïve (P<0.001, Paper II Fig. 4A). Importantly, an additional group of obese animals, studied following 11 days of intermittent NiAc (Inter. NiAc D11), showed no further development of tolerance (Inter. NiAc Day 11 *vs.* Inter. NiAc, P>0.05, Paper II Fig. 4A). During the Post Infusion Period, FFA rebound of various magnitude was observed in both lean and obese. Interestingly, the lean, but not obese, Cont. NiAc group also exhibited a rebound *vs.* saline (Fig. 9; P<0.001, Paper II Fig 4B), consistent with a previous study (Oh *et al.*, 2011). Remarkably, in the lean animals over the whole 8 hr observation period, none of the NiAc protocols reduced total FFA *AUC* (Paper II, Fig. 4C). In obese rats, total 8hr FFA *AUC* lowering was achieved only in the NiAc naïve group (-36%, P<0.001 *vs.* saline control, Paper II Fig. 4C), Thus, intermittent NiAc dosing retained FFA lowering efficacy, but the FFA rebound following NiAc cessation tends to cancel the net FFA lowering effect. In the obese Cont. NiAc group, total FFA *AUC* was actually increased *vs.* saline control (P<0.05, Paper II Fig 4C).

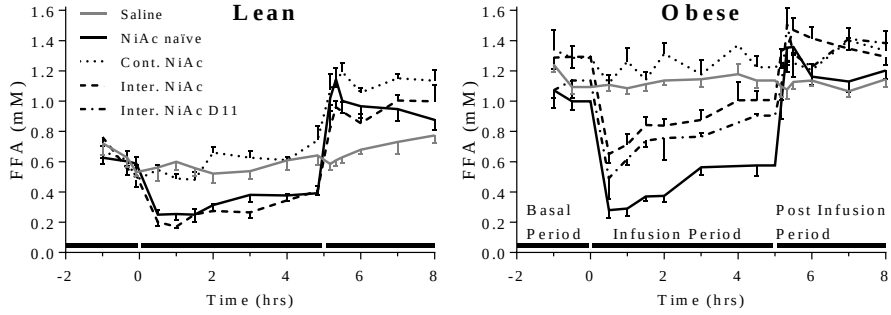

Figure 9. NiAc-induced plasma FFA responses in lean (left) and obese (right) following infusion of saline (Lean *n*=5, Obese *n*=12) or NiAc given either acutely (NiAc naïve) or following 5 days continuous (Cont. NiAc), intermittent (Inter. NiAc) or 11 days intermittent (Inter. NiAc D11) dosing. Data presented as mean ± SEM. Bottom black bars represent the Basal, Infusion and Post Infusion Periods, respectively.

Insulin and glucose: Compared to corresponding saline infused controls, plasma insulin concentrations were reduced during the Infusion Period (Fig. 10A-B) in lean NiAc naïve, Obese NiAc naïve and Obese Inter. NiAc ($P<0.05$, Paper II Fig. 6A). This occurred in absence of change in glycemia (Fig. 10C-D, $P>0.05$, Paper II Fig. 7A), suggesting an improvement in insulin sensitivity.

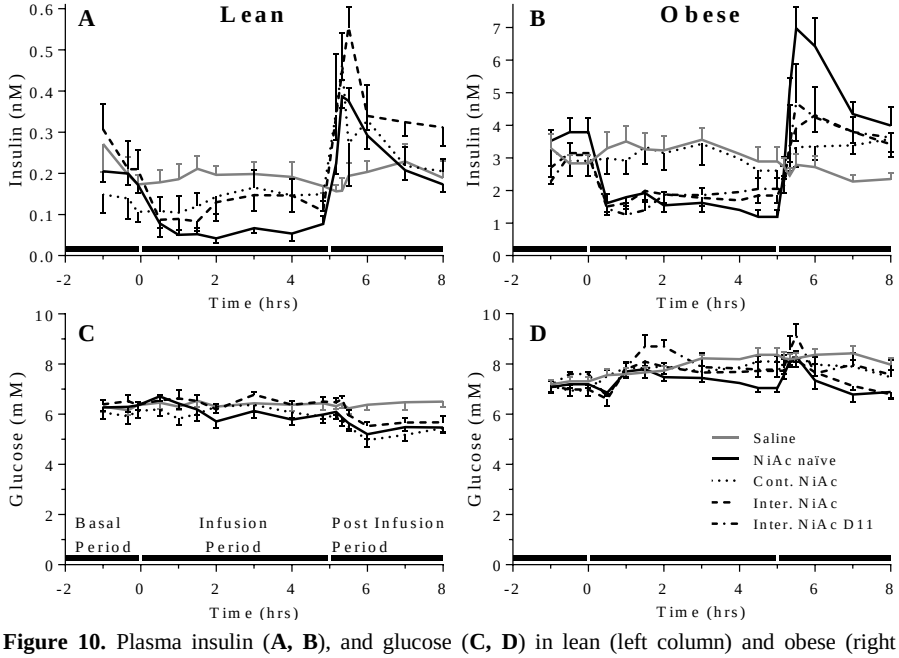

Figure 10. Plasma insulin (**A, B**), and glucose (**C, D**) in lean (left column) and obese (right column) after NiAc/saline infusion given acutely or after 5 days continuous (Cont. NiAc) or intermittent (Inter. NiAc) or 11 days intermittent (Inter. NiAc D11) dosing. Data presented as mean ± SEM. Bottom black bars represent the Basal, Infusion and Post Infusion Periods, respectively.

The rebound phenomenon was not just restricted to FFA. Insulin rebounds were observed in all NiAc dosed groups (Fig 18A-B) with the exception of Obese Cont. NiAc (Fig. 10B). As for FFA (described above), reduction in insulin *AUC* achieved during the Infusion Period tended to be cancelled during the Post Infusion Period, with the result that total 8hr insulin *AUC* is similar in all NiAc groups compared to respective saline control groups (Paper II Fig. 6C). NiAc succeeded in moderately lowering blood glucose in the Obese NiAc naïve group (-11%, P<0.001, Paper II Fig. 7C), although this effect was not maintained with either intermittent or continuous NiAc dosing.

Effects of alternating NiAc exposures on whole body insulin sensitivity: In the NiAc naïve group, glucose infusion rate (GIR) was markedly increased compared to saline infused controls (+92%, P<0.01). Importantly, the Inter. NiAc group also had an elevated GIR (+71%, P<0.05), similar in magnitude to the NiAc naïve group (P>0.05), compatible with a sustained insulin sensitization of the intermittent dosing approach. In stark contrast, upon continuous dosing, this effect was completely lost (P>0.05 *vs.* saline control, Fig. 11).

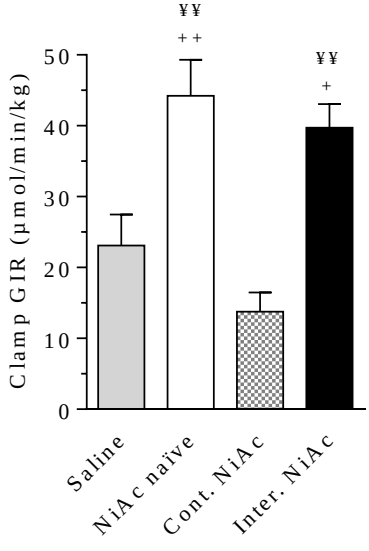

Figure 11. Glucose infusion rate (GIR) at clamp steady state in obese Zucker rats. $^{+}$P<0.05, $^{++}$P<0.01 *vs.* saline; $^{¥¥}$P<0.01 *vs.* Cont. NiAc. Data presented as mean ± SEM (n=6/group).

NiAc induced changes in adipose tissue gene expression: Overall there was no evidence that a coordinated alteration in expression of genes was responsible for the tolerance development in either lean or obese rats (Paper II Table 3).

Liver triglyceride (TG) content: In both lean and obese rats, NiAc (either intermittent or continuous exposure) had no significant impact on liver TG content (Paper II Table 4).

5.3 Paper III

Nicotinic acid exposure: Target plasma NiAc concentrations were similar at ~1 µM in both NiAc infused groups (Fig. 12A). By design, following pre-programmed initiation of the step-down protocol at 14:30, plasma NiAc levels declined gradually in the Day group, taking ~3.5 hr to reach acute *in vivo* IC_{50} for FFA lowering, ~0.070 µM (*vs.* 1.5 hr for abrupt termination Paper III Fig. 2A). NiAc exposure was much higher during the 13:00-19:00 time period in the Day *vs.* Night groups. Conversely, during the OGTT period (19:00-21:00), NiAc exposure was much higher in the Night *vs.* Day group.

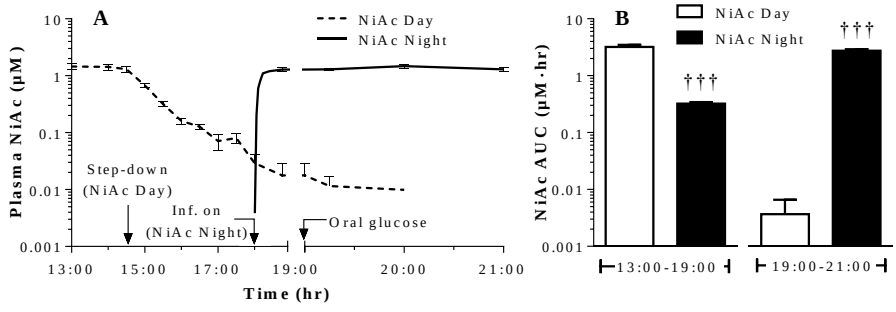

Figure 12. Study II, plasma NiAc concentrations on day 5 of programmed NiAc delivery, given either during fasting (NiAc Day) or feeding (NiAc Night) in obese Zucker rats †††P<0.001 *vs.* NiAc Day. Data presented as mean ± SEM (*n*=8/group).

Plasma FFA: As expected, in the Saline group the oral glucose load at 19:00, reduced FFA levels (Fig. 13A). Strikingly, Day *vs.* Night dosing had completely opposite effects on FFA levels during the OGTT. In the Day group FFA was increased ($P<0.05$), whereas in the Night group it was reduced ($P<0.01$) *vs.* Saline (Fig. 13B). Dosing NiAc to feeding resulted in 60% lower plasma FFA *vs.* daytime dosing ($P<0.001$).

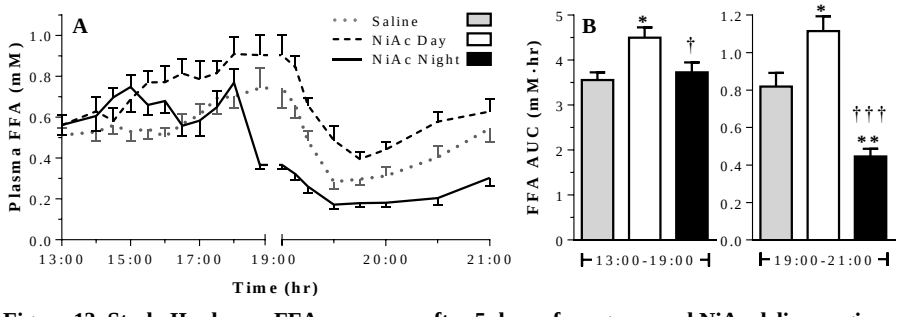

Figure 13. Study II, plasma FFA responses after 5 days of programmed NiAc delivery, given either during fasting (NiAc Day) or feeding (NiAc Night) in obese Zucker rats. *P<0.05, **P<0.01, *vs.* Saline; †††P<0.001 *vs.* NiAc Day. Data presented as mean ± SEM (*n*=8/group).

Plasma glucose: NiAc Night improved glycemia in the 13:00-19:00 fasting period *vs.* Day and Saline groups (P<0.01, Fig. 14B). Following glucose loading (OGTT), the Night group displayed a remarkable improvement in glucose control (19:00-21:00, Fig. 14A), with markedly improved glucose control *vs.* Day and Saline groups (-27% P<0.001, -22% P<0.01, respectively, Fig. 14B). After achieving peak levels at 15 min post glucose load, glucose levels were restored much more rapidly to baseline *vs.* Day and Saline groups.

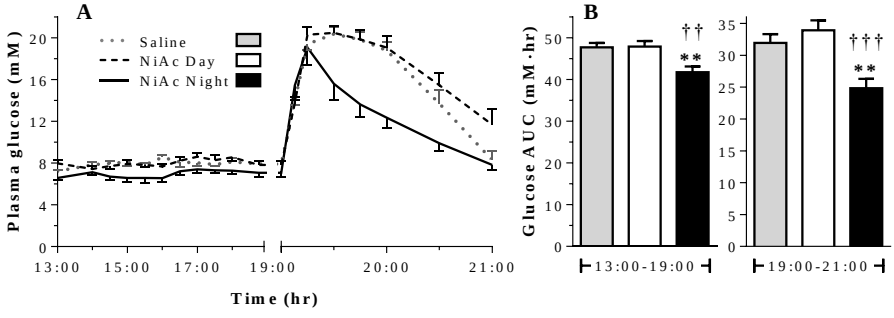

Figure 14. Study II, plasma glucose responses after 5 days of programmed NiAc delivery, given either during fasting (NiAc Day) or feeding (NiAc Night) in obese Zucker rats. Corresponding to the timing of breaking the fast (19:00) animals received an oral glucose load (4.1 g·kg$_{lbm}^{-1}$). **P<0.01, *vs.* Saline; ††P<0.01, †††P<0.001 *vs.* NiAc Day. Data presented as mean ± SEM (n=8/group).

Plasma insulin: During both the fasting and OGTT periods, NiAc timed to feeding exhibited lower insulin *vs.* NiAc Day (P<0.05, Fig. 15B). These data indicate that NiAc timed to feeding: 1) decreases insulin secretory burden *vs.* daytime dosing and 2) enhances insulin sensitivity, given the reduced fasting and OGTT levels of glycemia.

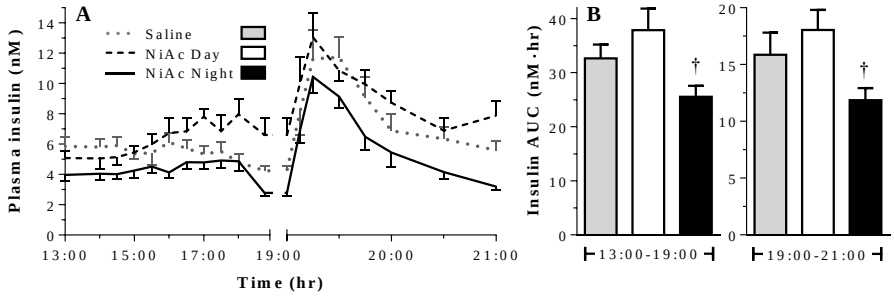

Figure 15. Study II, plasma insulin responses after 5 days of programmed NiAc delivery, given either during fasting (NiAc Day) or feeding (NiAc Night) in obese Zucker rats. †P<0.05 *vs.* NiAc Day. Data presented as mean ± SEM (n=8/group).

Plasma triglyceride (TG): During the 13:00-19:00 fasting period there was a tendency for the NiAc groups to exhibit lower plasma TG levels vs. Saline. During the OGTT period, NiAc dosed to feeding decreased TG $AUC_{(19:00\text{-}21:00)}$ vs. Saline ($P<0.05$ Fig. 16B). This was not observed with dosing to fasting

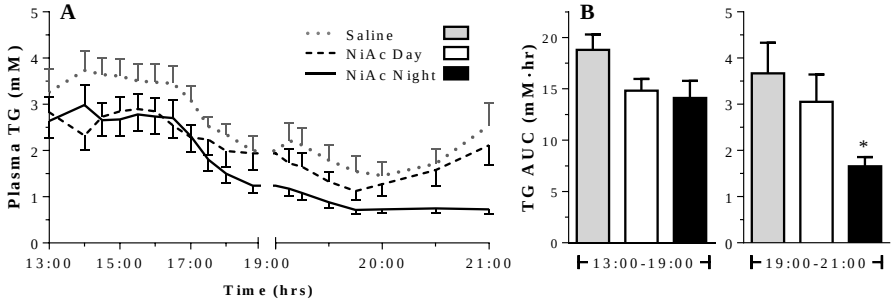

Figure 16. Study II, plasma TG responses after 5 days of programmed NiAc delivery, given either during fasting (NiAc Day) or feeding (NiAc Night) in obese Zucker rats. *P<0.05 *vs.* Saline. Data presented as mean ± SEM (*n*=8/group).

Liver and heart triglyceride (TG): Five days of programmed NiAc delivery, applied during nighttime feeding periods, dramatically reduced liver TG content *vs.* Saline and Day groups (*P*<0.01, and *P*<0.05, respectively). This was not the case with NiAc during daytime fasting periods, which had no effect on liver TG content *vs.* Saline. Heart TG content was markedly reduced in the Night *vs.* Day and Saline groups (*P*<0.001, *P*<0.05, respectively). In complete contrast, heart TG content was increased in the Day *vs.* Saline group (*P*<0.05, Fig. 17). Cardiac TG content across the groups paralleled the FFA exposures immediately prior to tissue collection (FFA $AUC_{(19:00\text{-}21:00)}$): linear regression $r^2 = 0.67$, *P*<0.0001.

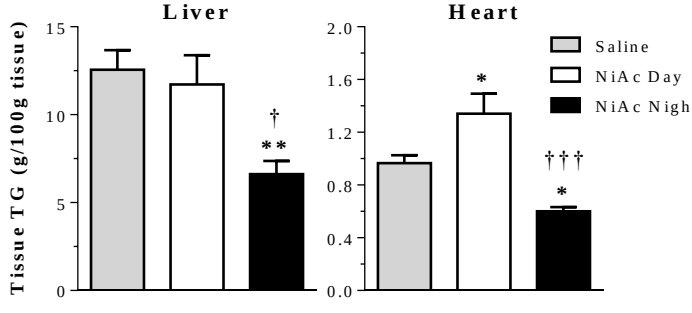

Figure 17 Study II, (left) liver and (right) heart triglyceride (TG) content following 5 days of programmed NiAc delivery, given either during fasting (NiAc Day) or feeding (NiAc Night) in obese Zucker rats. *P<0.05, **P<0.01 *vs.* Saline; †P<0.05, †††P<0.001 *vs.* NiAc Day. Data presented as mean ± SEM (n=8/group).

De novo lipogenesis (DNL) gene expression: Quantitative RT-PCR was conducted in liver samples to assess a potential involvement of a down-regulation in the DNL pathway in the observed reduction in liver TG in the Night group. Indeed, expression of two important master regulator genes, carbohydrate-responsive element-binding protein (ChREBP) and sterol regulatory element binding protein-1c (SREBP-1c), as well as four of their regulated genes directly involved in DNL, acetyl-CoA carboxylase 1 (ACC1), fatty acid synthase (FAS), fatty acid elongase 6 (Elovl6) and stearoyl-CoA desaturase-1 (SCD1) were downregulated compared to the saline control group. By contrast, hepatic expression of genes in the Day group were unchanged relative to the controls. The other major site of DNL in the body is adipose tissue. By contrast with the results in the liver, there was no significant treatment associated regulation of DNL genes in epididymal adipose tissue (Fig. 18).

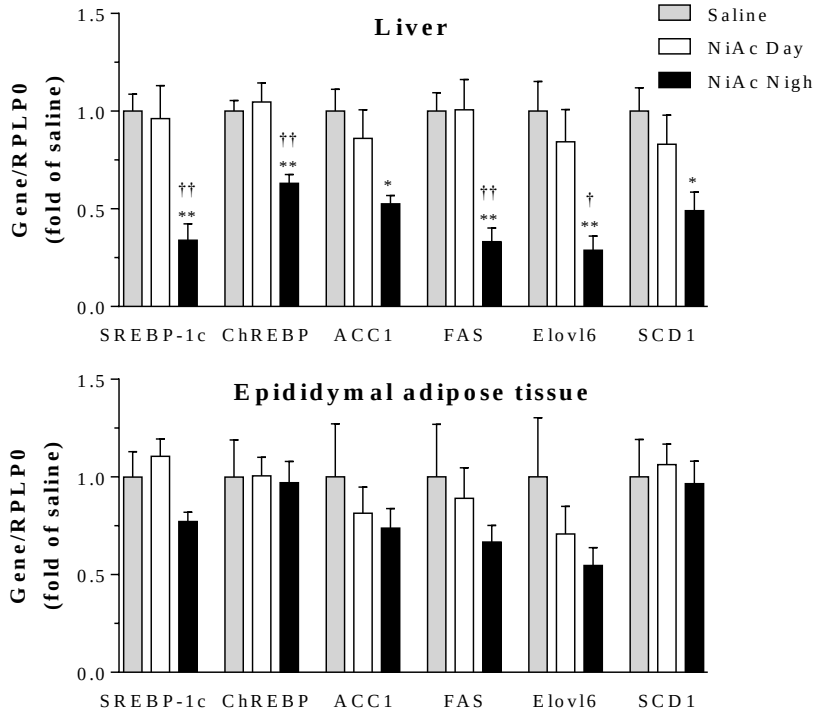

Figure 18. Study II, mRNA expression analysis of genes involved in de novo lipogenesis (DNL) in liver (top) and epididymal adipose tissue (bottom). Tissues were harvested following 5 days of programmed NiAc delivery, given either during fasting (NiAc Day) or feeding (NiAc Night) in obese Zucker rats. $^{*}P<0.05$, $^{**}P<0.01$ *vs.* Saline; $^{\dagger\dagger}P<0.01$ *vs.* NiAc Day. Data is normalized to housekeeping gene ribosomal protein large P0 (RPLP0) and presented as mean ± SEM (n=8/group).

5.4 Paper IV

The models were evaluated using visual predictive check (VPC) plots (Post *et al.*, 2008; Bergstrand *et al.*, 2011) and presented as the model predicted median individual with corresponding 90% prediction intervals, superimposed on observed experimental data (Fig. 19).

Pharmacokinetic model: The VPC plots capture the NiAc exposure-time course for all NiAc protocols (Fig. 7 & 8 Paper IV). The 90% prediction intervals covers all but 32 out of the 650 data points (~95%). Figure 19 (left column) shows 3 representative model predictions of plasma NiAc concentrations in lean rats, following different durations of NiAc infusions. The aim of modeling NiAc concentration-time data was to obtain individually fitted time-courses to co-drive the FFA concentration-time data. NiAc disposition parameter were estimated with relatively high precision (Table 2, Paper IV).

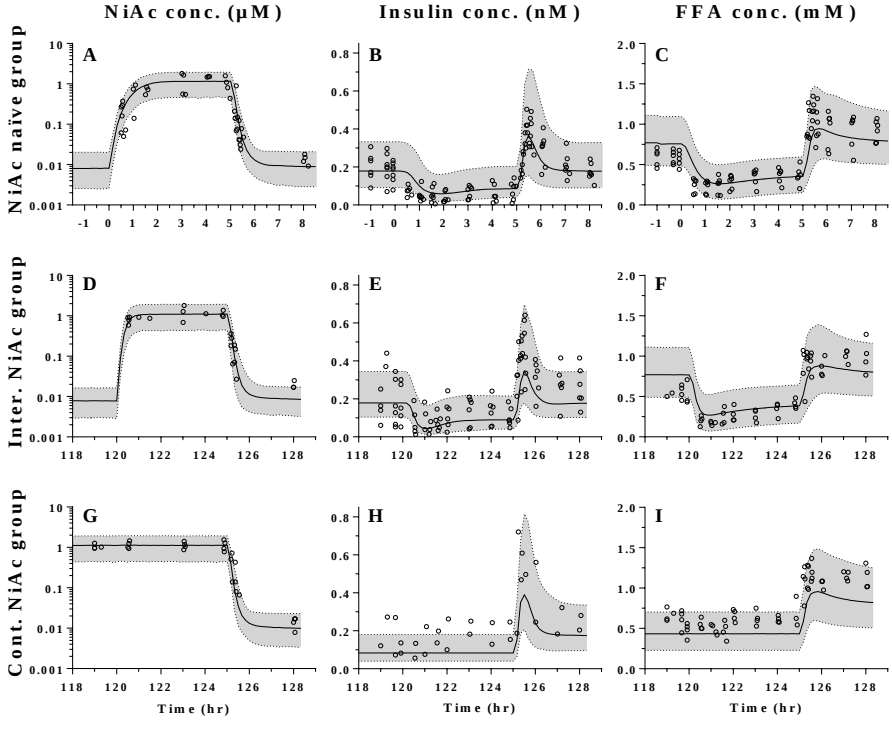

Figure 19. Population model fits of plasma concentrations of NiAc (left column), insulin (mid column) and FFA (right column) in lean rats. NiAc was given as a 5 hr s.c. infusion either acutely (NiAc naïve, top row) or following 5 days intermittent (Inter. NiAc, mid row) or continuous (Cont. NiAc, bottom row) NiAc dosing. Solid lines represent the predicted median individual and the grey area contained within the dashed lines represent the 90% prediction intervals. Open circles are the experimental observations.

Insulin model: The aim of modeling insulin concentration-time data was to obtain individually fitted time-courses to co-drive the FFA dynamics. The VPC plots shows that the model captures the general insulin-time course adequately with the predicted median individual following the course of the median data points (for all data see Fig. 7 & 8, Paper IV). For some NiAc protocols the model under-predicts the behavior of the mean (Fig. 7K & N and Fig. 8K & T, Paper IV). Overall the model was able to capture the main insulin observations in both lean and obese rats, including NiAc-induced insulin suppression, adaptation following long-term infusions and rebound upon infusion cessation. The 90% prediction intervals covered all but 144 of the 1834 data points (approximately 92%). Figure 19 (mid column) shows 3 representative model predictions of plasma insulin concentrations in lean rats, following different durations of NiAc infusions. Parameter estimates for the insulin model were determined with relatively high precision (Table 2, Paper IV).

FFA model: In lean rats, the VPC plots shows that the model captures the general FFA time-course adequately, with the predicted median individual following the course of the median data points (for all lean data see Fig. 7, Paper IV). Due to Paper IV being a work still in progress, at present, obese FFA parameter estimates has not yet been attained and precision in lean FFA parameter estimates are under assessment. However, in lean, the developed model structure allows for a system to be fully responsive to NiAc and insulin acutely (Fig. 19C) and following intermittent NiAc dosing (Fig. 19F). Over time tolerance develops with FFA returning towards baseline (Fig. 19I).

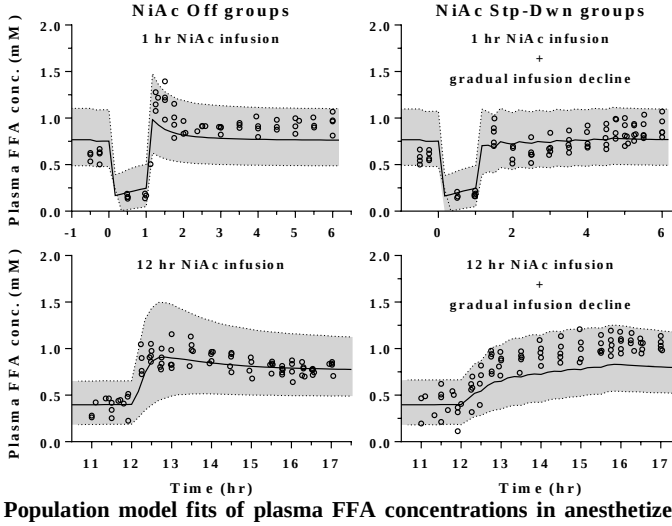

Figure 20. Population model fits of plasma FFA concentrations in anesthetized lean rats. NiAc was infused for 1 hr (A-B) or 12 hr (C-D), with abrupt (left column, NiAc Off) or gradual switch-off (right column, Stp-Dwn). Solid lines represent predicted median individual, grey area represent the 90% prediction intervals. Open circles are experimental observations.

The future potential and flexibility of the model structure to capture FFA dynamics following various NiAc infusion regimens are shown in Fig. 20. The model adequately captures the FFA responses in anesthetized lean rats following a constant rate NiAc infusion for either 1 hr or 12 hr followed by either abrupt termination (Fig. 20A & 20C) or gradual withdrawal (Fig 20B & 20D). The 90% prediction intervals cover essentially all experimental observations.

The ability of the FFA model to capture experimental data, was more evident on individual level, compared to the population median individual fits. The FFA model adequately captured individual FFA responses following acute, intermittent and continuous NiAc exposure (Fig. 21). Thus, inclusion of insulin along-side NiAc to co-drive the FFA response resulted in a model that captures FFA dynamics following a wide range of NiAc exposure durations, from 1 hr out to 5 days.

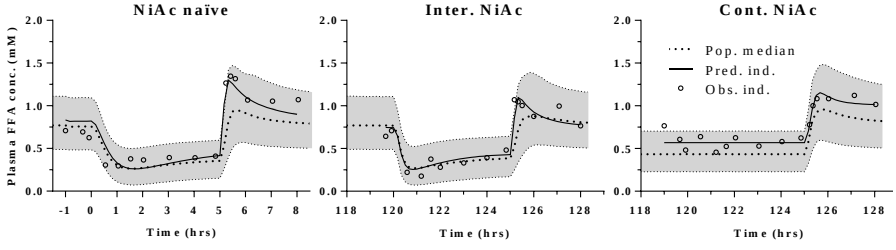

Figure 21. Representative model fits of individual plasma FFA concentrations. NiAc was given as a 5 hr s.c. infusion either acutely (NiAc naïve) or following 5 days intermittent (Inter. NiAc) or continuous (Cont. NiAc) NiAc dosing. Solid and dashed lines represents the individual and population fits, respectively. The grey area contained within the dotted lines represent the 90% population prediction intervals. Open circles are the individual experimental observations.

In this thesis Paper IV is in manuscript form and hence analysis and results are preliminary. The final parameter estimates, as well as estimate uncertainties, will upon completion add further knowledge and validity to the currently presented results.

6 Discussion

6.1 Background

This thesis explores the idea that a more comprehensive understanding of the relationship between pharmacokinetics (PK) and pharmacodynamics (PD) combined with knowledge of the physiologic regulation of metabolism (*i.e.* considering effects of key biomarkers) can be used to mitigate challenges such as tolerance and rebound phenomena, enabling invention of new drug therapies.

Available evidence suggest that a major driver of insulin resistance and metabolic disease is excessive accumulation of lipids in non-adipose tissues, including muscle and liver (Shulman, 2014; Lomonaco *et al.*, 2016; Krauss, 2004). An important source of these lipids is derived from circulating plasma FFA, released from adipose tissue via lipolysis (Frayn *et al.*, 2006). Therefore, a potential strategy for correcting metabolic disease is to suppress lipolysis and thereby reverse peripheral lipid accumulation.

NiAc potently inhibits lipolysis acutely, however, sustained NiAc exposures result in tolerance development and major FFA rebound upon abrupt drug withdrawal (Oh *et al.*, 2011; Carlson & Oro, 1962; Vega *et al.*, 2005). This may be one reason for the apparent worsening of glycemic control with NiAc (Aye *et al.*, 2014; Blond *et al.*, 2014; Koh *et al.*, 2014; Hu *et al.*, 2015; Ooi *et al.*, 2015; El Khoury *et al.*, 2016; Goldie *et al.*, 2016). Another important factor may be the currently used extended release dosing regimen; once daily at bedtime to minimize flush (NIASPAN®, Prescribing Information). FFA increases in the fasting state and it has recently been suggested that bedtime dosing might limit NiAc efficacy by triggering powerful counterregulatory mechanisms (Guyton *et al.*, 2015). It is not unreasonable that bedtime dosing might also be involved in the above mentioned glucose metabolic impairments. Moreover, an insulin-NiAc synergy on antilipolysis (Paper II) would favor

mealtime over bedtime dosing. Novel pharmacological approaches are needed to define dosing strategies to mitigate challenges such as tolerance and rebound in order to maximize FFA lowering.

In the present thesis, rather than focusing on the molecular mechanisms underlying rebound and tolerance, a macro-pharmacologic approach was used. A key principle has been the use of well-defined therapeutically relevant NiAc exposures, *i.e.* concentrations sufficient, but not excessive, for suppression of FFA in rats. Tailor-made, exposure profiles were produced with a low degree of invasiveness, using a programmable, implantable mini-pump. The physico-chemical properties of NiAc were ideal for this purpose. Furthermore, the rapid plasma half-life of NiAc offers the freedom to realize virtually any desired exposure profile. The metabolic consequences of FFA lowering experiments were performed in a translationally relevant preclinical model, the obese Zucker rat.

6.2 Paper I

The main purpose of Paper I was to assess and quantify differences in NiAc-induced FFA responses between lean Sprague Dawley and obese Zucker rats. Following acute NiAc administration, plasma FFA responses in obese rats were applied to an existing quantitative model. This feedback model was originally developed (Ahlström *et al.*, 2011) and challenged (Ahlström *et al.*, 2013) to describe NiAc-induced changes in FFA concentrations in lean rats. Given that lean rats often serve as a primary screening model during preclinical drug discovery, it is important to determine how drug-induced responses translates to an animal model of disease. Furthermore, a quantitative model that adequately describes a drug-induced response may have predictive value beyond the data from which it was developed. Thus, challenged and validated quantitative models may then serve as a valuable tool when designing new studies.

While NiAc effectively decreases plasma FFA concentrations to ~10% of baseline levels in lean (Ahlström *et al.*, 2013), corresponding infusion rate in obese produced a reduction to ~30% of baseline levels. Compared to lean, the onset of response was slower in obese rats, with more pronounced tolerance development during the period of constant NiAc exposure. Although an FFA rebound was observed following NiAc cessation, the magnitude was substantially less in obese compared to lean rats.

Insulins regulatory impact on FFA was modelled as a series of transit compartments, with the moderator in the first and last compartment representing a rapid (Stralfors *et al.*, 1984; Frayn *et al.*, 1994) and slow (Frayn

et al., 1994; Sadur & Eckel, 1982) regulation, respectively. Although the pharmacodynamic characteristics of the obese rats in Paper I differed from lean rats, the feedback model managed to successfully described the observed NiAc-induced FFA responses in obese rats. This included the elevated basal FFA level, pronounced tolerance development and the diminished rebound. Generally, obese parameters were estimated with high precision, with no apparent residual plot correlations. Compared to lean, R_0, k_{in} and p were increased by 44, 41 and 78 %, respectively, while k_{out} and γ were reduced by 64 and 84 %. The k_{tol} and IC_{50} were similar in both rat strains.

The concentration-response relationships at equilibrium differed between lean and obese rats, with obese displaying an upward shift and a shallower shaped curve, compared to lean. The extent of such shifts are important, as they demonstrate the impact of disease at equilibrium and, if ignored, may lead to erroneous dose predictions and consequently poorly designed studies.

In summary, the feedback model captured the acute experimental data consistently and NiAc-induced FFA responses was found to differ between lean and obese rats. The pronounced tolerance development, observed during constant drug exposure, suggests that 24 hr drug coverage may substantially reduce NiAc efficacy. Future studies beyond the acute dosing situation are needed to explore whether an optimized NiAc treatment regimen, *e.g.* intermittent dosing, could achieve durable FFA lowering.

6.3 Paper II

The standard pharmacological approach of engaging the target mechanism (GPR109A activation) for 24 hr/day fails in the case of NiAc, as prolonged exposure induces tolerance development, with FFA returning to pretreatment levels (Oh *et al.*, 2011). In paper II, it was hypothesized that by interspacing daily NiAc exposures with drug holidays, tolerance development might be circumvented and NiAc-induced FFA lowering efficacy would be retained. If true, non-adipose tissues (primarily liver and muscle) would then receive a reduced 24 hr average FFA exposure, resulting in reversed peripheral lipid accumulation and thereby improved metabolic control. This hypothesis was tested by comparing FFA lowering in lean Sprague Dawley and obese Zucker rats in response to a 5 hr NiAc infusion, in either NiAc-naïve (acute dosing) animals, or after 5 days of continuous (24 hr/day) or intermittent (12 hr/day) NiAc exposure. To assess treatment effects on insulin sensitivity, hyperinsulinemic-isoglycemic clamps were performed in obese Zucker rats. Additionally, the impact of the alternative dosing regimens on expression of selected adipose tissue genes involved in FFA mobilization were examined.

The intermittent drug holiday approach succeeded in retaining the ability of a therapeutically relevant NiAc exposure to induce FFA lowering. In lean rats, following intermittent (12 hr on/12 hr off) infusions for 5 days, the acute NiAc-induced FFA suppression was completely preserved. While in the obese rats there was a partial loss of FFA lowering efficacy, following 5 days intermittent dosing, importantly, this did not appear to be progressive with similar FFA lowering at 11 *vs.* 5 days. By contrast, continuous NiAc infusion for 5 days resulted in complete return of FFA to pre-treatment levels, consistent with previous findings (Oh *et al.,* 2011).

Markedly improved insulin sensitivity was seen in association with NiAc induced FFA lowering, either in NiAc naïve or previously intermittently dosed obese Zucker rats. Acute suppression of circulating FFA levels by NiAc in the obese animals was associated with reduced fasting hyperinsulinemia. Reduced insulin secretion might be explained by direct effects on the islets of either NiAc (Li *et al.,* 2011) or the fall in FFA level (Dobbins *et al.,* 1998). However, if the effect was only to decrease insulin secretion then levels of glycemia should have increased, which was not the case. Reduced fasting insulinemia in association with normoglycemia suggests instead that NiAc enhanced whole body insulin sensitivity. Indeed, this was confirmed by the elevated GIRs needed to maintain isoglycemia during the hyperinsulinemic clamps. In contrast to the intermittent group, continuous NiAc exposure did not enhance whole body insulin action.

Tissue lipid loading is determined both by acute circulating lipid levels (plasma FFA and TG) and the intracellular lipid stores. The ability of plasma FFA lowering alone, to significantly impact on total tissue fatty acid utilization has been clearly demonstrated by work showing that acute NiAc administration, in the fasting state, induces a major shift from whole body fat to carbohydrate oxidation, in association with the suppression of plasma FFA (Wang *et al.,* 2000). The intension was to reverse lipid overload, not just by reducing acute circulating FFA, but also by lowering tissue lipids via a reduction of net (24 hr average) FFA exposure. However, the AUC analysis suggests that the FFA rebound upon abrupt NiAc withdrawal, is of a magnitude sufficient to cancel the acute NiAc-induced FFA lowering in the intermittently dosed groups, despite the fact that this presumably only occurred once per day. Failure to lower net FFA exposure was also indicated by the lack of liver TG lowering. This may well explain why the enhancement in GIR was similar in the NiAc naïve and previously intermittently dosed groups.

Changes in adipose tissue gene expression of genes involved in liberating *vs.* storing FFAs, was not associated with the loss of FFA lowering during prolonged NiAc infusion. Previous studies in rats and mice have reported

substantial downregulation of PDE-3B, which the authors argued could be an important mechanism responsible for the return of FFA to pretreatment levels during prolonged NiAc exposure (Oh *et al.,* 2011; Heemskerk *et al.,* 2014). An explanation for the discrepancy between the present study and the previous work has not been found, although one possible cause might be that in the present study substantially lower NiAc doses were used, ~1/10th of those used in the earlier studies.

The rebound phenomena are different in lean vs. obese. To our knowledge, this is the first study to assess the detailed, time dependent response of plasma insulin and glucose in response to sudden NiAc withdrawal. In all cases where there was a FFA rebound, this was associated with an insulin rebound, with a particularly pronounced response in the obese NiAc naïve animals. This phenomenon may be the result of the well-known potentiation of glucose stimulated insulin secretion by long chain FFAs (Dobbins *et al.,* 1998). However, the surprisingly modest glycemic reductions in response to the insulin rebound, suggest that the complete explanation is likely more complex. Indeed, Vega *et al.* (Vega *et al.,* 2005) suggested that NiAc induced FFA lowering might trigger a counterregulatory response, perhaps to defend substrate supply, and NiAc has been shown to significantly alter levels of a number of hormones including glucagon and growth hormone (Quabbe HJ *et al.,* 1983). The mechanism of the loss of FFA lowering, in response to continuous NiAc exposure appears to be different in lean and obese animals. Thus in the lean animals, sudden withdrawal of NiAc after 5 days of uninterrupted exposure, induced a marked FFA rebound. This is consistent with the interpretation of Oh *et al.* (Oh *et al.,* 2011), that the return of FFA to pre-infusion levels represents the net effect of preserved NiAc action in the presence of an enhanced basal rate of lipolysis. By contrast, in obese Zuckers there was absolutely no evidence of FFA rebound indicating that lipolysis had become completely tolerant to NiAc *i.e.* complete tachyphylaxis.

Further refinements to NiAc dosing might achieve greater lipid lowering and insulin sensitization. To this end, fine-tuning of the infusion protocol and/or optimal timing of NiAc administration relative to food intake might mitigate these issue. Thus a programmed, more gradual decline in NiAc concentrations to terminate each infusion period might help to minimize the rebound. Alternatively, FFA rebound might be minimized if NiAc withdrawal is timed to occur in association with feeding/insulin administration. This might especially be the case if the proposal that FFA rebound involves a counter-regulatory response (Vega *et al.,* 2005) to defend substrate supply is correct.

In summary, the intermittent NiAc dosing strategy succeeded in retaining FFA lowering and improving insulin sensitivity in obese Zucker rats. While

these data suggest that FFA lowering is sufficient to improve insulin sensitivity, further refinements of the NiAc dosing regimen should be explored to more profoundly reverse lipid-overload induced insulin resistance.

6.4 Paper III

Further refinements to the NiAc dosing regimen were made in Paper III. To fully realize the potential of NiAc-induced antilipolysis on improving metabolic control, net FFA lowering must be achieved. To this end, the FFA rebound issue must be solved. The magnitude of the FFA rebound appears to be highly associated with NiAc's relatively short plasma half-life, thus if the washout period could be extended the FFA rebound issue might be mitigated (Ahlström *et al.,* 2013). Furthermore, timing of NiAc exposure with respect to feeding or fasting periods was not considered in Paper II. This is an important aspect since antilipolysis during fasting periods may invoke a more powerful counterregulatory response to defend the supply of the predominant oxidative fuel (Guyton *et al.,* 2015; Vega *et al.,* 2005). Hence NiAc exposure during feeding, which in rodents coincide with the nighttime period, might deliver most benefit to both glucose and FFA control. In Paper III these hypotheses were tested by comparing 5 days of fasting (daytime) *vs.* feeding (nighttime) NiAc exposure in obese Zucker rats. To entrain defined periods of feeding in these hyper-phagic animals, food was available during the nighttime periods only. In a pre-study (Study I, Paper III), the effects of abrupt *vs.* gradual NiAc withdrawal in either the basal fasted or glucose infused situation were compared. The rational was to assess which combination that delivered the minimal FFA rebound. This combination would then be deployed in the fasting *vs.* feeding study. Following the 5 day treatment period, metabolic control was assessed by an oral glucose tolerance test (OGTT) followed by tissue sampling to evaluate lipid unloading and potential effects on gene expression.

The timing and shape of the intermittent NiAc exposure profile have a major impact on metabolic control. Specifically, timing a well-defined NiAc exposure to feeding periods, terminated by an engineered gradual washout profile, results in marked improvements in the metabolic phenotype of the obese Zucker rat. These include plasma FFA and TG lowering, as well as, improved glycemic and lipid control. Perhaps most significantly, however, is the substantial peripheral tissue lipid unloading, implying a long term, fundamental improvement of metabolic control. Importantly, these effects are achieved without any measurable change in food intake or body weight (Fig. 3, Paper III). By contrast, these improvements are not observed when timing

NiAc exposure to fasting periods, with a 12 hr time-shifted but otherwise identical NiAc administration protocol.

Post-prandial NiAc exposure likely enhanced the FFA lowering during the treatment period, given the relative hyperinsulinemia associated with this state. Although the anti-lipolytic effects of both NiAc and insulin are mediated via adipocyte cAMP lowering, their combined effects are theoretically synergistic since NiAc, via GPR109A agonism, decreases cAMP formation (Tunaru *et al.*, 2003) while insulin increases cAMP breakdown, through enhanced phospho-diesterase-3B (PDE-3B) activity (DiPilato *et al.*, 2015). An insulin-NiAc synergy is not just a theoretical possibility, but was in fact seen in Paper II during the hyperinsulinemic clamps. The observed post-prandial FFA lowering is likely the primary mechanism driving the metabolic improvements resulting from dosing NiAc with feeding. Reduced FFA supply to the tissues should lower substrate competition with glucose (Wang *et al.*, 2000) and improve insulin sensitivity (Paper II) just when it is needed the most, *i.e.* during the influx of dietary carbohydrate in the post-prandial phase, resulting in reduced post-prandial hyperglycemia and hyperinsulinemia.

Evidence that NiAc timed to feeding succeeded in reducing 24 hr average FFA levels is provided by the tissue TG data. In the heart, TG stores parallel the FFA exposure during the period leading up to tissue collection, likely reflecting the quantitative dependence of this lipid pool on plasma FFA and its high turnover rate. Thus, the NiAc timed to feeding-induced reduction in FFA exposure during the OGTT is associated with a reduction in cardiac TG, in striking contrast to the opposite responses seen in the daytime dose group. This unloading of the much larger liver TG pool may result from both direct and indirect effects of the post-prandial FFA lowering. The latter from the longer term effects of repeated reductions in post-prandial hyperglycemia and hyper-insulinemia leading to reduced hepatic de novo lipogenesis (DNL). This path-way is heavily regulated via transcriptional control by glucose and insulin (Postic & Girard, 2008). Strong evidence for reduced hepatic DNL with NiAc timed to feeding is provided by the observed downregulation of the master regulatory genes, ChREBP and SREBP-1c, as well as four of their regulated genes directly involved in DNL, namely ACC1, FAS, Elovl6 and SCD1. The absence of a significant change in adipose DNL genes suggests that excess dietary glucose is partitioned away from the liver and into adipose tissue. Hepatic TG lowering might be an important driver of the observed reduction in post-prandial hypertriglyceridemia, which would also tend to reduce ectopic lipid accumulation and its negative consequences (Shulman, 2014).

In summary, the dosing regimen, in the context of a fixed daily dose, has a critical influence on whether or not NiAc improves metabolic control in the

obese Zucker rat. Specifically, Paper III demonstrate that timing a well-defined intermittent NiAc infusion protocol to feeding periods, is actually able to reverse ectopic lipid accumulation and improve lipid and glucose control. These data support the concept that antilipolysis, applied in conjunction with feeding, can be an effective means of reversing lipid overload induced insulin resistance and dyslipidemia.

6.5 Paper IV

A new model structure was implemented in order to capture the complete tolerance development following sustained NiAc exposures (observed in Paper II). The hypothesis was that a quantitative model built upon repeated dosing data more accurately would capture NiAc-induced FFA dynamics. NiAc has a major influence on insulin dynamics, with patterns resembling the FFA responses; acute reduction upon starting a NiAc infusion, tolerance development upon sustained NiAc exposures, with major rebound seen upon abrupt NiAc withdrawal (Paper II & III). Moreover, NiAc-induced FFA and insulin responses differed between lean and obese rats.

Given the potent endogenous antilipolytic effects of insulin (Arner *et al.*, 1981), and the large dynamic changes in insulin in response to NiAc, the model was expanded to include insulin dynamics as a co-driver of the FFA response, alongside NiAc kinetics. Linking FFA to insulin dynamics gives an opportunity to quantitatively determine insulin potency on FFA dynamics. The derived model can then be used as a tool to further optimize NiAc delivery to maximize FFA lowering and improve metabolic control.

The new model structure was needed in order to describe the rather unique transition of this system; from being fully sensitive to NiAc and insulin upon acute or intermittent dosing, into a system which at a later time point following sustained NiAc exposure, displays complete tolerance development. Interestingly, the tolerance development on response to continuous NiAc exposure appear to have different temporal aspects in lean *vs.* obese. Following 5 days of uninterrupted NiAc exposure, both lean and obese rats displayed a return of FFA to pretreatment levels, despite ongoing NiAc infusions. However, in lean rats a FFA rebound was seen upon NiAc cessation, consistently with a previous study (Oh *et al.*, 2011). In contrast, corresponding obese group did not produce a rebound upon NiAc cessation, indicating complete tolerance development. Thus, NiAc's efficacy to suppress FFA changes over time appears to have different temporal aspects in lean *vs.* obese. To derive a mathematical model able to capture these observations a hypothetical NiAc action compartment was incorporated into the model. In this compartment, NiAc's efficacy on

FFA/insulin is non-constant and allowed to change over time. This resulted in a model structure which was able to capture different degrees of NiAc efficacy with respect to time.

To become clinically meaningful, durable NiAc-induced FFA lowering must be established. Specifically, the daily net FFA exposure (24 h FFA exposure) must be reduced in order to limit the FFA being supplied to the liver. Thus, both tolerance and rebound need to be contained. Given the NiAc-induced FFA dynamics in the present data, clearly sustained NiAc exposures would not deliver this effect since a complete loss of efficacy occurs. In contrast, the intermittent dosing approach appears to contain tolerance development. However, at the end of the 12 h infusion, a major FFA rebound is seen, tending to cancel out the lowering achieved during the infusion periods. Whether there is an optimal length of exposure which would maximize the drug-induced effect while at the same time produce a minimal rebound, still needs to be explored. This is not a trivial task, not least due to the number of possible infusion profiles. To this end, the predictive power of a quantitative model, as the one presented here, can reduce the infinite number of imaginable infusion protocols down to a few plausible candidates.

Despite major changes in both insulin and FFA dynamics, plasma glucose concentrations remained remarkably stationary in the present study data (see paper II & Paper III, Glu- groups). Therefore, glucose was assumed to have minor influence on insulin and FFA dynamic seen in the present data. Hence, in order to reduce an already very complex system, an active decision was made to omit glucose dynamics in the present analysis. Given the remarkable impact of timing NiAc exposure to feeding *vs.* fasting (Paper III), future model generations will be extended to encompass glucose dynamics to further improve the predictive and translational value.

In summary, in the present study we developed a novel NiAc-FFA model, linking insulin dynamics to the release of FFA and quantitatively assessed insulin's potency and sensitivity on FFA dynamics. The current analysis is still in development and awaits the final FFA parameter estimates for obese rats, along with final FFA estimate uncertainties. Nonetheless, the model was found to have promising potential by displaying the ability to capture the FFA response time-courses following a variety of different NiAc infusion regimens, including long-term sustained NiAc exposures. Moreover, a different method for capturing tolerance was successfully incorporated into the pharmaco-dynamic models giving the model the flexibility required to capture complete tolerance development. It is anticipated that this model will be helpful to further optimize NiAc dosing regimens.

6.6 Design aspects for durable NiAc-induced antilipolysis

Therapeutically relevant NiAc exposures. The goal was to achieve a therapeutically relevant plasma NiAc concentration of ~1 µM, based on the concentration-response relationship for FFA lowering, delivering a close to maximal FFA suppression in substance naïve rats (Oakes *et al.,* 2013; Ahlstrom *et al.,* 2013). Data confirmed that this was the case. It is important to have a sufficient, but not excessive level of NiAc for two reasons. Firstly, loss of FFA lowering might theoretically be exacerbated by sustained supra-maximally effective levels of target engagement, *e.g.* by ligand-induced GPR109A desensitization and internalization (Li *et al.,* 2012). Secondly, excessive exposures can invoke additional complicating mechanisms beyond FFA lowering. Examples include: hepatic DGAT2 inhibition with an IC_{50} of 100 µM (Blond *et al.,* 2014; Ganji *et al.,* 2004); suppression of glucose mediated insulin secretion via direct activation of GPR109A in the islet observed at 100 µM (Li *et al.,* 2011; Chen *et al.,* 2015); downregulation of PDE-3B in adipose tissue reported at ~10-fold higher dose of NiAc than used in the current study (Oh *et al.,* 2011), but not occurring at the present dose level (Kroon *et al.,* 2015); and finally Toll-like receptor-4 signaling inhibition observed at 100 µM (Digby JE *et al.,* 2012).

Limiting tolerance. The standard pharmacological approach of engaging the target mechanism (GPR109A activation) for 24 hr/day fails in the case of NiAc, as prolonged exposure induces tolerance development, with FFA returning to pretreatment levels (Oh *et al.,* 2011). Therefore, intermittent dosing with 12 hr drug holidays were applied, resulting in containment, but not elimination of tolerance (Paper II). Thus, tolerance still developed when NiAc was applied in the fasting state; evident at the start of the experiment in the NiAc Day group (Paper III). Given the magnitude of tissue lipid unloading when NiAc was timed to feeding, extent of tolerance development was likely reduced in this group.

Minimizing FFA rebound. It was hypothesized that the FFA rebound is potentiated by a rapid decline of NiAc exposure towards the FFA lowering potency (~0.07 µM). Therefore (in Paper III), an infusion protocol terminated by a gradual withdrawal was implemented. This protocol prolonged the fall of plasma NiAc concentrations to 0.07 µM by ~2 hr *vs.* abrupt NiAc infusion termination. Indeed, FFA rebound was minimized, when combined with glucose loading. In contrast, when NiAc was gradually withdrawn under fasting conditions, FFA exposure was actually increased *vs.* saline control. These results suggest that timing NiAc withdrawal to the fed state will maximize FFA lowering. A caveat, however, is that withdrawal under conditions of glucose loading, worsens glycemic control (Paper III). To

minimize the impact of this effect, withdrawal was timed to occur late in the feeding period.

Timing NiAc administration to feeding. The potential for FFA lowering might in theory be greater in the fasting state. However, it has been suggested that antilipolysis in this situation (compared to the fed) may invoke a more powerful counterregulatory response to defend the supply of the predominant oxidative fuel (Guyton *et al.*, 2015; Vega *et al.*, 2005). Some evidence for this comes from a study in rats, where FFA was acutely lowered in the fasting state either by NiAc, an A_1 adenosine receptor agonist or insulin in combination with glucose infusion to maintain euglycemia (Oh *et al.*, 2012). Interestingly and in support of the above idea, increases of plasma adrenocorticotropic hormone (ACTH) and the lipolytic hormone corticosterone were attenuated with insulin/glucose (a situation somewhat akin to the fed state) compared to the other interventions. Another reason for applying NiAc during feeding is to exploit an insulin-NiAc synergy on antilipolysis. The clamp study results shows that insulin sensitization occurs when NiAc is present at sufficient levels for FFA lowering (Paper II). Based on the above factors, it is anticipated that NiAc application during the postprandial state would deliver greatest benefit to glucose and FFA control, which indeed is the case (Paper III).

6.7 Experimental paradigm – key experimental design features

Several features of the experimental design were key to being able to answer the principal questions of this work. *1) Properties of NiAc*. Chemical stability, high potency against GPR109A and high solubility allow infusion solutions of sufficient concentration to achieve target plasma NiAc exposures over the 5 day period, in spite of the small depot volume in the implanted pump. A very short plasma half-life (~2 min in the rat) provides the freedom to design and realize almost any desired NiAc exposure profile. *2) Programmable, implantable pump*. This device enables implementation of infusion protocols to reliably and precisely achieve the desired exposure profiles (shapes and timing) with a low degree of invasiveness. *3) Distinct feeding and fasting cycles*. A fundamental aspect to the design in Paper III was to time NiAc to either the fasting or feeding periods. To ensure that this occurred, well-defined feeding/fasting periods were entrained in the hyper-phagic obese Zucker rats, by restricting food availability to the 12 hr dark period only.

6.8 Clinical implications for the use of niacin

Could the present results have implications for the clinical use of niacin? Following the negative outcomes of two recent clinical trials (AIM-HIGH and HPS2-THRIVE (AIM-HIGH Investigators *et al.,* 2011; HPS2-THRIVE Collaborative Group, 2013; HPS2-THRIVE Collaborative Group *et al.,* 2014)) prescriptions have been declining (IMS Health Inc.,) and there is considerable debate about the value of niacin therapy (Guyton *et al.,* 2013; Toth *et al.,* 2015; Haynes & Rahimi, 2016). In addition, patient studies (with standard bedtime dosing) continue to report impairments in insulin sensitivity and/or glycemic control (Aye *et al.,* 2014; Blond *et al.,* 2014; Koh *et al.,* 2014; Hu *et al.,* 2015; Ooi *et al.,* 2015; El Khoury *et al.,* 2016) and even increased risk of new-onset diabetes (Goldie *et al.,* 2016). Is it possible that the standard dosing regimen with extended release niacin (the predominant formulation), including bedtime administration, plays a role in limiting clinical efficacy and inducing negative glycemic effects? Indeed, mealtime dosing has been suggested as an approach to augment the clinical efficacy of this drug (Guyton *et al.,* 2015; Usman MH *et al.,* 2012). Extended release niacin given immediately before an oral fat tolerance test suppressed post-prandial FFA and plasma TG excursions (Usman MH *et al.,* 2012). Consistent with the idea of mealtime over bedtime dosing, in Paper III, plasma TG lowering when treatment was timed to the OGTT and not when it was timed to fasting. Additionally, the results in Paper III suggest that mealtime dosing even has the potential to improve glycemic control.

It should be pointed out, that the specific exposure profile of NiAc used in our preclinical studies markedly differs from exposures achieved following oral niacin dosing in patients (Kesisoglou F *et al.,* 2014). An intermittent NiAc exposure profile equivalent to the ones used in the present studies, has to our knowledge not been applied in the clinic. This is important because different profiles may have different effects on average FFA levels. Several factors probably prevent present day ER formulations from reducing average FFA levels. 1) Dosing has not been designed to lower FFA, rather the goal has been to ameliorate dyslipidemia, via effects that may be independent of the FFA lowering mechanism (Lauring *et al.,* 2012). 2) While the ER formulation prolongs plasma NiAc exposure, maximum plasma NiAc levels are reduced compared to equivalent crystalline formulation doses (Kesisoglou F *et al.,* 2014), likely resulting in poor FFA suppression over a large fraction of the day (based on the relationship between circulating NiAc levels and FFA suppression, (Carlson *et al.,* 1968)). 3) In addition, clinical dosing is also associated with significant FFA rebound (Carlson & Oro, 1962; Lauring *et al.,* 2012). Therefore, simply giving currently available formulations at meal-time, may not reproduce the beneficial effects seen in Paper III. Nonetheless,

aside from timing, the data also indicate the importance of a prolonged drug washout to minimize the FFA rebound. In combination with mealtime dosing, prolonged drug washout might explain improvements in glucose control seen with acipimox (Vaag & Beck-Nielsen, 1992; Makimura *et al.,* 2015) but not niacin (Grundy *et al.,* 2002), given the longer plasma half-life of acipimox compared to niacin (Efthymiopoulos *et al.,* 1993; Gugler, 1978).

6.9 Implications for drug discovery

The results within this thesis have important general implications for the pharmacological approaches applied when assessing new modalities aimed to cure metabolic disease. The main project return deliveries, from a drug discovery perspective, are summarized in the bottom row of Table 1. In paper I, it was found that acute responses to NiAc differs substantially between lean and obese rats. Thus, in a preclinical drug discovery project, targeting a metabolic ailment, early screening of test compounds in a suitable disease animal model may be pivotal for sound conclusions and correct decision making. Although acute data is helpful and necessary to assess the proof of principle or target engagement, pharmacological intervention of metabolic pathways must include repeated dosing. When settling on level of exposures for repeated dosing in animals, it is often based on *in vitro* IC_{50}/EC_{50} data, with the aim of achieving a specific fold above this *in vitro* potency. However, very little, if any, attention is given to various durations of daily exposures. In fact, it is often implied that drug coverage at this specific exposure is needed 24 hr/day for meaningful effects. Thus, based on the particular pharmacokinetic profile of the test compound a dosing rate is then set to achieve level of target engagement, which often will involve multiple daily doses to ensure complete drug coverage.

Paper II demonstrates that an around-the-clock exposure approach completely fails in the case of NiAc to deliver durable FFA lowering, due to comprehensive tolerance development. In contrast, drug efficacy is retained when hitting the target mechanism with therapeutically meaningful exposures for 12 hr/day. Although some loss of efficacy occurred between NiAc naïve and intermittently dosed obese animals (day 1 *vs.* 5), importantly, this did not appear to be progressive, with similar FFA lowering achieved day 5 *vs.* 11. Thus, in the case of durable NiAc-induced FFA lowering, interspacing daily exposures by drug holidays is essential for maintained drug efficacy.

Paper III revealed that the details of the intermittent exposure profile (timing and shape) plays a critical role in metabolic outcomes. Thus, fine-tuning of the intermittent exposure profile (shape) was needed in order to tackle the rebound phenomenon. This demonstrates the importance of careful

characterization of pharmacodynamic effects during washout. Specifically, effects stemming from exposures equivalent to predicted drug potency since rebound phenomena are most likely to be revealed at this level of exposures. Finding an optimal plasma half-life of a compound should thus not only be regarded as a means of establishing target exposures with minimal number of needed doses. The plasma half-life may also have significant impact on sustainable net effects, especially if rebound phenomena are involved.

Metabolism is a tightly regulated processes and does not simply occur at random. Interfering with systems that are fundamental for organism survival, with millions of evolutionary years in development, is not trivial. Simply "plugging a leaking hole" often does not work in metabolism, especially not when trying to restrain or activate a metabolic pathway for 24 hr/day. The data from Paper III clearly demonstrate that also timing of target engagement with respect to normal physiological conditions, such as feeding, may be essential for improved metabolic control. Completely different responses occurred when a 12 hr time-shifted, but otherwise identical NiAc dosing regimen was applied in conjunction with feeding/fasting. Thus, attention should be given to timing of drug exposure and normal physiological conditions that might influence drug-induced effects.

Modulation of a metabolic processes will undoubtedly result in buildup of pathway specific precursors or metabolites. Given the stringent regulation in normal metabolism, this buildup will likely activate backup pathways, cause cellular harm or trigger endogenous counterregulatory processes. Thus, duration, extent and timing of the target engagement mechanism may be of equal importance and crucial for successful discovery of new modalities acting on metabolic disease.

6.10 Future perspectives

Although repeated dosing was used in the present studies, ideally the 5-day treatment paradigm should be extended, in order to assess maintained efficacy and metabolic improvements. Given the relatively small depot volume for the dosing solution in the implantable pump, extending present protocols requires refills. Indeed, the pumps has this feature, however, it requires a percutaneous needle penetration for access to the refill septum situated on the pump. Thus, whether this could be done in the conscious state or requires anesthesia would need to be assessed. Given that anesthesia potentially disturbs metabolism, weekly refills with this approach may prove detrimental for improved metabolic control. The pump is also limited by its battery life. Thus, in obese rats the intermittent protocol with gradual washouts would use up the battery

after ~5 weeks and dosing solutions would need to be refilled several times. Although experimentally challenging, it can be done. Furthermore, in the present studies, tolerance development was contained but not fully circumvented. Thus, whether the intermittent dosing approach could be further optimized still needs to be explored. To this end, quantitative pharmacological approaches, using modeling should be used to predict both optimal exposure levels and durations, which would maximize FFA lowering and potentially further improve metabolic control. This exercise was indeed our intention with the development of the model in Paper IV. However, in the interest of time this activity did not make it into this thesis.

Whether improvements in metabolic control, seen in the present studies are unique to NiAc needs to be explored. Furthermore, can the results be repeated in other preclinical disease models? For example, using other GPR109A agonists, with distinct molecular chemistry *e.g.* acipimox, could shed light on whether GPR109A agonism *per se* indeed produces similar effects. Another important question is whether antilipolysis via different targets, *e.g.* GPR81 or the A_1 adenosine receptor, would result in similar effects. Again, this would be experimentally challenging but possible if drug candidates with adequate solubility, potency and pharmacokinetic properties could be identified.

An intriguing and important question is whether similar effects could be achieved in humans. This would, however, not be a trivial, given the need for well controlled NiAc exposures and present available oral formulations. However, a short duration experiment using *i.v.* infusions would likely suffice as a proof of principle.

7 Conclusions

The simple feedback turnover model adequately describes acute FFA responses to NiAc exposure and washout in metabolically healthy and diseased states. In obese animals, the model captures reduced efficacy, pronounced tolerance development and diminished rebound compared to lean animals at equivalent NiAc exposures. Importantly, the model aided in designing a gradual NiAc infusion termination protocol to minimize FFA rebound. The improved model is required for longer term experiments, capturing the development of complete tolerance in response to sustained NiAc exposure. An important endogenous regulator of lipolysis is insulin, which undergoes large dynamic changes in response to NiAc. The improved model therefore also incorporates the influence of insulin on FFA dynamics. It is anticipated that this model will be helpful to further optimize NiAc dosing regimens.

A major finding is the substantial influence that the NiAc exposure profile has on metabolic control. The strategy of around-the-clock NiAc exposure fails to deliver durable FFA lowering due to tolerance development. By contrast, an intermittent NiAc dosing strategy succeeds in retaining FFA lowering and acute insulin sensitizing effects. However, this approach does not reverse lipid-overload. This is achieved through a synergy between pharmacology and physiology by timing NiAc exposure to feeding periods which reverses peripheral lipid accumulation and profoundly improves lipid and glucose control. This thesis supports the concept that antilipolysis, applied in conjunction with feeding, can be an effective means of reversing lipid overload-induced insulin resistance and dyslipidemia. Pharmacological principles for treating metabolic disease may require careful fine-tuning (timing and shape) of drug exposures.

In conclusion, a macro-pharmacologic approach can succeed in identifying a rational NiAc exposure that circumvents the problems of rebound and tolerance and profoundly improves metabolic control in a preclinical model of

the metabolic syndrome. The work shows the power of a multi-disciplinary drug discovery approach, using a comprehensive understanding of the relationship between pharmacokinetics and pharmacodynamics combined with knowledge of the physiologic regulation of metabolism.

Populärvetenskaplig sammanfattning

Sockersjuka (typ 2-diabetes) är en mycket alvarligt sjukdom som idag drabbar hundratals miljoner människor. Mycket tyder på att inlagring av fett i skelettmuskulatur och lever, istället för i fettväv, är en grundläggande orsak till varför insulin förlorar sin effekt (s.k. insulinresistens); en starkt bidragande faktor för att utveckla typ 2-diabetes. Hos sjuka individer läcker det fett ifrån fettväven i form av fria fettsyror, och bidrar till fettinlagringen i muskel och lever.

Nikotinsyra är ett läkemedel som ökar det goda och sänker det dåliga fettet i blodet och används för att minska risken för hjärtsjukdomar. På kort sikt kan nikotinsyra även sänka koncentrationen av fria fettsyror i blodet, genom att stänga av dess läckage från fettväven. För att uppnå en önskad effekt på längre sikt och på så vis kunna hjälpa patienter måste två problem lösas: om nikotinsyra ges oavbrutet tappar den sin effekt (toleransutveckling) och försvinner det ur kroppen för snabbt, sker en mycket hastig och kraftig ökning av fria fettsyror i blodet; ett fenomen som kallas för rebound.

I denna avhandling undersöks idén att en mer omfattande förståelse av förhållandet mellan farmakokinetik (hur blodhalter av läkemedel ändras över tid) och farmakodynamik (vad läkemedel har för effekter) i kombination med kunskap om den normala regleringen av ämnesomsättningen (metabolismen), kan användas för att motverka tolerans och rebound och möjliggöra nya effektiva läkemedelsbehandlingar. Arbetet utfördes i en tvärvetenskaplig utvecklingsmiljö vid AstraZeneca R&D Gothenburg. Metabola konsekvenser av nikotinsyra-inducerad fettsyresänkning bedömdes i en experimentell modell, den feta Zucker-råttan, som har en rubbad ämnesomsättning mycket lik den hos typ 2-diabetiker. En högteknologisk programmerbar mini-pump var kirurgiskt inopererad och möjliggjorde för skräddarsydda doseringsprofiler och fullständig kontroll över blodkoncentrationen av nikotinsyra. Genom att lägga in resultaten i matematiska modeller kunde effekter från olika typer av doseringar förutsägas och användas för att förbättra doseringsprofilen. En

enkel modell fungerade bra för korta behandlings perioder medan en mer komplex modell behövdes för långvarig behandling.

Detta avhandlingsarbete visar på det stora inflytande som doseringsprofilen av nikotinsyra har på metabol kontroll. När koncentrationen av nikotinsyra hölls konstant, dygnet runt, skedde en fullständig återgång av fettsyrorna till ursprungsnivån, på grund av toleransutveckling. Vid en intermittent doseringsstrategi, när nikotinsyra tillfördes 12 timmar per dag, kvarstod en fettsyresänkande effekt, samt en förbättrad insulinkänslighet. För att uppnå en bestående effekt, kraftfull nog för att minska fettinlagring i muskel och lever, krävdes en samverkan mellan farmakologi och fysiologi. Genom att låta ett optimerat doseringsprotokoll sammanfalla med födointag, istället för fasta, upptäcktes en kraftigt reducerad fettinlagring i lever, samt en markant förbättrad metabol kontroll. Denna avhandling stödjer grundtanken om att farmakologiskt inducerad fettsyresänkning i samband med föda, kan vara ett effektivt sätt att reducera fettinlagring i vävnader med en ökad effekt av insulin och förbättrad ämnesomsättning.

Sammanfattningsvis har vi, i en djurexperimentell modell av metabol sjukdom, identifierat en doseringsprofil för nikotinsyra, och när det bör ges, som motverkar problemen med toleransutveckling och rebound och avsevärt förbättrar ämnesomsättningen. Arbetet visar på kraften hos ett tvärvetenskapligt tillvägagångssätt i en läkemedelsutvecklingsmiljö applicerat på en omfattande förståelse kring förhållandet mellan farmakokinetik och farmakodynamik i kombination med kunskap om den fysiologiska regleringen av ämnesomsättningen.

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Acknowledgements

The work presented in this thesis was collaboratively funded by AstraZeneca R&D Gothenburg and the Swedish University of Agricultural Sciences (SLU), Uppsala. I would like to acknowledge the Department of Biomedical Sciences and Veterinary Public Health, Division of Pharmacology and Toxicology at SLU as well as the CVMD iMed Bioscience Diabetes and DMPK Departments at AstraZeneca R&D Gothenburg, for supporting this project.

I wish to express my sincere gratitude to everyone who has in one way or another contributed to this thesis. Especially, I would like to thank:

Prof. Johan Gabrielsson and Dr. Nicholas D. Oakes, my supervisors, for your encouragement and guidance, for all the great science discussions and for your enthusiasm whenever new results were produced.

Johan, for sharing your PK/PD knowledge, for initiating and supporting this project and for tirelessly pushing me forward.

Nick, for taking me in as a part of your team and for sharing your *in vivo* pharmacology knowledge, for always showing me support and for opening my eyes to the field of metabolism.

Pia Thalén and Ann Kjellstedt, both of you have truly been the greatest teachers. Thank you for sharing your profound knowledge in the field of advanced experimental animal work. Our discussions in "the green couch" were always very fruitful.

Kristina Wallenius, for always given me great encouragement, sharing your knowledge, taking your time to review manuscripts and for always lending a hand in the lab.

Christine Ahlström, for introducing me to AZ in the first place, for your continued support and for letting me in on your *in vivo* experiments; I'm hooked!

Robert Andersson, for the collaboration with Paper IV.

Jeremie Boucher, for sharing your knowledge and taking your time to thoroughly review and give input on my manuscripts.

Bader Zarrouki, for always taking an interest in new results and given me great encouragement and sharing your knowledge.

Stefan Hallén, for always taking an interest in my data on antilipolysis.

Arne Olsén, for teaching me how to run qRT-PCR and for a great collaboration and the swift gene expression analysis in Paper III.

Anne-Cristine Carlson, Lena Amrot Fors and Anna Lindblom for your help and support when I struggled with the Pentra analyses.

The LAS team at AZ: Lise, Weronica, Kajsa, Fermen, Annica, Marita and everyone else, for your outstanding support with the daily care of the animals.

Tania Baccega, for a great job and help during the surgeries and experiments of Paper III.

Therese Hagstedt, for sharing your great surgical skills.

Daniel Karlsson, for always being close to laughter and for lending a hand in the lab.

My brother and sister in law PO and Mia, with families, for believing in me and for your friendship.

Pappa, for always believing in me and giving me support and for always taking an interest in my work.

My sister Jennie with family for your support, for believing in me and for your friendship.

Mamma and Tommy, for always giving me encouragement and taking great interest in my work, for your unconditional help whenever I have asked. Truly, without your support and love this work would not have been possible.

Clara and Edith, my two nuggets of gold, Daddy is almost done!

Helene, my love, for being the best mother our children ever could have, for always believing in me, and for the freedom to pursue this path. Love you!