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Impact of autonomic nervous system activity and interscapular fat on human glucose metabolism

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Vosseler, Andreas

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Dekan: Professor Dr. B. Pichler

1. Berichterstatter: Professor Dr. M. Heni
2. Berichterstatter: Professor Dr. Dr. P. Ruth

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Abbreviations

AUC	area under the curve
BAT	brown adipose tissue
bpm	beats / breaths per minute
BR	breathing rate
DAkkS	Deutsche Akkreditierungsstelle
DI	disposition index
DZD	Deutsches Zentrum für Diabetesforschung
ECG	electro cardiography / cardiogram
e.g.	exempli gratia
et al.	et altera
F	degree of freedom
fig.	figure
HF	high frequency
HR	heart rate
HRV	heart rate variability
HZ	Hertz
i.e.	id est
ICcE	individual calibration control evaluation
IFG	impaired fasting glucose
IGT	impaired glucose tolerance
ISI	insulin sensitivity index
LF	low frequency
mg/dL	milligram per deciliter
min	minute
MRI	magnetic resonance imaging
MRT	Magnetresonanz-Tomographie
ms	millisecond
mv	millivolt
n	number
NEFA	non-esterified fatty acids
ng/dL	nanogram per deciliter

NGT	normal glucose tolerance
OGTT	oral glucose tolerance test
p	probability value
pmol/l	picomol per liter
RESPI	Effekte der Modulation des autonomen Ne**R**vensyst**E**ms durch Atmung**S**manöver auf die **P**ostprandiale Stoffwechselregulat**I**on beim Menschen
RMSSD	root mean square of successive differences
RQ	Respiratory quotient
SCAT	subcutaneous adipose tissue
SD	standard deviation
SDB	slow deep breathing
SE	standard error
SGLT2	Sodium glucose cotransporter 2
taVNS	transcutaneous auricular vagus nerve stimulation
VAT	visceral adipose tissue
VNS	vagus nerve stimulation
WAT	white adipose tissue
WHO	World Health Organization (WHO)
z.B.	zum Beispiel

1 Introduction

In 2021, the world was celebrating the 100th anniversary of the discovery of insulin. Since the groundbreaking findings of Frederick Banting and Charles Best, insulin has saved millions of lives and its pharmaceutical journey is still ongoing. In former times, when insulin therapy was in its infancy, diabetes was not a widespread disease yet. So far, the prevalence of diabetes mellitus has increased within a few decades and its incidence is still rising. By now, diabetes mellitus has become a pandemic disease with more than 460 million affected people around the world (Saeedi et al., 2019).
Diabetes is not only associated with economic burdens that challenge the health systems of all countries, but also causes co-morbidities and multiple severe complications for people with diabetes, often resulting in a dramatic loss of life expectation (Saeedi et al., 2019).

The World Health Organization (WHO) subsumes different types of diabetes mellitus in its classification (Irvine, 1977) that includes type 1 and type 2 diabetes, other specific types as well as gestational diabetes. Recently, a more detailed and pathophysiology-based approach for the classification of common diabetes has been proposed (Ahlqvist et al., 2018). The authors defined five clusters of diabetes patients with significant differences in pathophysiological aspects as well as associations with complications. Of note, these clusters consider the major pathogenetic determinants of diabetes, namely insulin secretion and insulin sensitivity. However, this novel concept still has to be tested for its prognostic and therapy-guiding value in appropriate prospective trials and has therefore not yet been implemented in clinical routine care.

Of all classical diabetes types, type 2 diabetes is the most common accounting for around 90 % of all diabetes cases in the world (Saeedi et al., 2019). Though, the pathogenesis of type 2 diabetes is not fully understood, there is a strong association with environmental triggers as obesity, lack of physical activity, high-caloric diet, increasing age and ethnicity, as well as genetic and epigenetic predisposition (DeFronzo et al., 2015).

However, type 2 diabetes is a heterogeneous disease with different pathophysiological phenotypes and different trajectories towards complications and success of treatment (Tuomi et al., 2014).

1.1 Pathogenetic determinants of type 2 diabetes mellitus

In the early pathogenesis of type 2 diabetes, reduced insulin sensitivity does not result in impaired glucose tolerance due to compensatory increased insulin secretion from the pancreatic beta cells that is sufficient to compensate the reduced insulin action (DeFronzo, 2004). Thus, insulin resistance in muscle, liver and adipose tissues is attended by upregulated insulin secretion. However, the increase of insulin secretion can cause a depletion of beta cell function over time, with consecutively impaired insulin secretion resulting in an impaired fasting glucose (IFG), impaired glucose tolerance (IGT) and finally overt diabetes (Stumvoll et al., 2007). According to the criteria of the American Diabetes Association (ADA), IFG is defined as fasting plasma glucose levels of 5.6 mmol/l to 6.9 mmol/l, IGT as 2-h values in the oral glucose tolerance test of 7.8 mmol/l to 11.0 mmol/l (American Diabetes Association, 2014).

In addition to genetic and epigenetic factors, there are different determinants that are related to insulin resistance and the pathogenesis of type 2 diabetes, including obesity and lack of physical activity (Matthaei et al., 2000) as well as gut microbiota composition (Delzenne and Cani, 2011) and further components of the metabolic syndrome (Mayans, 2015). Besides medication and nutritional imbalance, dysfunctional adipose tissue (Freeman and Pennings, 2021) and the autonomic nervous system (Schlaich et al., 2015) are believed to have an impact on glucose metabolism.
In accordance with this pathophysiologic understanding, the improvement of insulin sensitivity and insulin secretion appears to be a major target for the prevention and therapy of type 2 diabetes (Chiasson and Rabasa-Lhoret, 2004).

Figure 1 visualizes the relationship between insulin sensitivity and insulin secretion in persons with and without diabetes.

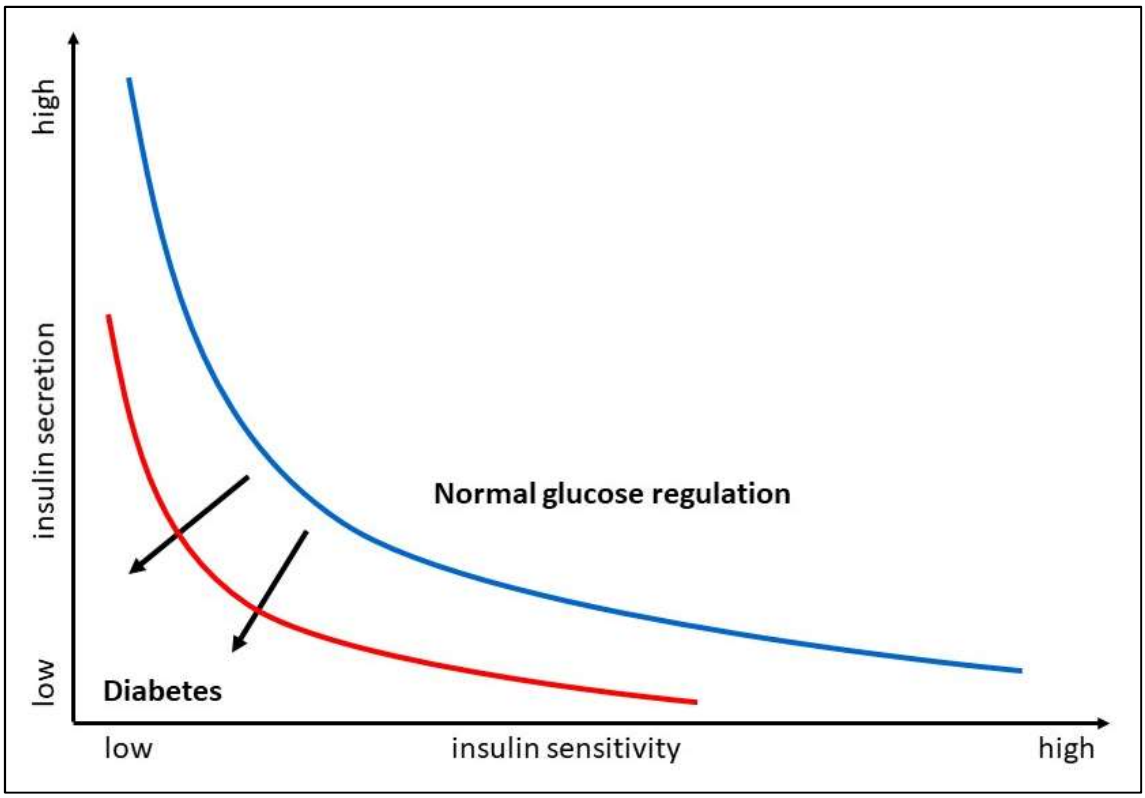

Figure 1: The relationship between insulin sensitivity and insulin secretion in non-diabetic and diabetic persons (Fritsche and Stefan, 2021).

1.1.1 The autonomic nervous system and its contribution to glucose metabolism

One important contributor to the regulation and synchronization of insulin secretion and insulin sensitivity is the autonomic nervous system (Schlaich et al., 2015) with its two antagonistic proportions, the sympathetic and parasympathetic branch.

Established surrogates for the activity of the autonomic nervous system are the heart rate and heart rate variability. Heart rate variability (HRV) shows the

balance fluctuation of sympathetic and vagal activation (Bootsma et al., 1994) and can be assessed by electrocardiography.
One established variable of the heart rate variability is the RMSSD (root mean square of successive differences) that reflects the vagal activity (Stein et al., 1994). Decreased heart rate variability is associated with cardiovascular diseases and diabetes and appears to be a risk factor of mortality after myocardial infarction (Stein et al., 1994) and the lowering of risk profiles is linked to an increase of heart rate variability (Thayer et al., 2010).
Further, it has been reported that increased sympathetic activity is closely linked to insulin resistance and the resulting hyperinsulinemia (Lambert et al., 2010). This in turn leads to impaired glucose tolerance, a pre-stage of type 2 diabetes. Moreover, sympathetic nerve activity is upregulated and parasympathetic activity is reduced in persons with prediabetes and diabetes (Carnethon et al., 2003). Beyond that, insulin resistance is not only found in peripheral tissues but also in the brain (Heni et al., 2015), the upstream regulator of the autonomic nervous system.

In humans, the autonomic nervous system can be modulated by administrating intranasal insulin to the brain and thereby improves insulin sensitivity in peripheral organs via parasympathetic nerve activation (Heni et al., 2014). This is well in line with earlier findings in rodents that especially highlighted the role of the vagus nerve in the brain-derived regulation of peripheral glucose metabolism (Berthoud et al., 1990).
Another approach to modulate the autonomic nervous system in a non-pharmacological and non-invasive manner is the electrical stimulation of the vagus nerve. Non-invasive vagus nerve stimulation has different effects on the human body as it reduces sympathetic nerve activity (Clancy et al., 2014), improves multiple cognitive functions (Dietrich et al., 2008; Marshall et al., 2004; Neuser et al., 2020) and changes the heart rate variability (Murray et al., 2016) showing an activation of the autonomic nervous system.
Besides the surgical implantation of pulse generators to stimulate the vagus nerve that are used, e.g., for the therapy of drug-resistant epilepsy (Ben-

Menachem, 2002) and chronic treatment-resistant depression (Howland, 2014), the vagus nerve can also be stimulated non-invasively. By using an electrode that is placed at the outer ear, non-invasive transcutaneous vagus nerve stimulation affects the auricular branch of the vagus nerve (Ellrich, 2011). Neither of these approaches have yet been sufficiently investigated in relation to systemic glucose metabolism.

Another approach to target the autonomic nervous system in a non-invasive manner are specific breathing patterns. The central nervous system can also be affected by deep breathing maneuvers. Slow deep breathing is known to modulate the activity of the autonomic nervous system (Russo et al., 2017) and increases the activity of the parasympathetic nerve (Pal et al., 2004) and cardiac vagal activity (Kromenacker et al., 2018). Deep breathing can also reduce blood pressure and heart rate (Mori et al., 2005). The activation of the parasympathetic nerve by slow deep breathing could therefore be an approach to affect insulin sensitivity or insulin secretion.

1.1.2 Body fat distribution and its contribution to glucose metabolism

Body fat distribution is very different between individuals and its location is crucial for the impact of adipocytes on systemic metabolism (Tchkonia et al., 2013) and the pathogenesis of multiple diseases (Després Jean-Pierre, 2012).
The distribution of body fat is also a well-described contributor to the pathogenesis of insulin resistance and diabetes (Freeman and Pennings, 2021). Especially the venous blood of visceral fat is drained via the portal vein directly to the liver (Ibrahim, 2010), where factors released from this fat depot induce the synthesis and release of pro-inflammatory cytokines that can cause detrimental effects like vascular inflammation (Frayn, 2000; Ibrahim, 2010) and increase the risk for multiple diseases (Britton et al., 2013).

Different fat compartments were studied over the last decades. Especially visceral fat and liver fat (Stefan, 2020), but also other non-classical fat depots like pancreatic fat (Ishibashi et al., 2020) and perivascular fat (Meijer et al., 2011) are of interest in the aetiopathogenesis of type 2 diabetes. Besides these, interscapular fat is a fat compartment, that was shown to be positively correlated with insulin resistance in humans (Thamer et al., 2010). Interscapular fat is localized in the neck area and its content depends on different factors like age, body mass index (BMI), total adipose tissue, visceral and subcutaneous tissue mass (Li et al., 2014).

In addition to the localization of fat depots, there are functionally and histologically different types of adipocytes: brown adipose tissue, white adipose tissue and beige adipose tissue (brown-in-white) (Frühbeck et al., 2009; Shapira and Seale, 2019). These different types of fat have antagonistic characteristics as white adipose tissue stores excess energy and brown adipose tissue contributes to heat production (Saely et al., 2012). Brown adipose tissue is more prominent in newborns in the interscapular region (Lidell et al., 2013). In contrast, brown fat mass in adults is located cervical, paravertebral, mediastinal or perirenal (Becher et al., 2021; Jones et al., 2017) and was not detected in the interscapular area (Betz and Enerbäck, 2015). Brown adipose tissue can be activated by cold exposure in adults (Cypess et al., 2013). Cold-induced activation of brown fat is inversely correlated with BMI and age in adult humans (Cypess et al., 2009). Further, activated brown fat was shown to be associated with lower blood glucose and improved insulin sensitivity in humans (Chondronikola, 2020; Matsushita et al., 2014).

1.2 Overview of the research project

The research project summarized in this cumulative thesis consists of three related manuscripts that report on investigation of the two main pathogenetic aspects of type 2 diabetes, insulin sensitivity and insulin secretion. To study this highly complex interplay of human metabolism, two relevant determinants were

specifically addressed: the autonomic nervous system and body fat distribution. In figure 2, a schematic overview of the research project is given.

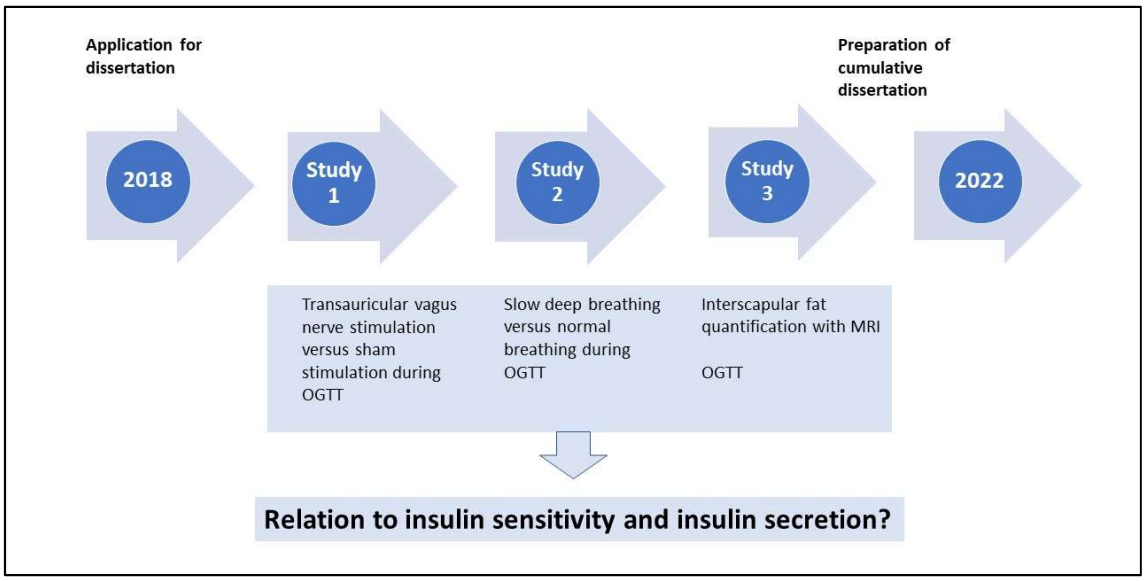

Figure 2: Schematic overview of the research project. OGTT: oral glucose tolerance test; MRI: magnetic resonance imaging.

The first study of the research project (Vosseler et al., 2020) investigated the attempt to modulate the autonomic nervous system by non-invasive vagus nerve stimulation and assessed its potential influence on insulin sensitivity and insulin secretion. In this trial, a transcutaneous auricular vagus nerve stimulation (taVNS) was performed during an oral glucose tolerance test (OGTT) in 15 young healthy men (mean age 24 years (SD±3)) and was compared to sham stimulation. In the postprandial state, insulin secretion and sensitivity are relevant for a quick and healthy response to the glucose challenge. The insulin response after meal ingestion is modulated by the autonomic nervous system (Ahrén and Holst, 2001). The parasympathetic nervous system is crucial for postprandial metabolism as increased activation of the parasympathetic nerve enhances insulin secretion and insulin sensitivity (Heni et al., 2014; Lindmark et al., 2003).

Additionally, energy expenditure was assessed using indirect calorimetry to investigate the effect of the modulation of the autonomic nervous system on postprandial energy expenditure.
The localization of the stimulation electrodes and the stimulation device are presented in figure 3.

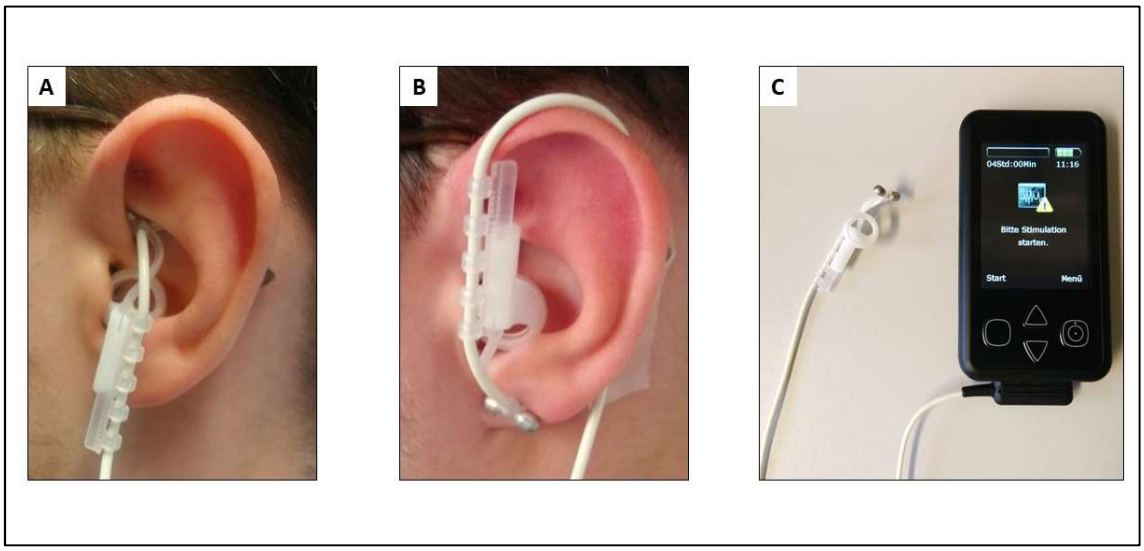

Figure 3: Stimulation of the auricular branch of the vagus nerve (A) and sham stimulation of the ear lobe (B). Electrical stimulation device Nemos by Cerbomed (C).

In the second clinical trial (Vosseler et al., 2021), slow deep breathing maneuvers versus normal breathing during an OGTT was applied in 15 young healthy men (mean age 27 years (SD± 8)), again, in an attempt to modulate the balance of the autonomic nervous system.
The breathing rate for slow deep breathing and normal breathing was paced by moving bars on a computer (fig. 4) and the respiration depth was assessed by a respiration belt around the chest. The activity of the autonomic nervous system was monitored by analyzing heart rate variability and insulin sensitivity and secretion was assessed by indices calculated from an OGTT. The resting energy expenditure was calculated from indirect calorimetry measurements.

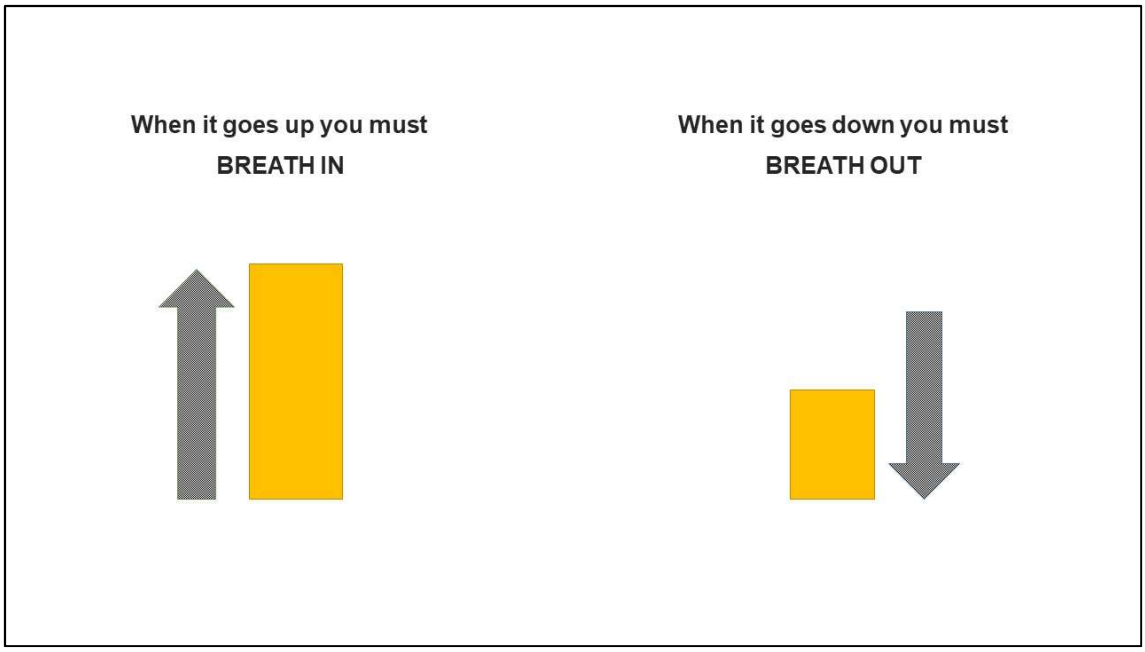

Figure 4: Slow deep breathing and normal breathing frequency indicated with moving bars for inspiration and expiration with the software Affect 4.0.

The third research work (Vosseler et al., 2022) analyzed interscapular fat that was assessed by magnetic resonance imaging (MRI). The study included 822 persons (females and males, mean age 46 years (SD ± 15)) from ongoing studies with different glucose tolerance status (normal glucose tolerance, impaired fasting glucose, impaired glucose tolerance). Oral glucose tolerance tests were performed in all participants. In this study that was based on earlier reports on a link between interscapular fat and insulin sensitivity (Thamer et al., 2010), we re-addressed the potential link between this specific fat depot and human metabolism in much more detail. The focus of this trial was on glucose tolerance, insulin secretion and insulin sensitivity. Interscapular fat mass localization in a normal-weight or obese person is shown in figure 5.

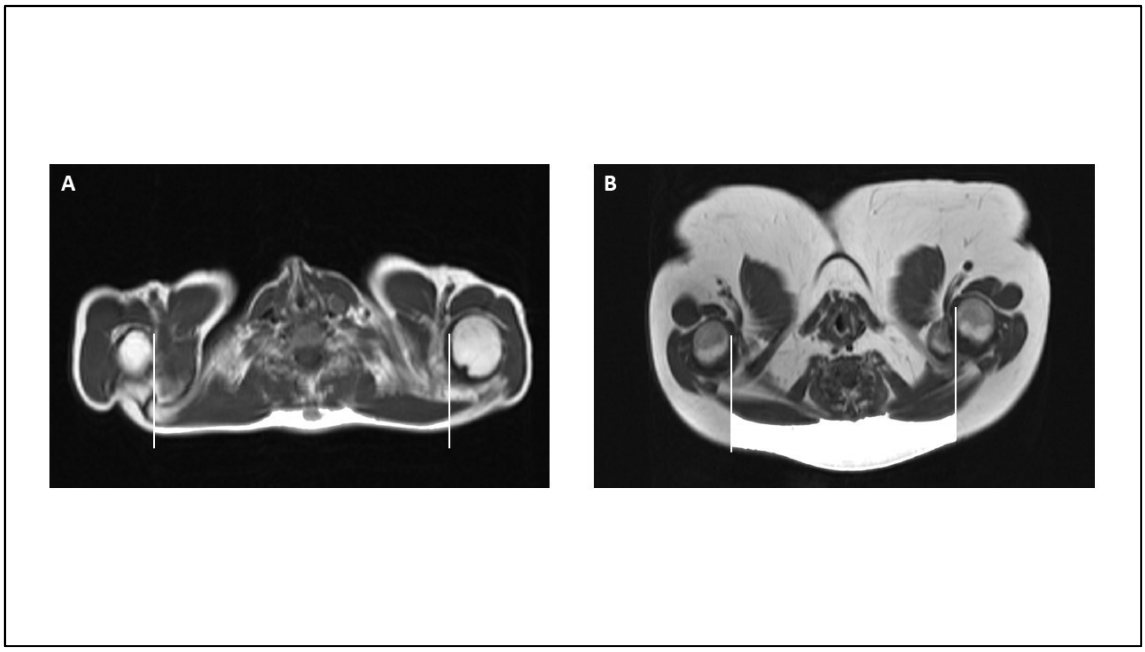

Figure 5: Interscapular fat mass (highlighted white section) in a normal-weight person (A) and obese person (B).

1.3 Research questions

The research questions of this thesis refer to the main pathogenetic aspects of type 2 diabetes, insulin resistance and insulin secretion, and potential clinical consequences:

- Can insulin sensitivity or insulin secretion be improved by modulating the autonomic nervous system in healthy persons?

- Is interscapular fat a clinically relevant fat depot with regard to insulin sensitivity and insulin secretion?

- What are the clinical implications of these three studies in terms of insulin sensitivity and insulin secretion as pathophysiological aspects for the prevention or treatment of type 2 diabetes?

Hereinafter, the three aforementioned studies are presented and the research questions are discussed. The methodology chosen to investigate the phenomenon will be critically reflected upon and explanations of the findings as well as implications for future studies and clinical practice will be given.

2 Results and discussion

2.1 Publication 1: No modulation of postprandial metabolism by transcutaneous auricular vagus nerve stimulation: a cross-over study in 15 healthy men

Authors: **Andreas Vosseler***, Dongxing Zhao*, Louise Fritsche, Rainer Lehmann, Konstantinos Kantartzis, Dana M. Small, Andreas Peter, Hans-Ulrich Häring, Andreas L. Birkenfeld, Andreas Fritsche, Robert Wagner, Hubert Preißl, Stephanie Kullmann, Martin Heni.
*contributed equally

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No modulation of postprandial metabolism by transcutaneous auricular vagus nerve stimulation: a cross-over study in 15 healthy men

Andreas Vosseler[1,2,3,6], Dongxing Zhao[2,6], Louise Fritsche[1,2,3], Rainer Lehmann[4], Konstantinos Kantartzis[1,2,3], Dana M. Small[5], Andreas Peter[3,4], Hans-Ulrich Häring[1,2,3], Andreas L. Birkenfeld[1,2,3], Andreas Fritsche[1,2,3], Robert Wagner[1,2,3], Hubert Preißl[2,3], Stephanie Kullmann[2,3] & Martin Heni[1,2,3,4]✉

Experimental evidence suggests a crucial role of the autonomic nervous system in whole body metabolism with major regulatory effects of the parasympathetic branch in postprandial adaptation. However, the relative contribution of this mechanism is still not fully clear in humans. We therefore compared the effects of transcutaneous auricular vagus nerve stimulation (taVNS, Cerbomed Nemos) with sham stimulation during an oral glucose tolerance test in a randomized, single-blind, cross-over design in 15 healthy lean men. Stimulation was performed for 150 min, 30 min before and during the entire oral glucose tolerance test with stimulation cycles of 30 s of on-phase and 30 s of off-phase and a 25 Hz impulse. Heart rate variability and plasma catecholamine levels were assessed as proxies of autonomic tone in the periphery. Neither analyzed heart rate variability parameters nor plasma catecholamine levels were significantly different between the two conditions. Plasma glucose, insulin sensitivity and insulin secretion were also comparable between conditions. Thus, the applied taVNS device or protocol was unable to achieve significant effects on autonomic innervation in peripheral organs. Accordingly, glucose metabolism remained unaltered. Therefore, alternative approaches are necessary to investigate the importance of the autonomic nervous system in postprandial human metabolism.

The autonomic nervous system modulates systemic metabolism through the innervation of peripheral organs[1]. This appears to be of special importance in the postprandial setting when rapid adaptations in various tissues are crucial for a healthy response to this metabolic challenge. While this is achieved predominantly via direct cellular action of postprandial factors like insulin, the autonomic nervous system appears to modulate and coordinate those effects[2–4]. More specifically, the parasympathetic nervous system is important for postprandial metabolism, as increased parasympathetic nerve activity leads to improved insulin sensitivity, insulin secretion and glucose tolerance[2,5,6].

The major parasympathetic nerve, the vagus nerve innervates the pancreas, the hepatic portal system, and most of the gastrointestinal tract. Vagal efferents stimulate pre- and postprandial insulin secretion from the pancreas[1,7] as well as hepatic insulin sensitivity and insulin clearance[8]. As most of these results are derived from studies in rodents, the relative contribution of the autonomic nervous system for glucose metabolism in humans is still not fully understood.

[1]Department of Internal Medicine IV, Division of Diabetology, Endocrinology and Nephrology, University Hospital Tübingen, Otfried-Müller-Str. 10, 72076 Tübingen, Germany. [2]Institute for Diabetes Research and Metabolic Diseases of the Helmholtz Center Munich at the University of Tübingen, Tübingen, Germany. [3]German Center for Diabetes Research (DZD e.V.), Neuherberg, Germany. [4]Institute for Clinical Chemistry and Pathobiochemistry, Department for Diagnostic Laboratory Medicine, University Hospital Tübingen, Tübingen, Germany. [5]Modern Diet and Physiology Research Center, Department of Psychiatry, Yale University, New Haven, CT, USA. [6]These authors contributed equally: Andreas Vosseler and Dongxing Zhao. ✉email: martin.heni@med.uni-tuebingen.de

n	15
Age (years)	24 (± 3)
BMI (kg/m^2)	22.9 (± 3.01)
Body fat content (%)	13.7 (± 2.9)
HbA1c (mmol/mol; %)	33.7 (± 2.6); 5.2 (± 0.2)
Waist-to-hip ratio	0.83 (± 0.04)

Table 1. Subject characteristics. Values are given as mean ± SD.

Non-invasive transcutaneous auricular vagus nerve stimulation (taVNS) is an approach to stimulate the auricular branch of the vagus nerve through the outer ear in humans. TaVNS can potentially activate organs indirectly via vagal afferent or directly by activation of the vagal efferents from the ear to peripheral organs[9].

TaVNS is successfully used for the therapy of drug-resistant epilepsy[10].

TaVNS activates the vagal afferents and influences various brain functions[11]. Parasympathetic vagal afferent signals are integrated in the nucleus of the solitary tract and further ascend to hypothalamus[12,13] and striatum[14]. These brain regions also integrate and process central signals and in turn regulate the vagal efferent. Though, evidence about the effects of taVNS on the vagal efferent is sparse, taVNS may serve as an interesting non-invasive intervention that may influence vagal efferent activities.

A prior study suggested that taVNS over 15 min regulates cardiac branches of the vagal efferent. For example, heart rate variability (HRV), an indicator of the vagal efferent activity of the cardiac branches, was immediately changed by taVNS, in a direction that points to a shift from sympathetic to parasympathetic tone[15]. In line, Badran et al., reported decreased heart rate and attenuate heart rate rebound during taVNS compared to sham stimulation[16].

Furthermore, 30 min of taVNS reduced gastric frequency, i.e. the rhythmic contractions of the stomach[13].

In contrast, 14 min of taVNS did not modulate the autonomic tone to visceral organs up to 120 min post stimulation in a recent study[17].

Thus, most previous studies indicated that taVNS affects vagal outflow to the periphery and might therefore be a useful tool to experimentally modulate autonomic regulations in the body.

We now aimed to study the effect of immediate modulation of parasympathetic tone by taVNS with Cerbomed NEMOS on whole-body metabolism during an oral glucose challenge and hypothesized improved insulin sensitivity and insulin secretion. Therefore, we designed a randomized, placebo-stimulation controlled, single blind, cross-over study to test effects of vagus nerve stimulation on systemic metabolism and energy expenditure.

Results

The clinical characteristics of the fifteen healthy men who were included in the current study are presented in Table 1. In a cross-over design they received taVNS versus sham stimulation (Fig. 1). Endocrine and metabolic results are shown in Fig. 2. Detailed results of the linear mixed model analyses can be found in Table 2.

Effects of taVNS on peripheral vagal activity. Mean heart rate decreased after the oral glucose tolerance test (OGTT) (main effect of time, $p < 0.0001$). Though, mean heart rate did not differ between taVNS and sham stimulation (main effect of condition, $p = 0.88$), nor was there a time-by-condition interaction ($p = 0.22$).

Likewise, root mean square of successive differences (RMSSD) as an indicator for vagal tone did not change over time (main effect of time, $p = 0.15$). Moreover, RMSSD did not differ between taVNS and sham conditions (main effect of condition, $p = 0.39$). However, there was a significant time-by-condition interaction ($p = 0.025$), but post hoc contrasts did not show significant differences between conditions at any time point (all $p_{Holm} > 0.1$).

Low frequency to high frequency ratio (LF/HF) indicates sympathovagal balance, and higher LF/HF indicates dominance of sympathetic activity, and vice versa. We found that LF/HF increased after OGTT (main effect of time, $p < 0.0001$). However, LF/HF did not differ between conditions (main effect of condition, $p = 0.86$) and there was no time-by-condition interaction ($p = 0.33$).

As a second approach to address peripheral autonomic tone, we analyzed plasma catecholamines, the effectors of the sympathetic nervous system. Plasma noradrenaline levels decreased over time ($p = 0.016$). There was no significant time effect for adrenaline ($p = 0.16$). There was no statistically significant difference between taVNS and sham stimulation (adrenaline $p = 0.20$, noradrenaline $p = 0.26$) and no time-by-condition interaction was detected (adrenaline $p = 0.73$, noradrenaline $p = 0.84$).

Effects of taVNS on whole-body glucose metabolism. Glucose excursions during the OGTT where comparable between taVNS and sham stimulation ($AUC_{glucose}$ $p = 0.1$). Hence, glucose tolerance assessed as plasma glucose 2 h after initiation of OGTT, was not different between the two conditions ($p = 0.4$).

As readout for insulin secretion of the pancreatic beta cells, we analyzed serum insulin and C-peptide. In both conditions, there was no difference between serum insulin and C-peptide concentrations (main effect of condition, insulin $p = 0.41$, C-peptide $p = 0.092$).

Neither insulin sensitivity (ISI Matsuda $p = 0.9$; NEFA ISI $p = 0.8$) nor insulin secretion (Disposition index $p = 0.2$) was statistically significantly different between taVNS and sham condition.

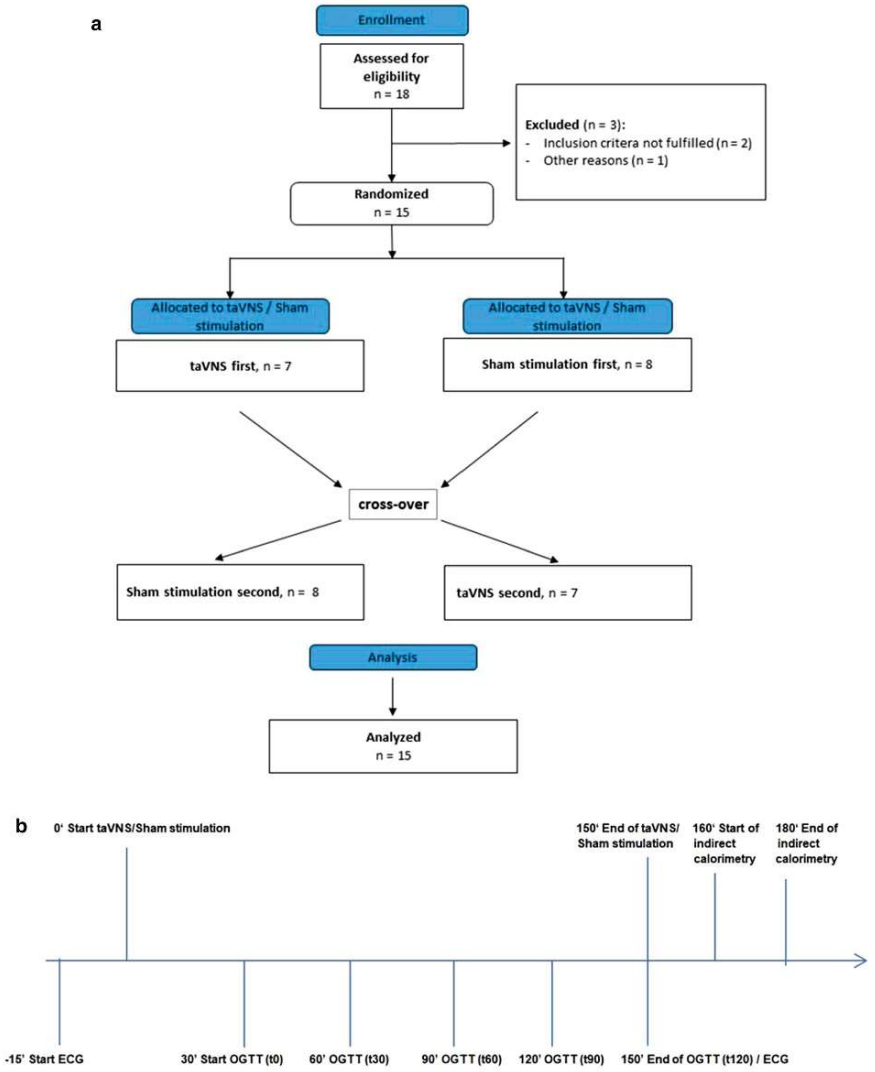

Figure 1. (**a**) Overview of the course of the study. (**b**) Schematic overview of the experiment. Electrocardiogram (ECG) was continuously recorded from timepoint -15 min before starting transcutaneous vagus nerve stimulation (taVNS) until the end of oral glucose tolerance test (OGTT) at 150 min. taVNS and sham stimulation were performed from timepoint 0 to 150 min. Start of OGTT was 30 min after initiation of taVNS/sham stimulation with intake of 75 g glucose. The last blood sample of OGTT was taken 2 h thereafter at timepoint 150 min. Indirect calorimetry was assessed after completion of the OGTT starting at 160 min.

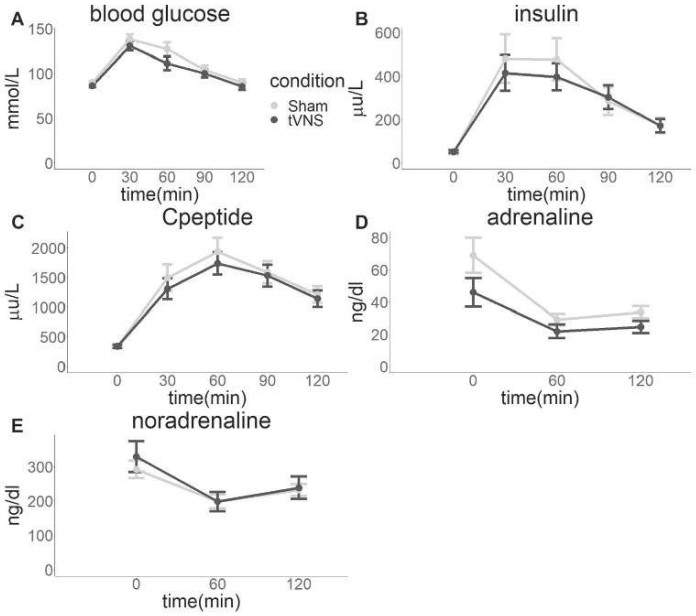

Figure 2. Endocrine and metabolic results. (**A**) Blood glucose, (**B**) serum insulin and (**C**) C-peptide increases during the oral glucose tolerance test (OGTT) that started at 0 min. Plasma adrenaline (**D**) and noradrenaline (**E**) decreased. However, transcutaneous vagus nerve stimulation (taVNS) did not significantly influence any of the hormone levels. Presented are means, error bars indicate standard errors.

Effects of taVNS on resting energy expenditure and post-load substrate oxidation. Resting energy expenditure was comparable between conditions ($p=0.9$). In the postprandial condition 2 h after OGTT, the preferential energy source was glucose in both conditions as indicated by an RQ around 1.0 ($p=0.5$).

Discussion

In the current study, we measured effects of a non-invasive vagus nerve stimulation on sympathetic and parasympathetic responses during an oral glucose tolerance test in healthy men. We found no significant influence of our stimulation approach on the tested parameters for autonomic balance. Accordingly, none of the analyzed glycemic traits were changed by the stimulation. Insulin sensitivity, insulin secretion, glucose tolerance and resting energy expenditure during the oral glucose tolerance tests did not differ between vagus stimulation and sham condition. TaVNS did not change sympathetic or parasympathetic tone to the heart as there were no detectable effects on heart rate variability. Finally, plasma catecholamines, the neurotransmitters of the sympathetic nervous system, were also unaffected by the stimulation. Thus, our data do not support major taVNS effects of taVNS on peripheral tissues.

However, taVNS is a well validated tool for vagal afferent stimulation that affects different brain functions[11,15,18–20]. These functions though were not able to modulate efferent outflows. Therefore, vagal afferent/efferent interaction appears to be unaffected by our current approach. How this interaction is regulated in detail is still not fully understood and should be further investigated in mechanistic studies at the molecular level.

One reason for the ineffectiveness of taVNS with the Nemos device could be that its vagal afferent stimulation in the auricular branch did not influence efferent activity towards the body.

Furthermore, an experiment in rodents suggested that a brain-gut communication occurs by directly stimulating the right vagus nerve[14] whereas in our study stimulation electrodes were placed in the left ear due to safety concerns when stimulating the right ear in humans.

The stimulation electrode can be applicated in different regions at the outer ear (tragus, concha or cymba concha), however, it is not fully known which region and which ear is the most reliable for taVNS as the innervation of the auricular branch of the vagus nerve is still not fully clear[21].

Main effects	Degrees of freedom	F	p
Mean heart rate			
Time	4,56	14.60	< 0.0001
Condition	1,14	0.03	0.88
Time-by-condition interaction	4,55	1.47	0.22
RMSSD			
Time	4,56	1.76	0.15
Condition	1,14	0.80	0.39
Time-by-condition interaction	4,55	3.04	0.025
LF/HF ratio			
Time	4,56	9.69	< 0.001
Condition	1,14	0.03	0.86
Time-by-condition interaction	4,55	1.18	0.33
Adrenaline			
Time	1,41	2.05	0.16
Condition	1,41	1.72	0.20
Time-by-condition interaction	1,41	0.12	0.73
Noradrenaline			
Time	1,41	6.37	0.016
Condition	1,41	1.32	0.26
Time-by-condition interaction	1,41	0.04	0.84
Blood glucose			
Time	3,97	29.77	< 0.0001
Condition	1,97	0.85	0.36
Time-by-condition interaction	1,97	0.03	0.85
Insulin			
Time	3,97	26.83	< 0.0001
Condition	1,97	0.67	0.41
Time-by-condition interaction	1,97	0.39	0.76
C-peptide			
Time	3,97	41.72	< 0.0001
Condition	1,97	2.89	0.092
Time-by-condition interaction	1,97	0.59	0.44
Time point (min)	**taVNS**	**Sham**	
Mean heart rate (bpm)			
−15	73.7 ± 2.7	74.2 ± 3.2	
0	66.8 ± 2.4	68.6 ± 2.5	
30	70.5 ± 2.3	72.3 ± 2.3	
60	73.4 ± 2.1	72.2 ± 2.4	
90	75.2 ± 2.1	73.4 ± 2.6	
120	73.5 ± 1.9	75.1 ± 2.4	
RMSSD (ms)			
−15	33.3 ± 3.2	33.3 ± 4.1	
0	48.2 ± 5.0	39.8 ± 3.9	
30	45.9 ± 3.8	44.2 ± 4.6	
60	39.6 ± 3.0	39.6 ± 3.8	
90	37.2 ± 2.4	38.7 ± 3.3	
120	46.5 ± 4.7	36.6 ± 2.7	
LF/HF ratio			
−15	2.6 ± 0.2	2.7 ± 0.3	
0	3.2 ± 0.4	2.9 ± 0.4	
30	2.8 ± 0.2	3.3 ± 0.4	
60	6.2 ± 0.9	5.4 ± 0.8	
90	4.6 ± 0.2	4.5 ± 0.5	
120	2.7 ± 0.2	3.1 ± 0.4	
Continued			

Time point (min)	taVNS	Sham	
Adrenaline (ng/dL)			
0	45.8 ± 8.9	68.9 ± 11.0	
60	21.8 ± 4.3	29.0 ± 3.6	
120	24.5 ± 3.7	33.7 ± 3.8	
Noradrenaline (ng/dL)			
0	329 ± 45	292 ± 25	
60	198 ± 28	198 ± 20	
120	238 ± 33	232 ± 17	
Blood glucose (mg/dL)			
0	86.4 ± 1.9	89.8 ± 1.6	
30	130.7 ± 5.2	138.3 ± 5.2	
60	111.3 ± 7.6	127.0 ± 7.6	
90	100.1 ± 4.6	104.1 ± 4.9	
120	85.7 ± 4.0	89.9 ± 3.4	
Insulin (pmol/l)			
0	51.9 ± 6.9	47.8 ± 8.2	
30	414.7 ± 82.4	480.6 ± 111.3	
60	397.7 ± 62.3	477.9 ± 96.8	
90	303.3 ± 55.0	285.7 ± 63.9	
120	171.3 ± 31.0	172.3 ± 34.6	
C-peptide (pmol/l)			
0	338.3 ± 29.4	338.7 ± 36.0	
30	1308.8 ± 176.9	1493.2 ± 226.1	
60	1737.3 ± 190.9	1937.5 ± 234.5	
90	1530.8 ± 186.1	1588.2 ± 185.3	
120	1139.3 ± 137.6	1208.7 ± 139.0	

Table 2. Results of linear mixed model analysis on the effects of transcutaneous vagus nerve stimulation on heart rate variability and hormones. *RMSSD* root mean square of successive differences; *LF/HF ratio*: low frequency to high frequency ratio.

As the optimal stimulation frequency, intensity and duration for the intended effects are unknown, we might not have picked optimal parameters. Furthermore, potential technical issues as well as electrode size and fit could have limited potential effects.

An increased stimulation duration could possibly show an effect of taVNS on glucose metabolism as Huang et al. showed positive effects of taVNS on glucose metabolism during a period of 12 weeks in persons with metabolic syndrome[22].

Clancy et al. reported increased heart rate variability in response to taVNS, indicating a shift from sympathetic to parasympathetic predominance in a much larger cohort compared to ours[15]. In addition, the stimulation protocol and trial setting of our current study was different from Clancy et al., who performed taVNS with another device, different stimulation protocol and included female and male participants whereas in our study only male persons were included. Their device stimulated for 15 min continuously[15], whereas we used a repetitive sequence with 30 s of stimulation followed by 30 s pause for a longer period of time. Furthermore, no metabolic challenge was applied in the study of Clancy et al.[15].

In contrast to Clancy's positive results, Borges et al. could not detect any difference between transcutaneous auricular vagus nerve stimulation and sham stimulation on cardiac vagal activity in 61 healthy men[23].

Thus, possible slight effects of the stimulation might have been masked by stronger effects of the metabolic alterations in our study.

Although vagus stimulation did not affect plasma catecholamine courses, there was a time effect of adrenaline and noradrenaline in both conditions. Adrenaline and noradrenaline levels were higher at time point 0 and declined during the OGTT. This could reflect the well-known shift from sympathetic towards parasympathetic tone in the postprandial state[1,24,25]. However, as we did not study catecholamines without glucose intake, we cannot exclude other potential contributors to this response.

In conclusion, auricular vagus nerve stimulation with the Nemos device had no major acute effects on the autonomic regulation of peripheral organs and did therefore neither alter insulin sensitivity, insulin secretion, resting energy expenditure nor sympathovagal balance. The physiological significance of the autonomic nervous system for glucose metabolism in humans must therefore be investigated with alternative approaches, e.g. either by biofeedback paradigmes or pharmacologically.

Methods

Fifteen male healthy volunteers at an age between 18 and 31 years were included (further details are provided in Table 1). Body mass index (BMI) was between 19.3 and 25.2 kg/m^2, body fat content was between 9.5 and 22.5% as measured by bioelectrical impedance testing (BIA 101 by Akern Srl, Florence, Italy) and estimated with Cyprus 2.7 Body Composition Analysis Software (RJL Systems, Michigan, USA). Subject characteristics are shown in Table 1.

The sample size (n = 15) provides 80% power to detect effect size f = 0.35 when setting the alpha-level to 0.05 (calculated with Gpower 3.1).

The study protocol was approved by the ethics committee of the medical faculty of the University Tübingen. Written informed consent was obtained from all study participants and all research was performed in accordance with relevant guidelines/regulations. The study was pre-registered at clinicaltrials.gov (NCT03615209; 03/08/2018).

An overview of the course of the study is given in Fig. 1.

Non-invasive transcutaneous vagal stimulation was applied with the Cerbomed NEMOS device, an earpiece with titanium electrodes that is placed in the cymba conchae of the left external ear and in upside down position (ear lobe) for sham stimulation . The device stimulates the auricular branch of the nervus vagus with a mild electrical current with stimulation cycles of 30 s of on-phase and 30 s of off-phase and a 25 Hz impulse.

Auricular vagus nerve stimulation (and sham stimulation, respectively) was performed 30 min before and during the entire OGTT (150 min overall) in randomized cross-over design in the morning of two different days with 5 to 16 days washout period in a randomized single-blind design.

Stimulation procedure was done according to the protocol of Frangos et al.[11]. Stimulus intensity was adjusted by the participants from 0.1 mA in 0.1 mA increments until the person reported a "tingling" sensation, but no pain[11]. Stimulation intensity was 2.5 mA ± SD 0.9 in taVNS and 3.2 mA ± SD 1.5 in sham condition.

All persons underwent an oral glucose tolerance test. After an overnight fast, participants ingested a solution containing 75 g glucose over 5 min (Accu-Chek Dextrose OGT, Roche). Before, and 30, 60, 90 and 120 min after glucose ingestion, blood samples were obtained following standard procedures[26]. Oral glucose tolerance test was performed to address dynamic insulin secretion from the pancreatic beta cells and insulin sensitivity from plasma glucose, C-peptide and insulin responses after ingestion of glucose solution. OGTT is a frequently used tool to assess insulin secretion and insulin sensitivity and can furthermore assess glucose tolerance[27].

HbA1c was tested at baseline, serum insulin levels, C-peptide, plasma glucose and free fatty acids were measured at all five timepoints. All measurements were performed in a routine diagnostic laboratory that is accredited with the German accredited body (DAkkS). Glucose values were measured directly using a bedside glucose analyzer (Biosen C-line, EKF-diagnostic GmbH, Barleben, Germany). Serum insulin and C-peptide levels were determined by an immunoassay with ADVIA Centaur XP Immunoassay System (Siemens Healthineers, Eschborn, Germany).

Plasma concentrations of total non-esterified fatty acids (NEFA) were measured with an enzymatic method (WAKO Chemicals, Neuss, Germany). HbA1c measurements were performed using Tosoh glycohemoglobin analyzer HLC-723G8 (Tosoh Bioscience, Tokyo, Japan).

Insulin sensitivity and insulin secretion was calculated from the OGTT as described previously[28].

Plasma catecholamines adrenaline and noradrenaline were measured at baseline and 60 and 120 min after glucose ingestion. Catecholamines were analyzed with high performance liquid chromatography (HPLC) using a commercial kit (Kits No 5000, Chromsystems, Grafelfingen, Germany). Adrenaline and noradrenaline were isolated from plasma prior to chromatographic separation by solid phase extraction. After sample pre-treatment, an isocratic HPLC analysis was performed with a flow rate of 1 ml/min and a total run time of 20 min. The limit of detection (LOD) for adrenaline is 3 ng/dl, and for noradrenaline 10 ng/dl.

Electrocardiogram (ECG) recordings were performed to analyze heart rate variability as a surrogate parameter for autonomic nerve activation and parasympathetic projections to the heart. ECG was continuously recorded 15 min before starting the electric stimulation until the end of OGTT. HRV analysis was done in 10 min intervals in resting state, before the start of the stimulation and 5 min before blood extraction. ECG was recorded with Biopac MP 36 (Biopac Systems, Inc., Goleta, CA) and analyzed with Matlab (Mathworks, Inc. USA).

Standard ECG electrodes were attached to the chest wall. ECG was recorded at 2.5 kHz and transduced, amplified and filtered with a low pass filter at 25 Hz and a high pass filter at 1 Hz. The data were visually inspected for artifact correction in Artiifact[29]. Data with less than 2 min continuous measurement (uninterrupted by movement artifacts) were excluded. Inter-beat intervals calculated from visually inspected data were then processed in Artiifact to correct for ectopic peaks. Root mean square of successive differences (RMSSD) in inter-beat intervals was calculated in the time domain as an indicator of the vagal tone. High-frequency (0.15–0.50 Hz, HF) component and low-frequency (0.05–0.14 Hz, LF) was calculated in the frequency domain, and the low-frequency (LF/HF) ratio was calculated as an indicator of the vagal tone[30,31].

ECG was recorded for 5 min at baseline and every 15 min after glucose intake. HRV parameters collected for each 5 min time bins were then analyzed in mixed effect models.

Resting energy expenditure was assessed after completion of the OGTT and the taVNS stimulation. Energy expenditure after vagus nerve and sham stimulation was calculated by indirect calorimetry measurements with Vyntus CPX (Vyaire Medical, Illinois, USA). O_2 consumption and CO_2 production was measured for 15 min. To correct for monitor-specific deviations and eliminate the influence of inherent variability of the device on the measurement results, individual calibration control evaluation (ICcE) was applied[32].

Statistical analysis was performed using SAS 9.4 (SAS Institute, Cary, NC). All results are presented as mean ± SD. $p < 0.05$ was considered statistically significant.

Measurements for major outcomes were subtracted from baseline measurements and boxcox-transformed if needed to fulfill the assumption of normally distributed residuals. Mixed effect models were performed on that data, with main effects of time, taVNS, and their interaction effects as fixed effects, the subjects with random intercepts, and the visit order as a dummy variable. Denominator degrees of freedom were estimated using the default method in SAS, which is a containment method. The variance–covariance structure providing the best fit was chosen based on the minimum value of Akaike's Information Criterion (AIC). Significant interaction effects were followed by post hoc contrasts at each time point between conditions, with stepdown Bonferroni (Holm) correction for multiple testing. Metabolic results and the resting energy expenditure and respiratory quotient were analyzed using paired *t* tests between conditions.

Data availability

The data are not publicly available due to them containing information that could compromise research participant privacy/consent.

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References

1. Teff, K. L. Visceral nerves: vagal and sympathetic innervation. *JPEN J. Parenter. Enteral. Nutr.* **32**, 569–571 (2008).
2. Heni, M. *et al.* Central insulin administration improves whole-body insulin sensitivity via hypothalamus and parasympathetic outputs in men. *Diabetes* **63**, 4083–4088 (2014).
3. Begg, D. P. & Woods, S. C. Interactions between the central nervous system and pancreatic islet secretions: a historical perspective. *Adv. Physiol. Educ.* **37**, 53–60 (2013).
4. Ahrén, B. & Holst, J. J. The cephalic insulin response to meal ingestion in humans is dependent on both cholinergic and noncholinergic mechanisms and is important for postprandial glycemia. *Diabetes* **50**, 1030–1038 (2001).
5. Lindmark, S., Wiklund, U., Bjerle, P. & Eriksson, J. W. Does the autonomic nervous system play a role in the development of insulin resistance? A study on heart rate variability in first-degree relatives of Type 2 diabetes patients and control subjects. *Diabet. Med.* **20**, 399–405 (2003).
6. Perin, P. C., Maule, S. & Quadri, R. Sympathetic nervous system, diabetes, and hypertension. *Clin. Exp. Hypertens.* **23**, 45–55 (2001).
7. Strubbe, J. H. & Steffens, A. B. Neural control of insulin secretion. *Horm. Metab. Res.* **25**, 507–512 (1993).
8. Macedo, M. P. *et al.* Risk of postprandial insulin resistance: the liver/vagus rapport. *Rev. Endocr. Metab. Disord.* **15**, 67–77 (2014).
9. Butt, M. F., Albusoda, A., Farmer, A. D. & Aziz, Q. The anatomical basis for transcutaneous auricular vagus nerve stimulation. *J. Anat.* **236**, 588–611 (2020).
10. Stefan, H. *et al.* Transcutaneous vagus nerve stimulation (t-VNS) in pharmacoresistant epilepsies: a proof of concept trial. *Epilepsia* **53**, e115-118 (2012).
11. Frangos, E., Ellrich, J. & Komisaruk, B. R. Non-invasive access to the vagus nerve central projections via electrical stimulation of the external ear: fMRI evidence in humans. *Brain Stimul.* **8**, 624–636 (2015).
12. Berthoud, H.-R. Vagal and hormonal gut–brain communication: from satiation to satisfaction. *Neurogastroenterol. Motil.* **20**, 64–72 (2008).
13. Teckentrup, V. *et al.* Non-invasive stimulation of vagal afferents reduces gastric frequency. *Brain Stimul.* **13**, 470–473 (2020).
14. Han, W. *et al.* A neural circuit for gut-induced reward. *Cell* **175**, 665-678.e23 (2018).
15. Clancy, J. A. *et al.* Non-invasive vagus nerve stimulation in healthy humans reduces sympathetic nerve activity. *Brain Stimul.* **7**, 871–877 (2014).
16. Badran, B. W. *et al.* Short trains of transcutaneous auricular vagus nerve stimulation (taVNS) have parameter-specific effects on heart rate. *Brain Stimul.* **11**, 699–708 (2018).
17. Gancheva, S. *et al.* Constant hepatic ATP concentrations during prolonged fasting and absence of effects of Cerbomed Nemos® on parasympathetic tone and hepatic energy metabolism. *Mol. Metab.* **7**, 71–79 (2017).
18. Marshall, L., Mölle, M., Hallschmid, M. & Born, J. Transcranial direct current stimulation during sleep improves declarative memory. *J. Neurosci.* **24**, 9985–9992 (2004).
19. Neuser, M. P. *et al.* Vagus nerve stimulation increases vigor to work for rewards. *bioRxiv* 789982 (2019). https://doi.org/10.1101/789982.
20. Dietrich, S. *et al.* A novel transcutaneous vagus nerve stimulation leads to brainstem and cerebral activations measured by functional MRI. *Biomed. Tech. (Berl)* **53**, 104–111 (2008).
21. Badran, B. W. *et al.* Tragus or cymba conchae? Investigating the anatomical foundation of transcutaneous auricular vagus nerve stimulation (taVNS). *Brain Stimul.* **11**, 947–948 (2018).
22. Huang, F. *et al.* Effect of transcutaneous auricular vagus nerve stimulation on impaired glucose tolerance: a pilot randomized study. *BMC Complement. Altern. Med.* **14**, 203 (2014).
23. Borges, U., Laborde, S. & Raab, M. Influence of transcutaneous vagus nerve stimulation on cardiac vagal activity: not different from sham stimulation and no effect of stimulation intensity. *PLoS ONE* **14**, e0223848 (2019).
24. Lu, C.-L., Zou, X., Orr, W. C. & Chen, J. D. Z. Postprandial changes of sympathovagal balance measured by heart rate variability. *Dig. Dis. Sci.* **44**, 857–861 (1999).
25. Strubbe, J. H. Parasympathetic involvement in rapid meal-associated conditioned insulin secretion in the rat. *Am. J. Physiol.* **263**, R615-618 (1992).
26. Pacini, G. & Mari, A. Methods for clinical assessment of insulin sensitivity and β-cell function. *Best Pract. Res. Clin. Endocrinol. Metab.* **17**, 305–322 (2003).
27. Stumvoll, M. *et al.* Use of the oral glucose tolerance test to assess insulin release and insulin sensitivity. *Diabetes Care* **23**, 295–301 (2000).
28. Wagner, R. *et al.* A novel insulin sensitivity index particularly suitable to measure insulin sensitivity during gestation. *Acta Diabetol.* **53**, 1037–1044 (2016).
29. Kaufmann, T., Sütterlin, S., Schulz, S. M. & Vögele, C. ARTiiFACT: a tool for heart rate artifact processing and heart rate variability analysis. *Behav. Res. Methods* **43**, 1161–1170 (2011).
30. Appelhans, B. M. & Luecken, L. J. Heart rate variability as an index of regulated emotional responding. *Rev. Gen. Psychol.* **10**, 229–240 (2006).
31. Quintana, D. S. *et al.* Reduced heart rate variability in schizophrenia and bipolar disorder compared to healthy controls. *Acta Psychiatr. Scand.* **133**, 44–52 (2016).

32. Schadewaldt, P., Nowotny, B., Strassburger, K., Kotzka, J. & Roden, M. Indirect calorimetry in humans: a postcalorimetric evaluation procedure for correction of metabolic monitor variability. *Am. J. Clin. Nutr.* **97**, 763–773 (2013).

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Author contributions
A.V. researched and analyzed data and drafted the manuscript. D.Z. analyzed data and contributed to discussion. L.F., R.L., K.K. and A.P. researched data and contributed to discussion. H.U.H., A.B., A.F., R.W., H.P., S.K. and D.M.S. contributed to discussion. M.H. contributed to analyses, supervised the project and contributed to discussion. All authors approved the final version of the manuscript prior to submission.

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Competing interests
The authors declare no competing interests.

Additional information
Correspondence and requests for materials should be addressed to M.H.

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2.2 Publication 2: Slow deep breathing modulates cardiac vagal activity but does not affect peripheral glucose metabolism in healthy men

Authors: **Andreas Vosseler**, Dongxing Zhao, Julia Hummel, Ali Gholamrezaei, Sarah Hudak, Konstantinos Kantartzis, Andreas Peter, Andreas L. Birkenfeld, Hans-Ulrich Häring, Robert Wagner, Hubert Preißl, Stephanie Kullmann, Martin Heni.

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Slow deep breathing modulates cardiac vagal activity but does not affect peripheral glucose metabolism in healthy men

Andreas Vosseler[1,2,3], Dongxing Zhao[2], Julia Hummel[1,2,3], Ali Gholamrezaei[5], Sarah Hudak[1,2,3], Konstantinos Kantartzis[1,2,3], Andreas Peter[2,3,4], Andreas L. Birkenfeld[1,2,3], Hans-Ulrich Häring[1,2,3], Robert Wagner[1,2,3], Hubert Preißl[2,3], Stephanie Kullmann[2,3] & Martin Heni[1,2,3,4]✉

Parasympathetic nervous system innervates peripheral organs including pancreas, hepatic portal system, and gastrointestinal tract. It thereby contributes to the regulation of whole-body glucose metabolism especially in the postprandial state when it promotes secretion of insulin and enhances its action in major target organs. We now aimed to evaluate the effect of parasympathetic modulation on human glucose metabolism. We used slow deep breathing maneuvers to activate the parasympathetic nervous system and tested for effects on metabolism during an oral glucose tolerance test in a randomized, controlled, cross-over trial in 15 healthy young men. We used projections towards the heart as a readout for parasympathetic activity. When analyzing heart rate variability, there was a significant increase of RMSSD (root mean square of successive differences) when participants performed slow deep breathing compared to the control condition, indicating a modulation of parasympathetic activity. However, no statistically significant effects on peripheral glucose metabolism or energy expenditure after the glucose tolerance test were detected. Of note, we detected a significant association between mean heart rate and serum insulin and C-peptide concentrations. While we did not find major effects of slow deep breathing on glucose metabolism, our correlational results suggest a link between the autonomic nervous system and insulin secretion after oral glucose intake. Future studies need to unravel involved mechanisms and develop potential novel treatment approaches for impaired insulin secretion in diabetes.

Insulin resistance of peripheral tissues combined with impaired insulin secretion from pancreatic β-cells are the major pathomechanisms that cause type 2 diabetes[1–4]. One of the main effects of insulin in target tissues is to promote uptake of glucose into the cells. Liver, skeletal muscle and adipose tissue are considered major insulin-sensitive target tissues. By contrast, glucose is absorbed into the brain in an insulin-independent manner. However, insulin receptors are densely expressed in most parts of the central nervous system[5]. Central nervous insulin action modulates the metabolism throughout the body and increases cardiac vagal outputs[6]. Postprandial factors like insulin are perceived by the human brain and induce signals that modulate glucose metabolism via the parasympathetic nervous system[6–8].

The vagus nerve as major parasympathetic nerve innervates peripheral organs including the pancreas, the hepatic portal system, and most of the gastrointestinal tract. Pancreatic beta cells communicate with vagal sensory neurons in mice[9]. Insulin secretion from the pancreas in pre- and postprandial state is stimulated by vagal efferents[10,11]. Likewise, hepatic insulin sensitivity and insulin clearance is improved upon vagal activation[12].

[1]Division of Endocrinology, Diabetology and Nephrology, Department of Internal Medicine, Internal Medicine IV, University Hospital Tübingen, Otfried-Müller-Str. 10, 72076 Tübingen, Germany. [2]Institute for Diabetes Research and Metabolic Diseases of the Helmholtz Centre Munich at the University of Tübingen, Tübingen, Germany. [3]German Center for Diabetes Research (DZD), Neuherberg, Germany. [4]Department for Diagnostic Laboratory Medicine, Institute for Clinical Chemistry and Pathobiochemistry, University Hospital Tübingen, Tübingen, Germany. [5]Pain Management Research Institute, Faculty of Medicine and Health, The University of Sydney, Sydney, Australia. ✉email: martin.heni@med.uni-tuebingen.de

	Main effects	Degrees of freedom	F	p
Breathing rate	Time	28,779	10.99	< 0.0001
	Condition	1,28	16.46	0.0004
	Time-by-condition interaction	28,779	6.71	< 0.0001
Respiration depth	Time	28,779	3.33	< 0.0001
	Condition	1,28	1.97	0.17
	Time-by-condition interaction	28,779	1.54	0.038
Mean heart rate	Time	28,806	10.99	< 0.0001
	Condition	1,29	0.05	0.82
	Time-by-condition interaction	28,806	0.71	0.86
RMSSD	Time	28,806	6.73	< 0.0001
	Condition	1,29	0.10	0.75
	Time-by-condition interaction	28,806	1.42	0.073
Insulin	Time	5,144	198.32	< 0.0001
	Condition	1,29	0.33	0.57
	Time-by-condition interaction	5,144	1.41	0.22
C-peptide	Time	5,144	203.64	< 0.0001
	Condition	1,29	0.02	0.90
	Time-by-condition interaction	5,144	1.18	0.32
Blood glucose	Time	5,144	52.93	< 0.0001
	Condition	1,29	0.00	0.96
	Time-by-condition interaction	5,144	1.10	0.36

Table 1. Results of mixed model analysis on the effects of slow deep breathing on respiration, heart rate (variability) and hormones.

Accordingly, parasympathetic nervous system modulates glucose metabolism in postprandial state by enhancing insulin sensitivity, insulin secretion and glucose tolerance[6,13,14].

In a previous study, we investigated the effect of non-invasive transauricular vagus nerve stimulation versus sham stimulation on glucose metabolism during an oral glucose tolerance test[15]. However, the applied methodology was unable to exert major effects on vagus nerve and had therefore no major effects on glucose metabolism. We now aimed to investigate the activation of the parasympathetic nervous system with another approach. Slow deep breathing (SDB) exercise was found to increase parasympathetic activity[16,17], decrease heart rate [18] and alters heart rate variability (HRV) in healthy persons.

We hypothesize that slow deep breathing exercise can modulate peripheral glucose metabolism by alternating vagal responses. To investigate this, we tested effects of slow deep breathing exercise versus normal breathing on insulin sensitivity, insulin secretion, glucose tolerance, resting energy expenditure, and parasympathetic nervous system by analyzing heart rate and heart rate variability.

Results

Breathing. We first checked compliance to and efficacy of the breathing instructions. As instructed, the breathing rate significantly decreased during paced-train in the slow deep breathing condition (paced-train vs. pre-train, $p_{Holm} = 0.0003$), but not in control condition ($p_{Holm} = 0.53$), and the difference between the two study conditions was significant ($p_{Holm} = 0.0002$). Moreover, respiration depth significantly increased during paced-train in both slow deep breathing and control conditions ($p_{Holm} = 0.0003$ and 0.01, respectively). The increase of respiration depth during paced-train was significantly higher in the slow deep breathing compared to the control condition ($p_{Holm} = 0.002$) (supplementary table 1). The results of mixed model analysis on the effects of slow deep breathing on respiration, heart rate variability and glucose metabolism are presented in Table 1.

Effects of slow deep breathing on heart rate variability as a proxy for autonomic modulation. Mean heart rate (HR) did not differ between the conditions ($p = 0.82$) and there was no significant time-by-condition interaction ($p = 0.86$) (Fig. 1).

Post hoc contrasts revealed that RMSSD increased in slow deep breathing condition during paced-train cycle compared to pre-train cycle, i.e. when no specific breathing maneuver was performed ($p_{Holm} = 0.0003$). This was not the case in the control condition ($p_{Holm} = 0.16$). Moreover, the difference in RMSSD between the conditions was statistically significant ($p_{Holm} = 0.0003$).

The complete results from the mixed effect models are presented in Table 1. Table 2 displays the results of post hoc contrasts (paced-train cycle vs. pre-train cycles).

Effects of slow deep breathing on whole-body glucose metabolism. Neither fasting glucose, nor post-load glucose were significantly different between the breathing conditions (fasting glucose $p = 0.96$, post-

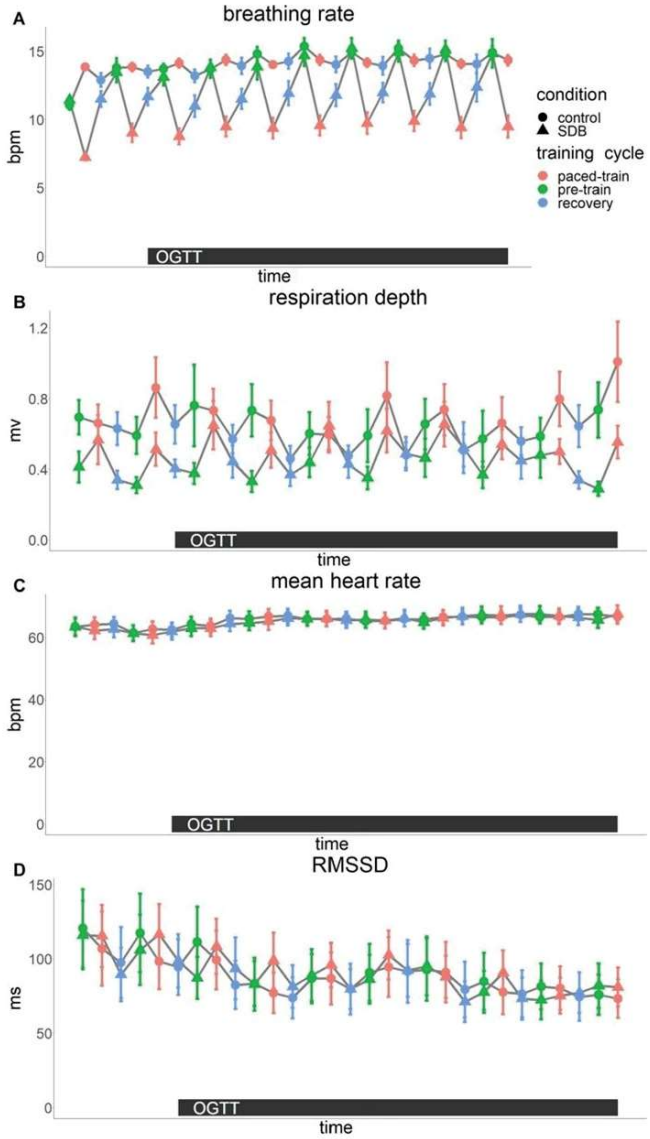

Figure 1. Breathing rate (**A**) and respiration depth (**B**), mean heart rate (**C**) and root mean square of successive differences (RMSSD, **D**). Breathing rate was significantly lower during paced-train compared to pre-train in the slow deep breathing (SDB) condition ($p < 0.0005$) but not in the control condition (not significant, NS). Respiration depth (averaged by each participant and each condition) was significantly higher during paced-train compared to pre-train in both control and SDB conditions, and the differences between conditions were significant. Mean heart rate (averaged by each participant and each condition) was not significantly different between paced-train and pre-train in either condition. RMSSD increased significantly during paced-train compared to pre-train in SDB condition, but not in control condition, and the differences between the two conditions were significant. OGTT=oral glucose tolerance test; bpm=breaths per minute (A) or beats per minute (C); mv=millivolt; ms=millisecond.

	Post hoc contrasts	Degrees of freedom	t	p_{Holm}
Breathing rate	SDB	779	−17.57	0.0003
	Control	779	−0.63	0.53
	SDB vs. control	779	−5.36	0.0003
Respiration depth	SDB	799	6.94	0.0003
	Control	799	2.59	0.01
	SDB vs. control	799	3.33	0.002
RMSSD	SDB	806	5.25	0.0003
	Control	806	−1.74	0.16
	SDB vs. control	806	4.99	0.0003

Table 2. Results of post hoc contrasts (paced-train minus pre-train periods with normal respiration) on the effects of slow deep breathing on respiration and heart rate variability. *RMSSD* root mean square of successive differences.

load glucose $p = 0.26$). In line, two main determinants of blood glucose levels, insulin secretion and insulin sensitivity were unaffected, as Matsuda insulin sensitivity index (ISI Matsuda) and disposition index (DI) were comparable (ISI Matsuda $p = 0.11$, DI $p = 0.37$).

However, statistical models revealed a positive correlation between mean HR and both insulin ($p < 0.0001$) and C-peptide concentrations ($p < 0.0001$).

Effects of slow deep breathing on resting energy expenditure and post-load substrate oxidation. There was no difference in resting energy expenditure between slow deep breathing and normal breathing maneuvers ($p = 0.87$). Respiratory quotient (RQ) was around 1 during both visits, with no difference between conditions ($p = 0.88$).

Discussion

In this study, we investigated the effect of slow deep breathing versus normal breathing during an oral glucose tolerance test on autonomic nervous system activity, on whole-body glucose metabolism, as well as on resting energy expenditure.

Slow deep breathing was performed correctly by the study participants and this appears to have impacted parasympathetic activity as there was an increase of RMSSD upon slow deep breathing. The increase of RMSSD during slow deep breathing indicates a difference between cardiac vagal modulation in the deep breathing versus normal breathing condition.

However, this was not sufficient to introduce major effects on peripheral glucose metabolism or energy expenditure after oral glucose load. The time intervals of deep breathing may have been too short to induce a robust effect on vagal activation and may explain the absence of major metabolic effects. Furthermore, altering cardiac autonomic nervous system by breathing exercise does not necessarily influence its activity at the levels where glucose metabolism can be modulated.

In fact, slow deep breathing was previously shown to modulate autonomic tone when 60 healthy young volunteers practiced deep breathing versus fast breathing for three months[16]. However, this could also be a long-term effect that is not directly relied on slow deep breathing.

In line, Kromenacker et al. showed that slow deep breathing changes heart rate variability predominantly mediated by the parasympathetic nerve and is therefore an effective tool for cardiac vagal activation[28].

The applied protocol in our study did not change actual heart rate. A prolongation of slow deep breathing (e.g. for 15 min) could eventually be more effective in modulating autonomic tone and may then affect peripheral metabolism. Furthermore, autonomic tone and subsequently peripheral metabolism could also be modulated by pharmacological interventions in future studies.

Another reason for the lack of metabolic effects in our current study may be that slow deep breathing does only modulate specific brain centers that are responsible for the control of autonomic outflow towards the heart. Further autonomic outflows towards metabolic organs are likely under the control of additional brain centers that might not respond to altered breathing patterns[29].

The tested breathing variation in our current study had no effect on insulinemia. Though, additional analysis revealed a relation of mean heart rate and insulin and C-peptide concentrations regardless of breathing maneuvers or glucose intake. Previous findings affirm the crucial role of the autonomic nervous system for the regulation of insulin and whole-body glucose metabolism[6]. The association of serum C-peptide and insulin levels with heart rate indicates a presumed link between central modulation of insulin secretion and vagal activation of peripheral organs.

Due to the postprandial state (2 h after ingestion of glucose solution) during the measurement of the energy expenditure, RQ indicates glucose as preferential energy source. This preferred energy source was not shifted by the applied breathing protocol.

Our study has some limitations. We currently included only a limited number of participants and were therefore unable to detect smaller effects. Furthermore, only men were included and the breathing protocol might not be strong enough to introduce persistent effects on autonomic tone.

n	15
Age (years)	27 (± 8)
BMI (kg/m^2)	22.7 (± 1.8)
Body fat content (%)	14.0 (± 3.4)
HbA1c (mmol/mol; %)	34,1 (± 2.4); 5.3 (± 0.2)
Waist-to-hip ratio	0.83 (± 0.04)

Table 3. Subject characteristics. Values are given as mean ± SD.

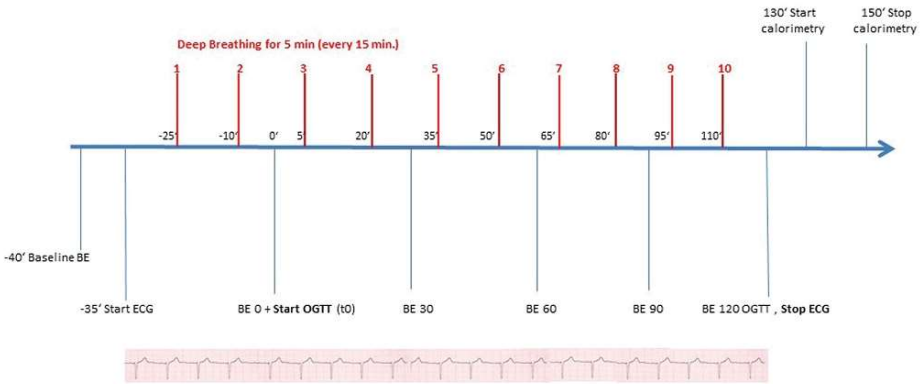

Figure 2. Schematic overview of the experiment. Baseline blood samples were obtained at time point − 40, OGTT was started 40 min after the first blood extraction (BE) and stopped 120 min after ingestion of the glucose solution. ECG recordings were performed 35 min before the start and during the OGTT. Slow deep breathing maneuvers and normal breathing respectively, were performed 25 min before the start of the OGTT for a duration of 5 min with 10 min breaks between each maneuver. Energy expenditure was measured after the end of the OGTT.

In conclusion, although slow deep breathing has a profound effect on cardiac autonomic activity, it has no major effect on autonomic innervation of other peripheral organs. Insulin secretion, insulin sensitivity and resting energy expenditure were therefore not affected by slow deep breathing in our study. This may be due to an insufficient vagal modulation by the applied slow deep breathing practice or cardiac specificity of vagal responses to this breathing exercise.

The mechanistic basis of the detected association of insulinemia with heart rate and its implication for glucose metabolism warrents to be investigated with further clinical trials. Such results could be the basis to develop novel treatment approaches for impaired insulin secretion in diabetes.

Methods

In our study, 15 male healthy volunteers between 20 and 55 years were included. Body mass index (BMI) was between 20.5 and 27.3 kg/m^2, body fat content was measured by bioelectrical impedance testing (BIA 101 by Akern Srl, Florence, Italy) and estimated with Cyprus 2.7 Body Composition Analysis Software (RJL Systems, Michigan, USA). Participants had a body fat content between 6.8 and 20.8 %. Detailed subject characteristics are presented in Table 3.

With an alpha-level of 0.05, our sample size (n = 15) provided 80% power to detect an effect size f = 0.35 (calculated with Gpower 3.1).

The study protocol was approved by the ethics committee of the medical faculty of the University Tübingen. Written informed consent was obtained from all study volunteers and all research was performed in accordance with relevant guidelines and regulations. The study was pre-registered at clinicaltrials.gov (NCT04150627; 01/11/2019).

A schematic overview of the study is shown in Figs. 2 and 3.

All study participants underwent a 75 g oral glucose tolerance test (OGTT) with blood samples obtained at − 40 min, before glucose ingestion (0 min) and 30, 60, 90 and 120 min after glucose ingestion (Accu-Chek Dextrose OGT, Roche). Glycated hemoglobin (HbA1c) was measured at baseline with Tosoh glycohemoglobin

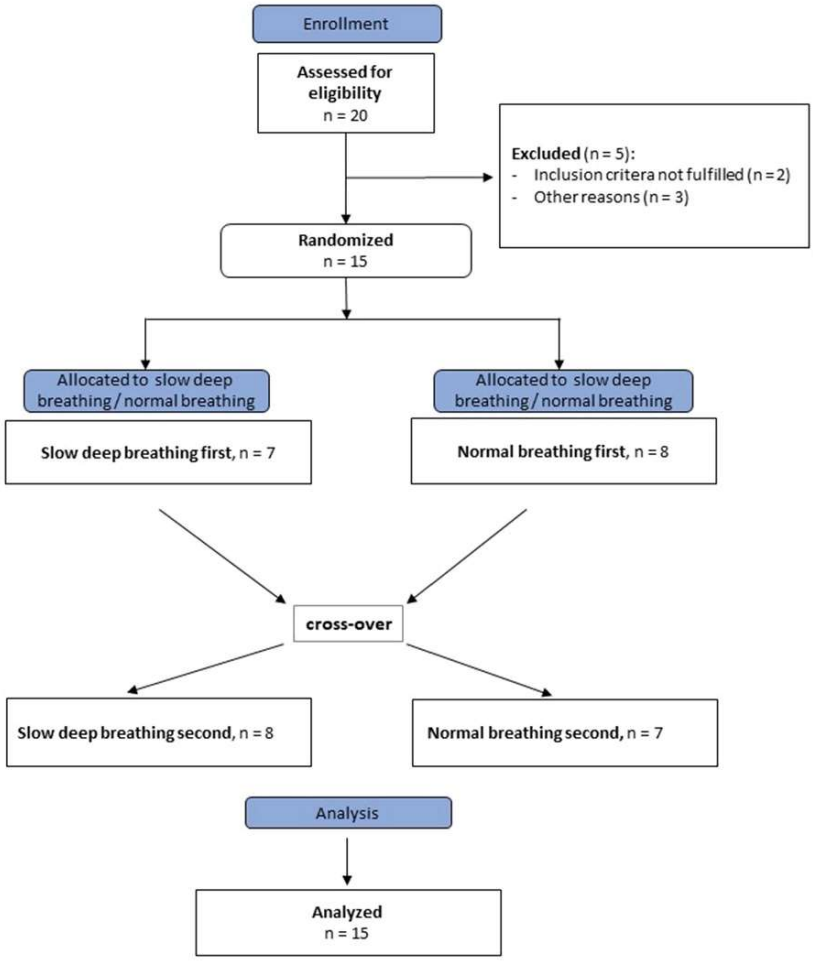

Figure 3. Overview of the course of the study.

analyzer HLC-723G8 (Tosoh Bioscience, Tokyo, Japan). Glucose, proinsulin and insulin levels, C-peptide, and non-esterified fatty acids (NEFA) were measured at all time points.

Serum pro-insulin, insulin and C-peptide concentrations were determined by an immunoassay with ADVIA Centaur XP Immunoassay System (Siemens Healthineers, Eschborn, Germany). Glucose measurements were done by hexokinase method with ADVIA XPT System (Siemens Healthineers, Eschborn, Germany). All measurements in this study were performed in a routine diagnostic laboratory that is accredited with the German accredited body (DAkkS).

Insulin secretion and insulin sensitivity were calculated from the oral glucose tolerance tests as described previously[19].

Slow deep breathing or normal breathing was performed twenty-five minutes before and during the entire OGTT in a randomized cross-over design at two different days in the morning.

Volunteers underwent the study in a supine position. Slow deep paced breathing maneuvers vs. control (paced-breathing) were carried out every 15 min for a period of 5 min. Affect 4.0 was used for visually displaying inhalation and exhalation cycles on a laptop with a moving bar for inhalation and exhalation in order to make it easy for the volunteers to follow paced breathing[30]. This also ensured compliance with the breathing rate,

inspiration to expiration ratio (1:2) and time intervals. In the Slow deep breathing condition, participants were instructed with 6 breaths per minute during paced breathing. In control condition, participants were instructed with 14 breaths per minute for 5-min followed by a 10-min interval with endogenous breath rate. Hence, a 15-min paced-breathing training cycle (5-min pre-train, 5-min paced-train and 5-min recovery) was repeated until the end of the visit.

Before the start of the experiment, all volunteers were trained in deep breathing and normal breathing maneuvers. Instructions for paced breathing and parameters were based on similar studies showing increased vagal modulation during slow deep breathing[21].

Breathing cycles were recorded with a respiration belt that was placed around volunteers' chest (Biopac, Systems Inc., Goleta, CA, USA). Respiration data were collected with Acknowledge Student Lab (Biopac, Systems Inc., Goleta, CA) at a sample rate of 1000 Hz. The depth was measured between the peak and the lowest point before the peak. Collected data were cut into 5-min bins in accordance to the training cycle for analysis in Matlab (Mathworks, Inc. USA). Data segments were preprocessed with a band-pass filter (0.05–1 Hz). Breathing rate (BR) and respiration depth were averaged in each time bin as indicators of volunteers' respirational activity.

Electrocardiogram (ECG) was recorded to analyze heart rate variability (HRV) as a parameter for sympatho-vagal activation. ECG was continuously recorded throughout the visit with Biopac MP 36 (Biopac Systems, Inc., Goleta, CA), and analyzed in Matlab. Collected data were then cut into 5-min bins in accordance to the training cycle for analysis. ECG was collected with a sampling rate at 1000 Hz. A band-pass filter at 0.5–35 Hz was applied on sampling data. Raw heart rate sampling data were visually inspected and corrected for artifacts in Artiifact[22], and analyzed in Matlab. Mean heart rate (HR) and root mean square of successive differences (RMSSD) in inter-beat intervals were calculated in the time domain as indicators of the vagal activity. Heart rate variability is mainly modulated by cardiac parasympathetic nerve activity as data of pharmacological blockade of the autonomic nervous system in animals[23,24] and humans[25,26] suggest. HRV parameters were determined for each 5-min time period.

Resting energy expenditure was measured after the OGTT and breathing maneuvers. Energy expenditure after deep breathing and normal breathing was calculated by indirect calorimetry measurements with Vyntus CPX (Vyaire Medical, Illinois, USA). Consumption of O_2 and output of CO_2 were measured for 20 min. An individual calibration control evaluation (ICcE) was applied, correcting for monitor-specific deviations and eliminating the influence of inherent variability of the device on measurement results[27].

Statistical analysis was performed using SAS 9.4 (SAS Institute, Cary, NC). $p < 0.05$ was considered statistically significant, and $p < 0.10$ is considered a trend. The data are presented as mean ± SE.

Major outcomes were boxcox-transformed. Mixed effect models were performed on respiration and HRV data with main effects of time, condition (slow deep breathing vs. control), and their interaction. Variance–covariance structure providing the best fit was chosen based on the minimum value of Akaike's Information Criterion (AIC). For breathing rate and respiration depth, planned contrasts were performed to compare between the pre-train and paced-train cycles. For HRV measurements, post hoc contrasts were performed when there was a trend or significant effect of time by condition interaction. The contrasts were performed for the slow deep breathing (SDB) day, the control day, or differences between days (SDB vs. control) respectively, with Bonferroni-Holm correction for multiple testing.

Hormonal results were also analyzed in mixed effect models with main effects of time, condition, and their interaction. Moreover, an additional explorative analysis was performed on hormones with main effect of condition and time, their interaction, and breathing rate, respiration depth, HR and RMSSD (all from the same cycles when the blood samples were collected) as covariates, and reported when covariates were significant.

Data availability

The data are not publicly available due to them containing information that could compromise research participant privacy/consent.

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References

1. DeFronzo, R. A. From the triumvirate to the ominous octet: A new paradigm for the treatment of type 2 diabetes mellitus. *Diabetes* **58**, 773–795 (2009).
2. DeFronzo, R. A. Insulin resistance, lipotoxicity, type 2 diabetes and atherosclerosis: the missing links. The Claude Bernard Lecture 2009. *Diabetologia* **53**, 1270–1287 (2010).
3. Kahn, S. E. The relative contributions of insulin resistance and beta-cell dysfunction to the pathophysiology of Type 2 diabetes. *Diabetologia* **46**, 3–19 (2003).
4. Staiger, H., Machicao, F., Fritsche, A. & Häring, H.-U. Pathomechanisms of type 2 diabetes genes. *Endocr. Rev.* **30**, 557–585 (2009).
5. Morton, G. J., Cummings, D. E., Baskin, D. G., Barsh, G. S. & Schwartz, M. W. Central nervous system control of food intake and body weight. *Nature* **443**, 289–295 (2006).
6. Heni, M. *et al.* Central insulin administration improves whole-body insulin sensitivity via hypothalamus and parasympathetic outputs in men. *Diabetes* **63**, 4083–4088 (2014).
7. Begg, D. P. & Woods, S. C. Interactions between the central nervous system and pancreatic islet secretions: A historical perspective. *Adv. Physiol. Educ.* **37**, 53–60 (2013).
8. Ahrén, B. & Holst, J. J. The cephalic insulin response to meal ingestion in humans is dependent on both cholinergic and noncholinergic mechanisms and is important for postprandial glycemia. *Diabetes* **50**, 1030–1038 (2001).
9. Makhmutova, M. *et al.* Pancreatic beta cells communicate with vagal sensory neurons. *Gastroenterology* https://doi.org/10.1053/j.gastro.2020.10.034 (2020).
10. Teff, K. L. Visceral nerves: Vagal and sympathetic innervation. *J. Parenter. Enter. Nutr.* **32**, 569–571 (2008).
11. Strubbe, J. H. & Steffens, A. B. Neural control of insulin secretion. *Horm. Metab. Res.* **25**, 507–512 (1993).

12. Macedo, M. P. *et al.* Risk of postprandial insulin resistance: The liver/vagus rapport. *Rev. Endocr. Metab. Disord.* **15**, 67–77 (2014).
13. Lindmark, S., Wiklund, U., Bjerle, P. & Eriksson, J. W. Does the autonomic nervous system play a role in the development of insulin resistance? A study on heart rate variability in first-degree relatives of Type 2 diabetes patients and control subjects. *Diabet. Med.* **20**, 399–405 (2003).
14. Perin, P. C., Maule, S. & Quadri, R. Sympathetic nervous system, diabetes, and hypertension. *Clin. Exp. Hypertens.* **23**, 45–55 (2001).
15. Vosseler, A. *et al.* No modulation of postprandial metabolism by transcutaneous auricular vagus nerve stimulation: A cross-over study in 15 healthy men. *Sci. Rep.* **10**, 20466 (2020).
16. Pal, G. K., Velkumary, S. & Madanmohan,. Effect of short-term practice of breathing exercises on autonomic functions in normal human volunteers. *Indian J. Med. Res.* **120**, 115–121 (2004).
17. Cottin, F., Papelier, Y. & Escourrou, P. Effects of exercise load and breathing frequency on heart rate and blood pressure variability during dynamic exercise. *Int. J. Sports Med.* **20**, 232–238 (1999).
18. Mori, H. *et al.* How does deep breathing affect office blood pressure and pulse rate?. *Hypertens. Res.* **28**, 499–504 (2005).
19. Wagner, R. *et al.* A novel insulin sensitivity index particularly suitable to measure insulin sensitivity during gestation. *Acta Diabetol.* **53**, 1037–1044 (2016).
20. Spruyt, A., Clarysse, J., Vansteenwegen, D., Baeyens, F. & Hermans, D. Affect 4.0: A free software package for implementing psychological and psychophysiological experiments. *Exp. Psychol.* **57**, 36–45 (2010).
21. Gholamrezaei, A., Diest, I. V., Aziz, Q., Vlaeyen, J. W. S. & Oudenhove, L. V. Influence of inspiratory threshold load on cardiovascular responses to controlled breathing at 0.1 Hz. *Psychophysiology* **56**, e13447 (2019).
22. Kaufmann, T., Sütterlin, S., Schulz, S. M. & Vögele, C. ARTiiFACT: A tool for heart rate artifact processing and heart rate variability analysis. *Behav. Res. Methods* **43**, 1161–1170 (2011).
23. Billman, G. E. The effect of heart rate on the heart rate variability response to autonomic interventions. *Front. Physiol.* **4**, 2 (2013).
24. Deutschman, C. S., Harris, A. P. & Fleisher, L. A. Changes in heart rate variability under propofol anesthesia: A possible explanation for propofol-induced bradycardia. *Anesth. Analg.* **79**, 373–377 (1994).
25. Challapalli, S., Kadish, A. H., Horvath, G. & Goldberger, J. J. Differential effects of parasympathetic blockade and parasympathetic withdrawal on heart rate variability. *J. Cardiovasc. Electrophysiol.* **10**, 1192–1199 (1999).
26. Martinmäki, K., Rusko, H., Kooistra, L., Kettunen, J. & Saalasti, S. Intraindividual validation of heart rate variability indexes to measure vagal effects on hearts. *Am. J. Physiol.-Heart Circ. Physiol.* **290**, H640–H647 (2006).
27. Schadewaldt, P., Nowotny, B., Strassburger, K., Kotzka, J. & Roden, M. Indirect calorimetry in humans: a postcalorimetric evaluation procedure for correction of metabolic monitor variability. *Am. J. Clin. Nutr.* **97**, 763–773 (2013).
28. Kromenacker, B. W., Sanova, A. A., Marcus, F. I., Allen, J. J. B. & Lane, R. D. Vagal mediation of low-frequency heart rate variability during slow yogic breathing. *Psychosom. Med.* **80**, 581–587 (2018).
29. Iversen, S., Iversen, L. & Saper, C. B. The autonomic nervous system and the hypothalamus. 16.

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Author contributions
A.V. researched and analyzed data and drafted the manuscript. D.Z. analyzed data and contributed to discussion. J.H., A.G., K.K., S.H. and A.P. researched data and contributed to discussion. A.B., H.U.H., R.W., H.P. and S.K. contributed to discussion. M.H. contributed to analyses, supervised the project and contributed to discussion. All authors approved the final version of the manuscript prior to submission.

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Competing interests
The authors declare no competing interests.

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Correspondence and requests for materials should be addressed to M.H.

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2.3 Publication 3: Interscapular fat is associated with impaired glucose tolerance and insulin resistance independent of visceral fat mass

Authors: **Andreas Vosseler**, Jürgen Machann, Louise Fritsche, Christian Kübler, Andreas Peter, Hans-Ulrich Häring, Norbert Stefan, Andreas L. Birkenfeld, Andreas Fritsche, Robert Wagner, Martin Heni.

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Interscapular fat is associated with impaired glucose tolerance and insulin resistance independent of visceral fat mass

Andreas Vosseler[1,2,3], Jürgen Machann[2,3,4], Louise Fritsche[1,2,3], Christian Kübler[1,2,3], Hans-Ulrich Häring[1,2,3], Andreas L. Birkenfeld[1,2,3], Norbert Stefan[1,2,3], Andreas Peter[3,5], Andreas Fritsche[1,2,3], Robert Wagner[1,2,3], Martin Heni[1,2,3,5]

1. Department of Internal Medicine, Division of Diabetology, Endocrinology and Nephrology, University Hospital Tübingen, Tübingen, Germany
2. Institute for Diabetes Research and Metabolic Diseases of the Helmholtz Centre Munich at the University of Tübingen, Tübingen, Germany
3. German Center for Diabetes Research (DZD), Neuherberg, Germany.
4. Section of Experimental Radiology, Department of Diagnostic and Interventional Radiology, University Hospital Tübingen, Tübingen, Germany
5. Institute for Clinical Chemistry and Pathobiochemistry, Department for Diagnostic Laboratory Medicine, University Hospital Tübingen, Tübingen, Germany

Corresponding author

Prof. Martin Heni, MD
University Hospital Tübingen, Internal Medicine IV
Otfried-Müller-Str. 10
72076 Tübingen
Germany

E-mail: martin.heni@med.uni-tuebingen.de
Phone: +49 7071 29-82714
Fax: +49 7071 29-2784

Abstract

Dysregulated body fat distribution is a major determinant of various diseases. Adipose tissue in different localizations of the body appear to have protective or detrimental properties. Particularly increased visceral fat mass and ectopic lipids in the liver are linked to metabolic disorders such as insulin resistance and type 2 diabetes. Furthermore, interscapular fat is considered to be a non-classical, but metabolically active, fat compartment.

In this study, we measured interscapular fat mass and investigated its relationship with glucose tolerance, insulin sensitivity and insulin secretion in 822 subjects with a wide range of body mass index (BMI) and different glucose tolerance status. Magnetic resonance imaging was used to quantify body fat depots and an oral glucose tolerance test (OGTT) was performed to determine glucose metabolism.

Elevated interscapular fat mass was positively associated with age, BMI, total body, visceral and subcutaneous adipose tissue mass. High interscapular fat mass associated with elevated fasting glucose levels, glucose levels at 2 hours during the OGTT, glycated hemoglobin, as well as with insulin resistance, independently of sex, age, total body and visceral fat mass.

In conclusion, interscapular fat might be a highly specific fat compartment with potential impact on glucose metabolism and the pathogenesis of diabetes mellitus. Since this depot is assessible by ultrasound, it could represent a feasible target to quantify metabolic risk in the future.

Introduction

There are large inter-individual differences in the distribution of fat across the body. This distribution of adipose tissue appears to be crucial for human health as it is believed to contribute to the pathogenesis of various diseases[1,2]. Body fat distribution partly depends on sex and age[1,2]. The majority of body fat (around 80% of total body fat) is stored as subcutaneous adipose tissue (SCAT), most prominently around the abdomen as well as in the subscapular, gluteal and femoral areas[3], with potentially distinct impact dependent on its location[4,5].

The second major fat compartment, visceral adipose tissue (VAT), accounts for up to 20 % of total body fat content[6]. In contrast to subcutaneous fat, the venous blood of visceral adipose tissue is drained directly towards the liver via the portal vein[6]. Factors released from this fat compartment activate immune functions both directly in adipose tissue and in the liver. In response, cytokines are released with unfavorable impact on the entire body e.g. vascular inflammation[6,7] that increases the risk for cardiovascular diseases[8,9]. Furthermore, visceral fat is associated with multiple complications such as hypertension, dyslipidemia, insulin resistance and type 2 diabetes[10,11]. Visceral fat is also a predictor of all-cause mortality[12,13].

In addition to increased visceral fat mass, other fat compartments might also have a negative impact on glucose and lipid metabolism. Particularly fat accumulation in the liver and fat that is localized in the neck area, are related to insulin resistance and cardiovascular diseases in humans [14–20].

Besides different fat locations, there are functionally and histologically different types of adipocytes that are categorized as white adipose tissue (WAT) and brown adipose tissue (BAT), as well as brown-in-white, so called beige fat[21,22].

Though, BAT accounts only for a small proportion of adipose tissue[23]. The two fat types appear to have antagonistic characteristics as white adipose tissue stores excess energy and brown adipose tissue primarily converts stored energy to heat[24]. While brown adipose tissue substantially contributes to the total amount of fat in rodents, the proportion of brown adipose tissue is much smaller in humans[25]. This type of fat is more prominent in newborns but can be activated by cold exposure also in adults[26] where it inversely correlates with BMI[27] and glucose metabolism[28,29]. In

infants, brown adipose tissue is present in the interscapular region[30], while in adults it appears to be mainly located elsewhere[31].

In contrast to theoretical beneficial characteristics of brown adipose tissue in terms of glucose metabolism, we previously detected an association of increased interscapular fat mass with insulin resistance in 168 subjects[17]. In line, the neck region in whole-body MR images was highlighted in over half of the cases by machine learning as important to detect diabetes in a recent study[32]. In contrast to visceral adipose tissue, that can be precisely quantified only by expensive approaches or approaches that relay on radiation, interscapular fat is assessable by easier approaches like ultrasonography[33,34].

In the present study, we extended our previous work[17] and reevaluated this non-classical fat depot to study possible links of interscapular fat with glucose metabolism in a much larger cohort.

Methods

In our study we could now include 822 volunteers, 510 females and 312 males. The study participants were recruited from the ongoing TUEFF-study between 2011 and 2020[32]. All volunteers gave written informed consent and the study was approved by the Ethics Committee of the University Hospital of Tübingen, Germany, and all research was conducted in accordance with relevant guidelines and regulations.

The volunteers had an increased risk for the development of type 2 diabetes due to at least one risk factor (BMI >27 kg/m^2 or obesity, impaired fasting glucose or glucose tolerance, previous gestational diabetes or type 2 diabetes in first-grade relatives)[35]. An oral glucose tolerance test (OGTT) was performed after 10 hours of overnight fast. All volunteers underwent the OGTT with 75 g dextrose (Accu-Chek Dextrose OGT, Roche). Blood samples were obtained before and 30, 60, 90 and 120 minutes after glucose ingestion.

Baseline blood samples included glycohemoglobin A1c (HbA1c) that was measured with Tosoh glycohemoglobin analyzer HLC-723G8 (Tosoh Bioscience, Tokyo, Japan).

Pro-insulin, insulin, C-peptide and cortisol levels were determined by ADVIA Centaur XP Immunoassay System (Siemens Healthineers, Eschborn, Germany). Glucose measurements were analyzed by hexokinase method with ADVIA XPT System

(Siemens Healthineers, Eschborn, Germany). All measurements were performed in a routine diagnostic laboratory accredited with the German accredited body (DAkkS).

Insulin sensitivity was estimated from oral glucose tolerance tests as described by Matsuda and DeFronzo[38] (ISI Matsuda). NEFA-based insulin sensitivity index (NEFA-ISI) was calculated including BMI, serum insulin and non-esterified fatty acids (NEFA) as proposed by Wagner et al.[37]. First phase insulin secretion was calculated as proposed by Stumvoll et al.[38]: $1283 + 1.829 \times Insulin_{30} - 138.7 \times Glucose_{30} + 3.772 \times Insulin_{0}$.

Body fat compartments and liver fat content were quantified by magnetic resonance imaging (MRI) and proton magnetic resonance spectroscopy (^{1}H-MRS), respectively[39]. In brief, a T1-weighted fast spin echo sequence was applied to acquire images from the whole body in a total measurement time of approximately 20 minutes. From these, visceral adipose tissue (between hips and thoracic diaphragm) and subcutaneous adipose tissue (between hips and shoulders) were quantified using an automated fuzzy c-means algorithm and orthonormal snakes[40]. Additionally, interscapular fat area in the neck was segmented between the humeral heads as shown in figure 1. Liver fat was assessed in the posterior part of segment 7 applying a single voxel STEAM technique and calculating the ratio from fat (methylene + methyl signals) and the sum of water and fat resonances.

Statistical analysis on $\log_e$-transformed data was performed using JMP 14 (SAS Institute, Cary, NC). Two-sided t-tests were performed to compare variables. Data were furthermore tested with multivariate models. The data are presented as mean ± SE, $p < 0.05$ is considered statistically significant. Regression coefficient is presented as standardized β.

Results

The average age of the participants was 46 years (SD ± 15) and the body mass index (BMI) was 29.4 kg/m2 (SD ± 6.3). The basic anthropometrics are displayed in table 1. 50.9% of the volunteers (n=418) had a normal glucose tolerance (neither impaired fasting glucose nor impaired glucose tolerance), 24.9% (n=205) had an impaired fasting glucose (IFG, fasting plasma glucose levels 5.6 mmol/l to 6.9

mmol/l), 96 were categorized as impaired glucose tolerance (IGT, 2-h values in the oral glucose tolerance test of 7.8 mmol/l to 11.0 mmol/l) and 103 had both IFG and IGT, according to ADA's criteria[41]. The metabolic characterization of our cohort is shown in table 2.

Interscapular fat mass was higher in females than in males, both unadjusted (β=0.07±0.629; p=0.04) and adjusted for total adipose tissue mass (β=-0.07±0.002, p=0.002). However, after additional adjustment for visceral fat mass, sex was no longer associated with interscapular fat (β=-0.05±0.503; p=0.10). Interscapular fat was positively associated with age (β=0.12±0.029; p=0.0005). Furthermore, it was positively correlated with BMI (β=0.82±0.007; p<0.0001), total adipose tissue (β=0.82±0.017; p<0.0001), visceral adipose tissue (β=0.53±0.004; p<0.0001) and with subcutaneous adipose tissue (β=0.84±0.007; p<0.0001), detailed results are displayed in table 3 and figure 2.

We next analyzed associations of interscapular fat with glucose metabolism (table 4). This fat compartment was positively correlated with fasting glucose (β=0.26±0.002; p<0.0001). This relationship remained significant after adjustment for sex and total adipose tissue (β=0.14±0.002; p=0.008), as well as after additional adjustment visceral adipose tissue (β=0.12±0.002; p=0.02). Of note, both interscapular and visceral fat were independently associated with fasting glucose (β=0.23±0.015; p=0.0002).

Interscapular fat was also associated with 2 hours glucose levels during the OGTT (β=0.24±0.003; p<0.0001). Again, this relationship was independent of sex, age, total and visceral fat content (β=0.24±0.005; p<0.0001). In line, there were positive associations of interscapular fat with $AUC_{glucose}$ during the OGTT (β=0.30±0.006; p<0.0001;) as well as with HbA1c (β=0.21±0.009; p<0.0001) in univariate models. These associations remained statistically significant after adjustment for sex, age, total and visceral fat content ($AUC_{glucose}$ during the OGTT: β=0.29±0.010; p<0.0001; HbA1c: β=0.13±0.013; p=0.02).

In search for potential contributors to the relation of interscapular fat and glycemia, we next analyzed the two major determinants of blood glucose – insulin sensitivity and insulin secretion. Interscapular fat content was negatively associated with insulin sensitivity (ISI Matsuda: β=-0.51±0.013; p<0.0001; NEFA ISI: β=-0.66±0.003; p<0.0001) as shown in figure 3. This association remained significant after

adjustment for sex, age and total adipose tissue mass (ISI Matsuda: β=-0.27±0.023; p<0.0001; NEFA ISI: β=-0.30±0.005; p<0.0001) as well as after additional adjustment visceral adipose tissue content (ISI Matsuda: β=-0.22±0.02; p<0.0001; NEFA ISI: β=-0.27±0.005; p<0.0001).

Only in a univariate model, insulin secretion assessed from both C-peptide and glucose concentrations was positively associated with interscapular fat ($AUC_{C-pep0-30/AUCgluc0-30}$: β=0.11±0.123; p=0.001; adjusted for ISI Matsuda). However, after adjustment for sex, age, total and visceral adipose tissue mass, there was no significant association of insulin secretion with interscapular fat ($AUC_{C-pep0-30/AUCgluc0-30}$: β=-0.04±0.187; p=0.45; adjusted for ISI Matsuda).

First phase insulin secretion assessed from both serum insulin and glucose concentrations was positively correlated with interscapular fat again only in univariate models (β=0.16±1.458; p<0.0001; adjusted for ISI Matsuda). However, there was no correlation of first phase insulin secretion with interscapular fat after adjustment for sex, age, total and visceral adipose tissue content (β=-0.03±2.171; p=0.61; adjusted for ISI Matsuda).

There were no significant interactions of interscapular fat with serum cortisol (β=-0.06±0.321; p=0.11).

Discussion

We detected an association of higher amounts of interscapular fat with impaired glucose tolerance and insulin resistance. In addition, the link between interscapular fat and glycemia was robust and independent of the major potential confounders sex, age and total adiposity. Of note, the detected associations were independent of visceral fat mass. Though, there was no correlation of interscapular fat with insulin secretion in our multivariable models. The fact that the detected associations with glycemia remained significant after adjustment for visceral fat suggests that interscapular fat is not just a read-out of visceral fat mass.

Our data support the earlier hypothesis[17] that interscapular fat is linked to insulin resistance in humans. Thus, interscapular fat tissue might harbor cells that are metabolically active and, thereby, contribute to the pathogenesis of insulin resistance in the entire body. The negative effects of interscapular fat on glucose metabolism

could be caused by pro-inflammatory adipokines, similarly to what has been described for other non-classical fat compartments, like perivascular fat[42].

The main driver of the development of detrimental fat depots such as interscapular fat is obesity that also induces excess fat depots that lead to insulin resistance via cellular mechanisms[43].

In addition, elevated cortisol levels can contribute to insulin resistance[44]. Especially fat in the neck area is a symptom of pathological hypercortisolism and might therefore be a relevant parameter in accordance with interscapular fat. Though, interscapular fat was not associated with serum cortisol in our study. Since we measured cortisol only once in the morning, it is still possible that one pathophysiologic link could be subclinical hypercortisolism that would only be detectable by more measurements over the day or by specific suppression tests[45,46].

The association of interscapular fat and insulin resistance in our study was consistent with previous findings with MR-derived quantification[17] or simpler anthropometric estimates[47,48]. In line, interscapular fat was associated with additional risk factors for insulin resistance like age, increased BMI and body fat compartments.

While the amount of interscapular fat was higher in women, this sex difference was not independent of total and visceral fat content. This observation is well in line with known differences in body fat distribution between sexes[3] but argues against a sex-specific effect specifically for interscapular fat.

Interscapular fat might be a clinically relevant risk factor of impaired glucose tolerance, independent of visceral fat mass, that is in turn associated with an increased risk of cardiovascular disease[49,50].

Furthermore, insulin secretion was not linked to interscapular fat after adjustment for potential confounders.

In line with previous observation in very large cohorts[31], our data argue against a predominance of highly active brown fat in the interscapular region as protective properties of this fat depot on glucose metabolism are missing. In addition, the association of interscapular fat with insulin resistance has an opposing direction to the characteristics of brown fat in other studies[51,52].

Even though it appears unlikely that interscapular fat represents larger amounts of brown adipose tissue, a limitation of our study is that the histological type of the adipose tissue of interscapular fat was not examined. In addition, potential risk factors for the accumulation of interscapular fat stay unclear.

In conclusion, interscapular fat is associated with insulin resistance and fasting glucose as well as 2-hours glucose from the OGTT. It seems to be an important fat compartment concerning glucose metabolism, independent of visceral fat mass. Further studies may elucidate its consequence for the pathogenesis of insulin resistance and dysglycemia. Since this compartment is assessable by widely available approaches like ultrasound[33,34], it could represent a feasible target to quantify metabolic risk in clinical practice in the future.

Author Contributions

AV researched and analyzed data and drafted the manuscript. JM researched and analyzed data and contributed to discussion. LF, CK, AP, NS, AF and RW researched data and contributed to discussion. HUH and AB contributed to discussion. MH contributed to analyses, supervised the project and contributed to discussion. All authors approved the final version of the manuscript prior to submission.

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Conflict of interest

The authors do not have conflicts of interest that are directly relevant to the contents of this study.

Data Availability

The data are not publicly available due to them containing information that could compromise research participant privacy/consent.

References

1. Després, J.-P. & Lemieux, I. Abdominal obesity and metabolic syndrome. *Nature* **444**, 881–887 (2006).
2. Blüher, M. & Laufs, U. New concepts for body shape-related cardiovascular risk: role of fat distribution and adipose tissue function. *Eur Heart J* **40**, 2856–2858 (2019).
3. Karastergiou, K., Smith, S. R., Greenberg, A. S. & Fried, S. K. Sex differences in human adipose tissues – the biology of pear shape. *Biology of Sex Differences* **3**, 13 (2012).
4. Stefan, N. Causes, consequences, and treatment of metabolically unhealthy fat distribution. *Lancet Diabetes Endocrinol* **8**, 616–627 (2020).
5. Tchkonia, T. *et al.* Mechanisms and metabolic implications of regional differences among fat depots. *Cell Metab* **17**, 644–656 (2013).
6. Ibrahim, M. M. Subcutaneous and visceral adipose tissue: structural and functional differences. (2010).
7. Frayn, K. N. Visceral fat and insulin resistance — causative or correlative? *British Journal of Nutrition* **83**, S71–S77 (2000).
8. Britton, K. A. *et al.* Body fat distribution, incident cardiovascular disease, cancer, and all-cause mortality. *J Am Coll Cardiol* **62**, 921–925 (2013).
9. Després Jean-Pierre. Body Fat Distribution and Risk of Cardiovascular Disease. *Circulation* **126**, 1301–1313 (2012).
10. Björntorp, P. Abdominal obesity and the development of noninsulin-dependent diabetes mellitus. *Diabetes/Metabolism Reviews* **4**, 615–622 (1988).
11. Wajchenberg, B. L. Subcutaneous and Visceral Adipose Tissue: Their Relation to the Metabolic Syndrome. *Endocrine Reviews* **21**, 697–738 (2000).
12. Kuk, J. L. *et al.* Visceral fat is an independent predictor of all-cause mortality in men. *Obesity (Silver Spring)* **14**, 336–341 (2006).

13. McNeely, M. J., Shofer, J. B., Leonetti, D. L., Fujimoto, W. Y. & Boyko, E. J. Associations Among Visceral Fat, All-Cause Mortality, and Obesity-Related Mortality in Japanese Americans. *Diabetes Care* **35**, 296–298 (2012).
14. Laakso, M., Matilainen, V. & Keinänen-Kiukaanniemi, S. Association of neck circumference with insulin resistance-related factors. *International Journal of Obesity* **26**, 873–875 (2002).
15. Dixon, J. B. & O'Brien, P. E. Neck circumference a good predictor of raised insulin and free androgen index in obese premenopausal women: changes with weight loss. *Clinical Endocrinology* **57**, 769–778 (2002).
16. Stefan, N., Häring, H.-U. & Schulze, M. B. Metabolically healthy obesity: the low-hanging fruit in obesity treatment? *The Lancet Diabetes & Endocrinology* **6**, 249–258 (2018).
17. Thamer, C. *et al.* Interscapular fat is strongly associated with insulin resistance. *J Clin Endocrinol Metab* **95**, 4736–4742 (2010).
18. Wagner, R. *et al.* Pathophysiology-based subphenotyping of individuals at elevated risk for type 2 diabetes. *Nat Med* **27**, 49–57 (2021).
19. Preis, S. R. *et al.* Neck Circumference as a Novel Measure of Cardiometabolic Risk: The Framingham Heart Study. *J Clin Endocrinol Metab* **95**, 3701–3710 (2010).
20. Dai, Y., Wan, X., Li, X., Jin, E. & Li, X. Neck circumference and future cardiovascular events in a high-risk population--A prospective cohort study. *Lipids Health Dis* **15**, 46 (2016).
21. Frühbeck, G., Becerril, S., Sáinz, N., Garrastachu, P. & García-Velloso, M. J. BAT: a new target for human obesity? *Trends in Pharmacological Sciences* **30**, 387–396 (2009).
22. Shapira, S. N. & Seale, P. Transcriptional control of brown and beige fat development and function. *Obesity (Silver Spring)* **27**, 13–21 (2019).
23. Wronska, A. & Kmiec, Z. Structural and biochemical characteristics of various white adipose tissue depots. *Acta Physiol (Oxf)* **205**, 194–208 (2012).
24. Saely, C. H., Geiger, K. & Drexel, H. Brown versus white adipose tissue: a mini-review. *Gerontology* **58**, 15–23 (2012).

25. Nedergaard, J. & Cannon, B. How brown is brown fat? It depends where you look. *Nature Medicine* **19**, 540–541 (2013).

26. Cypess, A. M. *et al.* Anatomical localization, gene expression profiling and functional characterization of adult human neck brown fat. *Nature Medicine* **19**, 635–639 (2013).

27. Cypess, A. *et al.* Identification and Importance of Brown Adipose Tissue in Adult Humans. *The New England Journal of Medicine* **360**, 1509–1517 (2009).

28. Matsushita, M. *et al.* Impact of brown adipose tissue on body fatness and glucose metabolism in healthy humans. *Int J Obes (Lond)* **38**, 812–817 (2014).

29. Chondronikola, M. The role of brown adipose tissue and the thermogenic adipocytes in glucose metabolism: recent advances and open questions. *Current Opinion in Clinical Nutrition & Metabolic Care* **23**, 282–287 (2020).

30. Lidell, M. E. *et al.* Evidence for two types of brown adipose tissue in humans. *Nature Medicine* **19**, 631–634 (2013).

31. Becher, T. *et al.* Brown adipose tissue is associated with cardiometabolic health. *Nature Medicine* **27**, 58–65 (2021).

32. Dietz, B. *et al.* Diabetes detection from whole-body magnetic resonance imaging using deep learning. *JCI Insight* e146999 (2021) doi:10.1172/jci.insight.146999.

33. Tsai, W.-C., Yang, S.-H. & Chen, H.-C. 3372: Interscapular area muscle thickness measurement by ultrasonography. *Ultrasound in Medicine and Biology* **32**, P253 (2006).

34. Ribeiro, G. *et al.* A-Mode Ultrasound Reliability in Fat and Muscle Thickness Measurement. *J Strength Cond Res* (2020) doi:10.1519/JSC.0000000000003691.

35. Ley, S. H., Schulze, M. B., Hivert, M.-F., Meigs, J. B. & Hu, F. B. Risk Factors for Type 2 Diabetes. in *Diabetes in America* (eds. Cowie, C. C. et al.) (National Institute of Diabetes and Digestive and Kidney Diseases (US), 2018).

36. Matsuda, M. & DeFronzo, R. A. Insulin sensitivity indices obtained from oral glucose tolerance testing: comparison with the euglycemic insulin clamp. *Diabetes Care* **22**, 1462–1470 (1999).

37. Wagner, R. *et al.* A novel insulin sensitivity index particularly suitable to measure insulin sensitivity during gestation. *Acta Diabetol* **53**, 1037–1044 (2016).

38. Stumvoll, M. *et al.* Use of the oral glucose tolerance test to assess insulin release and insulin sensitivity. *Diabetes Care* **23**, 295–301 (2000).

39. Machann, J. *et al.* Follow-up whole-body assessment of adipose tissue compartments during a lifestyle intervention in a large cohort at increased risk for type 2 diabetes. *Radiology* **257**, 353–363 (2010).

40. Würslin, C. *et al.* Topography mapping of whole body adipose tissue using A fully automated and standardized procedure. *J Magn Reson Imaging* **31**, 430–439 (2010).

41. American Diabetes Association. Diagnosis and Classification of Diabetes Mellitus. *Diabetes Care* **37**, S81–S90 (2014).

42. Almabrouk, T. a. M., Ewart, M. A., Salt, I. P. & Kennedy, S. Perivascular fat, AMP-activated protein kinase and vascular diseases. *British Journal of Pharmacology* **171**, 595–617 (2014).

43. Achike, F. I., To, N.-H. P., Wang, H. & Kwan, C.-Y. Obesity, metabolic syndrome, adipocytes and vascular function: A holistic viewpoint. *Clin Exp Pharmacol Physiol* **38**, 1–10 (2011).

44. Purnell, J. Q. *et al.* Enhanced cortisol production rates, free cortisol, and 11β-HSD-1 expression correlate with visceral fat and insulin resistance in men: effect of weight loss. *American Journal of Physiology-Endocrinology and Metabolism* **296**, E351–E357 (2009).

45. Di Dalmazi, G., Pasquali, R., Beuschlein, F. & Reincke, M. Subclinical hypercortisolism: a state, a syndrome, or a disease? *Eur J Endocrinol* **173**, M61-71 (2015).

46. Chiodini, I. Clinical review: Diagnosis and treatment of subclinical hypercortisolism. *J Clin Endocrinol Metab* **96**, 1223–1236 (2011).

47. Stabe, C. *et al.* Neck circumference as a simple tool for identifying the metabolic syndrome and insulin resistance: results from the Brazilian Metabolic Syndrome Study. *Clin Endocrinol (Oxf)* **78**, 874–881 (2013).

48. Polymeris, A. & Papapetrou, P. D. Anthropometric indicators of insulin resistance. *Hormones (Athens)* (2021) doi:10.1007/s42000-021-00296-0.

49. Ceriello, A. Impaired glucose tolerance and cardiovascular disease: the possible role of post-prandial hyperglycemia. *Am Heart J* **147**, 803–807 (2004).

50. DECODE Study Group, the European Diabetes Epidemiology Group. Glucose tolerance and cardiovascular mortality: comparison of fasting and 2-hour diagnostic criteria. *Arch Intern Med* **161**, 397–405 (2001).

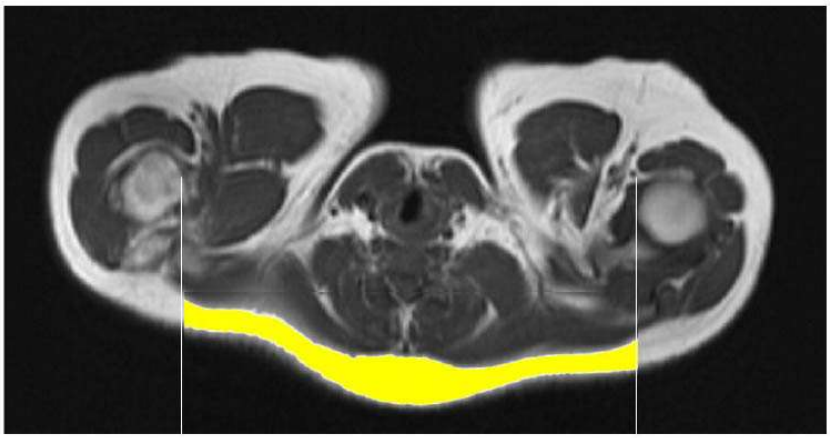
51. Maliszewska, K. & Kretowski, A. Brown Adipose Tissue and Its Role in Insulin and Glucose Homeostasis. *International Journal of Molecular Sciences* **22**, 1530 (2021).

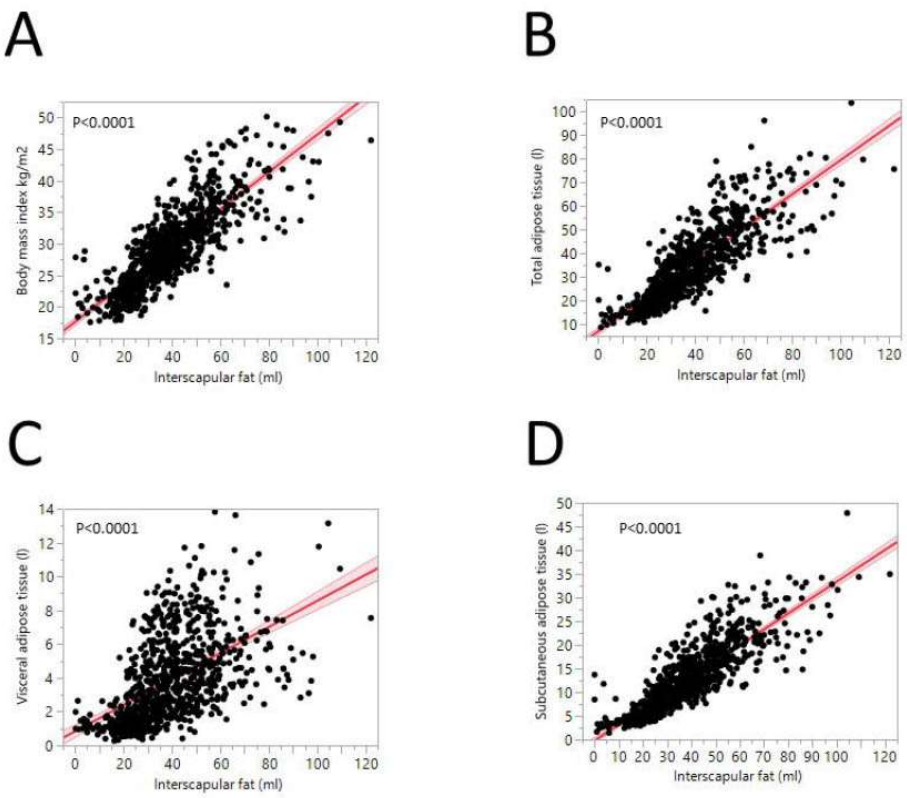
52. Gavaldà-Navarro, A., Villarroya, J., Cereijo, R., Giralt, M. & Villarroya, F. The endocrine role of brown adipose tissue: An update on actors and actions. *Rev Endocr Metab Disord* (2021) doi:10.1007/s11154-021-09640-6.

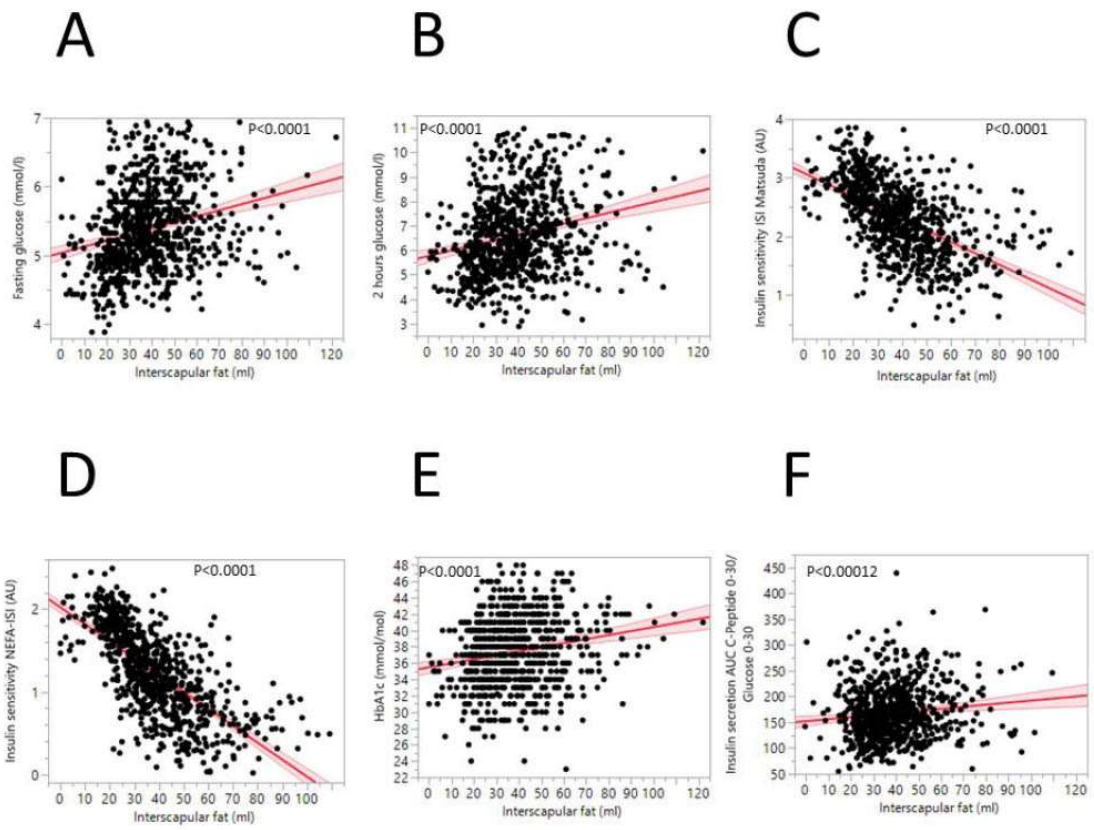
Figure 1: Interscapular fat depot
Magnetic resonance image of the interscapular fat depot, area in the neck was segmented between the humeral heads (highlighted section).

Figure 2: Correlation of interscapular fat with body mass index and fat depots
Positive correlation of interscapular fat with body mass index (A), total adipose tissue (B), visceral adipose tissue (C) and subcutaneous adipose tissue (D) in a univariate model, n=822. Lines represent fit lines ±CI.

Figure 3: Correlation of interscapular fat with parameters of glucose metabolism
Positive correlation of interscapular fat with fasting glucose (A), 2 hours glucose from the oral glucose tolerance test (B), insulin sensitivity index ISI Matsuda (C), insulin sensitivity index NEFA ISI (D) and glycated hemoglobin HbA1c in an univariate model, n=822. Lines represent fit lines ±CI.






A
P<0.0001
Fasting glucose (mmol/l)
Interscapular fat (ml)
B
P<0.0001
2 hours glucose (mmol/l)
Interscapular fat (ml)
C
P<0.0001
Insulin sensitivity ISI Matsuda (AU)
Interscapular fat (ml)
D
P<0.0001
Insulin sensitivity NEFA-ISI (AU)
Interscapular fat (ml)
E
P<0.0001
HbA1c (mmol/mol)
Interscapular fat (ml)
F
P<0.00012
Insulin secretion AUC C-Peptide 0-30/ Glucose 0-30
Interscapular fat (ml)

3 Discussion

In this work, potential pathogenetic determinants of type 2 diabetes were investigated. The work was focused on insulin sensitivity and insulin secretion which were studied in three clinical trials. These studies applied electrical vagus nerve stimulation and breathing maneuvers to modulate the autonomic nervous system, and studied body fat distribution with focus on interscapular fat and its contribution to glucose metabolism.
In the following sub-chapters, the three clinical trials of this research project are discussed and conclusions are drawn regarding clinical implications of the study results.

3.1 Non-invasive vagus nerve stimulation and its impact on glucose metabolism

This work addressed the potential influence of the autonomic nervous system on systemic glucose metabolism. To modulate the parasympathetic branch of the autonomic nervous system, non-invasive vagus nerve stimulation versus sham stimulation was performed in 15 healthy young men in a cross-over design (Vosseler et al., 2020). Systemic glucose metabolism was quantified using concurrent oral glucose tolerance tests. However, insulin sensitivity and insulin secretion were not affected by the applied vagus nerve stimulation and sham stimulation. Further, resting energy expenditure was not different between both conditions. Heart rate, as a proxy for cardiac autonomic activity, was not different between the two stimulation conditions, indicating ineffectiveness of the applied stimulation technique.
In contrast to these null findings, non-invasive vagus nerve stimulation was shown to increase vagus nerve activity (Clancy et al., 2014; Pal et al., 2004) and affect heart rate (Badran et al., 2018b) and blood pressure (Zamotrinsky et al., 2001) in previous studies. Therefore, this stimulation technique could potentially

affect peripheral organs like the pancreas as it was considered in the concept of this study.

Different discussion points for the ineffectiveness of the applied vagus nerve stimulation are presented in the manuscript (Vosseler et al., 2020): For instance, the stimulation of the auricular branch of the vagus nerve may not have been able to generate efferent outflows of the central nervous system towards peripheral organs. Physical factors like the location of the electrode, the stimulation intensity, duration and frequency are potential limitations of the applied vagus nerve stimulation. Further, the limited number of included participants (n=15) might have made it impossible to detect smaller effect sizes. Though, the study was sufficiently powered to detect effect sizes of f = 0.35 (80% power, α=0.05).

In line to our finding, a recent study by Gancheva et al. (Gancheva et al., 2017) could not detect a modulatory effect of the autonomic nervous system on peripheral organs by non-invasive vagus nerve stimulation.

In summary, non-invasive vagus nerve stimulation and sham stimulation had no detectable effects on glucose metabolism in the performed trial.

3.2 Slow deep breathing maneuvers and their effects on glucose metabolism

Since our vagus stimulation approach in the first study was not able to significantly modulate the autonomic nervous system and could therefore not clarify the impact of autonomic nervous system on systemic metabolism, we next aimed for an alternative approach to modulate autonomic activity. In this follow-up trial (Vosseler et al., 2021), 15 healthy young men performed slow deep breathing versus normal breathing maneuvers in a cross-over design. Peripheral glucose metabolism was again quantified using oral glucose tolerance tests.

In this study, insulin sensitivity and insulin secretion remained unaffected by the performed breathing maneuvers. Moreover, slow deep breathing did not change resting energy expenditure. In contrast to this null findings, parasympathetic

activity was increased during slow deep breathing though it did not alter peripheral glucose metabolism.
Unexpectedly, our study showed a correlation of mean heart rate and C-peptide and insulin levels regardless of breathing condition and glucose concentration. This correlation could be a surrogate for the influence of the autonomic nervous system on insulin secretion as it is known that the autonomic nervous system contributes to the regulation of insulin (Teff, 2008).
In a previous study (Kromenacker et al., 2018), slow deep breathing over three months was shown to be effective in modulating the autonomic tone in 60 participants. However, the modulation of the autonomic tone could be a long-term effect that was not caused by the performed breathing maneuvers. Disregarding a long-term effect of slow deep breathing, extended deep breathing periods may have been more effective in the modulation of the autonomic tone and impact on peripheral glucose metabolism in this work.
Further, slow deep breathing may modulate brain centers that effect the heart but not metabolic organs. While altering cardiac autonomic nervous system by slow deep breathing, its activity is not necessarily influenced at the levels where glucose metabolism can be modulated.

Potential limitations of the study are again the limited sample size and a selection bias as only male persons were included. In this pilot trial, we did not include women to avoid well known effects of the menstrual cycle on autonomic nervous system activity (Brar et al., 2015) that are difficult to control for and might have masked potential effects of the breathing intervention. Furthermore, sex-specific regulation of heart rate variability is suspected (Huang et al., 2013).
Moreover, the study protocol and the duration of slow deep breathing may have not been appropriate to induce strong effects on autonomic tone and glucose metabolism.

3.3 Interscapular fat and glucose metabolism

In the third study (Vosseler et al., 2022), the influence of one specific non-classical fat depot – interscapular fat – on systemic glucose metabolism was investigated. Interscapular fat is located in the neck area and was shown to be associated with insulin resistance (Thamer et al., 2010).

Body fat distribution was quantified by magnetic resonance imaging and oral glucose tolerance tests were performed in 822 women and men with an average age of 46 years and different status of glucose tolerance.

Interscapular fat was found to be directly associated with insulin resistance and 2 hours glucose levels during the OGTT that is a well-established predictor of cardiovascular diseases and mortality (e.g. DECODE Study Group, the European Diabetes Epidemiology Group., 2001). There was no association of interscapular fat with insulin secretion.

The adverse effects of interscapular fat on glucose metabolism could potentially result from adipokines that cause inflammation as known from other fat compartments like perivascular fat (Almabrouk et al., 2014). The association of interscapular fat with insulin resistance in this trial is in line with previous findings in MR-based body fat quantification (Thamer et al., 2010) and neck circumference (Polymeris and Papapetrou, 2021; Stabe et al., 2013). The development of detrimental excess fat depots such as interscapular fat may largely be caused by obesity. These excess fat depots trigger inflammatory processes that lead in turn to multiple complications as insulin resistance (Achike et al., 2011).

A limitation of this study is that the histological type of fat in the interscapular region was not examined whereas there are different types of adipose tissue with different effects on metabolism (Saely et al., 2012). The negative effects of interscapular fat on glucose metabolism seen in this study argue against a predominance of highly active brown fat in this location according to previous findings in large cohort studies (Becher et al., 2021; Betz and Enerbäck, 2015).

In conclusion, interscapular fat seems to be a clinically relevant risk factor of insulin resistance and impaired glucose tolerance and might be a relevant target for stratifying diabetes risk. Besides magnetic resonance imaging, other

techniques, i.e. ultrasound, should be used for the quantification of interscapular fat in the future.

3.4 Concept of the research project

This research project summarizes three studies that addressed the two main pathophysiological mechanisms in the development of type 2 diabetes mellitus – insulin sensitivity and insulin secretion. The current work aimed to clarify potential impact of the autonomic nervous system and of a specific type of body fat distribution with accumulation of adipose tissue in the interscapular compartment.

In the first clinical study, the applied non-invasive vagus nerve stimulation could not robustly deflect autonomic nervous system activity and had therefore neither effects on insulin sensitivity nor insulin secretion.
Based on this negative result of non-invasive vagus nerve stimulation, another approach to modulate the autonomic nervous system was applied.

In the follow-up experiment, slow deep breathing was now able to modulate parasympathetic nerve activity, but did neither alter insulin sensitivity nor insulin secretion. Consequently, no effects on plasma glucose were achieved. However, a link between mean heart rate and C-peptide and insulin levels was detected, regardless of breathing condition and glucose concentration. This uncovered relationship could indicate a link between central modulation of insulin secretion and parasympathetic activation of organs in the periphery.

The investigation of body fat distribution in the third study of this research project revealed a link between interscapular fat and insulin sensitivity and glucose tolerance, whereas interscapular fat was not associated with insulin secretion. Thus, the link between interscapular fat and impaired glucose metabolism is most likely caused by the first of two major pathomechanisms for diabetes – systemic insulin resistance.

The three studies vary in the number of included participants due to their differences in design: While the experimental vagus nerve stimulation and slow deep breathing study included 15 persons (based on appropriate sample size calculations for this cross-over design), 822 persons were examined in the correlative interscapular fat study. Beyond that, only men were included in the vagus nerve (taVNS) and slow deep breathing (RESPI) experiments, whereas female and male participants were selected for the interscapular fat trial.
The average age was different in the conducted trials: 24 years (SD±3) in taVNS vs. 27 years (SD± 8) in RESPI vs. 46 years (SD ± 15) in the interscapular fat study.
The first two experimental studies aimed for healthy individuals. Therefore, the glucose tolerance status in the first and second trial were normal, whereas in the third study different glucose status were present, ranging from normal glucose tolerance to impaired fasting glucose (IFG) and impaired glucose tolerance (IGT) or both, IFG and IGT.

There are some limitations of the performed studies: the relatively limited number of participants included in the taVNS and RESPI study might have hindered detection of smaller effects. However, the trials were powered to detect clinically relevant effects.
A limitation of the interscapular fat study may be that the histological specification of the fat compartments was not investigated. It was furthermore focused on one specific non-classical fat depot (interscapular fat) and did not investigated further non-classical fat depots (e.g., perivascular fat, pancreatic fat, renal sinus fat) that might have additional or complementary effects.

3.5 Conclusions

The tested non-invasive vagus nerve stimulation and slow deep breathing paradigms were not able to enhance insulin sensitivity or secretion in the presented trials. To investigate the effect of these specific stimulation techniques

on the autonomic nervous system and glucose metabolism, different study protocols may be of interest in future studies. Further, other treatments to modulate the autonomic nervous system could be applied to continue this line of research and further clarify the importance of autonomic nervous system for human glucose metabolism.

Previously, other approaches for the modulation of the central nervous system were studied, e.g. pharmacological interventions with intranasal insulin were able to enhance insulin sensitivity in peripheral organs (Heni et al., 2014). Moreover, a recent pharmacological study (Kullmann et al., 2021) enhanced hypothalamic insulin sensitivity by SGLT2-inhibition (sodium glucose cotransporter 2) with positive effects on whole-body metabolism in patients with pre-diabetes.

In conclusion, the modulation of the autonomic nervous system still has the potential to be an important target for the prevention of type 2 diabetes. Therefore, non-pharmacological approaches to modulate the autonomic nervous system need to be tested in future studies.

Interscapular fat was found to be associated with insulin resistance and impaired glucose tolerance. In line, previous studies showed an association of interscapular fat with insulin resistance and negative effects of dysregulated body fat as visceral fat, liver fat (Nguyen-Duy et al., 2003), pancreatic fat and perivascular fat (Ferrara et al., 2019). Body fat distribution is a relevant aspect in the pathogenesis of type 2 diabetes (Frayn, 2000; Wajchenberg, 2000). Moreover, interscapular fat was found to be a relevant fat depot for metabolic risk. This could be of clinical importance as a reliable prediction of the disease is still of great interest for a timely and targeted screening as well as an early treatment strategy. In contrast to the expensive and labor-intense magnetic resonance imaging that was applied in this scientific study, other approaches may be easier and more cost-efficient to apply in the practice. The quantification of interscapular fat could be done by ultrasonography (Tsai et al., 2006) and might be implemented in the clinical practice for the risk stratification of metabolic disorders in the future.

In conclusion, this research project identified interscapular fat as an important fat compartment that likely predicts metabolic risk in the future. Transcutaneous auricular vagus nerve stimulation and slow deep breathing had no effect on insulin secretion and insulin sensitivity. Thus, the detailed contribution of the autonomic nervous system for human metabolism still remains open. Nevertheless, modulating the autonomic nervous system appears to be a promising research area to improve glucose metabolism in the future.

4 Summary

In this thesis, three studies investigated potential regulators of insulin sensitivity and insulin secretion, the two main pathophysiological contributors in the development of type 2 diabetes. The work was focused on the impact of the autonomic nervous system and body fat distribution on insulin sensitivity and insulin secretion.

The research project addressed the questions if insulin sensitivity or insulin secretion can be improved by modulating the autonomic nervous system in healthy persons and if interscapular fat is a clinically relevant fat depot with regard to insulin sensitivity, insulin secretion and glucose metabolism.

The modulation of the autonomic nervous system was performed with *(i)* non-invasive vagus nerve stimulation and *(ii)* slow deep breathing maneuvers in two randomized, controlled, cross-over studies, each including 15 healthy young men. The investigation of interscapular fat's association with insulin sensitivity and insulin secretion was done in 822 women and men with different glucose tolerance status.

Non-invasive vagus nerve stimulation revealed no effect on insulin secretion and insulin sensitivity in the conducted trial (Vosseler et al., 2020). However, this does not exclude an impact of the autonomic nervous system, as autonomic tone was unexpectedly not changed by the stimulation device.

In the second trial (Vosseler et al., 2021), cardiac vagal activity was mildly modulated during slow deep breathing but no effects on insulin secretion and

sensitivity were found. While this argues against a clinically relevant effect of the changes in autonomic activity achieved by the applied paradigm, it is quite possible that prolongation of the slow deep breathing may have been more effective and might potentially have induced metabolic effects.
The third study (Vosseler et al., 2022) showed that interscapular fat mass was directly associated with insulin resistance and impaired glucose tolerance, independently of visceral fat. Interscapular fat was not associated with insulin secretion.

In conclusion, the applied techniques to modulate the autonomic nervous system, non-invasive vagus nerve stimulation and slow deep breathing, were not appropriate to exert major effects on insulin sensitivity and insulin secretion in the performed trials. In contrast, other approaches, i.e. pharmacological interventions, were shown to be effective in modulating the autonomic nervous system with consecutive effects on whole-body glucose metabolism (D'Alessio et al., 2001; Heni et al., 2014; Kullmann et al., 2021).

The autonomic nervous system is a major target for the development of therapeutic strategies in the prevention and therapy of type 2 diabetes mellitus. Therefore, novel non-invasive approaches to modulate the autonomic nervous system should be tested and optimized in future studies.

Interscapular fat was found to be associated with insulin resistance and impaired glucose tolerance. It could be a clinically relevant predictor for metabolic risk and may be easier quantified with other techniques (e.g. ultrasonography) in the clinical practice in the future.

5 German summary – Zusammenfassung

Die vorliegende Arbeit befasst sich mit zwei Hauptmechanismen der Pathogenese des Typ-2-Diabetes: Insulinresistenz und Insulinsekretionsstörung. Um das komplexe Zusammenspiel dieser beiden Faktoren zu untersuchen, wurden zwei Determinanten ausgewählt, die für die Insulinsensitivität und Insulinsekretion bedeutend sind: Das autonome Nervensystem und die Körperfettverteilung.

Das Forschungsprojekt untersucht die Frage, ob die Insulinsensitivität bzw. die Insulinsekretion durch Modulation des vegetativen Nervensystems bei gesunden Menschen verbessert werden kann und ob interskapulares Fett ein klinisch relevantes Fettdepot in Bezug auf Insulinsensitivität, Insulinsekretion und Glukosestoffwechsel ist.

Die Modulation des autonomen Nervensystems wurde mit nicht-invasiver Vagusnerv-Stimulation und langsamer vertiefter Atmung in zwei randomisierten, kontrollierten, Cross-Over-Studien mit jeweils 15 gesunden jungen Männern durchgeführt.

Der Zusammenhang von interskapularem Fett mit der Insulinsensitivität und Insulinsekretion wurde bei 822 Frauen und Männern mit unterschiedlichen Glukosetoleranzstatus untersucht.

Die nicht-invasive Vagusnerv-Stimulation zeigte in der durchgeführten Studie keinen Einfluss auf die Insulinsekretion und die Insulinsensitivität (Vosseler et al., 2020) .

In der zweiten Studie (Vosseler et al., 2021) wurde die kardiale Vagusaktivität während der langsamen vertieften Atmung leicht moduliert, eine Auswirkungen auf die Insulinsekretion und die Insulinsensitivität konnte jedoch nicht festgestellt werden. Dies spricht zwar gegen einen klinisch relevanten Effekt der langsamen vertieften Atmung auf den Glukosestoffwechsel in der durchgeführten Studie, jedoch hätte eine längere Dauer der langsamen vertieften Atmung möglicherweise Auswirkungen auf den Metabolismus induzieren können.

Die dritte Studie (Vosseler et al., 2022) zeigte einen vom viszeralen Fettgehalt unabhängigen Zusammenhang der Interskapular-Fettmasse mit Insulinresistenz und eingeschränkter Glukosetoleranz. Eine Assoziation von interskapularem Fett und Insulinsekretion konnte hingegen nicht gezeigt werden.
Eine Limitation dieser Studie besteht darin, dass die histologische Art der Fettmasse nicht untersucht wurde. Zudem blieb eine mögliche Modulation des autonomen Nervensystems durch die nicht-invasive Vagusnerv-Stimulation und die langsame vertiefte Atmung in den durchgeführten Studien ohne relevante Auswirkung auf die Insulinsensitivität und -sekretion. Im Gegensatz dazu konnte in vorherigen Arbeiten (D'Alessio et al., 2001; Heni et al., 2014; Kullmann et al., 2021) eine Modulation des autonomen Nervensystems mit anderen Ansätzen (z.B. medikamentösen Interventionen) festgestellt werden, welche sich auf den Glukosestoffwechsel im ganzen Körper auswirkten.

Das autonome Nervensystem ist ein wichtiger Ansatzpunkt für die Entwicklung von Therapie-Strategien für die Prävention von Diabetes mellitus Typ 2 und seiner Behandlung. In zukünftigen Studien sollten daher neue Ansätze zur nicht-invasiven Modulation des autonomen Nervensystems entwickelt werden.
Interskapularer Fettgehalt ist mit Insulinresistenz und eingeschränkter Glukosetoleranz assoziiert und könnte ein klinisch relevanter Prädiktor für metabolisches Risiko sein und zukünftig mit anderen Techniken (z.B. Ultraschall) einfacher quantifiziert werden.

6 References

Achike, F.I., To, N.-H.P., Wang, H., Kwan, C.-Y., 2011. Obesity, metabolic syndrome, adipocytes and vascular function: A holistic viewpoint. Clin Exp Pharmacol Physiol 38, 1–10. https://doi.org/10.1111/j.1440-1681.2010.05460.x

Ahlqvist, E., Storm, P., Käräjämäki, A., Martinell, M., Dorkhan, M., Carlsson, A., Vikman, P., Prasad, R.B., Aly, D.M., Almgren, P., Wessman, Y., Shaat, N., Spégel, P., Mulder, H., Lindholm, E., Melander, O., Hansson, O., Malmqvist, U., Lernmark, Å., Lahti, K., Forsén, T., Tuomi, T., Rosengren, A.H., Groop, L., 2018. Novel subgroups of adult-onset diabetes and their association with outcomes: a data-driven cluster analysis of six variables. Lancet Diabetes Endocrinol 6, 361–369. https://doi.org/10.1016/S2213-8587(18)30051-2

Ahrén, B., Holst, J.J., 2001. The Cephalic Insulin Response to Meal Ingestion in Humans Is Dependent on Both Cholinergic and Noncholinergic Mechanisms and Is Important for Postprandial Glycemia. Diabetes 50, 1030–1038. https://doi.org/10.2337/diabetes.50.5.1030

Almabrouk, T. a. M., Ewart, M.A., Salt, I.P., Kennedy, S., 2014. Perivascular fat, AMP-activated protein kinase and vascular diseases. British Journal of Pharmacology 171, 595–617. https://doi.org/10.1111/bph.12479

American Diabetes Association, 2014. Diagnosis and Classification of Diabetes Mellitus. Diabetes Care 37, S81–S90. https://doi.org/10.2337/dc14-S081

Appelhans, B.M., Luecken, L.J., 2006. Heart Rate Variability as an Index of Regulated Emotional Responding. Review of General Psychology 10, 229–240. https://doi.org/10.1037/1089-2680.10.3.229

Badran, B.W., Brown, J.C., Dowdle, L.T., Mithoefer, O.J., LaBate, N.T., Coatsworth, J., DeVries, W.H., Austelle, C.W., McTeague, L.M., Yu, A., Bikson, M., Jenkins, D.D., George, M.S., 2018a. Tragus or cymba conchae? Investigating the anatomical foundation of transcutaneous auricular vagus nerve stimulation (taVNS). Brain Stimul 11, 947–948. https://doi.org/10.1016/j.brs.2018.06.003

Badran, B.W., Mithoefer, O.J., Summer, C.E., LaBate, N.T., Glusman, C.E., Badran, A.W., DeVries, W.H., Summers, P.M., Austelle, C.W., McTeague, L.M., Borckardt, J.J., George, M.S., 2018b. Short trains of transcutaneous auricular vagus nerve stimulation (taVNS) have parameter-specific effects on heart rate. Brain Stimul 11, 699–708. https://doi.org/10.1016/j.brs.2018.04.004

Becher, T., Palanisamy, S., Kramer, D.J., Eljalby, M., Marx, S.J., Wibmer, A.G., Butler, S.D., Jiang, C.S., Vaughan, R., Schöder, H., Mark, A., Cohen, P., 2021. Brown adipose tissue is associated with cardiometabolic health. Nature Medicine 27, 58–65. https://doi.org/10.1038/s41591-020-1126-7

Begg, D.P., Woods, S.C., 2013. Interactions between the central nervous system and pancreatic islet secretions: a historical perspective. Adv Physiol Educ 37, 53–60. https://doi.org/10.1152/advan.00167.2012

Ben-Menachem, E., 2002. Vagus-nerve stimulation for the treatment of epilepsy. Lancet Neurol 1, 477–482. https://doi.org/10.1016/s1474-4422(02)00220-x

Berthoud, H. -r, n.d. Vagal and hormonal gut–brain communication: from satiation to satisfaction. Neurogastroenterology & Motility 20, 64–72. https://doi.org/10.1111/j.1365-2982.2008.01104.x

Berthoud, H.R., Fox, E.A., Powley, T.L., 1990. Localization of vagal preganglionics that stimulate insulin and glucagon secretion. Am J Physiol 258, R160-168. https://doi.org/10.1152/ajpregu.1990.258.1.R160

Betz, M.J., Enerbäck, S., 2015. Human Brown Adipose Tissue: What We Have Learned So Far. Diabetes 64, 2352–2360. https://doi.org/10.2337/db15-0146

Billman, G.E., 2013. The effect of heart rate on the heart rate variability response to autonomic interventions. Front. Physiol. 4. https://doi.org/10.3389/fphys.2013.00222

Björntorp, P., 1988. Abdominal obesity and the development of noninsulin-dependent diabetes mellitus. Diabetes/Metabolism Reviews 4, 615–622. https://doi.org/10.1002/dmr.5610040607

Blüher, M., Laufs, U., 2019. New concepts for body shape-related cardiovascular risk: role of fat distribution and adipose tissue function. Eur Heart J 40, 2856–2858. https://doi.org/10.1093/eurheartj/ehz411

Bootsma, M., Swenne, C.A., Van Bolhuis, H.H., Chang, P.C., Cats, V.M., Bruschke, A.V., 1994. Heart rate and heart rate variability as indexes of sympathovagal balance. American Journal of Physiology-Heart and Circulatory Physiology 266, H1565–H1571. https://doi.org/10.1152/ajpheart.1994.266.4.H1565

Borges, U., Laborde, S., Raab, M., 2019. Influence of transcutaneous vagus nerve stimulation on cardiac vagal activity: Not different from sham stimulation and no effect of stimulation intensity. PLOS ONE 14, e0223848. https://doi.org/10.1371/journal.pone.0223848

Brar, T.K., Singh, K.D., Kumar, A., 2015. Effect of Different Phases of Menstrual Cycle on Heart Rate Variability (HRV). J Clin Diagn Res 9, CC01–CC04. https://doi.org/10.7860/JCDR/2015/13795.6592

Britton, K.A., Massaro, J.M., Murabito, J.M., Kreger, B.E., Hoffmann, U., Fox, C.S., 2013. Body fat distribution, incident cardiovascular disease, cancer, and all-cause mortality. J Am Coll Cardiol 62, 921–925. https://doi.org/10.1016/j.jacc.2013.06.027

Butt, M.F., Albusoda, A., Farmer, A.D., Aziz, Q., 2020. The anatomical basis for transcutaneous auricular vagus nerve stimulation. Journal of Anatomy 236, 588–611. https://doi.org/10.1111/joa.13122

Carnethon, M.R., Golden, S.H., Folsom, A.R., Haskell, W., Liao, D., 2003. Prospective Investigation of Autonomic Nervous System Function and the Development of

Type 2 Diabetes. Circulation 107, 2190–2195. https://doi.org/10.1161/01.CIR.0000066324.74807.95

Ceriello, A., 2004. Impaired glucose tolerance and cardiovascular disease: the possible role of post-prandial hyperglycemia. Am Heart J 147, 803–807. https://doi.org/10.1016/j.ahj.2003.11.020

Challapalli, S., Kadish, A.H., Horvath, G., Goldberger, J.J., 1999. Differential Effects of Parasympathetic Blockade and Parasympathetic Withdrawal on Heart Rate Variability. Journal of Cardiovascular Electrophysiology 10, 1192–1199. https://doi.org/10.1111/j.1540-8167.1999.tb00295.x

Chiasson, J.-L., Rabasa-Lhoret, R., 2004. Prevention of type 2 diabetes: insulin resistance and beta-cell function. Diabetes 53 Suppl 3, S34-38. https://doi.org/10.2337/diabetes.53.suppl_3.s34

Chiodini, I., 2011. Clinical review: Diagnosis and treatment of subclinical hypercortisolism. J Clin Endocrinol Metab 96, 1223–1236. https://doi.org/10.1210/jc.2010-2722

Chondronikola, M., 2020. The role of brown adipose tissue and the thermogenic adipocytes in glucose metabolism: recent advances and open questions. Current Opinion in Clinical Nutrition & Metabolic Care 23, 282–287. https://doi.org/10.1097/MCO.0000000000000662

Clancy, J.A., Mary, D.A., Witte, K.K., Greenwood, J.P., Deuchars, S.A., Deuchars, J., 2014. Non-invasive vagus nerve stimulation in healthy humans reduces sympathetic nerve activity. Brain Stimul 7, 871–877. https://doi.org/10.1016/j.brs.2014.07.031

Cottin, F., Papelier, Y., Escourrou, P., 1999. Effects of exercise load and breathing frequency on heart rate and blood pressure variability during dynamic exercise. Int J Sports Med 20, 232–238. https://doi.org/10.1055/s-2007-971123

Cypess, A., Lehman, S., Williams, G., Tal, I., Rodman, D., Goldfine, A., Kuo, F., Palmer, E., Tseng, Y.-H., Doria, A., Kolodny, G., Kahn, R., 2009. Identification and Importance of Brown Adipose Tissue in Adult Humans. The New England Journal of Medicine 360, 1509–1517. https://doi.org/10.1056/NEJMoa0810780

Cypess, A.M., White, A.P., Vernochet, C., Schulz, T.J., Xue, R., Sass, C.A., Huang, T.L., Roberts-Toler, C., Weiner, L.S., Sze, C., Chacko, A.T., Deschamps, L.N., Herder, L.M., Truchan, N., Glasgow, A.L., Holman, A.R., Gavrila, A., Hasselgren, P.-O., Mori, M.A., Molla, M., Tseng, Y.-H., 2013. Anatomical localization, gene expression profiling and functional characterization of adult human neck brown fat. Nature Medicine 19, 635–639. https://doi.org/10.1038/nm.3112

Dai, Y., Wan, X., Li, Xin, Jin, E., Li, Xueqi, 2016. Neck circumference and future cardiovascular events in a high-risk population--A prospective cohort study. Lipids Health Dis 15, 46. https://doi.org/10.1186/s12944-016-0218-3

D'Alessio, D.A., Kieffer, T.J., Taborsky, G.J., Havel, P.J., 2001. Activation of the parasympathetic nervous system is necessary for normal meal-induced insulin secretion in rhesus macaques. J. Clin. Endocrinol. Metab. 86, 1253–1259. https://doi.org/10.1210/jcem.86.3.7367

DECODE Study Group, the European Diabetes Epidemiology Group., 2001. Glucose tolerance and cardiovascular mortality: comparison of fasting and 2-hour diagnostic criteria. Arch Intern Med 161, 397–405. https://doi.org/10.1001/archinte.161.3.397

DeFronzo, R.A., 2010. Insulin resistance, lipotoxicity, type 2 diabetes and atherosclerosis: the missing links. The Claude Bernard Lecture 2009. Diabetologia 53, 1270–1287. https://doi.org/10.1007/s00125-010-1684-1

DeFronzo, R.A., 2009. From the Triumvirate to the Ominous Octet : A New Paradigm for the Treatment of Type 2 Diabetes Mellitus. Diabetes 58, 773–795.

DeFronzo, R.A., 2004. Pathogenesis of type 2 diabetes mellitus. Med Clin North Am 88, 787–835, ix. https://doi.org/10.1016/j.mcna.2004.04.013

DeFronzo, R.A., Ferrannini, E., Zimmet, P., Alberti, K.G.M.M., 2015. International Textbook of Diabetes Mellitus. Wiley-Blackwell.

Delzenne, N.M., Cani, P.D., 2011. Gut microbiota and the pathogenesis of insulin resistance. Curr Diab Rep 11, 154–159. https://doi.org/10.1007/s11892-011-0191-1

Després Jean-Pierre, 2012. Body Fat Distribution and Risk of Cardiovascular Disease. Circulation 126, 1301–1313. https://doi.org/10.1161/CIRCULATIONAHA.111.067264

Després, J.-P., Lemieux, I., 2006. Abdominal obesity and metabolic syndrome. Nature 444, 881–887. https://doi.org/10.1038/nature05488

Deutschman, C.S., Harris, A.P., Fleisher, L.A., 1994. Changes in Heart Rate Variability Under Propofol Anesthesia: A Possible Explanation for Propofol-Induced Bradycardia. Anesthesia & Analgesia 79, 373–377.

Di Dalmazi, G., Pasquali, R., Beuschlein, F., Reincke, M., 2015. Subclinical hypercortisolism: a state, a syndrome, or a disease? Eur J Endocrinol 173, M61-71. https://doi.org/10.1530/EJE-15-0272

Dietrich, S., Smith, J., Scherzinger, C., Hofmann-Preiss, K., Freitag, T., Eisenkolb, A., Ringler, R., 2008. [A novel transcutaneous vagus nerve stimulation leads to brainstem and cerebral activations measured by functional MRI]. Biomed Tech (Berl) 53, 104–111. https://doi.org/10.1515/BMT.2008.022

Dietz, B., Machann, J., Agrawal, V., Heni, M., Schwab, P., Dienes, J., Reichert, S., Birkenfeld, A.L., Häring, H.-U., Schick, F., Stefan, N., Fritsche, A., Preissl, H., Schölkopf, B., Bauer, S., Wagner, R., 2021. Diabetes detection from whole-body

magnetic resonance imaging using deep learning. JCI Insight e146999. https://doi.org/10.1172/jci.insight.146999

Dixon, J.B., O'Brien, P.E., 2002. Neck circumference a good predictor of raised insulin and free androgen index in obese premenopausal women: changes with weight loss. Clinical Endocrinology 57, 769–778. https://doi.org/10.1046/j.1365-2265.2002.01665.x

Ellrich, J., 2011. Transcutaneous vagus nerve stimulation. European Neurological Review 6, 254–256.

Ferrara, D., Montecucco, F., Dallegri, F., Carbone, F., 2019. Impact of different ectopic fat depots on cardiovascular and metabolic diseases. J Cell Physiol 234, 21630–21641. https://doi.org/10.1002/jcp.28821

Frangos, E., Ellrich, J., Komisaruk, B.R., 2015. Non-invasive access to the vagus nerve central projections via electrical stimulation of the external ear: fMRI evidence in humans. Brain Stimul 8, 624–636. https://doi.org/10.1016/j.brs.2014.11.018

Frayn, K.N., 2000. Visceral fat and insulin resistance — causative or correlative? British Journal of Nutrition 83, S71–S77. https://doi.org/10.1017/S0007114500000982

Freeman, A.M., Pennings, N., 2021. Insulin Resistance, in: StatPearls. StatPearls Publishing, Treasure Island (FL).

Fritsche, A., Stefan, N., 2021. Epidemiologie, Ätiologie und Pathogenese des Typ-2-Diabetes, in: Häring, H.-U., Gallwitz, B., Müller-Wieland, D., Badenhoop, K., Meier, J.J., Usadel, K.-H., Mehnert, H. (Eds.), Diabetologie in Klinik und Praxis. Georg Thieme Verlag KG. https://doi.org/10.1055/b000000048

Frühbeck, G., Becerril, S., Sáinz, N., Garrastachu, P., García-Velloso, M.J., 2009. BAT: a new target for human obesity? Trends in Pharmacological Sciences 30, 387–396. https://doi.org/10.1016/j.tips.2009.05.003

Gancheva, S., Bierwagen, A., Markgraf, D.F., Bönhof, G.J., Murphy, K.G., Hatziagelaki, E., Lundbom, J., Ziegler, D., Roden, M., 2017. Constant hepatic ATP concentrations during prolonged fasting and absence of effects of Cerbomed Nemos® on parasympathetic tone and hepatic energy metabolism. Mol Metab 7, 71–79. https://doi.org/10.1016/j.molmet.2017.10.002

Gavaldà-Navarro, A., Villarroya, J., Cereijo, R., Giralt, M., Villarroya, F., 2021. The endocrine role of brown adipose tissue: An update on actors and actions. Rev Endocr Metab Disord. https://doi.org/10.1007/s11154-021-09640-6

Gholamrezaei, A., Diest, I.V., Aziz, Q., Vlaeyen, J.W.S., Oudenhove, L.V., 2019. Influence of inspiratory threshold load on cardiovascular responses to controlled breathing at 0.1 Hz. Psychophysiology 56, e13447. https://doi.org/10.1111/psyp.13447

Han, W., Tellez, L.A., Perkins, M.H., Perez, I.O., Qu, T., Ferreira, J., Ferreira, T.L., Quinn, D., Liu, Z.-W., Gao, X.-B., Kaelberer, M.M., Bohórquez, D.V., Shammah-

Lagnado, S.J., de Lartigue, G., de Araujo, I.E., 2018. A Neural Circuit for Gut-Induced Reward. Cell 175, 665-678.e23. https://doi.org/10.1016/j.cell.2018.08.049

Heni, M., Kullmann, S., Preissl, H., Fritsche, A., Häring, H.-U., 2015. Impaired insulin action in the human brain: causes and metabolic consequences. Nature Reviews Endocrinology 11, 701–711. https://doi.org/10.1038/nrendo.2015.173

Heni, M., Wagner, R., Kullmann, S., Veit, R., Mat Husin, H., Linder, K., Benkendorff, C., Peter, A., Stefan, N., Häring, H.-U., Preissl, H., Fritsche, A., 2014. Central insulin administration improves whole-body insulin sensitivity via hypothalamus and parasympathetic outputs in men. Diabetes 63, 4083–4088. https://doi.org/10.2337/db14-0477

Howland, R.H., 2014. Vagus Nerve Stimulation. Curr Behav Neurosci Rep 1, 64–73. https://doi.org/10.1007/s40473-014-0010-5

Huang, F., Dong, J., Kong, J., Wang, H., Meng, H., Spaeth, R.B., Camhi, S., Liao, X., Li, X., Zhai, X., Li, S., Zhu, B., Rong, P., 2014. Effect of transcutaneous auricular vagus nerve stimulation on impaired glucose tolerance: a pilot randomized study. BMC Complementary and Alternative Medicine 14, 203. https://doi.org/10.1186/1472-6882-14-203

Huang, W.-L., Chang, L.-R., Kuo, T.B.J., Lin, Y.-H., Chen, Y.-Z., Yang, C.C.H., 2013. Gender differences in personality and heart-rate variability. Psychiatry Res 209, 652–657. https://doi.org/10.1016/j.psychres.2013.01.031

Ibrahim, M.M., 2010. Subcutaneous and visceral adipose tissue: structural and functional differences. Obes Rev 11, 11–18. https://doi.org/10.1111/j.1467-789X.2009.00623.x

Irvine, W.J., 1977. Classification of diabetes mellitus. Lancet 1, 1202–1203. https://doi.org/10.1016/s0140-6736(77)92735-0

Ishibashi, C., Kozawa, J., Hosakawa, Y., Yoneda, S., Kimura, T., Fujita, Y., Fukui, K., Iwahashi, H., Shimomura, I., 2020. Pancreatic fat is related to the longitudinal decrease in the increment of C-peptide in glucagon stimulation test in type 2 diabetes patients. J Diabetes Investig 11, 80–87. https://doi.org/10.1111/jdi.13108

Iversen, S., Iversen, L., Saper, C.B., 2000. The Autonomic Nervous System and the Hypothalamus. Principles of neural science 4, 960–981, 16.

Jones, T.A., Reddy, N.L., Wayte, S.C., Adesanya, O., Dimitriadis, G.K., Hutchinson, C.E., Barber, T.M., 2017. Brown fat depots in adult humans remain static in their locations on PET/CT despite changes in seasonality. Physiol Rep 5, e13284. https://doi.org/10.14814/phy2.13284

Kahn, S.E., 2003. The relative contributions of insulin resistance and beta-cell dysfunction to the pathophysiology of Type 2 diabetes. Diabetologia 46, 3–19. https://doi.org/10.1007/s00125-002-1009-0

Karastergiou, K., Smith, S.R., Greenberg, A.S., Fried, S.K., 2012. Sex differences in human adipose tissues – the biology of pear shape. Biology of Sex Differences 3, 13. https://doi.org/10.1186/2042-6410-3-13

Kaufmann, T., Sütterlin, S., Schulz, S.M., Vögele, C., 2011. ARTiiFACT: a tool for heart rate artifact processing and heart rate variability analysis. Behav Res Methods 43, 1161–1170. https://doi.org/10.3758/s13428-011-0107-7

Kromenacker, B.W., Sanova, A.A., Marcus, F.I., Allen, J.J.B., Lane, R.D., 2018. Vagal Mediation of Low-Frequency Heart Rate Variability During Slow Yogic Breathing. Psychosom Med 80, 581–587. https://doi.org/10.1097/PSY.0000000000000603

Kuk, J.L., Katzmarzyk, P.T., Nichaman, M.Z., Church, T.S., Blair, S.N., Ross, R., 2006. Visceral fat is an independent predictor of all-cause mortality in men. Obesity (Silver Spring) 14, 336–341. https://doi.org/10.1038/oby.2006.43

Kullmann, S., Hummel, J., Wagner, R., Dannecker, C., Vosseler, A., Fritsche, L., Veit, R., Kantartzis, K., Machann, J., Birkenfeld, A.L., Stefan, N., Häring, H.-U., Peter, A., Preissl, H., Fritsche, A., Heni, M., 2021. Empagliflozin Improves Insulin Sensitivity of the Hypothalamus in Humans With Prediabetes: A Randomized, Double-Blind, Placebo-Controlled, Phase 2 Trial. Diabetes Care dc211136. https://doi.org/10.2337/dc21-1136

Laakso, M., Matilainen, V., Keinänen-Kiukaanniemi, S., 2002. Association of neck circumference with insulin resistance-related factors. International Journal of Obesity 26, 873–875. https://doi.org/10.1038/sj.ijo.0802002

Lambert, G.W., Straznicky, N.E., Lambert, E.A., Dixon, J.B., Schlaich, M.P., 2010. Sympathetic nervous activation in obesity and the metabolic syndrome—Causes, consequences and therapeutic implications. Pharmacology & Therapeutics 126, 159–172. https://doi.org/10.1016/j.pharmthera.2010.02.002

Ley, S.H., Schulze, M.B., Hivert, M.-F., Meigs, J.B., Hu, F.B., 2018. Risk Factors for Type 2 Diabetes, in: Cowie, C.C., Casagrande, S.S., Menke, A., Cissell, M.A., Eberhardt, M.S., Meigs, J.B., Gregg, E.W., Knowler, W.C., Barrett-Connor, E., Becker, D.J., Brancati, F.L., Boyko, E.J., Herman, W.H., Howard, B.V., Narayan, K.M.V., Rewers, M., Fradkin, J.E. (Eds.), Diabetes in America. National Institute of Diabetes and Digestive and Kidney Diseases (US), Bethesda (MD).

Li, H.-X., Zhang, F., Zhao, D., Xin, Z., Guo, S.-Q., Wang, S.-M., Zhang, J.-J., Wang, J., Li, Y., Yang, G.-R., Yang, J.-K., 2014. Neck circumference as a measure of neck fat and abdominal visceral fat in Chinese adults. BMC Public Health 14, 311. https://doi.org/10.1186/1471-2458-14-311

Lidell, M.E., Betz, M.J., Leinhard, O.D., Heglind, M., Elander, L., Slawik, M., Mussack, T., Nilsson, D., Romu, T., Nuutila, P., Virtanen, K.A., Beuschlein, F., Persson, A., Borga, M., Enerbäck, S., 2013. Evidence for two types of brown adipose tissue in humans. Nature Medicine 19, 631–634. https://doi.org/10.1038/nm.3017

Lindmark, S., Wiklund, U., Bjerle, P., Eriksson, J.W., 2003. Does the autonomic nervous system play a role in the development of insulin resistance? A study on heart rate

variability in first-degree relatives of Type 2 diabetes patients and control subjects. Diabet. Med. 20, 399–405.

Lu, C.-L., Zou, X., Orr, W.C., Chen, J.D.Z., 1999. Postprandial Changes of Sympathovagal Balance Measured by Heart Rate Variability. Dig Dis Sci 44, 857–861. https://doi.org/10.1023/A:1026698800742

Macedo, M.P., Lima, I.S., Gaspar, J.M., Afonso, R.A., Patarrão, R.S., Kim, Y.-B., Ribeiro, R.T., 2014. Risk of postprandial insulin resistance: The liver/vagus rapport. Rev Endocr Metab Disord 15, 67–77. https://doi.org/10.1007/s11154-013-9281-5

Machann, J., Thamer, C., Stefan, N., Schwenzer, N.F., Kantartzis, K., Häring, H.-U., Claussen, C.D., Fritsche, A., Schick, F., 2010. Follow-up whole-body assessment of adipose tissue compartments during a lifestyle intervention in a large cohort at increased risk for type 2 diabetes. Radiology 257, 353–363. https://doi.org/10.1148/radiol.10092284

Makhmutova, M., Weitz, J., Tamayo, A., Pereira, E., Boulina, M., Almaça, J., Rodriguez-Diaz, R., Caicedo, A., 2020. PANCREATIC BETA CELLS COMMUNICATE WITH VAGAL SENSORY NEURONS. Gastroenterology. https://doi.org/10.1053/j.gastro.2020.10.034

Maliszewska, K., Kretowski, A., 2021. Brown Adipose Tissue and Its Role in Insulin and Glucose Homeostasis. International Journal of Molecular Sciences 22, 1530. https://doi.org/10.3390/ijms22041530

Marshall, L., Mölle, M., Hallschmid, M., Born, J., 2004. Transcranial direct current stimulation during sleep improves declarative memory. J. Neurosci. 24, 9985–9992. https://doi.org/10.1523/JNEUROSCI.2725-04.2004

Martinmäki, K., Rusko, H., Kooistra, L., Kettunen, J., Saalasti, S., 2006. Intraindividual validation of heart rate variability indexes to measure vagal effects on hearts. American Journal of Physiology-Heart and Circulatory Physiology 290, H640–H647. https://doi.org/10.1152/ajpheart.00054.2005

Matsuda, M., DeFronzo, R.A., 1999. Insulin sensitivity indices obtained from oral glucose tolerance testing: comparison with the euglycemic insulin clamp. Diabetes Care 22, 1462–1470.

Matsushita, M., Yoneshiro, T., Aita, S., Kameya, T., Sugie, H., Saito, M., 2014. Impact of brown adipose tissue on body fatness and glucose metabolism in healthy humans. Int J Obes (Lond) 38, 812–817. https://doi.org/10.1038/ijo.2013.206

Matthaei, S., Stumvoll, M., Kellerer, M., Häring, H.U., 2000. Pathophysiology and pharmacological treatment of insulin resistance. Endocr Rev 21, 585–618. https://doi.org/10.1210/edrv.21.6.0413

Mayans, L., 2015. Metabolic Syndrome: Insulin Resistance and Prediabetes. FP Essent 435, 11–16.

McNeely, M.J., Shofer, J.B., Leonetti, D.L., Fujimoto, W.Y., Boyko, E.J., 2012. Associations Among Visceral Fat, All-Cause Mortality, and Obesity-Related Mortality in Japanese Americans. Diabetes Care 35, 296–298. https://doi.org/10.2337/dc11-1193

Meijer, R.I., Serne, E.H., Smulders, Y.M., van Hinsbergh, V.W.M., Yudkin, J.S., Eringa, E.C., 2011. Perivascular adipose tissue and its role in type 2 diabetes and cardiovascular disease. Curr. Diab. Rep 11, 211–217. https://doi.org/10.1007/s11892-011-0186-y

Mori, H., Yamamoto, H., Kuwashima, M., Saito, S., Ukai, H., Hirao, K., Yamauchi, M., Umemura, S., 2005. How does deep breathing affect office blood pressure and pulse rate? Hypertens Res 28, 499–504. https://doi.org/10.1291/hypres.28.499

Morton, G.J., Cummings, D.E., Baskin, D.G., Barsh, G.S., Schwartz, M.W., 2006. Central nervous system control of food intake and body weight. Nature 443, 289–295. https://doi.org/10.1038/nature05026

Murray, A.R., Atkinson, L., Mahadi, M.K., Deuchars, S.A., Deuchars, J., 2016. The strange case of the ear and the heart: The auricular vagus nerve and its influence on cardiac control. Autonomic Neuroscience, Central and peripheral nerve influence on cardiac function in health and disease 199, 48–53. https://doi.org/10.1016/j.autneu.2016.06.004

Nedergaard, J., Cannon, B., 2013. How brown is brown fat? It depends where you look. Nature Medicine 19, 540–541. https://doi.org/10.1038/nm.3187

Neuser, M.P., Teckentrup, V., Kühnel, A., Hallschmid, M., Walter, M., Kroemer, N.B., 2020. Vagus nerve stimulation boosts the drive to work for rewards. Nat Commun 11, 3555. https://doi.org/10.1038/s41467-020-17344-9

Neuser, M.P., Teckentrup, V., Kühnel, A., Hallschmid, M., Walter, M., Kroemer, N.B., 2019. Vagus nerve stimulation increases vigor to work for rewards. bioRxiv 789982. https://doi.org/10.1101/789982

Nguyen-Duy, T.-B., Nichaman, M.Z., Church, T.S., Blair, S.N., Ross, R., 2003. Visceral fat and liver fat are independent predictors of metabolic risk factors in men. Am J Physiol Endocrinol Metab 284, E1065-1071. https://doi.org/10.1152/ajpendo.00442.2002

Pacini, G., Mari, A., 2003. Methods for clinical assessment of insulin sensitivity and β-cell function. Best Practice & Research Clinical Endocrinology & Metabolism, Carbohydrate Metabolism 17, 305–322. https://doi.org/10.1016/S1521-690X(03)00042-3

Pal, G.K., Velkumary, S., Madanmohan, 2004. Effect of short-term practice of breathing exercises on autonomic functions in normal human volunteers. Indian J. Med. Res. 120, 115–121.

Perin, P.C., Maule, S., Quadri, R., 2001. Sympathetic nervous system, diabetes, and hypertension. Clin. Exp. Hypertens. 23, 45–55. https://doi.org/10.1081/ceh-100001196

Polymeris, A., Papapetrou, P.D., 2021. Anthropometric indicators of insulin resistance. Hormones (Athens). https://doi.org/10.1007/s42000-021-00296-0

Preis, S.R., Massaro, J.M., Hoffmann, U., D'Agostino, R.B., Levy, D., Robins, S.J., Meigs, J.B., Vasan, R.S., O'Donnell, C.J., Fox, C.S., 2010. Neck Circumference as a Novel Measure of Cardiometabolic Risk: The Framingham Heart Study. J Clin Endocrinol Metab 95, 3701–3710. https://doi.org/10.1210/jc.2009-1779

Purnell, J.Q., Kahn, S.E., Samuels, M.H., Brandon, D., Loriaux, D.L., Brunzell, J.D., 2009. Enhanced cortisol production rates, free cortisol, and 11β-HSD-1 expression correlate with visceral fat and insulin resistance in men: effect of weight loss. American Journal of Physiology-Endocrinology and Metabolism 296, E351–E357. https://doi.org/10.1152/ajpendo.90769.2008

Quintana, D.S., Westlye, L.T., Kaufmann, T., Rustan, Ø.G., Brandt, C.L., Haatveit, B., Steen, N.E., Andreassen, O.A., 2016. Reduced heart rate variability in schizophrenia and bipolar disorder compared to healthy controls. Acta Psychiatr Scand 133, 44–52. https://doi.org/10.1111/acps.12498

Ribeiro, G., de Aguiar, R.A., Penteado, R., Lisbôa, F.D., Raimundo, J.A.G., Loch, T., Meira, Â., Turnes, T., Caputo, F., 2020. A-Mode Ultrasound Reliability in Fat and Muscle Thickness Measurement. J Strength Cond Res. https://doi.org/10.1519/JSC.0000000000003691

Russo, M.A., Santarelli, D.M., O'Rourke, D., 2017. The physiological effects of slow breathing in the healthy human. Breathe (Sheff) 13, 298–309. https://doi.org/10.1183/20734735.009817

Saeedi, P., Petersohn, I., Salpea, P., Malanda, B., Karuranga, S., Unwin, N., Colagiuri, S., Guariguata, L., Motala, A.A., Ogurtsova, K., Shaw, J.E., Bright, D., Williams, R., IDF Diabetes Atlas Committee, 2019. Global and regional diabetes prevalence estimates for 2019 and projections for 2030 and 2045: Results from the International Diabetes Federation Diabetes Atlas, 9th edition. Diabetes Res Clin Pract 157, 107843. https://doi.org/10.1016/j.diabres.2019.107843

Saely, C.H., Geiger, K., Drexel, H., 2012. Brown versus white adipose tissue: a mini-review. Gerontology 58, 15–23. https://doi.org/10.1159/000321319

Schadewaldt, P., Nowotny, B., Strassburger, K., Kotzka, J., Roden, M., 2013. Indirect calorimetry in humans: a postcalorimetric evaluation procedure for correction of metabolic monitor variability. Am. J. Clin. Nutr. 97, 763–773. https://doi.org/10.3945/ajcn.112.035014

Schlaich, M., Straznicky, N., Lambert, E., Lambert, G., 2015. Metabolic syndrome: a sympathetic disease? Lancet Diabetes Endocrinol 3, 148–157. https://doi.org/10.1016/S2213-8587(14)70033-6

Shapira, S.N., Seale, P., 2019. Transcriptional control of brown and beige fat development and function. Obesity (Silver Spring) 27, 13–21. https://doi.org/10.1002/oby.22334

Spruyt, A., Clarysse, J., Vansteenwegen, D., Baeyens, F., Hermans, D., 2010. Affect 4.0: a free software package for implementing psychological and psychophysiological experiments. Exp Psychol 57, 36–45. https://doi.org/10.1027/1618-3169/a000005

Stabe, C., Vasques, A.C.J., Lima, M.M.O., Tambascia, M.A., Pareja, J.C., Yamanaka, A., Geloneze, B., 2013. Neck circumference as a simple tool for identifying the metabolic syndrome and insulin resistance: results from the Brazilian Metabolic Syndrome Study. Clin Endocrinol (Oxf) 78, 874–881. https://doi.org/10.1111/j.1365-2265.2012.04487.x

Staiger, H., Machicao, F., Fritsche, A., Häring, H.-U., 2009. Pathomechanisms of type 2 diabetes genes. Endocr. Rev. 30, 557–585. https://doi.org/10.1210/er.2009-0017

Stefan, H., Kreiselmeyer, G., Kerling, F., Kurzbuch, K., Rauch, C., Heers, M., Kasper, B.S., Hammen, T., Rzonsa, M., Pauli, E., Ellrich, J., Graf, W., Hopfengärtner, R., 2012. Transcutaneous vagus nerve stimulation (t-VNS) in pharmacoresistant epilepsies: a proof of concept trial. Epilepsia 53, e115-118. https://doi.org/10.1111/j.1528-1167.2012.03492.x

Stefan, N., 2020. Causes, consequences, and treatment of metabolically unhealthy fat distribution. Lancet Diabetes Endocrinol 8, 616–627. https://doi.org/10.1016/S2213-8587(20)30110-8

Stefan, N., Häring, H.-U., Schulze, M.B., 2018. Metabolically healthy obesity: the low-hanging fruit in obesity treatment? The Lancet Diabetes & Endocrinology 6, 249–258. https://doi.org/10.1016/S2213-8587(17)30292-9

Stein, P.K., Bosner, M.S., Kleiger, R.E., Conger, B.M., 1994. Heart rate variability: a measure of cardiac autonomic tone. Am. Heart J. 127, 1376–1381.

Strubbe, J.H., 1992. Parasympathetic involvement in rapid meal-associated conditioned insulin secretion in the rat. Am. J. Physiol. 263, R615-618. https://doi.org/10.1152/ajpregu.1992.263.3.R615

Strubbe, J.H., Steffens, A.B., 1993. Neural control of insulin secretion. Horm. Metab. Res. 25, 507–512. https://doi.org/10.1055/s-2007-1002162

Stumvoll, M., Goldstein, B.J., van Haeften, T.W., 2007. Pathogenesis of type 2 diabetes. Endocr Res 32, 19–37. https://doi.org/10.1080/07435800701743810

Stumvoll, M., Mitrakou, A., Pimenta, W., Jenssen, T., Yki-Järvinen, H., Haeften, T.V., Renn, W., Gerich, J., 2000. Use of the oral glucose tolerance test to assess insulin release and insulin sensitivity. Diabetes Care 23, 295–301. https://doi.org/10.2337/diacare.23.3.295

Tchkonia, T., Thomou, T., Zhu, Y., Karagiannides, I., Pothoulakis, C., Jensen, M.D., Kirkland, J.L., 2013. Mechanisms and metabolic implications of regional differences among fat depots. Cell Metab 17, 644–656. https://doi.org/10.1016/j.cmet.2013.03.008

Teckentrup, V., Neubert, S., Santiago, J.C.P., Hallschmid, M., Walter, M., Kroemer, N.B., 2020. Non-invasive stimulation of vagal afferents reduces gastric frequency. Brain Stimul 13, 470–473. https://doi.org/10.1016/j.brs.2019.12.018

Teff, K.L., 2008. Visceral Nerves: Vagal and Sympathetic Innervation. Journal of Parenteral and Enteral Nutrition 32, 569–571. https://doi.org/10.1177/0148607108321705

Thamer, C., Machann, J., Staiger, H., Müssig, K., Schwenzer, N., Ludescher, B., Machicao, F., Claussen, C., Fritsche, A., Schick, F., Stefan, N., Häring, H.-U., 2010. Interscapular fat is strongly associated with insulin resistance. J Clin Endocrinol Metab 95, 4736–4742. https://doi.org/10.1210/jc.2009-1942

Thayer, J.F., Yamamoto, S.S., Brosschot, J.F., 2010. The relationship of autonomic imbalance, heart rate variability and cardiovascular disease risk factors. International Journal of Cardiology 141, 122–131. https://doi.org/10.1016/j.ijcard.2009.09.543

Tsai, W.-C., Yang, S.-H., Chen, H.-C., 2006. 3372: Interscapular area muscle thickness measurement by ultrasonography. Ultrasound in Medicine and Biology 32, P253.

Tuomi, T., Santoro, N., Caprio, S., Cai, M., Weng, J., Groop, L., 2014. The many faces of diabetes: a disease with increasing heterogeneity. Lancet 383, 1084–1094. https://doi.org/10.1016/S0140-6736(13)62219-9

Vosseler, A., Machann, J., Fritsche, L., Kübler, C., Peter, A., Häring, H.-U., Stefan, N., Birkenfeld, A.L., Fritsche, A., Wagner, R., Heni, M., 2022. Interscapular fat is associated with impaired glucose tolerance and insulin resistance independent of visceral fat mass | medRxiv [WWW Document]. URL https://www.medrxiv.org/content/10.1101/2022.02.07.22270571v1 (accessed 2.10.22).

Vosseler, A., Zhao, D., Fritsche, L., Lehmann, R., Kantartzis, K., Small, D.M., Peter, A., Häring, H.-U., Birkenfeld, A.L., Fritsche, A., Wagner, R., Preißl, H., Kullmann, S., Heni, M., 2020. No modulation of postprandial metabolism by transcutaneous auricular vagus nerve stimulation: a cross-over study in 15 healthy men. Sci Rep 10, 20466. https://doi.org/10.1038/s41598-020-77430-2

Vosseler, A., Zhao, D., Hummel, J., Gholamrezaei, A., Hudak, S., Kantartzis, K., Peter, A., Birkenfeld, A.L., Häring, H.-U., Wagner, R., Preißl, H., Kullmann, S., Heni, M., 2021. Slow deep breathing modulates cardiac vagal activity but does not affect peripheral glucose metabolism in healthy men. Sci Rep 11, 20306. https://doi.org/10.1038/s41598-021-99183-2

Wagner, R., Fritsche, L., Heni, M., Fehlert, E., Stefan, N., Staiger, H., Häring, H.-U., Fritsche, A., 2016. A novel insulin sensitivity index particularly suitable to

measure insulin sensitivity during gestation. Acta Diabetol 53, 1037–1044. https://doi.org/10.1007/s00592-016-0930-5

Wagner, R., Heni, M., Tabák, A.G., Machann, J., Schick, F., Randrianarisoa, E., Angelis, M.H. de, Birkenfeld, A.L., Stefan, N., Peter, A., Häring, H.-U., Fritsche, A., 2021. Pathophysiology-based subphenotyping of individuals at elevated risk for type 2 diabetes. Nat Med 27, 49–57. https://doi.org/10.1038/s41591-020-1116-9

Wajchenberg, B.L., 2000. Subcutaneous and Visceral Adipose Tissue: Their Relation to the Metabolic Syndrome. Endocrine Reviews 21, 697–738. https://doi.org/10.1210/edrv.21.6.0415

Wronska, A., Kmiec, Z., 2012. Structural and biochemical characteristics of various white adipose tissue depots. Acta Physiol (Oxf) 205, 194–208. https://doi.org/10.1111/j.1748-1716.2012.02409.x

Würslin, C., Machann, J., Rempp, H., Claussen, C., Yang, B., Schick, F., 2010. Topography mapping of whole body adipose tissue using A fully automated and standardized procedure. J Magn Reson Imaging 31, 430–439. https://doi.org/10.1002/jmri.22036

Zamotrinsky, A.V., Kondratiev, B., de Jong, J.W., 2001. Vagal neurostimulation in patients with coronary artery disease. Auton Neurosci 88, 109–116. https://doi.org/10.1016/S1566-0702(01)00227-2

7 Declaration and Contributions of other authors

7.1 Declaration

This work was carried out in the University Hospital in Tübingen, Medizinische Klinik IV, under the supervision of Prof. Dr. med. Martin Heni. The clinical trials were designed in cooperation with Prof. Dr. med. Martin Heni and performed by me and in cooperation with the Studienzentrale Diabetologie.
Herewith I, Andreas Vosseler, declare, that I wrote this thesis by myself, and that I did not use any sources other than those I have indicated. I also assure that I have only submitted this thesis in this and no other doctoral procedure. Further, I declare I have contributed to the major part of the following publications that are included in this work entitled *"Impact of autonomic nervous system activity and interscapular fat on human glucose metabolism"*. All co-authors agreed to the contributions to the publications listed in 7.2.

09.03.2022 _____ _____
Date *Signature Andreas Vosseler*

7.2 Contributions of other authors

Publication 1: No modulation of postprandial metabolism by transcutaneous auricular vagus nerve stimulation: a cross-over study in 15 healthy men

Authors: **Andreas Vosseler***, Dongxing Zhao*, Louise Fritsche, Rainer Lehmann, Konstantinos Kantartzis, Dana M. Small, Andreas Peter, Hans-Ulrich Häring, Andreas L. Birkenfeld, Andreas Fritsche, Robert Wagner, Hubert Preißl, Stephanie Kullmann, Martin Heni.
*contributed equally

Andreas Vosseler: research concept, selection of methods, recruitment of participants, planning of study visits, preparation of vagus nerve stimulation,

performance of study visits (blood extractions, instruction of the participants in using the vagus nerve stimulation device, ecg recording, blood pressure measurements, blood glucose measurements during the oral glucose tolerance test, documentation in the case report forms), data acquisition, data analysis, analysis of heart rate variability, interpretation of results, manuscript writing, manuscript editing.

Dongxing Zhao: selection of methods, preparation of vagus nerve stimulation, data analysis, analysis of heart rate variability, interpretation of results, preparation of manuscript.

Louise Fritsche: recruitment of participants, data acquisition, supervision of statistical analyses.

Rainer Lehmann: manuscript editing, catecholamine measurements.

Konstantinos Kantartzis: physical examination of the participants, supervision of participants, manuscript editing.

Dana M. Small, Andreas Peter, Hans-Ulrich Häring, Andreas L. Birkenfeld, Andreas Fritsche: manuscript editing.

Robert Wagner: physical examination of the participants, supervision of participants, data analysis.

Hubert Preißl: research concept, selection of methods, analysis of heart rate variability, interpretation of results, manuscript editing.

Stephanie Kullmann: research concept, selection of methods, preparation of vagus nerve stimulation, manuscript editing.

Martin Heni: research concept, selection of methods, preparation of vagus nerve stimulation, data analysis, analysis of heart rate variability, interpretation of results, manuscript editing.

Publication 2: Slow deep breathing modulates cardiac vagal activity but does not affect peripheral glucose metabolism in healthy men

Authors: **Andreas Vosseler**, Dongxing Zhao, Julia Hummel, Ali Gholamrezaei, Sarah Hudak, Konstantinos Kantartzis, Andreas Peter, Andreas L. Birkenfeld, Hans-Ulrich Häring, Robert Wagner, Hubert Preißl, Stephanie Kullmann, Martin Heni.

Andreas Vosseler: research concept, selection of methods, recruitment of participants, planning of study visits, preparation of breathing maneuvers, performance of study visits (blood extractions, instruction of the participants in breathing maneuvers, ecg recording, preparing participants with respiration belt, blood pressure measurements, blood glucose measurements during the oral glucose tolerance test, documentation in the case report forms), data acquisition, data analysis, analysis of heart rate variability, interpretation of results, manuscript writing, manuscript editing.

Dongxing Zhao: research concept, preparation of breathing maneuvers, data acquisition, data analysis, analysis of heart rate variability, interpretation of results, manuscript editing.

Julia Hummel: recruitment of participants, data acquisition, visit performance.

Ali Gholamrezaei: research concept, selection of methods, preparation of breathing maneuvers, manuscript editing.

Sarah Hudak: physical examination of the participants.

Konstantinos Kantartzis: physical examination of the participants, supervision of participants.

Andreas Peter: data analysis, manuscript editing.

Andreas L. Birkenfeld, Hans-Ulrich Häring, Robert Wagner: manuscript editing.

Hubert Preißl: research concept, selection of methods, analysis of heart rate variability, interpretation of results, manuscript editing.

Stephanie Kullmann: selection of methods, analysis of heart rate variability.

Martin Heni: research concept, selection of methods, data analysis, analysis of heart rate variability, interpretation of results, manuscript editing.

Publication 3: Interscapular fat is associated with impaired glucose tolerance and insulin resistance independent of visceral fat mass

Authors: **Andreas Vosseler**, Jürgen Machann, Louise Fritsche, Christian Kübler, Andreas Peter, Hans-Ulrich Häring, Norbert Stefan, Andreas L. Birkenfeld, Andreas Fritsche, Robert Wagner, Martin Heni.

Andreas Vosseler: research concept, selection of methods, data acquisition, performance of study visits (blood extractions, blood pressure measurements, blood glucose measurements during the oral glucose tolerance test, documentation in the case report forms), data analysis, interpretation of results, manuscript writing, manuscript editing.

Jürgen Machann: selection of methods, data acquisition, magnetic resonance imaging, data analysis, interpretation of results, manuscript editing.

Louise Fritsche: interpretation of results, manuscript editing.

Christian Kübler: data acquisition.

Andreas Peter: data acquisition, interpretation of results, manuscript editing.

Hans-Ulrich Häring: research concept, selection of methods, manuscript editing.

Norbert Stefan: research concept, selection of methods, magnetic resonance imaging, manuscript editing.

Andreas L. Birkenfeld: manuscript editing.

Andreas Fritsche: research concept, selection of methods, magnetic resonance imaging, manuscript editing.

Robert Wagner: selection of methods, data acquisition, data analysis, interpretation of results, manuscript editing.

Martin Heni: research concept, selection of methods, data acquisition, data analysis, interpretation of results, manuscript editing.

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