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FACTORS POTENTIALLY INFLUENCING PATHOGENETIC MECHANISMS AND HYPERGLYCEMIA IN PRE-DIABETES AND TYPE 2 DIABETES

- CLINICAL STUDIES IN HUMANS

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Factors potentially influencing pathogenetic mechanisms and hyperglycemia in pre-diabetes and type 2 diabetes - clinical studies in humans
THESIS FOR DOCTORAL DEGREE (Ph.D.)

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ABSTRACT

The pathogenetic mechanisms underlying type 2 diabetes (T2D) and prediabetes involve an interaction between β-cell dysfunction and insulin resistance (IR). The resulting hyperglycemia, as well as other clustered cardiovascular (CV) risk factors in T2D, constitutes a severe hazard for development of complications to the disease. To optimally treat these risk factors, it is vital to antagonize the mechanisms of the metabolic disorder. This thesis presents results from studies aiming to understand the mechanisms and effects of some interesting modes of intervention in subjects with T2D, prediabetes and IR.

Study I: The effects of exercise training for twelve weeks, with or without the addition of the α-glucosidase inhibitor acarbose, were examined in 48 subjects with T2D and moderate hyperglycemia. Exercise training augmented insulin sensitivity, and improved body composition and blood pressure, but glycemic control was unchanged. When exercise and acarbose were combined, glycemic control was significantly improved, in addition to similar benefits as with exercise alone. Moreover, the overall CV risk factor profile was probably improved with the combination therapy, suggesting it to be an interesting treatment alternative.

Study II: The associations between changes in mRNA expression in skeletal muscle of selected key genes, involved in muscle adaptation to exercise, and individual response to physical training were assessed in 19 individuals from study I. The expression of vascular endothelial growth factor (VEGF) was associated with change in insulin sensitivity and glycemic control. This could constitute a mechanism that contributes to the known variation in the individual adaptation to exercise.

Study III: The impact of dual endothelin-1 (ET-1) receptor blockade infusion was investigated in eleven males with IR. The study showed that the dual blockade increased glucose uptake in skeletal muscle, both in the basal and the insulin-stimulated state. The finding supports that endogenous ET-1 is important in regulating muscle glucose uptake in IR. Moreover, in vitro studies in cultured skeletal muscle cells demonstrated that ET-1 inhibits glucose uptake by a receptor dependent mechanism, indicating a direct impact on muscle cells by ET-1.

Study IV: Intervention with high-dose vitamin D_3 treatment for eight weeks was studied in 43 individuals with prediabetes or drug-naïve T2D, especially with respect to change in β-cell function. No significant effect was seen in first-phase insulin secretion, nor could we detect any effects on second-phase insulin secretion, IR or glycemic control. The study gives no support for treatment with vitamin D in subjects with abnormal glucose homeostasis.

In conclusion, combined treatment with exercise and acarbose proved superior to exercise alone. Further, a favorable response to physical training could involve increase in VEGF. In IR, ET-1 seems to be directly involved in muscle glucose uptake. And finally, we found no effect of vitamin D treatment on insulin secretion or IR in prediabetes and mild T2D.

POPULÄRVETENSKAPLIG SAMMANFATTNING PÅ SVENSKA

Mekanismerna bakom typ 2 diabetes (T2D) och förstadier till sjukdomen består av en kombination av minskad förmåga till insulinproduktion från bukspottskörteln och motstånd mot insulinets effekter i kroppen, så kallad insulinresistens. Detta orsakar förhöjt blodsocker, som tillsammans med andra riskfaktorer som är vanliga vid T2D, ger en påtagligt ökad risk för hjärtkärlsjukdom (t.ex. hjärtinfarkt och stroke). För att på bästa sett kunna behandla denna risk är det viktig att motverka mekanismerna bakom sjukdomen. Den här avhandlingen består av ett antal studier där jag har försökt förstå mekanismer och effekter av några olika intressanta behandlingar. Jag har undersökt dessa på personer med T2D, förstadier till diabetes samt hos dem med insulinresistens.

Studie I: Jag studerade effekten av fysisk träning, med eller utan tillägg av en medicin som fördröjer upptaget av socker från tarmen (akarbos). Träning ensamt minskade insulinresistens, kroppsfett och blodtryck, men inte blodsockret. När träning kombinerades med akarbos såg jag samma positiva effekter, men även en sänkning av blodsockret. Troligen så blev flera andra riskfaktorer också bättre med den kombinerade behandlingen, som därför verkar vara ett intressant sätt att behandla T2D.

Studie II: Olika personer får olika bra effekt av träning. Jag undersökte om det kunde bero av hur vissa gener kopplas på i musklerna. Jag fann att genen för en faktor som är viktig för att bilda nya kärl (VEGF) verkade vara viktig för om träningen gav mindre insulinresistens och bättre långtidssocker.

Studie III: Endothelin-1 (ET-1) är ett kroppseget ämne som framförallt drar ihop kärl. Genom att blockera effekten av endothelin-1 kunde vi se att upptaget av blodsocker i muskler ökade. I odlade muskelceller minskade också ET-1 sockerupptaget, vilket tyder på att ET-1 har en direkt effekt på cellerna.

Studie IV: Det finns en del forskning som har talat för att D-vitamin skulle kunna påverka blodsockret. Jag lottade personer till en hög dos D-vitamin eller placebo (overksam medicin). Undersökningen kunde inte visa att D-vitamin hade någon effekt på insulinproduktion, IR eller blodsockerkontroll.

Sammanfattningsvis så var kombinationsbehandling med träning och akarbos bättre än träning ensamt. Att få bra effekt av träning kan till viss del bero av faktorn VEGF i musklerna. ET-1 verkar ha en direkt effekt på blodsockerupptaget i muskelceller. Och slutligen så kunde jag inte se några positiva effekter av D-vitamin på mekanismer bakom blodsockerbalansen.

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IV. **Wagner H**, Alvarsson M, Mannheimer B, Degerblad M, Östenson CG.
No Effect of High-Dose Vitamin D Treatment on β-Cell Function, Insulin Sensitivity, or Glucose Homeostasis in Subjects With Abnormal Glucose Tolerance: A Randomized Clinical Trial. *Diabetes Care. 2016;39(3):345-52.*

CONTENTS

LIST OF ABBREVIATIONS

$1,25(OH)_2D$	1,25-dihydroxy-vitamin D_3
25(OH)D	25-hydroxy-vitamin D_3
ADA	American Diabetes Association
ANOVA	Analysis of variance
Apo	Apolipoprotein
BMI	Body mass index
BP	Blood pressure
CoV	Coefficient of variation
CT	Computerized tomography
CV	Cardiovascular
CVD	Cardiovascular disease
DI	Disposition index
DPP-4	Dipeptidyl peptidase-4
DR	Diabetic retinopathy
DXA	Dual-energy X-ray absorptiometry
ET-1	Endothelin-1
ET_A	Endothelin receptor A
ET_B	Endothelin receptor B
FBF	Forearm blood flow
FGU	Forearm glucose uptake
FHD	Family history of diabetes
HDL	High-density lipoprotein
HOMA	Homeostasis model of assessment
IFG	Impaired fasting glucose
IGT	Impaired glucose tolerance
IR	Insulin resistance

IRS1	Insulin receptor substrate 1
IVGTT	Intravenous glucose tolerance test
GIR	Glucose infusion rate
GLP-1	Glucagon-like petide-1
HbA1c	Glycated hemoglobin
LDL	Low-density lipoprotein
MAP-kinase	Mitogen-activated protein kinase
M/I	Insulin sensitivity index
NO	Nitric oxide
OAD	Oral antidiabetic drug
OGIS	Oral glucose insulin sensitivity
OGTT	Oral glucose tolerance test
PI3-kinase	Phosphatidylinositol-4,5-bisphosphate 3-kinase
PGC-1α	Peroxisome proliferator-activated receptor-gamma coactivator 1α
PTH	Parathyroid hormone
QUICKI	Quantitative insulin sensitivity check index
SDPP	Stockholm Diabetes Prevention Study
SEM	Standard error of the mean
SGLT2	Sodium-glucose cotransporter-2
T2D	Type 2 diabetes
VEGF	Vascular endothelial growth factor
VO_{2max}	Maximal oxygen uptake
WHO	World Health Organization

1 INTRODUCTION

1.1 TYPE 2 DIABETES AND PRE-DIABETES

Type 2 diabetes mellitus (T2D) is a state of hyperglycemia which, in combination with associated cardiovascular risk factors, constitutes a severe hazard of developing several health complications. It is by far the most common variant of diabetes, accounting for almost 90% of all diabetes in the world [1]. Both genetic and environmental factors are known to contribute to the development of the disease. Among environmental factors, obesity is considered the most important. In addition, physical inactivity, poor diet, tobacco and work stress can be mentioned among other independent risk factors [2-5]. The combination of heredity for T2D and obesity could be especially unfavorable, at least in men [6, 7].

Since hyperglycemia evolves gradually, and the fact that glucose levels just below the cut-off levels for T2D also are associated with increased risk of cardiovascular disease (CVD) and of developing manifest T2D [8, 9], pre-diabetic categories have been created. Impaired glucose tolerance (IGT) was acknowledged by the World Health Organization (WHO) already in 1985, but impaired fasting glucose (IFG) much later. If IFG and IGT co-exist, the risks of CVD and manifest diabetes are even further increased [8].

1.1.1 Definitions

The definitions and diagnosis of diabetes mellitus and pre-diabetes are based on plasma glucose levels. The cut-off levels have been lowered over time, in the aspiration of an earlier diagnosis to prevent complications. The current levels were set in 1999 [10]. Fasting plasma glucose levels ≥7.0 mmol/l and/or ≥11.1 mmol/l in the two-hour sample during an oral glucose tolerance test (OGTT) are the diagnostic cut-off levels for diabetes. Glycated hemoglobin (HbA1c) can also be used as a diagnostic tool according to new guidelines [11]. IFG is present if the fasting glucose level is 6.1-6.9 mmol/l. IGT is defined by a two-hour plasma glucose level of 7.8-11.0 mmol/l during an OGTT.

1.1.2 Pathogenesis

The pathogenesis of T2D is founded on two mechanisms:

- B-cell dysfunction or the insufficient capability of the β-cells in the pancreas to release sufficient amounts of insulin to maintain normoglycemia.
- Insulin resistance (IR) that infers a diminished ability of insulin to exert its effects on different target cells, including in the liver.

When there is a reduction in the secretion and action of insulin, the result will be a decreased glucose uptake in peripheral tissues, as well as an increased glucose output from the liver and dyslipidemia [12]. The two mechanisms contribute in different extents to hyperglycemia at the individual level. Further, in men with normal fasting glucose, both acute insulin response and IR interacted on the risk of acquiring manifest T2D 10-20 years later [13]. Moreover,

hyperglycemia and hyperlipidemia are hypothesized to per se further deteriorate β-cell function and cell responsiveness to insulin, referred as 'glucolipotoxicity' [14, 15].

1.1.2.1 B-cell function

A primary event in the disease is the reduced ability of the β-cells in the pancreas to produce and release sufficient amounts of insulin to maintain normoglycemia. When the insulin levels reached no longer can compensate for the increased demand due to IR, hyperglycemia will evolve. The first-phase insulin response seems to be most important in this respect [15]. The ability to secrete insulin decreases gradually during the course of the disease, exacerbating the hyperglycemic state [16]. Gene variants known to be associated with T2D are in a majority linked to β-cell dysfunction [17].

1.1.2.2 Insulin resistance

The other major pathogenetic element in T2D is the diminished ability of insulin to exert its effects on different cell types in the human body. Genetic susceptibility in the combination with several environmental factors contribute to IR [12, 14]. The environmental factors are largely in common with risk factors for T2D, like abdominal and truncal obesity [18] and physical inactivity [19]. Further, there is evidence that IR escalates over time [20]. On a general level, fuel overload and decreased energy turnover have been proposed as concepts [14, 21]. Several mechanisms have been found to be potentially important, of which some will be discussed below.

1.1.2.2.1 Skeletal muscle insulin resistance

Skeletal muscle accounts for approximately 85% of the insulin-mediated glucose uptake in the human body [22] and thus constitutes the most important tissue when IR is studied. As to mechanisms, especially accumulation of lipid derivatives, defects in insulin signaling, as well as impaired blood flow in muscle are thought to contribute. Another important mechanism involves mitochondrial function and defects in oxidative phosphorylation [23], which will be discussed in the exercise section (1.2.3). Moreover, fat tissue derived adipokines and cytokines, such as leptin, adiponectin, interleukin-6 and tumor necrosis factor-α has attracted much interest in this field [14], but will not be discussed in the scoop of this thesis.

1.1.2.2.1.1 Lipid accumulation

Skeletal muscle lipid content is associated to insulin resistance [24]. But muscle fat content is also high in well trained athletes, which have a high insulin sensitivity. This finding is referred to as 'the athlete paradox', and may point out that energy turnover is of greater importance than fat content [21]. Another related theory implicates that incomplete fat oxidation in the mitochondria results in mitochondrial dysfunction and subsequent IR, which was shown in a rodent model overfed with fat. Interestingly, exercise could reverse these metabolic defects [25]. Finally, several candidate pathways by which abnormalities in lipid metabolism could interfere with insulin signaling has been proposed [14].

1.1.2.2.1.2 INSULIN SIGNALING

The mechanisms of insulin signaling are complex. Insulin exerts its effects by binding to the insulin receptor. Simplified, this activates two major pathways: 1) Activation of the phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3-kinase) pathway, leading to increased translocation of the glucose transporter GLUT4 to the cell surface and increased glycogen synthesis, and 2) Activation of the mitogen-activated protein kinase (MAP-kinase) pathway, stimulating cell growth [26]. GLUT4 is recruited from intracellular storage vesicles to the plasma membrane. The molecular steps from the binding of insulin to the insulin receptor and the result of GLUT4 translocation have been extensively investigated, in the context of IR. Interference with signaling at three different levels have mostly been considered: 1) reduction in insulin receptor kinase, 2) decreased phosphorylation of insulin receptor substrate 1 (IRS1), and 3) reduced activation of PI3-kinase [12]. Especially, interest has been focused on different defects at the IRS1 level in the PI3-kinase pathway, but there are data suggesting that defects independent of IRS1 are of importance [27, 28].

1.1.2.2.1.3 BLOOD FLOW

Impaired blood flow is another corner stone in the pathogenesis of IR. If blood flow is reduced, the delivery of insulin and glucose to the target cells will be lower. Accordingly, there is an association between capillary density in human skeletal muscle and insulin sensitivity, measured by euglycemic clamp in non-diabetic subjects [29]. Capillary density is also associated with glucose tolerance [30, 31]. This suggests that the diffusion distance between the capillaries and muscle cells is of importance.

However, the contribution of diminished blood flow to IR has been difficult to estimate and has therefore been questioned [32]. One difficulty is that insulin per se has vasoactive properties and augments blood flow [33]. This is caused by a vasodilatory ability, that is nitric oxide (NO) dependent [34], and also by capillary recruitment [35]. In healthy men, ~ 20-30% of the insulin-mediated glucose uptake has been estimated to depend on the vasodilation induced by insulin [36]. Additionally, both vasodilation and capillary recruitment by insulin are probably impaired in T2D [37]. But there are conflicting results in studies. For example, local hyperinsulinemia in healthy and hypertensive individuals did not increase forearm blood flow, but augmented endothelium-dependent vasodilation (Taddei, Virdis et al. 1995). In healthy individuals, systemic but not local hyperinsulinemia raised forearm blood flow, suggesting that the vasodilatory property of insulin is not only mediated by direct action (Cardillo, Kilcoyne et al. 1998).

1.1.2.2.2 Hepatic insulin resistance

An impaired hepatic ability to react to insulin, i.e. hepatic insulin resistance, will result in an increased endogenous glucose production by the liver. This was showed to be the most important factor determining the glucose excursion during an OGTT, in middle-aged men

with different levels of glucose tolerance [38]. As to mechanisms, accumulation of lipid metabolites contributes to the hepatic IR, as in muscle tissue. A main regulator is malonyl coenzyme A, which by inhibition of carnitine palmitoyltransferase 1 decrease the import of fatty acids into the mitochondria [39]. In turn, this can lead to development of non-alcohol hepatic steatosis, which notably can be reversed in T2D subjects by a low-fat diet and weight reduction [40].

1.1.3 Cardiovascular risk factors

In addition to hyperglycemia, other cardiovascular risk factors such as hypertension, dyslipidemia and obesity are abundantly present in T2D. IR has long been regarded as the major underlying component of this cluster of risk factors [41]. For example, in Sweden the prevalence of pharmacologically treated hypertension in T2D subjects in primary care is 75% according to data from the National Diabetes Registry in Sweden. For dyslipidemia, the figure is 60% [42]. The constellation of these different elements have been considered as part of a syndrome, first named s*yndrome X* by Reaven [43]. Later, the terms *insulin resistance syndrome* or *metabolic syndrome* became predominant. Different components have been proposed to be included in the syndrome by different organizations. However, the major components consist of hyperglycemia, abdominal obesity, hypertension and dyslipidemia – all independent risk factors of CVD. But the expression *syndrome* has been questioned by major opinion leaders in the field [44]. Additionally, low cardiorespiratory fitness, measured as maximal oxygen uptake (VO_{2max}), is often present in T2D and is likewise an independent risk factor for CVD [45-47].

1.1.4 Complications

Besides the complications of acute severe hyperglycemia, the major concern in T2D has been the late complications that often develop after several years with the disease. The major complications can be divided into:

- Microangiopathy
    - Retinopathy
    - Nephropathy
    - Neuropathy
- Macroangiopathy
    - Ischemic heart disease
    - Cerebrovascular disease
    - Peripheral artery disease
- Neuropathy
    - Peripheral polyneuropathy
    - Autonomic neuropathy
    - Mono- or multifocal neuropathy
    - Compression neuropathy

Hyperglycemia induced oxidative stress in the endothelium is thought to be one of the main components, driving the development of these complications [48].

1.1.5 Burden of the disease

422 million people in the world were calculated to have diabetes in 2014 according to WHO [49]. The prevalence has doubled in the adult population since 1980, from 4,7% to 8,5% in 2014. It is difficult to assess the proportion of type 1 and type 2 diabetes in many international registries but the overall increase is founded on an increased prevalence of risk factors for T2D, such as overweight and physical inactivity. In recent years, the increase in diabetes prevalence has been greater in low and middle-income countries compared to high-income countries. In Sweden, the total prevalence of diabetes in 2012 was 4.7%, according to a recent nation-wide register-based study. During the years 2005 to 2013, a modest increase in prevalence and a simultaneous decrease in incidence was demonstrated [50].

The impact of T2D on morbidity and mortality is huge. The risk of different vascular diseases, including coronary heart disease, ischemic and hemorrhagic stroke and vascular death, is approximately doubled in diabetes patients [51]. These increased hazards are independent of other cardiovascular risk factors. In patients reaching end-stage renal disease, diabetes is regarded the primary cause in 12 to 66% in world-wide comparisons [52]. Proliferative diabetic retinopathy (DR), which is potentially vision-threatening, is prevalent in 7% of diabetes patients [53]. Further, rates of lower extremity amputations are 10-20 fold higher in populations with diabetes, in contrast to populations without [54]. Promisingly, the frequency of these complications are declining in several instances [49]. On the other hand, a recent meta-analysis showed that blindness and visual impairment due to DR had risen. In 2010, DR accounted for 2.6% of all blindness and 1.9% of all visual impairment worldwide [55].

1.1.6 Treatment

1.1.6.1 Target levels

The aim of the treatment in T2D is to lower, or if possible normalize, glucose levels. But the target level of HbA1c should be individualized, taking into account risk of hypoglycemia, disease duration, life expectancy, comorbidities, patient attitudes and resources etc. [56]. Moreover, other risk factors of CVD as obesity, physical inactivity, hypertension and dyslipidemia should be addressed. The target levels for these components have changed over time and are under constant evaluation and debate. The following treatment target levels have been proposed in Swedish National Guidelines from the National Board of Health and Welfare (Socialstyrelsen) and the American Diabetes Association in 2015 [57, 58] (**Table 1**).

	Socialstyrelsen 2015	ADA 2015
HbA1c	< 52 mmol/mol	< 53 mmol/mol
Blood pressure	< 140/85	< 140/90
LDL-cholesterol	Not used as target, treatment according to estimated total CVD risk	Not used as target, treatment according to estimated total CVD risk
Urine albumin/creatinine ratio	No recommendation	< 3.4 mg/mmol
Other	Smoke stop	Exercise > 150 min/week

Table 1. Treatment targets in diabetes proposed by the Swedish National Board of Health and Welfare (Socialstyrelsen) and the American Diabetes Association (ADA). Both organizations emphasize individualization of the target levels. LDL: low-density lipoprotein.

1.1.6.2 Lifestyle

Targeting lifestyle factors, such as improved diet and increased physical activity is the foundation of the treatment in T2D and is advocated in guidelines [59]. A Cochrane meta-analysis found that exercise alone lowered HbA1c by about 7 mmol/mol. Positive effects on visceral adipose tissue and triglycerides in blood were also detected [60]. Studies on diets are difficult to interpret due to the vast number of different diets used. For weight-loss, diets of low-carbohydrate, low-fat and calorie-restricted, as well as Mediterranean diets have proved efficacy up to two years [59].

More appropriate in a clinical perspective could be to evaluate the effects of combined lifestyle modification, including diet, physical activity and education. A meta-analysis on this subject, including16 studies, demonstrated significant benefits on HbA1c, body mass index (BMI) and blood pressure, but not on cholesterol levels [61].

In prevention, lifestyle intervention with diet and physical exercise has been shown to decrease the risk of progression from IGT to manifest diabetes [62, 63]. In two later studies, the relative progression rate could be reduced by 58% [64, 65], which has been evaluated as highly cost-effective in a society perspective [66]. Notably, the preventive effect was lower (28%) in an Indian study [67]. Moreover, it could be discussed if this reflects a true preventive effect or if the disease is masked due to an effective lifestyle treatment.

In the Look AHEAD trial, a great effort was made to evaluate if lifestyle modification could have an impact on cardiovascular morbidity and mortality in T2D. More than 5000 T2D subjects were randomized to intensive lifestyle modification, with the goal of a weight

reduction of > 7% and increased physical activity, or to a control group receiving basic lifestyle education. However, the study was terminated in advance after a mean follow-up of 9.6 years, since the probability of a significant group difference within the planned study-length of 13.5 years was less than 1% [68]. This was in spite of that the intensive lifestyle-modification group had a greater weight loss, fitness increase and a higher rate of remission of their diabetes than the control group after 4 years [69]. The study results were greatly discussed, especially concerning if the study was under-powered, since the reduction in weight and waist was moderate (6 % and 2 cm, respectively), and the fact that more intensive pharmacological treatment became clinical routine during the study period.

1.1.6.3 Pharmacological

1.1.6.3.1 Oral anti-diabetic agents (OADs)

Metformin is recommended in most guidelines as the first-line pharmacological treatment in T2D [56]. This is chiefly founded on the cardiovascular protective effect seen in a sub-group in the United Kingdom Prospective Diabetes Study (UKPDS) [70] Metformin has also been shown to decrease the relative risk of developing T2D in IGT subjects by 26-31% [65, 67]. Interestingly, one of the mechanisms by which metformin have its effects is by stimulating AMP-kinase [71].

Other OADs include sulfonylureas, glinides, acarbose, thiazolidinediones, dipeptidyl peptidase-4 (DPP-4) inhibitors and sodium-glucose cotransporter-2 (SGLT2) inhibitors. Special interest has lately been shown in the SGLT2 inhibitors, since one compound, empagliflozin, recently showed a pronounced cardio-protective effect [72].

1.1.6.3.1.1 ACARBOSE

Acarbose is an α-glucosidase inhibitor that decreases post-prandial hyperglycemia by delaying carbohydrate absorption from the small intestine [73]. It lowers HbA1c in mean by 9 mmol/l according to a Cochrane meta-analysis, without significant impact on body weight or serum lipids [74].
Further, in a meta-analysis of trials on acarbose treatment in T2D, acarbose was associated with a 35% reduction of CVD [75]. The evaluated outcomes were collected from adverse events registered in each study, and thus the studies included were in fact not designed for evaluation of cardiovascular (CV) events. Notably, acarbose treatment significantly improved several CV risk factors such as glycemic control, triglyceride levels, BMI and blood pressure. In a secondary analysis of the STOP-NIDDM trial, acarbose treatment was associated with a significant absolute risk reduction in cardiovascular events by 2.5% in a IGT population [76]. The trial was criticized for using a “modified” intention-to-treat group for the analysis, excluding subjects who discontinued very early [77, 78]. It has been proposed that the mechanism behind the benefits of acarbose on CV risks could be lowering of oxidative stress, secondary to the decrease of postprandial glucose by the compound [79]. Moreover, acarbose was also shown to decrease the progression from IGT to T2D in the STOP-NIDDM study [80]. However, the effect was smaller than in lifestyle intervention

trials. Further, when all study subjects were put on placebo at the end of the study and re-examined three months later, the progression to diabetes was rather high in the formerly acarbose-treated group.

1.1.6.3.2 Insulin

Insulin has long been an effective treatment option in T2D. Insulin is often necessary in long-standing disease when β-cell function has declined considerably. Different insulin formulations and adjustments to the insulin molecule have provided several variants for treatment with either short or long-acting properties.

1.1.6.3.3 Glucagon-like petide-1 (GLP-1) analogues

A fairly new treatment option is founded on the incretin hormone GLP-1. As opposed to the DPP-4 inhibitors, that achieve an increase in endogenous produced GLP-1, these agents create higher plasma levels by exogenous administration of modified variants of the hormone. They are at present made for subcutaneous injection only. Interestingly, some of these compounds have been attributed substantial cardio-protective effects in recent trials [81, 82].

1.1.6.4 Multifactorial treatment

Given the high frequency of several CV factors in T2D, the importance of multifactorial treatment is recommended by most organizations. The impact of such a strategy has been evaluated, establishing a great advantage in risk reduction in diabetes-related complications. In the Steno-2 trial, T2D subjects with microalbuminuria were intensively treated for tight glucose regulation, as well as optimal control of other CV risk factors. Compared to conventional treatment, the intensive multifactorial approach for 7.8 years significantly decreased the risk of cardiovascular disease (hazard ratio 0.47) and microvascular outcomes [83]. Observational data for an additional 13 years showed sustained benefits, as well as lower rates of total and cardiovascular mortality. A gain of nearly eight years in lifespan could be demonstrated [84].

1.2 PHYSICAL EXERCISE IN TYPE 2 DIABETES AND AT-RISK POPULATIONS

As mentioned before, physical activity has long been emphasized as part of the lifestyle treatment in T2D, and also as an element in preventing the disease in high risk subjects.

1.2.1 Effect on hyperglycemia and other cardiovascular risk factors

An early meta-analysis on the effect of exercise in T2D subjects revealed an average decrease in HbA1c of 7 mmol/mol, despite of the absence of weight loss [85]. In the two included studies also using diet co-intervention, the effect on HbA1c was more pronounced. A similar effect was seen in the Cochrane review mentioned previously [60]. The effect of exercise on insulin resistance is often pronounced [86]. There is some evidence that exercise may

improve β-cell function, when measured as disposition index during an intravenous glucose tolerance test [87].

Moreover, significant effects on abdominal (or visceral) obesity, serum triglycerides and blood pressure are often accomplished in exercise studies [60, 88].

1.2.1.1 Mode of exercise

No association between exercise intensity or volume and effect on HbA1c could be confirmed in two different reviews [85, 89]. However, later Boulé et al. did found evidence that exercise intensity was more important than exercise volume in improving VO_{2max} and HbA1c [90]. The highly intensive exercise in fairly young T2D subjects (mean age 45 years) at 75% of VO_2 peak, two sessions/week, and interval exercise one session/week in the study of Mourier et al. decreased HbA1c from 8.5 to 6.2% and increased VO_2 peak by 41% [91]. However, intense exercise might be hazardous and screening for CVD has been recommended in high risk individuals [92].

The most common exercise modalities studied in T2D and other populations are aerobic exercise, resistance training or the combination of both. In a meta-analysis, aerobic exercise alone, or in combination with resistance training, appeared more effective than resistance training alone. These modalities improved glycemic control, systolic blood pressure and triglycerides. Waist circumference was reduced by resistance training or the combined exercise [88]. The combined mode of exercise was also more effective in improving insulin sensitivity in sedentary adults [86].

1.2.1.2 Differences in response

The individual response to exercise training shows a large variation when measured as insulin sensitivity in a healthy sedentary population. In addition, men seem to respond better [93]. The response in respect of VO_{2max} also displays a wide variation [94]. Moreover, changes in gene expression are associated with the individual response to the exercise [95, 96]. It has been postulated that this individual response should be considered in studies evaluating effects on gene expression by physical exercise [94].

Moreover, there are studies showing that subjects with, or at-risk of, T2D respond less to exercise intervention than healthy individuals. Thus, first-degree relatives to subjects with T2D did not respond as well as those without heredity to a seven months' exercise program, when measured as VO_{2max}, weight and waist circumference, taking exercise volume into account [97]. This was associated to a lesser increase in expression of genes involved in oxidative phosphorylation and metabolism, compared to those without heredity [97].

1.2.2 Effects on cardiovascular outcomes

Observational data supports that an active lifestyle decreases the risk of adverse CV events and mortality in T2D [46, 98, 99]. A high level of physical activity at baseline, assessed by

pedometer, was associated with a less increase in measure of arterial stiffness after four years [100].

On the other hand, when subjects were randomized to intensive lifestyle modification in the Look AHEAD study, no impact was seen on hard end-points as CV morbidity or mortality [68].

1.2.3 Mechanisms

The mechanisms behind the positive impact of exercise on hyperglycemia and other cardiovascular risk factors are complex and not fully understood. Exercise training affects the expression of more than 500 genes [95]. Since most of the glucose uptake in the human body occurs in skeletal muscle, research has focused on understanding the mechanisms in this tissue.

Acute exercise induces translocation of GLUT4 through an insulin-independent mechanism. This was demonstrated in mice with a muscle-specific knock-out of the insulin receptor. These mice had a normal exercise-induced glucose uptake in muscle despite no responsiveness to insulin [101]. The GLUT4 translocation is probably mediated through several mechanisms. AMP-kinase [102] and Ca^{2+}/calmodulin-dependent protein kinases are of importance, as well as more downstream targets, such as AS160 and TBC1D1, that are shared with insulin signaling pathways [103].

In chronic exercise training, blood flow/angiogenesis as well as mitochondrial activity will be of importance. This will be discussed in the sections below.

1.2.3.1 Angiogenesis

Activation of angiogenesis in response to exercise training is probably of great importance for the different effects seen in metabolism. Early studies demonstrated that endurance training increases capillary density, or capillaries per fibre ratio, in human skeletal muscle [104]. This would in turn improve exchange of gas and substrate by increased surface area and blood residence time, as well as decreased diffusion distance. The favorable result of this could be increased glucose uptake, lipoprotein metabolism, insulin sensitivity and enhanced glucose tolerance [105].

1.2.3.1.1 Vascular endothelial growth factor (VEGF)

VEGF and its receptors are major angiogenetic factors [106]. There are several different isoforms of VEGF, where isoform A (VEGF-A) is the most relevant [107]. Henceforth, VEGF will be equivalent to VEGF-A in this text. VEGF exerts its effects by binding to the receptors VEGFR-1 and VEGFR-2 that are mainly expressed in endothelial cells [108]. Expression of the VEGF gene is greatly stimulated by exercise [109], especially in untrained muscle [110]. Blockade of the VEGF receptors reduce capillary density in rats [111]. Further evidence of the importance of VEGF was seen in a muscle-specific VEGF knock-out mouse-model that resulted in a 60% decrease in capillary density, accompanied by a 56% reduction in insulin stimulated glucose uptake [112].

1.2.3.1.2 Angiopoietin

The angiopoietin pathway has also been shown to be of importance, in association with VEGF, in the exercise-induced angiogenic response of skeletal muscle in rat [113] and in humans [114]. In humans, the angiopoietin related genes were especially activated in those who improved their VO_{2max} the most [94].

1.2.3.1.3 Other factors

Some other important factors and stimuli in exercise-induced angiogenesis include nitric oxide (NO), mechanical stress and perhaps hypoxia [105, 114]. Remodeling of the extracellular matrix, by in particular matrix metalloproteinases, is probably of importance as well [115].

1.2.3.2 Mitochondrial function and PGC-1α

Genes involved in oxidative phosphorylation and peroxisome proliferator-activated receptor-gamma coactivator 1α (PGC-1α) are downregulated in muscle of diabetic subjects [116], and likewise in those with heredity for T2D [23]. PGC-1α was first described as an important factor in mitochondrial thermogenesis [117]. Further studies proved PGC-1α to be involved in energy metabolism and muscle adaptation to exercise [118]. Exercise activates the AMP-kinase pathway, which in turn probably regulate and activate PGC-1α [119]. More recently, PGC-1α has also been shown to regulate angiogenesis and VEGF expression, in response to hypoxia and nutrient deprivation, in animal muscle [120]. This was also demonstrated in exercise-induced angiogenesis, where the effects of PGC-1α on VEGF expression were mediated through the estrogen-related receptor alpha [121, 122].

1.3 ENDOTHELIN-1 IN TYPE 2 DIABETES

The endothelin-1 (ET-1) system seems to be activated in conditions such as obesity, T2D and metabolic syndrome. This activation has a negative effect on endothelial function and insulin-induced vasodilatation, which seems to be normalized by blockade of ET receptors. Drugs targeting the ET-1 pathways could be interesting to prevent vascular complications in obesity-linked disorders [123].

1.3.1 Basic physiology

ET-1 is a potent vasoconstrictor peptide, first isolated from porcine aorta. ET is first released as the propeptide Big ET (1-38 amino acids), which is then cleaved into ET (1-21) and a C-terminal fragment (22-38) [124]. ET is present in three different isoforms, where ET-1 is considered the most important in regard to cardiovascular effects [125]. It is mainly produced by endothelial cells, but can be produced by several other cell types in pathophysiological conditions. It is considered as an important factor in the evolution of vascular dysfunction and CVD [126].

ET-1 exerts its effects via two different receptors, endothelin receptor A (ET_A) and B (ET_B) [125]. ET_A is predominantly present on vascular smooth muscle cells, where its activation induces vasoconstriction. In contrast, ET_B is mainly located on endothelial cells, but can also

be expressed on vascular smooth muscle cells. It mediates vasodilatation through release of NO and prostacyclin [127]. Hence, the effect of ET-1 will be a result of the balance between these two receptors. Normally, the net effect will be vasoconstriction by the ET_A receptor, that in part will be balanced by the vasodilatory effect of ET_B [126].

1.3.2 Endothelin-1 and insulin resistance

Research has suggested that ET-1 could be an important factor in insulin resistance. Circulating ET-1 levels are increased in subjects with insulin resistance and hyperglycemia [128]. Further, basal ET-1 levels were negatively correlated to total glucose uptake in a clamp study on men with T2D [129].

In rats, administration of ET-1 for 5 days led to a 30% reduction in total insulin-stimulated glucose disposal [130]. Likewise, ET-1 infusion in humans decreased insulin sensitivity by approximately 30%, without influencing blood flow in skeletal muscle [131]. Infusion of the precursor Big ET-1 also reduced insulin sensitivity in healthy humans [132].

1.3.3 Endothelin-1 and endothelial dysfunction

Endothelial dysfunction could be described as an imbalance between vasodilatory factors, where NO is most important, and vasoconstrictive factors, such as ET-1 [133], as well as pro-atherogenic oxygen-derived free radicals [134]. It is hypothesized that endothelial dysfunction is an early defect in the arteriosclerosis process. Endothelial dysfunction has been detected in subjects with T2D or insulin resistance, and also in those at risk of developing T2D [135].

Several mechanisms by which insulin resistance could be connected to endothelial dysfunction have been proposed, including influence on NO production, oxidant stress, ET, the renin-angiotensin system, as well as cytokines produced in adipose tissue [136].

1.3.4 Endothelin-1 receptor blockade

Blockade of the endothelin-1 receptors has been used to study the impact of the ET-1 system. Endothelium-dependent vasodilatation improved with ET_A receptor blockade in patients with atherosclerosis [137]. In obese individuals, selective ET_A receptor blockade enhanced insulin-stimulated glucose uptake, by increasing both blood flow and glucose extraction in the leg. Lean subjects did not show these effects [138]. Further, ET_A receptor blockade augmented blood flow in the forearm in T2D subjects more than in controls. Dual ET_A/ET_B blockade did not further increase this response [139]. Consequently, the ET-1 system seems to be of more importance for vasoconstriction in T2D than in non-diabetic subjects.

Dual ET_A/ET_B receptor blockade might be of greater advantage than selective blockade of the ET_A receptor. In subjects with insulin resistance, dual blockade increased endothelium-dependent vasodilatation. That was in contrast to selective ET_A receptor blockade, that had no effect compared to placebo [140]. Moreover, dual blockade acutely increased insulin sensitivity, measured by hyperinsulinemic clamp, in insulin resistant subjects. Conversely,

ET_A blockade did not show any effect [141]. But some studies have not proven dual blockade to be superior. As mentioned above, dual blockade in the study by Cardillo et al. did not augment the response on blood flow more than ET_A-blockade alone in T2D subjects [139].

A clinical placebo-controlled trial evaluating dual endothelin blockade (bosentan) have shown improved peripheral endothelial function in patients with long-standing T2D and microalbuminuria. No effects on glycemic indices were seen, but most of the subjects had more advanced treatment including insulin [142].

1.3.5 The complex interplay between insulin and endothelin-1

In the endothelium, insulin stimulates NO production by activation of the PI3-kinase dependent pathways, and thereby promoting vasodilatation. But ET-1 production, with its mainly vasoconstrictive effect, is also stimulated by insulin [143], but via the MAP-kinase dependent pathways [144]. Consequently, ET-1 levels in blood increased during hyperinsulinemic clamp in T2D individuals [129].

Interestingly, ET-1 can influence the insulin pathways. In arterial smooth muscle cells, ET-1 diminished PI3-kinase activity, suggesting interference with insulin signaling [145]. In adipocytes pre-treated with ET-1, insulin signaling through both the PI3-kinase and MAP-kinase pathways were impaired [146]. In later in vitro studies, the insulin resistance evoked by ET-1 seemed to involve the phosphatidylinositol 4,5-bisphosphate (PIP2)/actin system [147], which impaired GLUT4 translocation [148].

Insulin and ET-1 counteract each other in the vasoconstrictor system. In perfused hindlimb of rat, insulin blocked the vascular effects of ET-1 by its vasodilatory effect [149]. Moreover, in arteries from insulin-resistant rats, ET_A blockade normalized the vasodilatory response to insulin [150]. This suggest an increased activity of ET-1 in insulin resistance.

1.4 VITAMIN D IN TYPE 2 DIABETES

The inference of an impact of vitamin D status on T2D and related conditions emanates mainly from observational data. In cross-sectional observational studies, there is a fairly consistent association between low 25-hydroxy-vitamin D_3 (25(OH)D) levels and prevalence of T2D or the metabolic syndrome [151].

1.4.1 Basic vitamin D physiology

Classical effects of vitamin D includes regulation of calcium and phosphate homeostasis, as well as bone mineralization. Its active form 1,25-dihydroxy-vitamin D_3 ($1,25(OH)_2D$) binds to the nuclear vitamin D receptor and exerts its effects by influencing gene expression.

Sources of vitamin D are conversion in the skin of 7-dehydrocholesterol in two steps to vitamin D_3 by UV-light, or by foods containing or fortified with vitamin D_2 or D_3. Vitamin D_3 are subsequently 25-hydroxylated, primarily in the liver [152]. The serum levels of the product 25(OH)D are proportional to vitamin D intake, and has therefore long been regarded

as a good proxy of vitamin D status [153]. But there has been some doubt raised lately about the accuracy of that assumption [154]. The optimal serum concentration of 25(OH)D for different health outcomes has been much debated. Some authors have advocated higher levels, often above 75 nmol/l [155, 156], whereas Institute of Medicine (IOM) has advocated a more conservative approach [154].

The second step in the activation of vitamin D_3 is performed by 1α-hydroxylase, mainly in the kidney. This creates the active metabolite $1,25(OH)_2D$. The main catabolic step in deactivation of this compound creates 24,25-dihydroxy-vitamin D_3, which then is further degraded [152].

The classic target organs for $1,25(OH)_2D$ include the intestine, where calcium and phosphate absorption are enhanced. Further, vitamin D is important in the mineralization process of the skeleton, although probably not vital [157]. Finally, $1,25(OH)_2D$ suppresses the production of parathyroid hormone (PTH), whereas PTH stimulates 1α-hydroxylase in the kidney and thus increases formation of $1,25(OH)_2D$ [152].

1.4.2 New perspectives

Aside from the classical related disease rickets, vitamin D deficiency has lately been related to several other health issues. These include neuromuscular function and risk of falls, dental health and various cancers. Further, immune-related disorders as type 1 diabetes, multiple sclerosis, rheumatoid diseases, psoriasis and inflammatory bowel disease have been considered, as well as hypertension and T2D [155, 158]. But evidence of a clear role of vitamin D supplementation in these pathologic states are in most cases lacking [159]. Even in osteoporosis, the support for vitamin D treatment alone has little support according to a recent meta-analysis [160].

One of the basis of the interest in vitamin D originates from the fact that the receptor for the active metabolite $1,25(OH)_2D$ is present in more than 30 tissues [161], including pancreatic β-cells [162]. Moreover, the enzymatic conversion of 25(OH)D to $1,25(OH)_2D$ is possible in several locations, including the pancreatic β-cells [163]. Theoretically, the active $1,25(OH)_2D$ metabolite could thus be produced locally within the pancreatic islet and exert autocrine effects.

Another interesting aspect is that higher PTH levels have been associated to CVD. In the ULSAM cohort, PTH levels were positively associated to CV mortality in men [164]. An interaction with vitamin D status could be of importance for this finding. Notably, in a recent study evaluating metabolic effects after surgery for primary hyperparathyroidism, IR resistance measured by HOMA index was reduced six weeks postoperatively. Importantly, randomization to vitamin D treatment after surgery for one year did not influence IR or other CV risk factors in comparison to placebo, despite a higher 25(OH)D level and a lower PTH level in the vitamin D treated group [165].

1.4.3 Observational studies

Aside from the cross-sectional data of associations between 25(OH)D levels and T2D prevalence mentioned above [151], there are also prospective studies on the subject. In the large prospective Nurses´ Health Study cohort study, the risk of developing T2D was lower in subjects with a high intake of vitamin D or calcium, compared to those with a low intake [166]. A significant 40% reduction in relative risk of acquiring T2D were seen in a Finnish prospective cohort study, evaluating the impact of the highest and lowest quartiles of baseline 25(OH)D values. This finding suggested a protective effect of a high 25(OH)D level. Notably, the risk dropped to a trend when adjusting for multiple other risk factors for T2D [167]. However, in the Ely prospective study, levels of glucose tolerance, fasting insulin, insulin resistance (homeostasis model of assessment, i.e. HOMA index) and metabolic syndrome risk score were significantly lower in those with a high baseline 25(OH)D, even after adjustment for competing risk factors related to T2D [168]. In the Stockholm Diabetes Prevention Study (SDPP), high baseline 25(OH)D levels in men were protective in respect to development of T2D after 8-10 years. This relationship was seen in men with IGT, but not in those with normal glucose tolerance at baseline or in women [169]. A meta-analysis of 21 prospective studies confirmed the association, showing a significant hazard ratio of 0.62 when comparing risk of incident diabetes for those with high 25(OH)D levels compared to those with low levels at baseline. Adjustment for BMI and other related factors diminished the association slightly, but it remained significant [170].

1.4.4 Randomized intervention studies

Previous randomized studies are scarce. Randomization to 1000 mg of calcium and 400 IU vitamin D_3 supplement per day in post-menopausal women was not associated with a decreased risk of incident T2D during 7 years of follow-up [171].

1.4.5 Mechanistic studies

1.4.5.1 B-cell function

In vitro as well as in vivo animal studies in the 80´s suggested that vitamin D could have an impact on insulin release [172, 173]. This evoked several trials in human subjects. A vitamin D_3 dose of 2000 IU per day to subjects with vitamin D deficiency increased insulin secretion after an OGTT [174]. In vitamin D deficient Asians living in London and having slightly elevated glucose levels, a single dose of 100 000 IU vitamin D_3 augmented insulin and C-peptide response to an OGTT after 2-3 months. Notably, glucose tolerance was unchanged [175]. First-phase insulin secretion during an intravenous glucose tolerance test (IVGTT) increased significantly in females with T2D after one month of treatment with vitamin D_3 1332 IU per day [176]. Further, 3 weeks of treatment with 2 µg of active vitamin D (1α-$(OH)D_3$) increased total insulin response after an OGTT in T2D subjects [177]. A slight but significant increase in first-phase C-peptide levels after IVGTT were also seen in healthy subjects, taking 3 µg of active vitamin D ($1,25(OH)_2D$) for four days [178].

In contrast, four days of treatment with one µg of active vitamin D_3 to T2D subjects did not augment insulin response to a meal test [179]. In addition, 40 000 IU of vitamin D_3 once weekly for six months did not improve insulin secretion, calculated with HOMA index in T2D subjects [180]. In centrally obese men living in India, a high-dose treatment of three injections of 120.000 IU vitamin D_3, separated by 14 days, did not improve insulin secretion assessed by HOMA index [181].

1.4.5.2 Insulin sensitivity

There are a few reports of increased insulin sensitivity by vitamin D treatment. In the Indian study by Nagpal et al. mentioned above, insulin sensitivity measured by the OGTT-based 3-hour oral glucose insulin sensitivity (OGIS) index was significantly improved. Noteworthy, other indices of insulin sensitivity (HOMA, quantitative insulin sensitivity check index [QUICKI]) were not affected [181]. In a post-hoc analysis of a study focusing on bone-related outcomes, combined treatment with calcium and 700 IU vitamin D_3 slowed progression of insulin resistance over three years in those with IFG at baseline [182].

Conversely, most studies have not been able to detect this association. For example, the intravenous glucose tolerance test (IVGTT) based study by Borissova et al. and the six-month long study by Jorde et al. were neutral in this respect [176, 180]. In a subset of participants in the Women's Health Initiative study, HOMA index of insulin resistance did not differ between those allocated to calcium + vitamin D or placebo [171]. Several indices of insulin sensitivity, including the OGTT-based Insulin Sensitivity Index (ISI), were unaltered after two injections of 100 000 IU vitamin D_3 given to adults with vitamin D deficiency [183]. Moreover, one study using the euglycemic clamp could not find any benefit of active vitamin D treatment with two µg of α-25(OH)D per day in men with IGT. However, no control group was used [184]. Finally, 1.5 µg of active $1,25(OH)_2D$ for seven days did not affect the M-value, obtained by euglycemic clamp, in comparison with placebo in healthy males [185].

A cross-sectional study on overweight post-menopausal women gave support of a threshold effect of 25(OH)D level on glucose tolerance, fasting insulin and insulin resistance (HOMA index). The threshold level for the associations was set to 65 nmol/l [186].

1.5 MISSING INFORMATION

Physical exercise has been shown to positively affect several of the included risk factors but the additive effects of OADs to physical training is unknown.

Further, the response to physical exercise demonstrates a large individual variation. VEGF is a key player in angiogenesis and PGC-1α is a crucial factor in mitochondrial function. If these components thereby could promote improved insulin sensitivity by exercise, to a diverse extent in separate individuals, has not been studied.

ET-1 is another factor that could be important in glucose metabolism and insulin sensitivity. Blockade of the ET-1 receptors A and B (dual blockade) has been shown to increase glucose

uptake and insulin sensitivity in insulin resistant subjects, but it is unclear how ET-1 more precisely interferes with glucose metabolism.

Vitamin D could, according to epidemiological studies, have an important role in glucose homeostasis, and thereby be a putative agent for prevention of T2D in subjects with prediabetes or treatment of T2D. However, mechanistic studies in humans have yielded conflicting results as if vitamin D has effects on β-cell function and insulin sensitivity.

2 AIMS

To study, in patients with T2D and mild hyperglycemia, the effect of structured exercise training alone and in combination with acarbose treatment on glycemic control, body composition, and cardiovascular risk factor profile.

To investigate if associations exist between changes in expression of key genes and improved insulin sensitivity, VO_{2max} and glycemic control following exercise training in T2D, in the context of individual physiological adaptation.

To test the hypothesis that endogenous ET-1 contributes to impaired skeletal muscle glucose uptake in insulin-resistant subjects in vivo. Furthermore, to elucidate the direct actions of ET-1 on glucose metabolism and insulin signaling in skeletal muscle in vitro.

To investigate the effect of high-dose vitamin D treatment in people with prediabetes or drug-naïve T2D on β-cell function, insulin sensitivity, and glucose tolerance.

3 METHODOLOGICAL CONSIDERATIONS

3.1 STUDY SUBJECTS

3.1.1 Study I and II

We recruited study subjects from referrals to our outpatient diabetes daycare unit at Karolinska University Hospital, and by advertising in newspapers. Inclusion criteria included T2D diagnosis since at least 3 months, HbA1c <68 mmol/mol, age 45-60 years and BMI 25-30 kg/m^2. Permitted diabetes treatments were diet and no more than one oral antidiabetic drug (OAD). None of the study subjects took part in any regular exercise program with more than one training session per week.

3.1.2 Study III

3.1.2.1 In vivo study

We recruited sedentary males with IR, assessed by euglycemic-hyperinsulinemic clamp or HOMA index. Cut-off levels to categorize subjects as insulin resistant are arbitrary, but we used cut-off limits that have been recommended [187].

3.1.2.2 In vitro study

Muscle samples from m. rectus abdominis were collected from eight individuals during elective abdominal surgery. These subjects had no metabolic disorders.

3.1.3 Study IV

We recruited participants from the Stockholm Diabetes Prevention Program (SDPP), a prospective cohort study in Stockholm [188]. Subjects in SDPP were enrolled from five municipalities in Stockholm county during the period 1992 to 1998. A questionnaire was sent to all inhabitants aged 35-55, asking about present diabetes diagnosis and family history of diabetes (FHD). In the second step, subjects without diabetes but with FHD, together with randomly age- and sex-matched controls without FHD, were invited for an examination. This examination consisted of blood sampling, an OGTT, body measurements and a questionnaire about lifestyle. A baseline group of almost 8000 individuals was collected and re-invited 8-10 years later for a follow-up investigation, excluding those who were diagnosed with diabetes at baseline. Over 5500 subjects were re-examined by the same procedure as the baseline investigation. For the present study, we contacted by phone those who at follow-up in the years 2003–2006 were categorized as having prediabetes (IFG, IGT or both). Those who had the potential to meet eligibility criteria were invited to a screening visit.

Major inclusion criteria included: 1) IFG, IGT, IFG + IGT, or drug-naïve diabetes at the screening OGTT; 2) age 45 to 75 years, female or male; 3) BMI ≤32 kg/m^2; 4) HbA1c ≤63 mmol/mol); 5) fasting plasma glucose <9mmol/l and 6) serum 25(OH)D <75 nmol/l (below normal lab reference).

3.2 STUDY DESIGNS

3.2.1 Study I and II

After a wash-out period for 6 weeks of any OAD, the subjects were randomized to one of three interventions for a period of twelve weeks:

1. Aerobic/anaerobic exercise group training, for 50 minutes three times weekly.
2. Exercise (as in intervention 1) and acarbose treatment (as in intervention 3).
3. Acarbose treatment alone. The target dose was 100mg three times daily in conjunction with major meals. The dose was up-titrated during the first four weeks. Acceptable compliance according to pill-count was set to 75-120%.

Further, the study design included that 10 individuals within each intervention group were randomized to undergo muscle biopsies for gene expression analyses (study II). All assessments, including muscle biopsies, were performed before and after the twelve-week intervention period (**Figure 1**).

Included subjects were instructed to maintain their diet and exercise habits during the study period, except that of the allocated intervention.

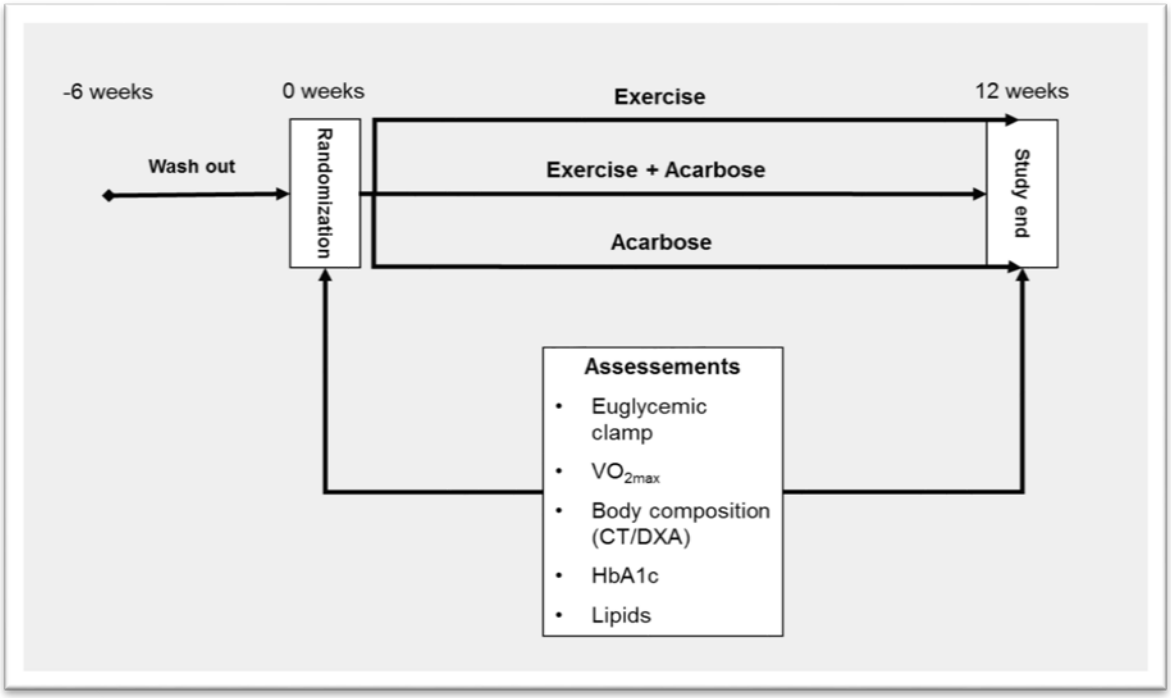


Figure 1. Study design and major assessments in study I and II. CT; computerized tomography, DXA; dual-energy X-ray absorptiometry.

3.2.2 Study III

Three different protocols were applied in the in vivo study on each study subject (see **Figure 2**). NaCl was given during minute -30 to minute 0 in all protocols. NaCl was also infused when necessary to balance the rate of infused volume in all three protocols.

- Protocol A: Infusion of BQ123 (ET_A blockade) and BQ788 (ET_B blockade) were given for 120 minutes. Insulin was infused from minute 60 to minute 120.
- Protocol B: Insulin was infused for 120 minutes.
- Protocol C: Insulin was infused for 135 minutes. ET-1 was infused from minute 60 to minute 135.

The investigations were separated by at least one week and were performed in random order, blinded to the subjects. To investigate possible time-dependent differences in protocol C, seven subjects were given a 15-minute prolonged ET-1 infusion.

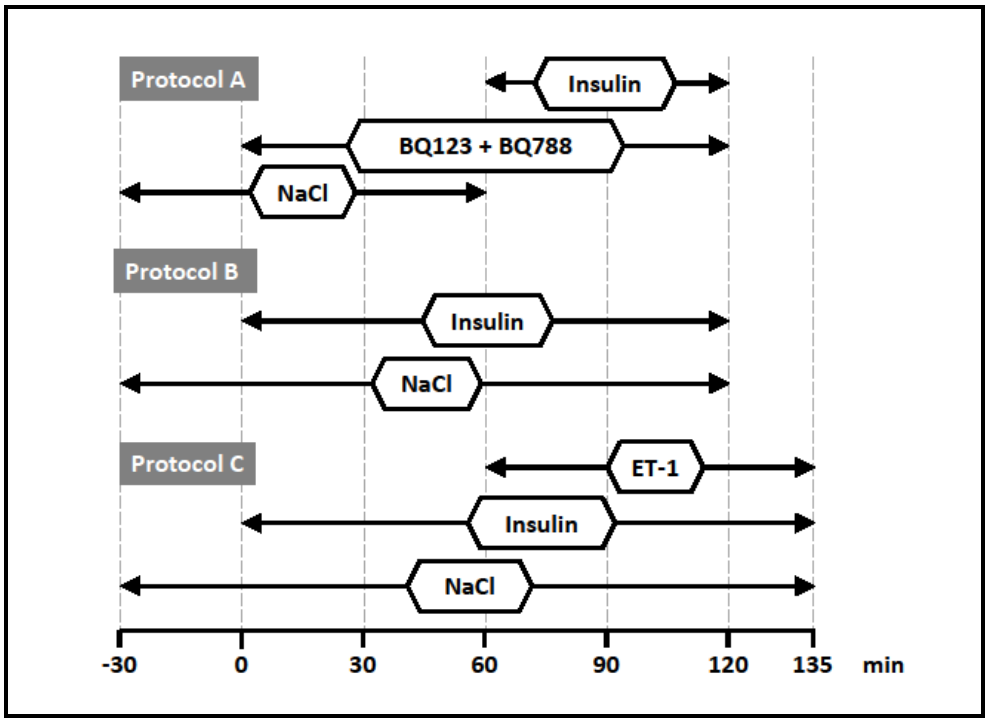


Figure 2. The three different protocols (A-C) in study III. *Reprinted with the kind permission of A. Shemyakin.*

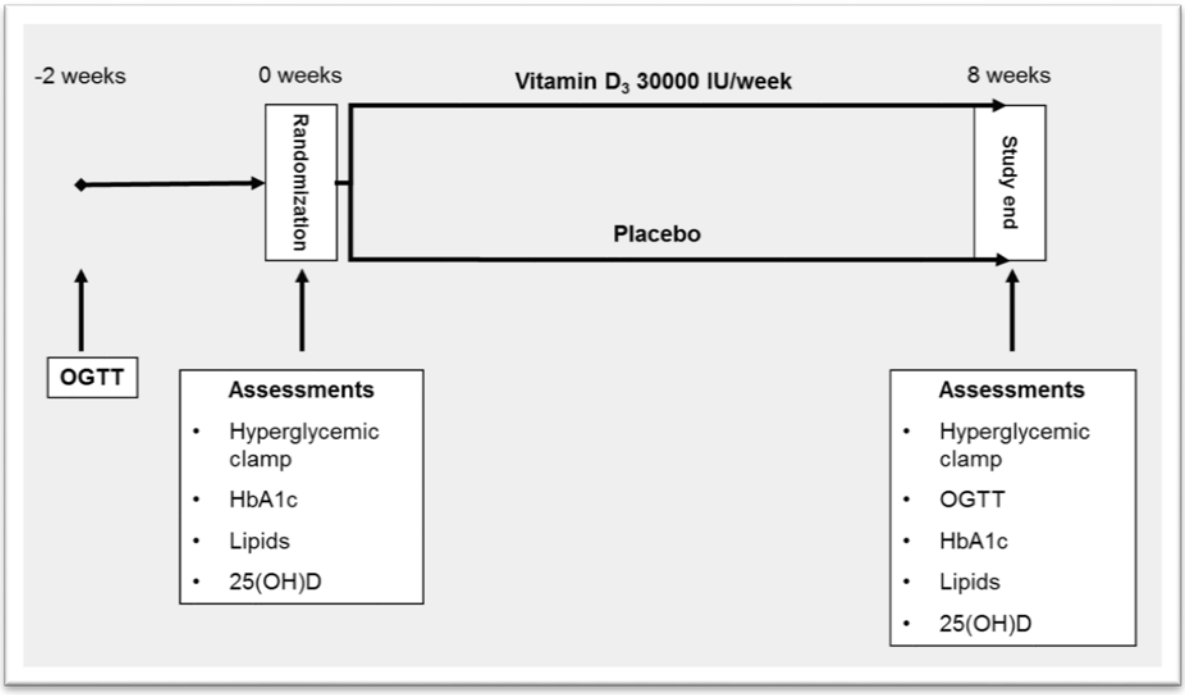
In the in vitro study, muscle biopsies from m. rectus abdominis were taken during planned abdominal surgery.

3.2.3 Study IV

This study was a randomized 1:1, parallel-group, double-blind, placebo-controlled study with an 8-week intervention period. Study centers at Karolinska University hospital and Södersjukhuset in Stockholm were used.

Primary outcome was the relative change in first-phase serum insulin secretion (0–12 min) during the hyperglycemic clamp investigation at study end, compared with baseline, between the two groups. Secondary end-points comprised relative change in second-phase insulin secretion (12–120 min), insulin sensitivity, and disposition index (DI) during the hyperglycemic clamp. Furthermore, secondary outcomes were change in glucose tolerance, change in fasting plasma glucose and HbA1c, and change in blood lipids between the two

groups. Moreover, change in 25(OH)D serum levels and safety end-points such as incidence of hypercalcemia and adverse events were assessed. Study design and major assessments are seen in **Figure 3.**



Figur 3. Study design and major assessments in study IV. OGTT; oral glucose tolerance test.

3.3 INTERVENTIONS

3.3.1 Exercise training (study I and II)

A combined aerobic and resistance group training was used. This exercise modality has been shown to probably be more effective than aerobic or resistance training alone [86, 88] on metabolic outcomes. Moreover, a group training concept of this modality is widely used and well known in Sweden, provided for instance by the organization Friskis & Svettis©. Physiotherapists supervised each training session. There were two exercise targets [189]:

1. An intensity of ≥50% of maximal exercise capacity, based on maximal heart rate during the exercise test at baseline, during ≥40 minutes.
2. An intensity of ≥80% during three periods of 3-4 minutes.

The participants were urged to reach an exertion of 13–15/20 on Borg's Rated Perceived Exertion scale during the heavier parts and 9–11/20 during the rest of the program [190]. Further, an online heart rate recording system was used (Activio, Stockholm, Sweden) to ascertain that exercise intensity was reached. This was applied at one of the weekly training sessions during weeks 1, 6 and 12 of the training period. This design comprised adjustment of the workload as the subjects became trained during the twelve-week period.

3.3.2 Acarbose treatment (study I and II)

The target dose of acarbose (Bayer AG) was 100 mg three times daily to be taken in conjunction with major meals. A total of 50 mg was added every week, reaching the dose 50 mg t.i.d. the third week. The fourth week the dose was increased to 100 mg t.i.d. If gastrointestinal side-effects occurred, the titration period was prolonged and/or the target dose reduced.

3.3.3 Endothelin-1, endothelin receptor blockade and insulin (study III)

ET-1 (Alexis Biochemicals, Lausen, Switzerland) was infused at the rate 20 pmol/min. BQ123 (ET_A receptor antagonist) and BQ788 (ET_B receptor antagonist) (both NeoMPS, Strasbourg, France) was infused at the rate 10 nmol/min. Insulin (Actrapid, 100 IU/ml; Novo Nordisk, Bagsværd, Denmark) was dissolved in 0.9% saline and blood immediately before use, and infused at the rate 0.05 mU x kg^{-1} x min^{-1}. All infusions were given by the brachial artery. Bosentan (dual ET_A/ET_B receptor blockade) (courtesy of Dr. Martine Clozel, Actelion Pharmaceuticals, Switzerland) was dissolved in double-distilled water, stored frozen at -80°C, and then diluted to the proper concentration in cell culture media on the day of the experiments.

3.3.4 Vitamin D (study IV)

Participants were randomly assigned to receive vitamin D_3 (Vigantol Oil©), 30 000 IU (1.5 ml/45 drops) given orally once weekly, or matching placebo oil. Merck KGaA (Darmstadt, Germany) manufactured vitamin D_3 and matching placebo, which were both delivered in identical dark bottles. The first dose was given at the randomization visit after study assessments. The fifth dose was given at the half-time visit. The other six doses were taken by the subject at home and study drug bottles were brought to the study center by each participant for compliance assessment.

3.4 ASSESSEMENTS

3.4.1 Oral glucose tolerance test (study IV)

After a fasting plasma glucose was obtained (0 minutes), 75 grams of glucose dissolved in water was given orally. Plasma glucose was obtained at 30 and 120 minutes after the glucose administration. The baseline investigation was performed at the screening visit. WHO criteria [10] were applied to determine glucose tolerance category according to:

- IFG = fasting plasma glucose 6.1–6.9 mmol/l
- IGT = 2-hour plasma glucose 7.8–11.0 mmol/l
- Diabetes = fasting plasma glucose ≥7.0 mmol/l and/or 2-hour plasma glucose ≥11.1 mmol/l)

3.4.2 Euglycemic-hyperinsulinemic clamp (study I, II and III)

In the studies we have used the clamp technique first described by DeFronzo in 1979 [191]. It is still considered as the golden standard when assessing insulin sensitivity. Other, less resource-demanding, methods have evolved, but are still compared against the clamp method [192]. The principle entails to raise insulin levels and add glucose intravenously to avoid hypoglycemia and maintain normoglycemia. The amount of glucose infused gives an estimate of insulin sensitivity (M).

The investigation was performed in the morning, after the subjects had fasted overnight. After the intervention period in studies I and II, the insulin clamp studies were repeated and performed 24–36 h after the last bout of exercise in intervention groups 1 and 2. Insulin (Actrapid; Novo Nordisk, Copenhagen, Denmark) was dissolved in saline and a small amount of the subject´s blood, to minimize insulin adherence to the infusion set. Insulin was infused at 1.0 mU x kg^{-1} x min^{-1} for 120 minutes. During the first 10 minutes, the insulin infusion rate was increased in a step-wise manner in order to faster reach steady-state concentrations of insulin. Glucose (200 mg/ml) was simultaneously infused intravenously at a variable rate to maintain the blood glucose concentration stable at 5.0 mmol/l. For this purpose, blood glucose was sampled every 5 to 10 minutes.

3.4.2.1 Calculations

Glucose infusion rate at the end of the clamp assessed insulin sensitivity (M), and was corrected for lean body mass as insulin-stimulated glucose uptake occurs primarily in skeletal muscle [22]. Insulin sensitivity index (M/I) was calculated by dividing M by the mean insulin levels (I) during steady state (study II).

3.4.3 Hyperglycemic clamp (study IV)

The hyperglycemic clamp, also described in 1979 by DeFronzo [191], is considered as the golden standard method when assessing β-cell function. It has also been proven to give a valid measurement of insulin resistance, with highly correlated R-values of approximately 0.85 in comparison with the hyperinsulinemic-euglycemic clamp [191, 193].

The investigation implies increasing plasma glucose from the baseline level by a glucose infusion. This will stimulate the endogenous insulin secretion, which will constitute a measure of β-cell function.

Glucose 200 mg/ml was infused intravenously for 120 minutes at a variable rate to increase plasma glucose to a target value of 6.9 mmol/l above the fasting glucose value. The first 14 minutes, a relatively high infusion rate was administered in a stepwise manner, based on estimated body surface, to faster reach a steady state. For the remainder of the investigation, the infusion rate was based on preset computer calculations, as described previously [191]. Arterialized blood for sampling of glucose and insulin levels were obtained from a retrograde inserted venous injection needle in a heated hand. Sampling was done every 2 min from minute 0 to 14, and thereafter every 5-10 minutes until the end of the investigation. Glucose

infusion rate (GIR) was calculated from records of the total amount of glucose infused every 5-10 minutes. Urine was collected for measurement of urinary glucose excretion during the investigation.

3.4.3.1 Calculations

First-phase insulin secretion was calculated as area under the curve of serum insulin during minutes 0–12 by the trapezoidal rule and was adjusted for the basal insulin level. The glucose stimuli will be of importance for the insulin response. As there are some random fluctuations in the glucose increase, especially in the beginning of the investigations, first-phase insulin responses (min 0-12) were adjusted for any difference in glucose stimuli (calculated as area under the curve above baseline) between the baseline and study-end clamps (Kanat, Mari et al. 2012).

Second-phase insulin secretion was calculated accordingly during minutes 12–120. Adjustment for glucose stimuli during the second phase was not necessary, as the glucose levels were stable during this period of the investigations. The basal insulin level was calculated as the mean of insulin measurements at minute -10 and minute 0. Insulin sensitivity index (GIR/mean insulin level [I]) was calculated during the last 30 min of the investigation as (GIR – urinary glucose excretion rate)/fat-free mass/mean insulin concentration x 100. The disposition index (DI), a measure of β-cell function in relation to insulin sensitivity, was calculated as insulin secretion x GIR/I during minutes 0–12 and 12–120, respectively.

3.4.4 Body composition (study I, II and IV)

In study I and II, fat-free mass, and total and truncal fat mass were measured by dual-energy X-ray absorptiometry (DXA) (Lunar DPX-L x-ray bone densitometer, Lunar Corp., Madison, WI). Computerized tomography assessed intra-abdominal fat area (Siemens Somatom Plus, Siemens Corp., New York, NY).

In study IV, a combined bioimpedance and weighing device (Tanita, Tokyo, Japan) was used, estimating total body fat and fat-free mass. This simpler technique was applied since fat free mass was mainly used in this study to correct clamp calculations for any individual change during intervention.

3.4.5 Physical fitness (study I and II)

Maximal workload and VO_{2max} were assessed during an exercise test using an electrically braked bicycle ergometer. The load was increased in a step-wise manner by 30 W every minute until exhaustion. A Jaeger Oxycon software program (Jaeger, Hoechberg, Germany) was used to make calculations on expired gases.

3.4.6 Step count (study IV)

A regular step counter was used by the subjects during seven days preceding the randomization and study-end visits. Step count per day was used to estimate any possible lifestyle change during the study.

3.4.7 Blood flow assessment and forearm glucose uptake (study III)

These investigations were done in the morning under fasting conditions and with no caffeine or nicotine use during the day of investigation. Percutaneous catheters were inserted into the brachial artery in the non-dominant arm for infusions and blood sampling, and in a deep vein in the ipsilateral arm for blood sampling. Forearm blood flow (FBF) was measured in both arms by venous occlusion plethysmography, a method considered as one of the golden standards when investigating vascular function. The method implies using a strain gauge applied around the forearm. The venous outflow is obstructed for 10 seconds in the upper arm by a cuff during recording, as well as the circulation of the hands by a wrist cuff inflated to 30 mmHg above systolic blood pressure. The volume of the forearm, assessed by the strain gauge, will be proportional to the arterial inflow. Arterial and deep venous blood samples were collected for assessment of forearm glucose uptake (FGU). During the investigations, the wrist cuff was inflated 2 minutes before blood sampling to avoid contribution from the hand circulation.

3.4.7.1 Calculations

The mean of 4-8 recordings during 2 min was used for FBF calculation. The ratio between the infused and the non-infused arm was utilized for calculations of change in baseline flow. FGU was calculated according to the formula: (arterial-venous glucose concentration) x blood flow x (1-hematocrit).

3.4.8 Plasma analyses

Blood or plasma glucose was determined by a glucose oxidase method (Yellow Springs Instrument, Yellow Springs, OH, USA), enabling fast sampling during the clamp investigations. Blood glucose values were recalculated to plasma values, if necessary. Plasma insulin was measured by an in-house radioimmunoassay method with inter- and intra-assay coefficient of variation (CoV) of 11.5–16.9% and 5.8–8.4%, respectively. In study III, plasma glucose was analyzed with the SYNCHRON LX system (Beckman Coulter, USA) and insulin by an electrochemiluminescence immunoassay (Roche Diagnostics, Germany). Plasma proinsulin was measured by a commercial radioimmunoassay (intra- and inter-assay CoV of 2.0% and 5.0%, respectively), and with a cross-reactivity for insulin and C-peptide <0.1%. HbA1c was analyzed by the high-performance liquid chromatography Mono-S method (intra- and inter-assay CoV 0.48% and 2.67%, respectively), which gives absolute values ~0.9 lower than the Diabetes Control and Complications Trial method. Mono-S values have been re-calculated to DCCT or IFCC (International Federation of Clinical Chemistry) units, when appropriate. In **Table 2**, the values expressed by the tree different standards can

be compared. Values in IFCC units (expressed as mmol/mol) are reported in the thesis. 25(OH)D was analyzed by competitive chemical immunoluminescence (LIAISON, intra- and inter-assay coefficient of variation of 5 and 8–11%, respectively). Blood lipids and apolipoproteins were analyzed by standard methods at the Karolinska University laboratory (Beckman Coulter, USA), as well as free calcium (ABL800 FLEX, Radiometer, Denmark). ET-1 was measured by a radioimmunoassay [194].

IFCC mmol/mol	Mono-S %	DCCT %
31	4.0	5.0
42	5.0	6.0
52	6.0	6.9
63	7.0	7.9
73	8.0	8.8
83	9.0	9.8

Table 2. Conversion between the three different standards for HbA1c. IFCC; International Federation of Clinical Chemistry, DCCT; Diabetes Control and Complications Trial

3.4.8.1 Season-adjusted vitamin D levels (study IV)

25(OH)D levels were season-adjusted, based on measurements in the SDPP cohort [169]. The calendar year was divided in four quarters (Q): November–January (Q1), February–April (Q2), May–July (Q3) and August–October (Q4). Q1was set as reference (no correction) and a correction value in nmol/L was added or subtracted to the measured value. The corrections were Q2 + 6.7, Q3 – 1.9, and Q4 – 13.7. The adjusted values are reported.

3.4.9 Cell experiments (study III)

3.4.9.1 Glucose uptake

Isotope-labeled glucose was used to measure glucose uptake. Muscle biopsies were collected in cold PBS supplemented with 1% penicillin-streptomycin solution. Satellite cells were isolated and cultured to form myotubes as described [195]. Myotubes were incubated in serum free medium overnight before each experiment. ET-1 (10 nM) or vehicle was added in the absence or presence of the dual ETA/ETB receptor antagonist bosentan (3 µM) for 24 h. Bosentan was always added 30 min before ET-1. Control cells were exposed to vehicle for the same length of time. Insulin (60 nM) was added for 30 min, by protocols. Overnight serumstarved myotubes (in the presence or absence of 10 nM ET-1) were stimulated with or

without insulin in Krebs buffer. Thereafter, cells were incubated with 10 µM 2-deoxy[3H]glucose (1µCi/ml) for 15 min at 37 °C. Each experiment was carried out on triplicate wells. Afterward, cells were rapidly rinsed four times with ice-cold PBS and solubilized with 1 ml 0.4 N NaOH. Then, 0.5 ml of lysate was transferred into scintillation vials and [3H] measured in a scintillation counter.

3.4.9.2 Protein expression

Western blot analysis was used to assess phosphorylation of IRS-1, Akt, AMPK and ERK and total expression of Akt and actin. An aliquot of muscle cell lysate (20 µg protein) was mixed in Laemmli sample buffer containing β-mercaptoethanol. Proteins were separated by 7.5% SDS-PAGE, transferred to polyvinylivenediflouride membrane (Millipore, Bedford, MA), and blocked in 7.5% nonfat dried milk in Tris-buffered saline with 0.02% Tween (TBST) for 2 h at room temperature. Membranes were incubated overnight at 4 °C with phospho-specific antibodies against phosphor IRS-1 Ser636 (1:1000), phospho-Akt Ser473 (1:1000), phospho ERK1/2, p42/44 MAP-kinase Thr202/Tyr204 (1:1000), phospho-AMP-kinase Thr172 (1:1000), Akt (1:1000), or pan-actin (1:1000, all from Cell Signaling Technology, Beverly, MA). After washing in TBST, the membranes were incubated with horseradish peroxidase antirabbit IgG for all the target proteins (1:25 000; Bio-Rad, Hercules, CA) for 1 h at room temperature, followed by additional washing. Proteins were visualized by enhanced chemiluminescence (Amersham, Arlington Heights, IL) and quantified using densitometry and Molecular Analyst Software (Bio-Rad, Richmond, CA).

3.4.10 Gene expression (study II and III)

3.4.10.1 Study II

Muscle biopsies were obtained from the m. vastus lateralis in resting state. At the end of the study, the biopsies were taken 24–48 h after the last bout of exercise. Quantification of mRNA expression of selected genes included VEGF and its receptors 1 and 2, Angiopoietin 1 and 2, and also PGC-1α. These genes were chosen according to their potential as key factors in exercise-induced muscle adaptation. Detection of mRNA was performed on an ABI-PRISM® 7700 Sequence Detector (Applied Biosystems Inc, Foster City, CA, USA). Oligonucleotide primers and TaqMan® probes were designed. 18S rRNA was selected as an endogenous control to correct for potential variations in RNA loading (4310893E, Applied Biosystems Inc.). All reactions were performed in 96-well MicroAmp Optical plates, using the ABI-PRISM® 7700. For every gene, all samples were amplified simultaneously in duplicate in one assay run. Relative quantification of the samples was carried out using dilution curves for each target gene analogous to a standard curve. The relative distribution of the targets was calculated for each individual. A threshold cycle (ΔCt) value was obtained by subtracting 18s Ct values from respective target gene Ct values. The relative expression of each isoform was then calculated by $2^{-\Delta-\Delta Ct}$ and presented as arbitrary units.

3.4.10.2 Study III

Myoblasts were cultured in six-well plates and at more than 90% confluence the differentiation to myotubes was initiated. Cells were stimulated with vehicle or ET-1 (10 nM) for 24 h before harvest. Five days after differentiation, myotubes were washed three times with RNase free PBS and harvested directly for RNA extraction (RNAeasy mini kit; QIAGEN, Crawley, UK). All RNA was DNase treated before reverse transcription (RQ1 RNase-free DNase; Promega, Southampton, UK). The mRNA concentrations of target genes were determined, and cDNA was prepared from total RNA samples using the TaqMan reverse transcription reagent. The quantification of polymerase chain reaction (PCR) products was analyzed by Real-Time PCR (TaqMan; Applied Biosystems, Foster City, CA) using the comparative cycle threshold method. All samples were analyzed in duplicate. The ABI Prism 7900 HT Sequence Detection System (Applied Biosystems) was used for analysis. Validated primers were purchased from Applied Biosystems (Maastricht, The Netherlands). Data were analyzed using GAPDH as a housekeeping gene.

3.5 STATISTICS

3.5.1 Studies I, II and IV

All continuous variables are presented as median (inter-quartile range). Analyses were primarily made on relative changes to adjust for baseline differences. Non-parametric methods were applied when possible due to small sample size. Thus, the Wilcoxon signed-rank test was used to test for within-group differences and the Kruskal-Wallis ANOVA or the Mann–Whitney U-test was used to analyze differences between groups. The two-tailed Fisher exact test was used for categorical variables. In study I (with three different intervention groups), Wilcoxon's rank-sum test was used for pairwise comparisons between groups when the overall Kruskal-Wallis ANOVA was significant. Multiple regression analysis was performed to adjust for age, sex, and HbA1c at baseline in study I, if necessary. In study II, relative change in gene expression was analyzed by multiple linear regression with sex and relative changes in insulin sensitivity index, VO_{2max} and HbA1c as independent variables. Therapy with acarbose was forced into the models, as we aimed to study the effect of exercise alone. All continuous variables were log-transformed before this parametric test. Statistical significance was set at a two-tailed $P < 0.05$. No adjustment for multiple testing was performed. Data processing was performed using STATISTICA data analysis software system (StatSoft Inc., versions 7, 8 and 10).

3.5.2 Study III

Data are presented as mean ± standard error of the mean (SEM). A power calculation based on a previous study [141] indicated that 10 individuals would suffice to detect a 30% difference in FGU at a 5% significance level. Analysis of variance (ANOVA) for repeated measurements was used to analyze change in FBF from baseline. Difference between the different protocols were evaluated by two-way ANOVA. Protein expression differences were assessed by Student´s *t* test. The significance level was set to $P < 0.05$.

3.6 ETHICS

Participants in all studies were informed about the studies before giving informed consent. The investigations were carried out in accordance with the Declaration of Helsinki and were approved by the ethics committee of the Karolinska Institute, Karolinska University Hospital or the regional human ethics review board in Stockholm.

4 RESULTS

4.1 STUDY I

4.1.1 Study subjects

Ninety-four subjects were screened, of which 62 met the inclusion criteria. A total of 14 subjects were excluded from the per-protocol analysis: seven subjects due to non-compliance with the intervention, two subjects due to intercurrent disease and five subjects due to dissatisfaction with the allocated intervention or lack of time to participate. Thus, 48 participants were eligible for the final analysis; exercise only n=17, combined treatment n=14, and acarbose only n=17.

In total, the participants were middle-aged (median 55.5 years) and moderately overweight with a median BMI of 27.7 kg/m^2. Women constituted 27% of the study population, and by chance the sex distribution was uneven between intervention groups, with more women in the combined treated group (43%). Median diabetes duration was three years and 60% were treated with one OAD, almost evenly distributed between metformin and sulfonylurea. One third of the participants had microalbuminuria, but only three subjects had a history of CVD. Treatment for hypertension and dyslipidemia were present in 38% and 15% of the participants, respectively.

Glycemic control included a moderately increased median HbA1c of 54 mmol/mol and blood lipids demonstrated LDL-cholesterol of 3.3 mmol/l and triglyceride levels of 1.5 mmol/l. A selection of basic characteristics, specified per intervention group, are displayed in **Table 3**.

	Exercise	Acarbose+Exercise	Acarbose
N (male/female)	17 (14 / 3)	14 (8 / 6)	17 (13 / 4)
Age (years)	54 (50-58)	57 (52-58)	54 (49-58)
N treatment diet/metformin/SU	6 / 4 / 7	7 / 4 / 3	6 / 8 / 3
N HT/DL treatment	7 / 2	6 / 2	5 / 3
BMI (kg/m^2)	28.7 (25.6-30.3)	28.7 (26.3-29.6)	27.4 (25.7-28.6)
Waist (cm)	99 (95-106)	103 (99-107)	100 (97-104)
Total fat (kg) DXA	21.7 (17.2-26.5)	23.0 (18.8-31.2)	23.0 (21.1-27.0)
Intraabdominal fat area (cm^2)	252 (158-319)	244 (202-292)	227 (173-261)
VO_{2max}/lean weight (ml/min/kg)	42.7 (39.6-47.5)	39.7 (36.2-41.6)	42.3 (40.6-45.0)
Fasting blood glucose (mmol/l)	7.8 (7.5-9.0)	6.7 (5.9-9.5)	7.3 (6.4-7.8)
HbA_{1c} (mmol/mol)	58 (53-63)	51 (42-58)	50 (42-58)
M-value/lean weight (ml/min/kg)	5.1 (3.9-7.2)	6.1 (3.5-9.3)	6.9 (6.3-10.2)
LDL (mmol/l)	3.2 (2.9-3.7)	3.2 (2.8-3.6)	3.5 (3.0-4.0)
Triglycerides (mmol/l)	1,4 (1,2-1,9)	1,4 (0,9-1,8)	1,6 (1,0-2,4)

Table 3. Basal characteristics in the three intervention groups in study I. Values are median (interquartile range). HT; hypertension, DL; dyslipidemia, DXA; dual-energy X-ray absorptiometry.

Compliance with the intervention was equal across intervention groups. Attendance at training sessions was 86 and 84% in the exercise and the combined treated group,

respectively, and compliance with acarbose treatment was 95 and 96% in the acarbose and the combined treated group, respectively.

4.1.2 Within group effects

4.1.2.1 Effects on body composition, physical fitness and glycemic control

The exercise-alone group demonstrated significant changes in body composition with reductions in BMI, waist, total fat and intraabdominal fat-area. VO_{2max} was unchanged but maximal workload was slightly enhanced (P = 0.005). The M-value increased by 92% (P = 0.02), but there was no effect on HbA1c (**Table 4**).

Likewise, the combined-treated group showed significant reduction in BMI, waist and measures of body fat. Both VO_{2max} and maximal workload increased (P = 0.046 and 0.03, respectively) and M-value was augmented by 56% (P=0.002). In contrast to the exercise-alone group, fasting blood glucose and HbA1c were significantly reduced (P = 0.048 and 0.002, respectively) (**Table 4**).

The treatment with acarbose alone had no significant effects on body composition, physical fitness or glycemic control. Fasting proinsulin was reduced significantly (P = 0.009), as well as in the combined-treated group (P= 0.01).

Insulin levels during the clamp investigations (steady-state) did not differ within or between the three different intervention groups.

4.1.2.2 Effects on blood pressure and lipid levels

Physical training alone resulted in a significant decrease in systolic blood pressure (BP) (140 to 135 mmHg, P = 0.01), but not in diastolic BP. Lipid levels were not affected. However, apolipoprotein(apo)B/apoA1 ratio was decreased (P =0.003) (**Table 4**).

The combined treatment resulted in a significant decrease in both systolic and diastolic BP (P = 0.001 and 0.02, respectively). Further, reductions were seen in total cholesterol (5.0 – 4.9 mmol/l, P = 0.04), total triglycerides (1.4 –1.0 mmol/l, P = 0.004), and apoB levels (1.08 – 1.01, P = 0.04). LDL and high density lipoprotein (HDL) cholesterol were unchanged (**Table 4**).

Acarbose treatment alone resulted in significant reductions in systolic and diastolic BP (P = 0.001 for both), as well as a small decrease in plasma HDL level. No other changes in lipid measurements were observed.

	Exercise (n=17)			Acarbose + Exercise (n=14)		
	Before	After	P	Before	After	P
BMI (kg/m^2)	28.7 (25.6-30.3)	28.4 (25.1-29.2)	0.01	28.7 (26.3-29.6)	27.6 (25.7-28.5)	0.004
Waist (cm)	99 (95-106)	99 (92-105)	0.04	103 (99-107)	100 (95-104)	0.008
Total fat (kg) DXA	21.7 (17.2-26.5)	19.3 (15.3-24.6)	0.002	23.0 (18.8-31.2)	20.5 (17.8-29.2)	0.001
Total abdominal fat area (cm^2)	454 (321-504)	406 (358-471)	0.02	455 (353-518)	403 (318-470)	0.002
Intraabdominal fat area (cm^2)	252 (158-319)	258 (130-284)	0.04	244 (202-292)	222 (154-263)	0.009
VO_{2max}/lean weight (ml/min/kg)	42.7 (39.6-47.5)	46.0 (41.5-47.6)	ns	39.7 (36.2-41.6)	44.2 (38.0-46.6)	0.046
Max workload (W)	210 (190-250)	230 (210-270)	0.005	190 (135-230)	190 (150-250)	0.03
Fasting blood glucose (mmol/l)	7.8 (7.5-9.0)	7.4 (6.7-8.5)	ns	6.7 (5.9-9.5)	6.4 (5.7-7.7)	0.048
HbA_{1c} (mmol/mol)	58 (53-63)	53 (49-58)	ns	51 (42-58)	47 (41-51)	0.002
M-value/lean weight (ml/min/kg)	5.1 (3.9-7.2)	9.8 (4.3-10.3)	0.02	6.1 (3.5-9.3)	9.6 (6.7-13.9)	0.002
Fasting proinsulin pmol/l	22.1 (14.9-26.2)	21.2 (15.0-25.1)	ns	20.7 (11.6-26.6)	15.7 (8.6-22.9)	0.01
Systolic BP (mmHg)	140 (135-150)	135 (120-140)	0.01	145 (140-155)	130 (120-135)	0.001
Diastolic BP (mmHg)	83 (75-88)	75 (75-80)	0.16	83 (75-95)	75 (75-80)	0.02
Total cholesterol (mmol/l)	4.8 (4.7-5.3)	4.7 (4.4-5.3)	0.18	5.0 (4.5-5.4)	4.9 (4.3-5.1)	0.04
LDL (mmol/l)	3.2 (2.9-3.7)	3.1 (2.9-3.4)	ns	3.2 (2.8-3.6)	3.2 (2.9-3.4)	ns
HDL (mmol/l)	1.0 (1.0-1.1)	1.0 (0.9-1.0)	ns	1.1 (0.9-1.3)	1.1 (0.9-1.2)	0.09
Triglycerides (mmol/l)	1,4 (1,2-1,9)	1.0 (0.7-1.8)	0.09	1,4 (0,9-1,8)	1.0 (0.7-1.4)	0.004
ApoB	1.11 (1.02-1.20)	1.03 (0.92-1.11)	0.049	1.08 (1.04-1.16)	1.01 (0.92-1.14)	0.04
ApoB/ApoA1 ratio	0.80 (071-0.87)	0.72 (0.61-0.83)	0.003	0.83 (0.64-0.86)	0.77 (0.64-0.88)	ns

Table 4. Within group effects of exercise and combined intervention in study I. Values are median (interquartile range). Wilcoxon signed-rank test was used. P values >0.2 are listed as not significant (ns). DXA; dual-energy X-ray absorptiometry, BP; blood pressure, Apo; apolipoprotein.

4.1.3 Between group effects

In the total study population, the relative decrease in HbA1c was significantly higher in men than in women (P = 0.03). As there was an imbalance in sex distribution across the intervention groups, adjustment for sex by multiple regression analysis was performed. Analyses showed that HbA1c was significantly lowered when acarbose was added to exercise (P = 0.04). Moreover, this regimen resulted in nearly significant reductions in several variables, including fasting plasma glucose (P = 0.07), systolic (P = 0.06) and diastolic (P = 0.07) BP, total fat area (P = 0.08) and apoB/apoA1 ratio (P = 0.09).

4.2 STUDY II

4.2.1 Study subjects

This study was designed to evaluate exercise training. Thus, subjects in study I randomized to intervention with exercise training, alone or in combination with acarbose, were qualified to participate. Of these subjects, 19 were randomized for muscle biopsy; n=10 on exercise training alone and n=9 on the combined intervention with exercise and acarbose. In total 16 subjects, 4 women and 12 men, completed the study per-protocol. Three participants, all men in the combination group were excluded from the analysis: two due to noncompliance and one that withdrew at will from the study. Basic characteristics and pre- and post-intervention findings are presented in **Table 5**.

4.2.2 Effects of intervention

Most clinical features related to T2D were improved by the intervention (**Table 5**). The two different intervention groups only differed in respect to a lower VO_{2max} at baseline in the combined treated group (P = 0.02). Otherwise, no significant differences between the intervention groups were seen at baseline or in relative change at the study end.

In contrast to the clinical variables, there was no significant change in expression of any of the genes studied by the intervention.

N (women/men)	16	(4/12)			
Age (yrs)	56	(51-58)			
Diabetes duration (yrs)	4.0	(2.0-5.5)			
N pre-study treatment – diet only/MET/SU	5/6/5				
	Before		**After**		**P**
Body mass index (kg/m^2)	28.3	(25.9-29.6)	27.5	(25.7-28.8)	0.03
Waist (cm)	99	(96-105)	99	(93-101)	0.01
Total fat DXA (%)	24.8	(20.9-31.4)	23.6	(20.4-29.8)	0.02
VO_{2max}/lean weight (ml min^{-1} kg^{-1})	40.7	(39.6-47.3)	46.3	(40.5-48.6)	0.005
HbA1c (mmol/mol)	57	(49-62)	51	(46-55)	0.001
Fasting plasma glucose (mmol/l)	8.4	(7.2-10.0)	8.2	(6.8-8.8)	0.07
M/lean weight (mg min^{-1} kg^{-1})	5.7	(4.2-7.5)	9.8	(7.2-10.9)	0.004
M/I (100 x mg min^{-1} kg^{-1} pmol^{-1} l^{-1})	1.38	(0.91-1.68)	2.35	(1.68-2.92)	0.004

Table 5. Basic characteristics and pre- and post-intervention findings in study II. Data are expressed as median (interquartile range). Wilcoxon signed-rank test was used. MET; metformin, SU; sulfonyl-urea, DXA; dual-energy X-ray absorptiometry, M/I; insulin sensitivity index.

	Beta	CI[a] for beta	P	Adj. R^{2b}
VEGF				0.52
M/I	0.69	0.27 – 1.11	0.004	
HbA1c	-0.46	-0.88 – (-0.04)	0.03	
Acarbose treatment	0.19	-0.24 – 0.63	0.4	
VEGF receptor-1				0.48
M/I	0.48	0.04 – 0.92	0.03	
HbA1c	-0.63	-1.07 – (-0.20)	0.009	
Acarbose treatment	0.15	-0.30 – 0.60	0.5	
PGC-1α				0.34
M/I	0.40	-0.11 – 0.91	0.11	
Acarbose treatment	0.51	0.005 – 1.01	0.048	
Female sex	-0.42	-0.92 – 0.09	0.09	

Table 6. Associations by multiple regression analyses in stud II. Relative changes in variables were used and continuous variables were log-transformed prior to analysis. Best subset according to Mallow's Cp was applied. [a]95% confidence interval. [b]Adjusted R^2 for whole model. CI; confidence interval, VEGF; vascular endothelial growth factor, M/I; insulin sensitivity index, PGC-1α; peroxisome proliferator–activated receptor γ coactivator-1α.

4.2.3 Associations by multiple regression analysis

Adjusted results by multiple regression revealed that the relative change in VEGF expression was positively associated with a relative increase in M/I (p = 0.004). In addition, a negative

association was found with relative change in HbA1c ($p = 0.03$). The same pattern was demonstrated in VEGF receptor-1 expression, which was positively correlated with an increase in M/I ($p = 0.03$) and a decrease in HbA1c ($p = 0.009$).

In contrast, change in PGC-1α expression showed no significant association with M/I ($p = 0.11$), and inclusion of HbA1c impaired these regression models.

No associations between gene expressions and VO_{2max} were found. Furthermore, change in expression of the other genes analyzed, i.e. VEGF receptor-2, Angiopoeitin 1 and 2, did not show any significant associations with the outcome variables. Acarbose therapy, that was forced into the models, differed to exercise alone only in the aspect of a larger relative increase in PGC-1α ($p = 0.048$).

Data for the significant associations found by multiple regression is shown in **Table 6**.

4.3 STUDY III

4.3.1 Study subjects

Eleven male subjects were recruited for the in vivo study. Mean age was 61 (± 3) years and subjects were overweight with a BMI of 28.4 (±1.6) kg/m^2. Six subjects had hypertension and two had suffered prior myocardial infarction. Fasting plasma glucose and HbA1c were 5.5 (±0.1) mmol/l and 38 (±2) mmol/mol, respectively. One subject did not undergo protocol B, as he withdrew at will after the second investigation.

For the in vitro study, eight subjects contributed with muscle biopsy material during elective surgery. They were free of metabolic diseases.

4.3.2 Glucose uptake

4.3.2.1 Protocol A

Infusion of ET receptor antagonists increased FGU from 5.1 (±1.0) at baseline to 8.3 (±1.1) µmol/min x 1000 ml ($P < 0.05$) at 60 minutes (**Figure 4, panel A**). The addition of insulin increased glucose uptake additionally to 15.7 (±1.8) µmol/min x 1000 ml ($P < 0.001$).

4.3.2.2 Protocol B

By insulin infusion, FGU was increased from 4.1 (±1.1) at baseline to 12.3 (±1.6) µmol/min x 1000 ml ($P<0.001$) at 60 minutes, when steady-state was attained. The FGU reached by dual ET-1 blockade in combination with insulin (protocol A) was significantly higher than insulin alone (protocol B) (**Figure 4, panel B**).

4.3.2.3 Protocol C

Infusion of insulin in protocol C increased FGU from 7.1 (±0.7) at baseline to 17.8 (±3.0) µmol/min x 1000 ml. The addition of ET-1 infusion did not influence FGU (16.5 (±2.0)

µmol/min x 1000 ml). Moreover, prolongation of the infusion with ET-1 for 15 min did not have any additional effect.

Neither basal plasma insulin levels, nor concentrations reached during insulin infusion, differed between the protocols.

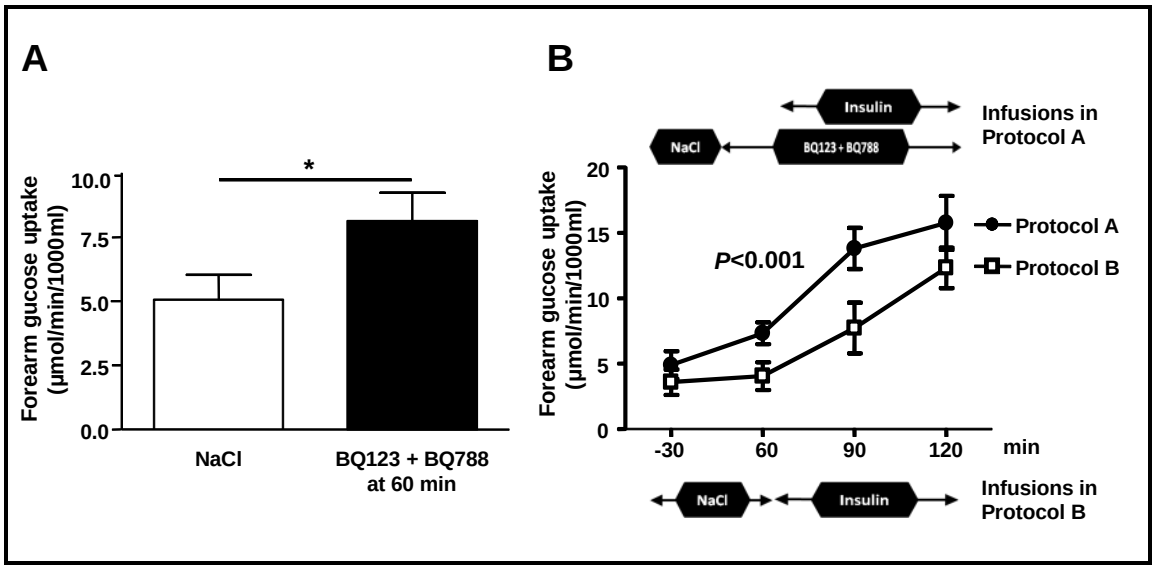


Figure 4. ***Panel A:*** FGU during NaCl infusion and after 60 min of ET_A/ET_B infusion in protocol A. *P<0.05 by Student´s *t* test. ***Panel B:*** FGU comparison of protocol A (ET_A/ET_B infusion without and with insulin co-infusion) and protocol B (insulin infusion alone). Two-way ANOVA including all time points was used. Data are means ± SEM (n=10). *Reprinted with the kind permission of A. Shemyakin.*

4.3.3 Blood flow

Basal FBF was similar in the three protocols.

4.3.3.1 Protocol A

FBF was increased by 30% with ET receptor blockade infusion (P<0.05). A further increase by 16% was measured when insulin was co-infused (P<0.05).

4.3.3.2 Protocol B

Insulin infusion alone did not show any impact on FBF.

4.3.3.3 Protocol C

ET-1 infusion reduced FBF by 38% when added to insulin infusion.

4.3.4 Glucose uptake in cultured cells

Incubation with ET-1 reduced both basal (P <0.05) and insulin-stimulated (P <0.01) glucose uptake. This effect could completely be counteracted by co-incubation with bosentan.

4.3.5 Insulin signaling in cultured cells

There was a non-significant trend of augmented insulin-stimulated phosphorylation of IRS-1 (P=0.13) by ET-1. This trend was inhibited by bosentan. ET-1 did not affect phosphorylation of Akt, AMP-kinase or ERK. Akt expression was not affected, neither was mRNA expression of GLUT1 and GLUT4 changed after ET-1 incubation.

4.4 STUDY IV

4.4.1 Study subjects

Among the 68 subjects screened for eligibility, 23 did not meet inclusion/exclusion criteria and one subject declined to participate. Of the 44 subjects randomized, one subject in the vitamin D group was excluded from the study due to initiation of oral corticosteroid treatment. Consequently, 43 subjects were available for the intention-to-treat analysis.

Basic characteristics of the study subjects are shown in **Table 7**. In total, the study population had a balanced sex distribution (47% females) and the median age was 67.3 years. BMI was 28.5 kg/m^2 and waist circumference 101 cm. Altogether, 58% of the study population was treated for hypertension and 35% for dyslipidemia. HbA1c was similar in the two groups: vitamin D group 43 (38–44) mmol/mol and placebo group 44 (42–46) mmol/mol, respectively. Season-adjusted 25(OH)D levels were also similar, 43 (36–50) and 43 (37–54) nmol/l respectively, in the vitamin D and placebo groups. Of all participants, 32 had prediabetes and 12 subjects were categorized as having diabetes; five randomized to vitamin and seven to placebo. Men had a slightly higher baseline 25(OH)D level than women, otherwise there were no significant sex differences.

4.4.2 Effects of vitamin D on outcomes

Main effects of vitamin D and placebo on study outcomes are presented in **Table 8**. The adjusted 25(OH)D level was doubled in the vitamin D group +42 (32-50) nmol/l, but remained unchanged in the placebo group. Concerning the primary end point of first-phase insulin secretion, there was a numerical increase in both groups; a tendency in the vitamin D group (+26%, P = 0.06) and significant increase in the placebo group (+33%, P = 0.02), with no difference between the two groups (P = 0.4). No changes in second-phase insulin secretion or insulin sensitivity, either within or between the groups were demonstrated. First-phase disposition index (DI) increased in both groups, again with no group difference in concordance with first-phase insulin secretion. In the vitamin D group, there was a tendency toward an increase in second-phase DI (P = 0.06), but with no statistical difference compared with placebo (P = 0.9).

HbA1c demonstrated a tendency toward a small reduction in the vitamin D group of -1 (-3 to 1) mmol/mol (P = 0.06), but with no significant difference versus placebo (P = 0.8). Other assessments of glycemia were unaltered. Glycemic tolerance category improved slightly in both groups, with no group difference.

	Overall		Vitamin D		Placebo	
N (females/males)	43	(20/23)	21	(9/12)	22	(11/11)
Age (years)	67.3	(64.0–68.5)	67.6	(63.4–68.8)	67.0	(64.7–68.5)
N IFG/IGT/IFG+IGT/Diab[a]	10/10/11/12		7/5/4/5		3/5/7/7	
N hypertension/dyslipidemia	25/15		12/5		13/10	
BMI (kg/m^2)	28.5	(25.7–29.8)	28.3	(24.5–29.4)	28.6	(26.4–29.9)
Waist (cm)	101	(95–107)	100	(94–106)	101	(95–112)
HbA1c (mmol/mol)	44	(41–45)	43	(38–44)	44	(42–46)
Fasting p-glucose (mmol/l)	6.3	(6.0–6.6)	6.3	(5.5–6.6)	6.3	(6.1–6.6)
2h p-glucose OGTT (mmol/l)	9.4	(7.5–10.3)	9.3	(7.1–9.7)	9.7	(8.2–10.3)
25(OH)D (nmol/l)	47	(36–55)	42	(35–55)	47	(42–53)
25(OH)D (nmol/l) adjusted[b]	43	(36–54)	43	(36–50)	43	(37–54)
PTH ng/l	59	(46–67)	62	(50–72)	55	(42–63)
Triglycerides (mmol/l)	1.3	(0.9–1.8)	1.3	(0.9–1.6)	1.4	(1.0–2.2)
LDL (mmol/l)	3.3	(2.7–3.8)	3.0	(2.7–3.8)	3.5	(3.1–3.8)
Step count/day	6300	(4359–8898)	5645	(4277–8316)	7099	(4414–9376)

Tabel 7. Basic characteristics in study IV. Continuous data are medians (interquartile range). [a]Glycemic tolerance category, [b]season-adjusted. IFG; impaired fasting glucose, IGT; impaired glucose tolerance, BMI; body mass index, OGTT: oral glucose tolerance test, PTH: parathyroid hormone, LDL; low-density lipoprotein.

The vitamin D group displayed a small increase in fat mass percent, a change being significant in group comparison. PTH was reduced by 13% in the vitamin D group, a tendency trend when the groups were compared (P = 0.07). Triglycerides decreased nearly significantly in the placebo group, this change being significant versus the vitamin D treated group (P = 0.02).

	Vitamin D				Placebo				Between group
	Baseline		Δ Study end	P^a	Baseline		Δ Study end	P^a	P^b
25(OH)D (nmol/l) adjusted[c]	43	(36–50)	+42 (32–50)	<0.001	43	(37–54)	0 (-7–11)	0.5	<0.001
$ISec_{0–12}$ ($mU·l^{-1}·min$)	168	(122–231)	+44 (-3–63)	0.06	137	(51–363)	+45 (-5–136)	0.02	0.4
$ISec_{12–120}$ ($mU·l^{-1}·min$)	5689	(2865–8118)	+547 (-1053–960)	0.79	4509	(3000–6508)	+56 (-934–1006)	0.5	0.8
GIR/I ($mg·min^{-1}·kg^{-1}·mU^{-1}·l·100$)	6.7	(4.7–11.4)	+0.3 (-0.3–1.4)	0.09	5.9	(4.6–9.7)	+0.5 (-0.5–1.8)	0.3	0.6
$DI_{0–12}$ ($mg·kg^{-1}·100$)	1449	(986–1922)	+512 (64–1082)	0.005	1113	(389–2324)	+260 (-7–963)	0.006	0.7
$DI_{12–120}$ ($mg·kg^{-1}·100$)	34464	(31500–42144)	+4608 (-2345–12360)	0.06	29157	(22429–40285)	+4210 (-4457–12524)	0.2	0.9
HbA1c (mmol/mol)	43	(38–44)	-1 (-3–1)	0.06	44	(42–46)	-1 (-3–1)	0.11	0.8
Fasting p-glucose	6.3	(5.5–6.6)	-0.1 (-0.5–0.4)	0.6	6.3	(6.1–6.6)	0.0 (-0.3–0.4)	0.9	0.8
2h p-glucose OGTT (mmol/l)	9.3	(7.1–9.7)	-0.5 (-1.4–1.0)	1.0	9.7	(8.2–10.3)	-0.9 (-1.5–1.8)	0.9	0.6

Tabel 8. Effects of vitamin D in study IV. Continuous data are medians (interquartile range). [a]Wilcoxon matched pairs test, [b]Mann–Whitney U test on relative changes, [c]season-adjusted. 25(OH)D; 25-OH-vitamin D_3, ISec0–12; first-phase insulin secretion, ISec12–120; second-phase insulin secretion, GIR/I; glucose infusion rate/mean insulin level, DI; disposition index, OGTT; oral glucose tolerance test.

4.4.3 Ancillary analyses in the vitamin D group

We observed a tendency that basal 25(OH)D levels were negatively correlated to the change in 25(OH) D at study-end (r = -0.38, P = 0.09) (**Figure 5, panel A**). To evaluate if low basal 25(OH) D levels predicted benefit in terms of first-phase insulin secretion, we performed a correlation of change in 25(OH)D and fold change in the outcome. No such association could be demonstrated (r = - 0.38, P = 0.34) (**Figure 5, panel B**). Further exploration of the data, where the vitamin D group were divided in high or low basal 25(OH)D levels (limit set to 50 nmol/l) could not reveal any association by this partition on the change in first-phase insulin secretion (P = 0.74) (**Figure 5, panel C**). Finally, no association between response on vitamin D treatment and 25(OH)D level at study-end could be found (**Figure 5, panel D**).

Moreover, there were no associations found between sex and any of the outcomes, and omitting subjects with diabetes did not alter the results.

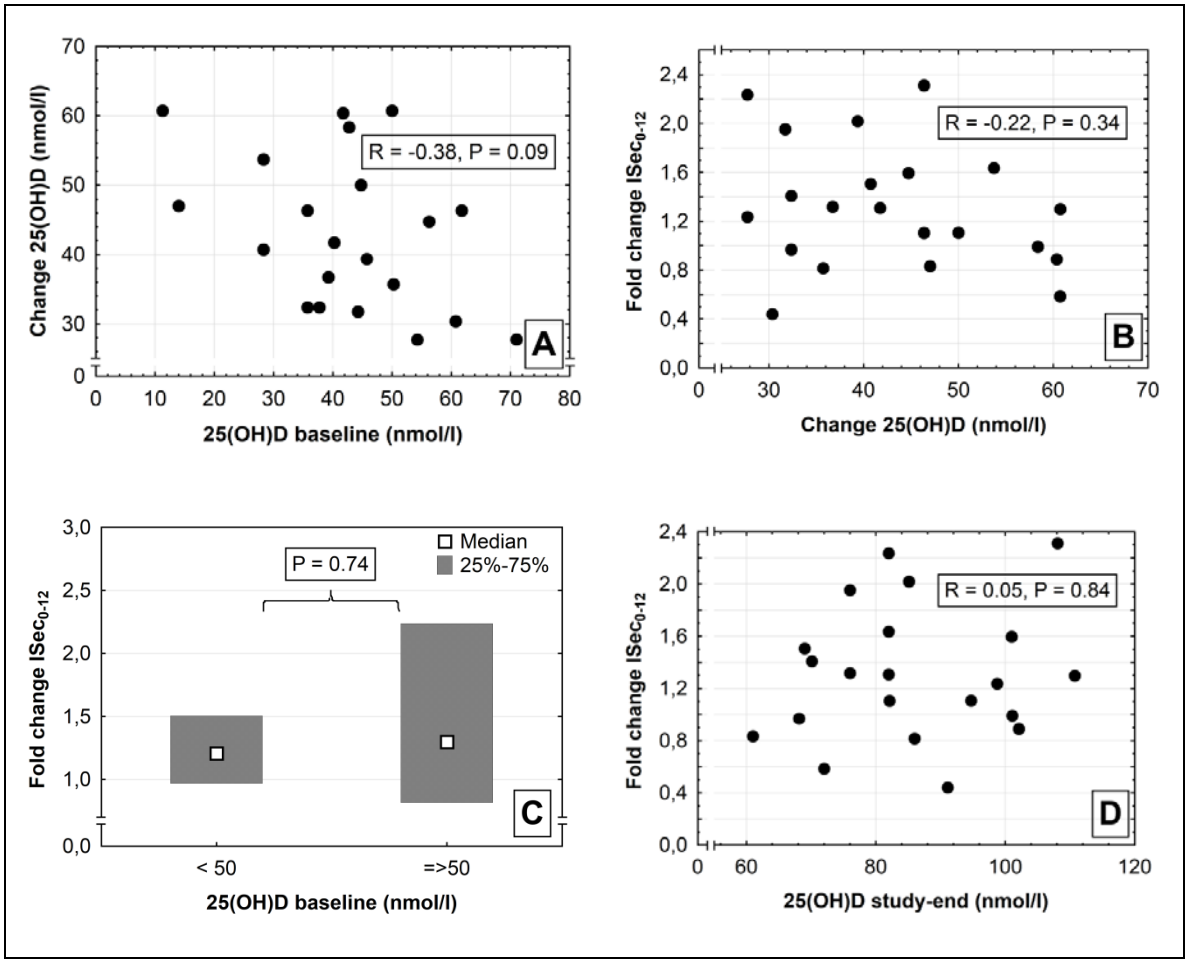


Figure 5, panel A–D. Ancillary analyses in the vitamin D group only. R and P values for correlations were calculated by Spearman rank R test. Mann-Whitney U test was used for group comparison (panel C). 25(OH)D; 25-hydroxy-vitamin D_3, ISec0–12; first-phase insulin secretion.

5 DISCUSSION

5.1 STUDY I

The main findings of the study were that in subjects with T2D and mild hyperglycemia, twelve weeks of exercise training augmented insulin sensitivity, and improved both body composition and blood pressure. Of note, there was no significant effect on glycemic control. Importantly, when exercise and acarbose were combined, glycemic control was significantly improved, and probably the overall CV risk factor profile. Acarbose treatment alone improved blood pressure and decreased the plasma proinsulin levels, considered a marker of diminished β-cell stress and improved β-cell function.

Studies on exercise in T2D have often been rather small, including our study, due to the resources demanded for implementation. Consequently, meta-analyses have been performed, demonstrating numerically similar effects on glycemic control as in our study [85, 196]. In the study of Boule et al., studies with a higher basal HbA1c tended to show greater improvements in glycemic control. Our study participants had a fairly mildly elevated Hba1c of 54 mmol/mol, making it more difficult to reach an improvement with exercise alone, in spite of a marked improvement of 92% in insulin sensitivity. The mode of exercise of moderate to high intensity was well tolerated in our sedentary middle-aged subjects. A higher intensity training might have attained more pronounced effects [91], but might have induced adverse events in our older study population.

We observed a reduction in plasma proinsulin levels during acarbose treatment, but no effect on insulin sensitivity. Theoretically, treatment with acarbose could improve both β-cell function and insulin sensitivity by reducing glucose toxicity. However, studies have yielded conflicting results. Some evidence points out that the compound could decrease β-cell stress, measured as proinsulin or proinsulin/insulin ratio [197-199]. However, short term acarbose treatment in older people with IGT did not improve β-cell function [200]. Further, in a study on IGT subjects, acarbose improved insulin sensitivity, measured as steady-state plasma glucose during an insulin suppression test [201]. In elderly T2D patients acarbose did not affect insulin release, but improved insulin sensitivity measured by hyperglycemic clamp [202]. On the other hand, in a study in patients with long standing diabetes, acarbose did not improve insulin sensitivity [203].

Efforts should be made to optimize CV risk factors, including hyperglycemia, and combinations of different treatment modalities will often be necessary. Acarbose, as an addition to exercise, was chosen since it does not pose any increased risk per se for hypoglycemia. Further, there is some evidence supporting CV benefits in subjects with IGT and T2D [75, 76], although there has been criticisms about the methods of analysis [77]. It is hypothesized that acarbose, by reducing post-prandial glucose excursions, could reduce the oxidative stress [79] thought to be one of the main components driving the development of diabetes complications [48]. Gastrointestinal side effects are common with acarbose treatment, but did not cause any premature discontinuation in our study. We used a quite

rapid dose titration scheme, which has been replaced by a slower scheme in updated recommendations [204]. Interestingly, the results of a large trial on CV outcomes are awaited in 2018 (Acarbose Cardiovascular Evaluation Trial, ClinicalTrials.gov identifier: NCT 00829660).

The strengths of the study comprise in particular the golden standard method used for assessment of insulin sensitivity (euglycemic clamp), and the stringent control of the exercise intensity. In group comparisons on cardiovascular risk factors, where the effects of the addition of acarbose to exercise were evaluated, there were only tendencies in several variables, except for the significant effect on glycemic control. However, since most of these factors did change in a favorable direction, it is fair to conclude that the CV risk factor profile probably was improved with this mode of intervention. The study population is regarded as fairly representative of the total diabetes population, based on register data demonstrating that 50% were treated with diet or OAD and had HbA1c <57 mmol/mol in Sweden at the time when the study was performed [205].

5.2 STUDY II

This study demonstrated, in subjects with T2D, that the variation in response to physical training, measured as inulin sensitivity and glycemic control, was associated with VEGF mRNA expression in muscle. This could constitute a mechanism that contributes to the known variation in the individual adaptation to exercise. Supportive of our findings are also the association between VEGF receptor 1 expression and the outcomes. This receptor is mainly expressed on endothelial cells and participates in mediating the effects of VEGF in muscle [108].

The changes in gene expression of VEGF and its receptor 1 were associated to change in HbA1c, statistically independent of insulin sensitivity. This could infer a positive effect on β-cell function by exercise training. There are some data supporting this theory in sedentary overweight subjects, showing an effect of moderate- to vigorous-intensity training, on disposition index during an IVGTT [87].

In addition to defects in insulin signaling, the vascular component is probably the other major factor behind IR. The number of capillaries is positively correlated to insulin action in muscle [31]. This was distinctively shown in mice, where a muscle specific deletion of VEGF resulted in a 60% reduction in capillary density, and as a consequence a 40-45% reduction in insulin-stimulated glucose uptake in muscle [112].

Adding complexity to the underlying mechanisms are the vasoactive properties of insulin, including vasodilatation and capillary recruitment. This augments the delivery of insulin and glucose to the muscle cells, which in turn increase the insulin-mediated glucose uptake [33, 206]. It has been proposed that regular exercise improves “vascular fitness” and improves capillary recruitment by insulin; this being one mechanism by which insulin sensitivity is increased by exercise [206].

We could not find any associations between PGC-1α expression and the degree of response to exercise. PGC-1α has mainly been connected to mitochondrial biogenesis, but is also thought to be involved in numerous additional adaptive processes [118, 120]. Moreover, in mice there are indications that this factor is involved in the regulation of VEGF expression by physical training, mediated by the nuclear receptor ERRα [122]. However, in our clinical study in humans, we could not detect any impact in physiological adaptation.

The method of assessing response to exercise gives the possibility to detect significant changes in factors, that would not have been noted at the group level. In this study, the changes in gene expression by the intervention were not significant when assessing the group in total, but became apparent when response was accounted for.

The number of subjects investigated in the present study was rather small, which could create a concern about power. Further, no endpoint effect such as microvascular blood flow or capillary density was assessed, which would be desirable in future studies. On the other hand, the associations found were statistically strong and advanced techniques were used for measurement of the outcomes. We therefore regard that the findings are interesting and warrant further research in this area.

5.3 STUDY III

The main finding of this study was that dual ET_A/ET_B blockade increased glucose uptake in skeletal muscle, both at the basal and the insulin-stimulated state, in insulin resistant subjects. This result supports that endogenous ET-1 is important in regulating muscle glucose uptake. Moreover, ET-1 inhibited glucose uptake in cultured skeletal muscle cells by a receptor dependent mechanism, indicating a direct impact on muscle cells by ET-1.

The co-infusion of ET_A/ET_B blockade and insulin created a more pronounced glucose uptake than insulin alone, which is concurrent with previous studies [138, 141]. Additionally, we showed that dual blockade enhanced glucose uptake per se. Thus, blockade of the ET-1 system in subjects with IR augments basal, and facilitates insulin-stimulated, glucose uptake.

In contrast, infused exogenous ET-1 did not affect insulin-stimulated glucose uptake. This is in opposition to earlier findings in healthy individuals [131, 132]. An important difference is that we investigated subjects with IR. The ET-1 system has been shown to be activated in IR and related conditions [129, 139, 140], and consequently exogenous ET-1 might be of minor importance in these states.

ET-1 demonstrated direct effects on glucose uptake in cultured muscle cells. This finding has also been seen in adipocytes [146]. This supports that the influence of the ET-1 system on glucose metabolism is beyond that of effects on blood flow alone. The inhibitory impact of ET-1 on glucose uptake was entirely reversed by dual receptor blockade, which was in conformity with the in vivo results of increased glucose uptake with ET_A/ET_B blockade infusion.

The in vitro investigation did not demonstrate any effect on IRS-1 or Akt phosphorylation, in contrast to previous studies [130, 145]. Further, we could not detect any impact of ET-1 on GLUT1 or GLUT4 expression. However, we did not investigate other important mechanisms in glucose transport, such as vesicular trafficking or membrane translocation. Therefore, it is possible that ET-1 could affect these cellular phenomena.

An important observation is that insulin infusion alone did not change FBF. Of note, when insulin was added to ET_A/ET_B receptor blockade, an increase in blood flow was seen. An explanation could be that the blockade restores insulin-dependent vasodilatation in IR, which would be in agreement with earlier findings [143, 150].

There are limitations in our study. First, we did not investigate ET-1 infusion without co-infusion with insulin. Thus, we cannot make any conclusions of the impact of ET-1 on basal FBF or FGU in the study subjects. Second, there was no control group of insulin sensitive individuals. The reason was the findings in a previous study, where dual blockade could not provoke any effect on vasodilatation in healthy individuals, which was in contrast to IR subjects [140]. And finally, w only investigated the effect of dual ET_A/ET_B receptor blockade, and not single ET-1 blockade. We chose this study design since previous studies on similar study subjects demonstrated favorable outcomes only after dual blockade [140, 141]. In a recent head-to-head study in subjects with T2D and coronary artery disease, the two modalities of ET blockade were compared. ET_A and dual receptor blockade improved endothelium-dependent vasodilatation to the same extent. Additionally, the dual blockade increased basal FBF, but ET_A-blockade did not [207].

5.4 STUDY IV

This study demonstrated no effect of high-dose vitamin D_3 treatment for eight weeks, in comparison with placebo, on first-phase insulin secretion in individuals with mildly abnormal glucose regulation. Neither could we detect any effects on second-phase insulin secretion, DI or in various measures of glycemic control.

The findings are in analogy with some recent intervention trials. The effect of high-dose (mean weekly dose 88 865 IU) vitamin D_3 intervention for one year was evaluated in pre-diabetic subjects with low 25(OH)D levels at baseline (55 nmol/l). No effects could be shown on glucose tolerance, nor on insulin secretion or insulin sensitivity with several different indices, including OGTT-based calculations. A small but significant difference in HbA1c of 2 mmol/mol, in favor of vitamin D treatment, was however present at the end of the study [208]. In addition, 20 000 IU vitamin D_3 weekly for one year in pre-diabetic individuals did not improve glycemia, nor HOMA or QUICKI indices in assessment of insulin resistance. Analyzing a sub-group with baseline 25(OH) D levels <50 nmol/l gave the same result [209]. This study will continue for a total study period of five years. In an Australian study on subjects at risk of T2D and with low vitamin D levels, calcium and target-based (25(OH)D >75 nmol/l) vitamin D_3 treatment for 6 months failed to affect different OGTT-based measures of insulin resistance or β-cell function. A post-hoc analysis in the ~50% of the

study subjects with pre-diabetes revealed a positive impact on insulin resistance measured by HOMA and Matsuda indices [210]. Moreover, giving 50 000 IU vitamin D_3 monthly to T2D subjects for six months did not improve HbA1c or other glycemic parameters, compared to placebo. Moreover, HOMA index of insulin resistance and β-cell function were unaltered. Worth considering was a small but significant decrease in HbA1c in severely vitamin D-deficient subjects (25(OH) D <30 nmol/l). However, this subgroup was very small [211]. Finally, a study with a very similar design as ours, but evaluating a vitamin D intervention of 24 weeks with OGTT-based measures, was recently accepted for publication. The outcome results were also in line with our findings, with no impact on glycemic control, β-cell function or insulin sensitivity during vitamin D treatment compared to placebo [212].

In opposition, intervention with 2000 IU vitamin D_3 for 16 weeks in subjects at risk for T2D improved first-phase insulin secretion and DI, assessed by a frequently-sampled IVGTT. There was a trend toward lower HbA1c level in the vitamin D-treated group compared to placebo. The trial also evaluated the effect of calcium supplementation, which had no impact on the outcomes [213].

In our ancillary analyses, we could not detect that either a low basal vitamin D level, or a large elevation of the vitamin D level by the treatment, were associated with the change in first-phase insulin secretion. Moreover, the impact of a possible threshold level was evaluated. The presence of a threshold level at 65 nmol/l in 25(OH)D has been suggested for glucose tolerance and IR [186]. However, the study-end level of 25(OH)D was not related to change in first-phase insulin secretion in our study, despite the fact that all but two subjects crossed this limit in the vitamin D treated group.

Limitations of our study include a rather small sample size. In planning the study, no adequate data were found that could constitute the basis of a power calculation. Moreover, the intervention period of eight weeks could be regarded as rather short to fully investigate the effects of vitamin D. On the other hand, study drop-out was minimal (one subject) and the risk of a change in lifestyle affecting our outcomes was low. A decrease in PTH were observed in the vitamin D group, which could be considered a surrogate for a metabolic effect of the vitamin D treatment. The strengths include the golden standard hyperglycemic clamp, which to our knowledge has not been used to investigate this area before.

Taken together, it is unlikely that vitamin D treatment has any substantial effect on glycemic control in pre-diabetes or T2D. It is plausible that vitamin D levels constitute a confounder in the fairly consistent observational findings of an association with T2D. Interestingly, in a Swedish prospective cohort study on individuals with T2D, low baseline 25(OH)D levels in men remained related to a higher all-cause mortality after adjusting for several CV risk factors, including HbA1c. In women, high PTH levels, but not vitamin D levels, showed an association with total mortality [214].

It is not entirely excluded that vitamin D intervention for a longer period of time might have a small impact on glycemic control, or could prevent T2D development in subjects at risk.

Interestingly, results of a study are awaited in 2017 that hopefully can elucidate these remaining questions [215].

6 CONCLUSIONS

Exercise training improved insulin sensitivity and body composition in mild T2D. With the addition of acarbose treatment, improved glycemic control was also attained. The general cardiovascular risk factor profile was probably improved more with the combination therapy than with exercise alone.

The individual response to exercise training in terms of improved insulin sensitivity was associated to change in VEGF expression. Moreover, decrease in HbA1c also showed this association. The individual adaptation to physical exercise can in part be dependent on VEGF expression, adding a potential target to influence IR.

ET-1 infusion decreased blood flow but had no effect on glucose uptake. Dual ET_A/ET_B blockade increased glucose uptake, both with and without inulin stimulation, and increased blood flow. In cell cultures, ET-1 impaired glucose uptake, an effect that could be counteracted by ET receptor blockade. This receptor-dependent mechanism by ET-1 on glucose metabolism could constitute a possible drug target.

Intervention with vitamin D had no effect on β-cell function, insulin sensitivity or glycemic control. Our findings do not support treatment with vitamin D in subjects with pre-diabetes or type 2 diabetes, neither in those with low vitamin D levels.

7 FUTURE PERSPECTIVES

Exercise training is obviously a powerful treatment option T2D. In this era of personalized medicine, it would be an importat advantage to be able to predict which individuals that would benefit the most by exercise intervention. Then great efforts should be made to provide this probably highly cost-effective treatment to these patients.

Acarbose has not been used frequently in diabetes treatment in Sweden. I believe that the criticism of the data on CV protection had an impact. But there are some promising findings with the compound, and if the awaited outcome study will show benefits with the treatment, more mechanistic studies on especially post-prandial hyperglycemia seem warranted. Moreover, acarbose could be an alternative in other group of patients, such as type 1 diabetes and diabetes in pregnancy.

The vascular contribution in IR is interesting. This could constitute possible drug targets, not the least since there is a lack in pharmacological options in affecting IR. The use of bosentan has been limited due to adverse effects, especially fluid retention. If other compounds can be developed, this would consequently be of interest for clinical trials.

The 'training pill' is still awaited. Noteworthy, reservatrol (a substance found in red wine) has shown to have some promising effects on IR, perhaps mediated by an effect on AMP-kinase,

PGC-1α and mitochondrial function [216]. If a compound with low toxicity could be found to influence these pathways, this would have a huge impact diabetes treatment.

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