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3. LÉKAŘSKÁ FAKULTA

Habilitační práce

Úloha větvených aminokyselin v rozvoji inzulínové rezistence

Branched Chain Amino Acids in Insulin Resistance Developement

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OBSAH:

PŘEDMLUVA

"Teorie jsou pouze hypotézami, verifikovanými více či méně četnými fakty …
ale ani tak nikdy konečné, nikdy k absolutní víře."

(Claude Bernard, Introduction à l'Étude de la Médecine Expérimentale, 1865)

Je tomu právě sto let, kdy byl objeven a následně popsán hormon inzulín. Záhy po jeho objevu začalo studium metabolických změn provázejících inzulínovou rezistenci rezonovat ve vědeckých kruzích. Ač je tento fenomén předmětem zkoumání již prakticky století, dostává se poslední desetiletí opět do popředí zájmu, tak jak se choroby spojené s inzulínovou rezistencí, zejména diabetes a nádory, stávají globální výzvou.

Předkládaná habilitační práce navazuje a dále významně rozpracovává vědecko-výzkumnou činnost, kterou jsem představil ve své práci dizertační. Od doby, kdy byla obhájena moje dizertační práce uplynuly čtyři roky. Mnoho nového bylo popsáno a já byl nucen řadu svých poznatků vidět v novém světle a nebo dokonce revidovat. V habilitační práci nepřináším jen soubor nových poznatků vlastního klinicko-fyziologického výzkumu, ale snažím se je také zasadit do kontextu abych poskytl ucelený vhled do problematiky.

Moje vědecko-výzkumná i klinická praxe je rozkročena mezi dva klinické obory – diabetologii a klinickou výživu. Právě strava se řadí mezi významné environmentální faktory rozvoje diabetu, ale také kardiovaskulárních onemocnění a onemocnění nádorových. Většina těchto chorob je ve svém průběhu spojena s poruchou funkce inzulínu, tzv. inzulínovou rezistencí. Funkce inzulínu je úzce spjata s intermediárním metabolizmem, buněčným růstem a v širším kontextu také zánětem, a proto je fenomén inzulínové rezistence společný řadě patologických stavů a je paradigmatem různých medicínských oborů.

Do těchto vod jsem byl nasměřován před více jak deseti lety svým školitelem prof. Michalem Andělem. První fyziologické modely, které jsem se učil a dále rozpracovával, byla clampová vyšetření a metody hodnocení mitochondriální respirace v kosterním svalu. Když jsem se postupně začal prokousávat metodologickou přípravou pro vlastní vědeckou práci, nastupoval jsem na pracoviště, které se nazývalo laboratoří funkčních testů, a ač za sebou mělo úctyhodnou vědeckou minulost, jeho další rozvoj byl limitován prostorově, personálně i dostupnými metodami. Během následujících pěti let se nám podařilo dostávat oddělení na stále lepši úroveň a díky mezinárodní spolupráci (zejména v rámci Česko-francouzské laboratoře pro studium obezity a mého EFSD mentorského programu pod vedením pracoviště OCDEM v Oxfordu) mu také vybudovat širší zakotvení ve vědecké komunitě. Díky tomu jsme se mohli stát partnery hned v několika evropských aplikacích, a stát u zrodu evropské sítě výzkumníků rostlinných diet. Nyní naše metodologické portfolio zahrnuje state-of-the-art metody oddělení klinického výzkumu od nepřímé kalorimetrie, spiroergometrie, přes funkční endokrinologické testy a clampová vyšetření s využitím stabilních izotopů, až po mikrodialýzu tukové tkáně a biopsie tuku a kosterního svalu.

Struktura samotného habilitačního spisu je členěna na teoretický úvod, východiska a tři kapitoly představující náš přínos k rozplétání složitého vztahu mezi inzulínovou rezistencí a cirkulujícími hladinami aminokyselin s větveným řetězcem. Kapitoly parafrázují práce již publikované, práce připravené k tisku a rozpracované, stejně jako abstrakta, která zazněla na odborných konferencích. Teoretický úvod rozpracovává problematiku funkce inzulínu a inzulínové rezistence, zejména ve vztahu k homeostáze glukózy v organizmu. Významnou tezí rozpracovanou v teoretickém úvodu je, že fenomenologicky je inzulínová rezistence jevem fyziologickým a v zásadě adaptivním. V textu představím fyziologické a patologické stavy, kterým je fenomem IR společný. Představím fyziologické modely, které v praxi používáme k jeho kvantifikaci. Tam, kde mohu text doložit vlastní expertízou tak namnoze učiním.

Následující tři kapitoly shrnují naše publikované výsledky. Korespondují s liniemi výzkumu, které v současnosti tvoří tři svébytné projekty, do kterých jsou zapojeni moji mladší kolegové, a která každá z nich má potenciál další vědecké proliferace. První kapitola přímo navazuje na linii výzkumu, který byl představen v rámci dizertace a vychází z hypotézy, že existuje kauzální závislost mezi dietním příjmem BCAA a inzulínovou rezistencí. Prokázali jsme, že BCAA mohou indukovat inzulínovou rezistenci u lidí, kteří mají bazálně jejich nízký příjem. Byli jsme první, kteří popsali, že dietní BCAA stimulují sekreci inzulínu cestou aktivace enteroinzulární osy, specificky cestou GLP-1. Druhá linie výzkumu, která přímo navazuje, se zaměřuje na složitý vztah mezi stravou, sekvestrací a produkcí BCAA v mikrobiotě střevní a inzulínovou rezistencí. Vskutku, BCAA jsou řazeny mezi významné signální molekuly ve vztahu mikrobiota – hostitel (řadí se mezi tzv. postbiotika). Třetí linie výzkumu si klade za cíl explorovat distribuční hypotézu vztahu BCAA a IR. Tedy, že vyšší cirkulující BCAA jsou podobně jako vyšší NEFA a glukóza odrazem substrátové redistribuce při inzulínové rezistenci. Specificky, že elevace BCAA je odrazem selhání inzulínu ve fyziologické supresi proteolýzy. Tento projekt využívá složitého fyziologického modelu inzulínové rezistence u pacientů s nádorovou kachexií.

Ač každý jeden projekt představuje vlastní závěry, mnoho otázek zůstává nezodpovězeno. Každá z představených linií výzkumu pokračuje dále a věřím, že v budoucnu přinese další stimulující závěry.

FOREWORD AND THANKS

"Theories are only hypotheses verified by more or less numerous facts – but never so final, never to be absolutely believed."

(Claude Bernard, Introduction à l'Étude de la Médecine Expérimentale, 1865)

It's been a hundred years since the hormone insulin was discovered and eventually described. Soon after, the study of metabolic derangements accompanying insulin resistance resonated in scientific circles. This phenomenon has been the subject of research for almost a century now, but it is gaining the prominence again last decades, as diseases associated with insulin resistance, especially diabetes and cancer, have become a global health care challenge.

The presented habilitation thesis is based on and further significantly develops the scientific research activity, which I presented in my doctoral work. Four years have passed since my doctoral thesis saw the light of day. Many new things were described, and I was forced to revise many of my findings. Although some theoretical starting points may overlap, especially in the general background, I always try to show them in new perspectives as scientific progress has shed light on them. Therefore, this work is original and provides an insight into the issue, presenting not only new experimental findings but also putting them in the context to give a comprehensive view.

My research and clinical practice stand apart between two fields of diabetology and clinical nutrition. After all, diet is one of the important environmental factors in the development of diabetes, cardiovascular disease and cancer. Most of these diseases are associated with an impairment of insulin function, so-called insulin resistance. Insulin function is closely related to intermediary metabolism, cell growth and, in a broader context, inflammation, and therefore the phenomenon of insulin resistance connects a number of pathological conditions and is a paradigm of several medical disciplines.

These were the waters into which I was directed more than ten years ago by my mentor prof. Michal Anděl. The first physiological models I learned and further developed were clamp studies and methods for assessing mitochondrial respiration in skeletal muscle. As I gradually began to go through the methodological preparation for my own scientific work, I started working at a facility called the laboratory of functional tests, and although it had had a remarkable scientific past, its further development was limited in space, personnel and available methods. Over the next five years, we have managed to bring the unit to the level that, thanks to international cooperation (especially within the Czech-French Laboratory for the Study of Obesity and my EFSD mentoring program led by OCDEM in Oxford), it has some international recognition. Thanks to this, we were able to become partners in several European applications, and to stand at the birth of a European network of plant diet researchers VESNA. Now our methodological portfolio includes state-of-the-art methods of clinical research from indirect calorimetry, spiroergometry, through functional endocrinological tests and clamp

studies using stable isotopes, to adipose tissue microdialysis and fat and skeletal muscle biopsies.

The structure of the text itself is divided into the theoretical introduction and experimental background and then three chapters showing our contribution in unraveling the complex relationship between insulin resistance and circulating levels of branched-chain amino acids. The chapters paraphrase works already published, works ready for submission and being developed, as well as abstracts that were presented at professional conferences. Our results are presented in a concise and comprehensive overview maintaining the logic of our scientific progress. The theoretical introduction elaborates the issue of insulin function and insulin resistance, especially in relation to glucose homeostasis in the body. An important thesis in the theoretical introduction is that phenomenologically, insulin resistance is a physiological response that is basically adaptive. In the text I will present physiological and pathological states that share the very same phenomenon of insulin resistance. I will present the physiological models that we use in practice to quantify it. Where I can substantiate the text with our own expertise, I will do so. The three chapters that follow summarize the lines of research that currently make up the three distinctive projects in which my younger colleagues are involved, and which each has the potential for further scientific proliferation. The first chapter is a direct follow-up to the line of research presented in the doctoral thesis and is based on the hypothesis that there is a causal relationship between BCAA dietary intake and insulin resistance. We have shown that BCAAs can induce insulin resistance in people who have its low basal intake. We were the first to report that BCAA show incretin effect, i.e. dietary BCAAs stimulate insulin secretion through activation of the enteroinsular axis, specifically through GLP-1. The second line of research, which is directly related, focuses on the complex relationship between diet, BCAA sequestration and production in the gut microbiota and insulin resistance. Indeed, BCAAs are classified as important signaling molecules in the microbiota-host relationship (they are among so-called postbiotics). The third line of research aims to explore the distribution hypothesis of the relationship between BCAA and IR. Thus, higher circulating BCAAs mirrors higher NEFA and glucose reflecting substrate redistribution in insulin resistance. Specifically, elevated circulating BCAA reflects insulin failure in the physiological suppression of proteolysis. This project uses a complex physiological model in patients with cancer cachexia.

Although each project presents its own conclusions, many questions remain unanswered. Each of the presented lines of research continues and would bring further stimulating conclusions in the future.

The projects' conduct would not be possible without the institutional and specific grant support, which I summarize below, and for which I am very grateful. Cooperation within the Center for Research on Diabetes, Metabolism and Nutrition of the 3rd Faculty of Medicine, which is supported by the faculty and the university, played and still has a crucial role in the implementation of the projects.

Scientific work is based on specific people, their exertions, enthusiasm and imagination. Although I am grateful to all my colleagues, many of whom we share authorship in common publications, I would like to thank some of them by name. My thanks go to Lenka

Rossmeislová, Jan Polák and Vladimír Štich, colleagues from the Institute of Pathophysiology of the Third Medical Faculty. Another thanks go to František Duška, head of the Department of Anesthesiology and Resuscitation, a man whose heart beats for the physiology of metabolism. They were all guides on my intricate research journeys. Thanks also go to my younger colleagues who work in the clinical research unit, because they are the ones who bring stimulating ideas and who continue their research in the department. I also thank all my fellow nurses who work in the department or helped us collecting clinical data: Blanka Horniková, Diana Čmuchálková, Tereza Rychtaříková, Zuzana Jindrová and Jana Hanková.

My big thanks go to Jana Potočková, the technical head of our clinical research unit. Thanks to her experience, her sense of honest work and her enthusiasm for physiology, we know that we stand on solid methodological foundations in every project in which she participates. Last but not least among my collaborators, I would like to pay gratitude to my teacher and mentor prof. Michal Anděl. He did ignite in me the fire for physiology. He was my guide on winding roads of both the clinical and experimental carrier. He is an exemplary physician and scientist of an ageless critical spirit.

Lastly, but with the utmost importance I am grateful to my family. To those who preceded me and live in me, and to my parents. And above all I am grateful to my wife and my kids. They are the deepest sources of my motivation and life enthusiasm.

I wrote this work myself. I formulated major hypotheses related to the scope and tried to find the answers in our results as well as in the literature. However, it is clear that this work was indirectly corroborated by all my co-workers. To show respect for this team collaboration I would use the plural number throughout the text.

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- Functional electrical stimulation cycling ergometry in critically ill: muscle phydsiology and long-term functional outcome (16-28663A, 2016-2019)
- Limited adipose tissue expandibility as a risk factor for Type 2. diabetes mellitus: the role of preadipocyt (16-28663A, 2016-2019)
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ABBREVIATIONS

4EBP-1	eukaryotic binding protein 1
AA	aminoacids
ACOX	acyl-CoA oxidase
AKT	protein kinase B
AT	adipose tissue
ATP	adenosin triphosphate
BCAA	branched chain amino acids
BCKDHA, BCKDHB	branched chain keto acid dehydrogenase A a B
BIA	bioimpedance
CD3g	cluster of differenciation 3, surface T lymfocyte antigen
CPT1b	kreatin-palmitoyl tranferase 1 β
DAG	diacylglycerol
DGAT-2	diglycerol acyl transferase
FASN	fatty acid syntase
FFA	free fatty acids
GLUT-4	glucose transporter 4
HEC	hyperinsulinemic euglycemic clamp
IS	insulin sensitivity
IR	insulin resistance
IRS	inuslin receptor substrate
IU	international units
mTOR	mammalian target of rapamycin
PPARγ	peroxisom proliferator activated receptor gamma
PLIN-1, PLIN-2	perilipins
ROS	reactive oxygen species
S6K1	p70-S6 kinase 1
SCD-1, SCD-5	stearoyl-CoA desaturase
SM	skeletal muscle
TAG	triglycerides
T2DM	type 2 diabetes mellitus
VO_2max	maximal oxygen consumption

1. THEORETICAL BACKGROUND

Obesity and type 2 diabetes (T2DM) are among major risk factors of atherosclerosis contributing greatly to excess not only in cardiovascular but also in cancer related [1] mortality worldwide [2,3]. Worldwide prevalence has reached pandemic proportions and projections are such that by 2025 there will be 438 million cases. Obviously, this represents a major societal and economic burden. In 2019 there were 4.2 million deaths attributable to diabetes with total cost reaching up to 760 billion US dollars, 4.5 % increase since 2017 [4]. Though the total amount of cases rises steeply, there are some signals that in the structurally developed countries the incidence has not risen since 2017 [5].

There were 862 thousand patients with diabetes in 2013 in the Czech Republic with population of 10 million. It is expected that this year 2020 the numbers would rise to reach 10 % prevalence in the population [6]. The actual incidence is about 20 thousand cases and there has been 2.5 times increase since 1980.

Morbidity and mortality of diabetes is related to both micro and macrovascular complications and cancer. In spite of the care and complex primary and secondary prevention programmes implemented in many countries, the life expectancy decreases up to 12 years in patients with diabetes [7].

Diabetes is a heterogenous group of nosological entities that share common osmotic symptomatology caused by hyperglycaemia. Though diabetes is perceived today as a complex intermediary metabolic disease, that does not exclude any major energy substrate (saccharides, lipids and proteins), in the clinical practice we focus mainly on glucoregulatory defects. Diagnosis of diabetes is made upon a repeated finding of fasting glycaemia above 7.0 mmol/L and/or random or 2nd hours OGTT glycaemia above 11.1 mmol/L in venous blood [8]. This practical simplification is based on several rationales. Diabetes mellitus has been empirically described based on presence of osmotic symptoms (i.e. polyuria and polydipsia) and passing extensive amounts of sweet urine. Of note, Διαβαινω, diabaino, or to pass through was a term first used by ancient Greek physicians. The adjective mellitus was added not sooner than in 17th century by English physician and anatomist Thomas Willis, describing taste of the urine “as if it has been mixed by honey” [9]. Glycaemia is very tightly regulated and shows very small interindividual variability, is readily accessible for laboratory analyses and its excursions correlate with metabolic compensation of diabetes and risk of future complications. Hyperglycaemia leads to tissue damage by changing structure of macromolecules, a mechanism called glucotoxicity. And last but not least, we use glucoregulatory loop (see below) as a physiological model to describe decreased response of tissues to insulin, so called insulin resistance.

Insulin resistance (IR) is a physiological phenomenon to describe decreased metabolic response to insulin. It will be described in detail in further chapters. When using IR in the text, it refers to decline in insulin sensitivity in a tissue in the broad sense of the term. When using a term “syndrome of IR”, it refers to metabolic syndrome, an entity characterised by cluster incidence of obesity, arterial hypertension, insulin resistance/diabetes and dyslipidaemia,

a state that contributes greatly to cardiovascular mortality. When talking about diabetes without any adjective, we refer to type 2 diabetes if not stated otherwise.

1.1. GLUCOSE HOMEOSTASIS

Macroergic compounds based on phosphate are bearers of energy in the organism as they are capable of releasing the energy through exergonic hydrolysis of its structure. ATP (adenosine triphosphate) is the most universal among these. These high-energy phosphates are being continuously consumed and regenerated. We use a term energy substrate to describe carbohydrate molecules that are used in the cell for the macroergic bonds regenesis. Energy substrates are basically derived from carbohydrates, lipids and proteins. Glucose is among these energy substrates. Dietary sources of glucose come from simple and complex saccharides that are hydrolysed by saccharidases from salivary and pancreatic gland (amylase) and intestinal brush oligosaccharidases. Mono- and disaccharides are consequently resorbed to enterocytes and released to portal circulation. Portal circulation bringing glucose to the pancreas washes beta cells and stimulates insulin release. This phenomenon represents an important glucostatic mechanism (see below). Glucose finally reaches systemic circulation, where all cell types in the organism may potentially utilize glucose. As glucose needs to be transported across cytoplasmatic membrane, cells use facilitated diffusion using transmembrane GLUT (glucose transporters) family carriers. There has been 14 specific types described so far, albeit only few of them has got defined glucose uptake properties [10]. For glucoregulatory purposes an important role is played by GLUT-4 transporter, that is expressed at skeletal and cardiac muscle cell and adipocyte membranes. GLUT-4 translocation to the cell surface is under control of insulin signalling cascade (see below) (Table 1).

Transporter	Function	K_m (mmol/l)	Tissue expression
GLUT-1	Basal glucose disposal	~1,5	Ubiquitous, namely neurons
GLUT-2	Glucose sensor, excretion of glucose	~17	β-cells, hepatocyte, tubular epithelia, enterocyte
GLUT-3	Basal glucose disposal	~1,5	Neurons, placenta, blood elements, ubiquitous
GLUT-4	Insulin stimulated glucose disposal	5	Skeletal muscle, cardiomyocyte, adipocyte

Table 1 Major glucoregulatory GLUT carriers. K_m is Michaelis-Menten's constant, that shows molar concentration needed for 50 % transport capacity saturation of the system. Adapted from [10].

Glucose serves as a starting substrate for glycolysis and aerobic phosphorylation. Glucose is unique among energy substrates as it can be utilized in anaerobic conditions. In the case the end products are two molecules of ATP and lactate per one molecule of glucose. Tissues that cannot rely on different sources of energy but glucose are termed glucose-dependent. Among these are namely neurons and cells without mitochondria. As per example the brain metabolises approximately 100 g of glucose per day. The role of glucose in the organism is far from being limited to the oxidative metabolism. It is substrate for many anaplerotic reactions as a source of carbon residue or a source for intermediates in Krebs' cycle [11]. It is a substrate

for pento-phosphate cycle (PPC) where it gives five carbon saccharides (ribose, a precursor for ribonucleic acid synthesis). In the oxidative part of PPC NADPH dehydrogenase reduces NADPH, a redox equivalent with reducing function in many synthetic pathways as well reducing glutathione, therefore working on antioxidative capacity of the cell. Taken all together, glucose is, per its functions both synthetic and ATP availability modulating, single most important and universal signal molecule in the organism [10].

Therefore, it is clear that glucose homeostasis needs to be tightly regulated. Indeed, levels of circulating glucose are the most tightly regulated when compared to other energy substrates. The key endocrine system for glucose metabolism regulation is the system of insulin and its contra regulatory hormones (glucagon, cortisol, catecholamines and growth hormone). Dominating metabolic effect of the system is directed by molar ratios of insulin to the contra regulatory counterparts, namely glucagon [12]. Insulin is produced and secreted in pulses from pancreatic islets' β-cells. It is therefore secreted to the portal circulation where it reaches the highest concentrations. Insulin is overall an anabolic hormone that show effects both metabolic and proliferative [13]. Insulin receptors are found at almost any cell type in the organism [14]. These are part of insulin-like growth factors receptors' family that share tyrosine-kinase activity as a common feature. Insulin receptor is a transmembrane heterodimer. Binding of ligand on the receptor leads to autophosphorylation of the intracellular part of the receptor and of the insulin receptor substrates (IRS). Downstream from the IRS is the signalling cascade of two major phosphorrylation pathways: 1/ phosphatidyl inositol triphosphate kinase (PI3K) and protein kinase B (aka AKT kinase) and 2/ ERK kinase cascade (extra cellular signal regulated kinase). The effects are mediated both via genomic (activation of transcriptional factors and gene expression) and non-genomic ways. There have been many effects of insulin described and it shows important tissue-specificity. For simplicity reasons these can be summarized as those metabolic (substrates' uptake, macromolecules synthesis) and mitogenic (growth, differentiation and antiapoptosis), i.e. effects on growth and regeneration of tissues are coupled with securing fuel needed for this purpose. Major intermediary "metabolic" signalling takes place in adipose tissue, skeletal muscle and liver [15] (Figure 1).

In the *liver*, glucose uptake is not dependent on transporter activation. Hepatocyte glucose uptake is mediated via insulin non-dependent GLUT-2 transporter. After entering the cell, the glucose is immediately phosphorylated by glucokinase. This keeps glucose intracellular concentration low thereby maintaining the concentration gradient. Glucose-6-phopsphate is directed further towards oxidation or any non-oxidative pathway. Insulin stimulates glycogen synthesis and inhibits its hydrolysis as well as gluconeogenesis therefore decreasing hepatic glucose output. By accelerating of glycolysis insulin increases availability of acetyl-CoA and malonyl-CoA, that are further oxidized or become precursors for fatty acids synthesis and de novo lipogenesis. Insulin directly stimulates the key enzyme acetyl-CoA carboxylase. Increased availability of malonyl-CoA inhibits FA transport to mitochondria (by inhibiting CPAT, carnitine-palmitoyl-acyl-transpherase), therefore decreasing their oxidation. FA are consequently subjected to de novo lipogenesis in the insulin stimulated state. Lower FA oxidation is coupled with ketogenesis inhibition. Further insulin activates proteosynthesis by activating downstream targets of mTOR (mammalian target of rapamycin) and inhibits proteolysis.

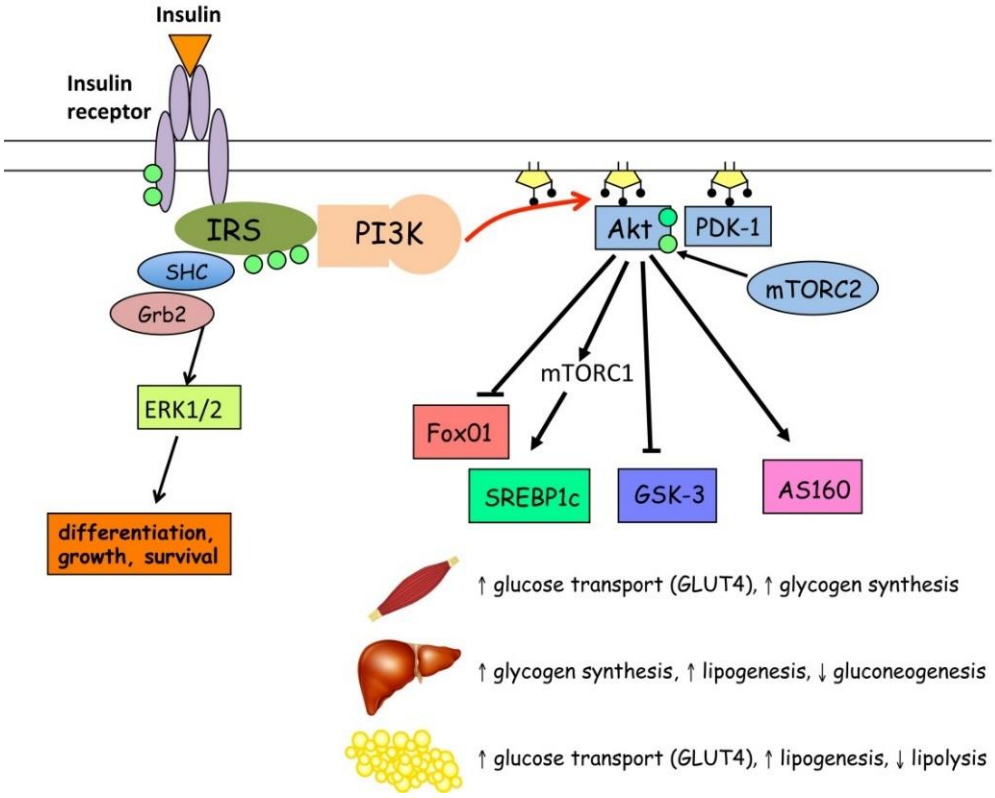


Figure 1 Schematic depiction of insulin signalling cascade. Adapted from [15].

Activation of insulin cascade in the ***skeletal muscle*** leads to GLUT-4 receptors translocation to sarcoplasmic membrane leading to an increase in a secondary active transport of glucose in the cells. Glucose is phosphorylated by hexokinase and directed either towards glycolysis or glycogen synthesis. Three carbon residues that are not oxidized may further become precursors for lipid synthesis [16] or may be released to the circulation and eventually hepatic gluconeogenic substrates. Insulin stimulates facilitated diffusion of amino acids to the skeletal muscle [17] and protein synthesis via mTOR activation [18].

In the ***adipose tissue*** insulin activates heparin sensitive lipoprotein lipase that hydrolyses triglycerides (TAG) from circulating lipoproteins. Glycerol and FA are consequently uptaken by adipocyte where TAG are resynthesized. Before the step FA may undergo further desaturation and/or elongation. These steps are related to insulin function as lower capacity of some desaturases was found in insulin resistant states [19]. Insulin blocks action of hormone sensitive lipase (it is inhibited by lower contra regulatory hormone action respectively) and effectively inhibit AT lipolysis [20].

Animals, as we humans are, have evolved in an environment that allowed only for discontinuous nutritional intake. For that purpose, we have adapted to a fasting – refeeding cycle. During refeeding we replete, during fasting we deplete. Therefore, two extreme states can be distinguished in terms of metabolic setting of the glucoregulatory loop: postprandial (after substrate ingestion) and postabsorptive (fasting). The hormonal and vegetative regulation relates to the cycle. The above-mentioned description of insulin metabolic effects *de facto*

corresponds to the insulin stimulated, i. e. postprandial metabolic setting. Postprandial period is metabolically characterized by synthesis of energy stores (glycogen, lipids and visceral/ skeletal muscle with a certain reserve). These stores are gradually depleted when the absorption of substrates from the intestine is terminated. On the contrary as post absorptive period and fasting starts insulin secretion diminishes, relative amount of circulating contra regulatory hormones increases and organism is ready to catabolise its energy reserves. To keep glycaemia stable, escalating hepatic glucose production (glycogenolysis and gluconeogenesis) ensues. Extrahepatic glucose output may reach up to 30 % supply of glucose to the extracellular pool, as it was shown in an anhepatic experiment in humans [21]. This comprises mainly of kidney tubular epithelia and intestinal enterocytes.

In the adipose tissue hormone sensitive lipase is deblocked, lipolysis is accelerated with a consequently increased flux of FA and glycerol to the liver. FA are further oxidized, released in the form of VLDL lipoproteins to the periphery and excess overflowing acetyl-CoA becomes a precursor for ketone bodies synthesis (aceto-acetate, β-hydroxybutyrate and acetone). Glycerol then substrate for gluconeogenesis. Ketone bodies are released to the circulation to become an alternative energy substrate for glucose-dependent tissues. There is an increased degradation of fibrillar proteins in the visceral and skeletal muscle and a release of glucogenic and ketogenic amino-acids to the circulation. Even in long term fasting, a minimal insulin release is maintained holding back both hepatic glucose production and AT lipolysis to prevent hyperglycaemia and ketoacidosis.

Insulin sensitivity reflects to what extent the effects in the tissues are. Nonetheless, only some of these effects are within the reach of an experimental observation. It is conceivable to set up a physiological model to assess insulin sensitivity/responsivity to skeletal muscle glucose disposal, hepatic glucose output suppression or AT lipolysis suppression. Moreover, insulin sensitivity is tissue specific and fluctuates over day and over life. When limiting to the three major metabolic organs, the most sensitive is AT, followed by liver. Relatively most resistant is skeletal muscle glucose disposal (in details below).

Insulin secretion. β-cells of pancreatic Langerhans' islets are responsible for synthesis and secretion of insulin. Insulin is formed as a precursor molecule of preproinsulin. Further post-translational modifications in endoplasmic reticulum and Golgi apparatus give rise to the two-strain molecule of the insulin itself. Connecting peptide (C-peptide) is formed in the equimolar ration to insulin. Insulin synthesis and release is under control of ATP availability and therefore a substrate flux to the cell. Insulin is disposed in the secretion granula, ready for exocytosis on a stimulus. Mechanism of the stimulus is complex and there are several pathways that converge on increasing intracellular availability of Ca^{2+}, that leads to microtubular/microfilament shift of the vesicles. The primary stimulus for the secretion is energy substrates availability, namely glucose, but also ketone bodies, FA and some AA. Besides substrate stimulation, vegetative stimulation also applies [22]. Insulin is secreted in pulses in a total dose of 20-40 IU per day. The stable diurnal trend in secretion is called basal and postprandial surges of secretion are termed stimulated.

Stimulated insulin secretion. Insulin secretion fluctuates during the day so that glycaemia is maintained within the normal range under normal circumstances. In the postprandial period, aka stimulated, insulin is released in excessive amount to stimulate glucose disposal from the extracellular pool (R_d) and block hepatic glucose production (R_a). Contrary, in the post absorptive period, a decrease in insulin levels prevents glucose disposal (R_d) and increases hepatic glucose output (R_a) [23]. This counter-regulatory relationship is depicted in Figure 2.

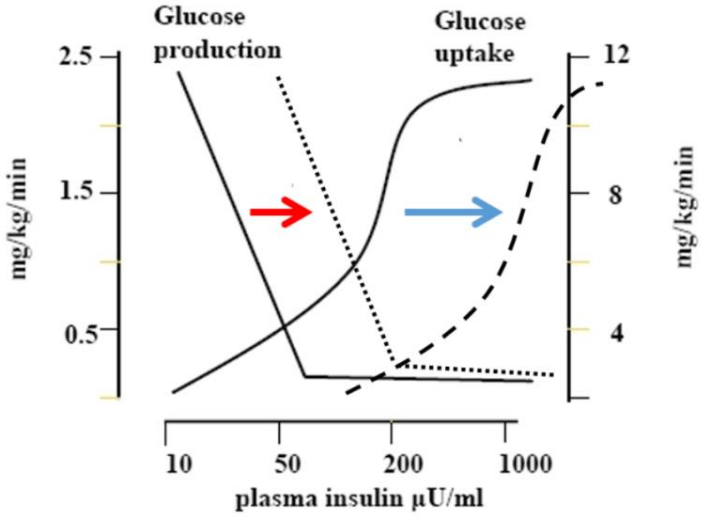
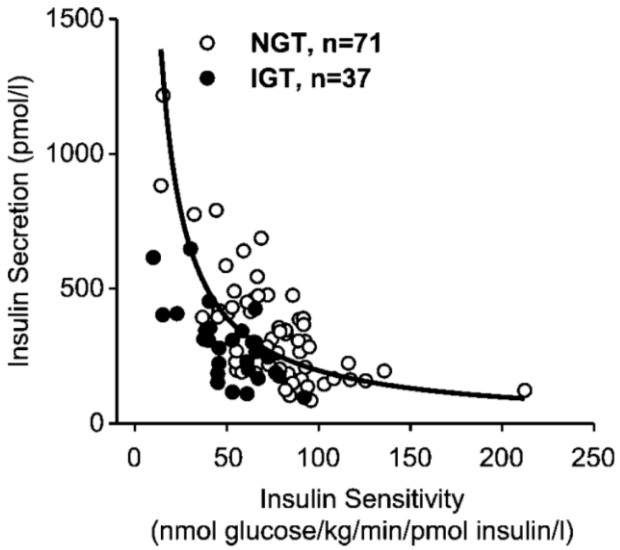


Figure 2 Relationship between glucose production (appearance) and disposal (disappearance) from the extracellular pool depending on insulin concentrations. Arrows depicts insulin resistance, the state where higher insulineamia is needed to elicit effects on glucose production inhibition and skeletal muscle disposal stimulation. According to [23,24].

Insulin sensitivity in peripheral tissues is fluctuating during longer postabsorptive states. It is modified by many factors. For instance day period (IS is usually lower before morning, the effect that can lead to so called dawn phenomena in diabetic patients [25]), acute illnesses, stress or physical activity [26]. β-cells adjust their insulin release as per changes in insulin sensitivity to maintain euglycaemia. Should we assess IS in one subject over day we get different values of glucose disposal. And should we plot these against corresponding values of insulin secretion, we get a hyperbolic relationship sensitivity vs. secretion [27]. This ratio is expressed as so-called disposition index and describes a dynamic function of glucoregulatory loop. When the glucose tolerance is maintained, a person moves up and down at his/her hyperbolic curve: changes in sensitivity are mirrored by changes in secretion [28]. Once the glucose tolerance worsens the hyperbole shifts left and downwards as depicted on Figure 3.

A.



B.

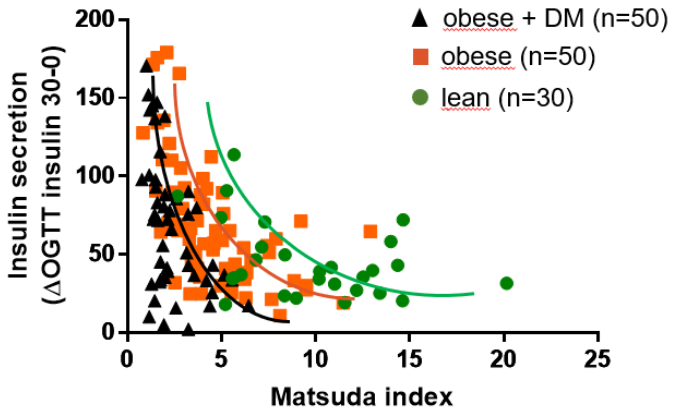


Figure 3 The hyperbolic relation in between sensitivity to insulin (A. expressed as M-value per insulinaemia; B. expressed as Matsuda index [29] and insulin secretion (A. as per arginin stimulation test; B. expressed as 30 min change in insulin levels after 75 g of glucose orally). A. Adapted from [26] and B our own observation from an ongoing trial TRIEMA NCT NCT03710850.

1.2. PHYSIOLOGICAL MODELS FOR INSULIN SENSITIVITY ASSESSEMENT

Here we provide an overview of different methods for insulin sensitivity assessment, in details dealing with a physiological model of glucose clamp, showing basic operating procedure and potential draw-backs of the method. All supported by our own expertise and data.

1.2.1. Introduction

When describing how to assess insulin sensitivity it is of note that sensitivity is defined by insulin's biological effects on dependent substrate metabolic pathways. Measured effects are according to physiological model glucose uptake, oxidative and non-oxidative metabolism of glucose, inhibition of lipolysis and lipid oxidation, decreasing hepatic glucose production and inhibition of proteolysis. There exist quite a few approaches to assessment of insulin sensi-

tivity at the whole-body level *in vivo* differing in its operator demands, each yielding a different information. For the reasons given above (see Chapter 1.1.), the focus here is on assessment of insulin sensitivity in terms of glucose production and disposal.

Assessment of insulin effects *in vivo* dates back to 1930s, once the insulin was discovered. In 1936 Himsworth showed that concomitant insulin and glucose administration leads to plasma glucose fall in ketotic diabetes patients. Contrary obese non-ketotic patients experienced a steep increase in glucose levels [30]. Since then many others attempted to standardize insulin effects. But it was not before radioimmune assay for insulin development that insulin sensitivity indices could be derived from simultaneous measuring of insulin and glucose after glucose challenge as reviewed by [31]. High insulin levels when compared to glycaemia were interpreted as insulin insensitivity. The major drawback of this conclusion is that glycaemia and insulinaemia are dependent variables, moreover in a complex relationship with contra regulatory hormones. This should be taken into consideration, when balancing feasibility of the test and its evidential value.

With analogy to other endocrine systems we can classify tests as being static and dynamic. Static tests rely on single fasting parameters (usually glucose and insulin/C-peptide). As liver is responsible for 90% glucose input into the extracellular pool in fasting state these tests assess dominantly hepatic IS [32]. Moreover, as fasting glycaemia and insulinemic are dependent variables, the latter with high interindividual variability, these test are generally considered reliable in epidemiological large cohort studies [33]. Dynamic tests are designed to disturb the feedback loop of glycaemia – insulin secretion. They can be further divided into those that perturb the feedback loop by testing to what extent a change in the signal intensity (i.e. insulin response to given glycaemia change) corresponds to a change in the physiological effect, or eliminating physiological control over the whole feedback loop (clamp studies, see below). An overview of different methods is depicted in Table 2. Among all the methods, glucose clamp, though elaborate and operator highly dependent, is considered gold standard for assessing insulin sensitivity [34].

Test	Details of the physiological model	Insulin secretion assessment	Complexity
Static tests			
HOMA	Empirical index of fasting insulin sensitivity	yes	+
QUICKI	Empirical index of fasting insulin sensitivity	yes	+
Dynamic tests			
2h-OGTT	Glucose change and AUC at given insulin levels; insulin stimulated glucose uptake (i.e. skeletal muscle)	yes	++
Insulin tolerance test	Rate of glucose disappearance	no	+++
Insulin sensitivity test	Estimate of glucose uptake at fixed insulin levels	no	++++
IV glucose tolerance test	Estimate of fractional glucose clearance	yes	+++
Hyperinsulinemic euglycemic clamp	Estimate of glucose uptake (skeletal muscle) at defined insulin levels	no	++++
Forearm/leg glucose clamp	Arterio-venous differences per plasma perfusion; exact measure of skeletal bed glucose uptake	no	+++++

Table 2 Methods for insulin sensitivity assessment. Adapted from [31]

1.2.2. Glucose clamp

Glucose clamps are considered as a gold standard in insulin sensitivity assessment [31] and has been also extensively used in bioequivalence studies for different insulin compounds [35]. As explained bellow, clamp's output, i.e. glucose infusion rate (GIR), equals the pharmacodynamic effects of insulin.

Though similar in practical conduct, there are variants of the clamp approach depending on the output of the test. The nomenclature is such: for peripheral insulin sensitivity output, euglycemic test is the one of choice. When assessing beta cells response, i.e. insulinogenic response, hyperglycaemic clamps are used and for contraregulatory response to hypoglycaemia, hypoglycaemic ones are used. Pancreatic clamp is a term for the test when infusing also somatostatin to block insulin release. As hepatic clamp an examination where isotope dilution techniques is added on top of the clamp to assess hepatic glucose output. The focus here is on the euglycemic clamps as these were widely implemented in our own research.

The general idea of the examination is that by giving exogenous insulin a hyperinsulinemic plateau is created. Depending on the insulin dose this may reach supra-/physiologic levels. The "classical" dose (1 mIU.kg^{-1}.min^{-1}) was empirically chosen so that it produces hyperinsulinaemia corresponding roughly to postprandial insulinaemia in healthy subjects. To maintain euglycaemia (4.5-5.5 mM) variable continuous glucose infusion is administered. For graphical summary see Figure 4.

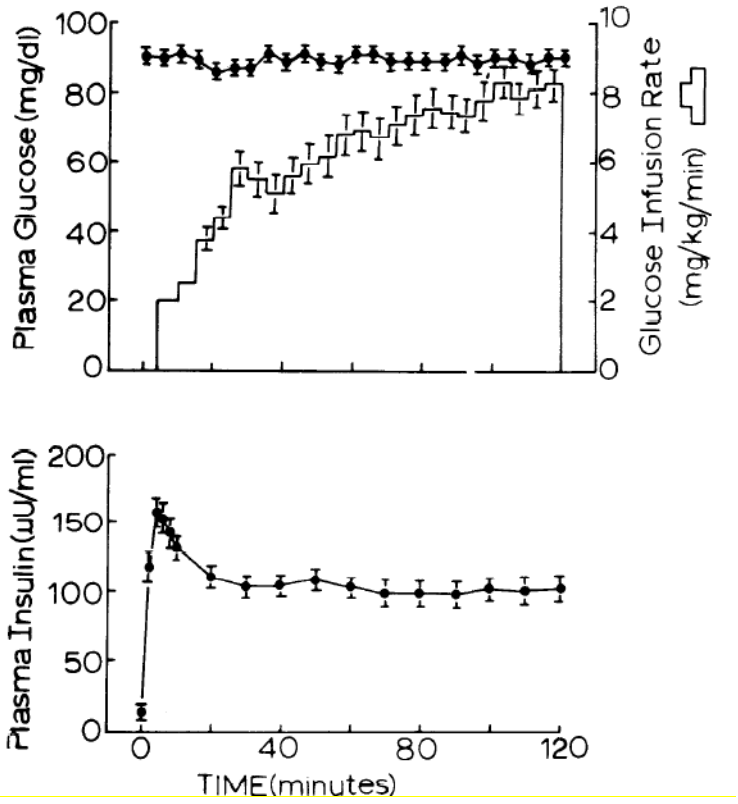


Figure 4: 120 min course of hyperinsulinemic euglycemic clamp from original work of DeFronzo [36]. Primed continuous infusion of insulin produces stable hyperinsulinemic plateau. To maintain euglycaemia, variable incremental dose of exogenous glucose is administered.

Under the assumption that endogenous glucose production as well as insulin secretion is completely inhibited (which may not always be the case, see below) the measure of net insulin action is then glucose infusion rate (glucose metabolised, M-value), the only contributor of glucose into the glucose space. In other words, the amount of glucose needed to maintain euglycaemia at a given hyperinsulinaemia. Therefore, the term hyperinsulinemic, euglycemic clamp (HEC) is used for this particular examination. The procedure was firstly developed by Andres and others [37] and further elaborated by DeFronzo in his seminal work Glucose Clamp Technique [36]. As described earlier, in the fasting state, R_a of glucose is driven by gluconeogenesis namely in liver (~95 %) and other extrahepatic tissues. When insulin is given (or secreted as in postprandial state), glucose production is suppressed and glucose is redistributed to insulin sensitive tissues, namely skeletal muscle (~80 %). Therefore, hyperinsulinemic conditions predispose the test to assess namely skeletal muscle glucose disposal.

M-value equals glucose utilization when stable hyperinsulinaemia and glycaemia is maintained. This is usually achieved after 60-90 min of primed insulin infusion during so called steady state, where glucose infusion rate is stable, and the feedback loop is in an equilibrium. Then glucose infused in the glucose pool equals glucose R_d, i.e. glucose disposal from extracellular space to tissues. In reality, glycaemia is almost never ideally clamped at a stable value. Therefore so called space correction (SC) was developed by DeFronzo [36]. It is an adjustment of M-value for oscillations in steady state glycaemias, i.e. situation where stable GIR does not match perfectly glucose utilization. Besides space correction urinary loss of glucose should be accounted for. This might be negligible in HEC in normo-glycemic subject but is not the case for hyperglycemic patients or isoglycemic clamps where renal threshold for reabsorption may be exceeded. Therefore, another urinary correction (UC) is applied. M-value then equals: M=GIR-SC-UC. For details on calculation we refer to the primary source [36].

Operating procedure. The operating procedure of the clamp requires a 12-h overnight fasting. In the morning antecubital vein is cannulated for simultaneous infusion of glucose, insulin and potassium compound (see below). The other cannula is inserted depending on protocol either in the artery or retrogradely in a dorsal hand vein for sampling of an arterialized blood. The insulin dose depends on the protocol and the information that is to be obtained. The "classic" dose 1 $mIU.kg^{-1}.min^{-1}$ (for BMI out of 20-25 range 40 $mIU.m^2.min^{-1}$) has been widely used in many studies. Though when examining extremely insulin resistant subjects it is common to use twice or three times of the dose (120 $mIU.m^2.min^{-1}$ was used for instance when examining critically ill patients, see Figure 12). On the other hand, as lipolysis inhibition is substantially more sensitive than glucose disposal, when assessing lipolysis inhibition (as per decrease in circulating NEFA/glycerol) we use lower doses (up to 10 $mIU.m^2.min^{-1}$). Different doses in the same subject are used for assessing insulin responsivity curve (see Figure 5).

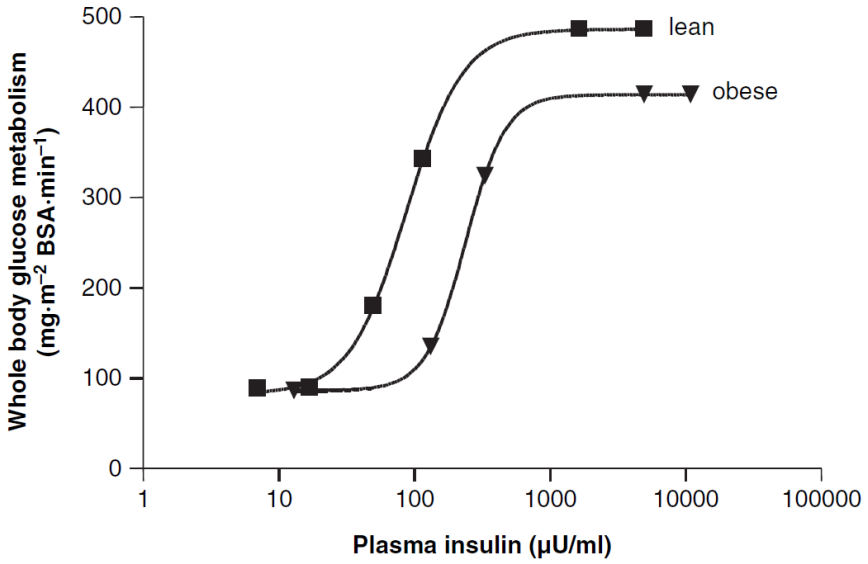


Figure 5 When performing clamp studies with different insulin doses at the same subject, dose response curve may be fitted. Here in obese patients, lower insulin sensitivity (right shift of the curve) and insulin unresponsiveness (down shift) is observed when compared to lean subjects. Graph from [31,38].

Prerequisites and limitations of clamp studies

M-value has got good reproducibility as was repeatedly shown [31,39] with relatively low coefficient of variance ~10 % [40]. Examples of different M-values across literature is depicted in Table 3.

On the other hand, it shows high inter-individual variability, therefore, a comparator group is a necessity and no accepted cut-offs for normal glucose disposal are widely accepted. Moreover, besides being time consuming and operator dependent, there are several assumptions that are taken into consideration.

Metabolic characteristics	M ($mg.kg^{-1}.min^{-1}$)	Reference
Long distance runners	10.0 ± 0.8	Yki-Jarvinen et al. 1987 [41]
Vegans with normal weight	9.6 ± 2.4	Gojda et al. 2017 [42]
Healthy subjects with normal weight	7.1 ± 2.4	Gojda et al. 2017 [42]
Healthy subjects with normal weight	7.1 ± 2.1	Ferrannini et al. 1997 [43]
First degree T2DM relatives	6.6 ± 0.5	Pratipanawatr et al. 2001 [44]
Obese, otherwise healthy subjects	5.5 ± 2.0	Ferrannini et al. 1997 [43]
Elderly	3.8 ± 0.5	Fink et al. 1983 [45]
Patients with T2DM and resistant hypertension	3.2 ± 1.5	Gojda et. al. 2013 unpublished
Patients with T2DM	2.7 ± 0.4	Doberne et al. 1982 [46]

Table 3 Different M-values according to metabolic status of population studied. Adapted from [31]

Arterialization of blood. To obtain valid indices as glucose disappearance, one must be sure that blood is sampled before reaching its target insulin-sensitive tissue, in this case the skeletal muscle. As glucose uptake in the tissue is a variable depending not only on insulin levels but also on sensitivity of tissues to insulin [47].

Therefore, venous blood glucose concentration equals arterial BGC minus tissue glucose uptake. Obviously, skeletal glucose uptake differs among individuals as well as among experi-

mental group. If this is not considered, relying on venous BGC yields variable yet inherent error. A prove for this comes from arterio-venous studies, when at the same time arterial and venous blood is sampled throughout the clamp. As depicted in Figure 6 AV difference may vary considerably. Clamping glucose concentration at the venous level results in higher arterial levels in the sensitive subject. The difference in arterial glucose concentrations is proportional to the difference in insulin sensitivity and thus systematically impacts on the determination of insulin sensitivity [31]. Of note, when clamping a homogenous group in terms of insulin sensitivity (healthy volunteers), glucose disposal is the same when comparing venous, capillary or arterial blood [48]. This should be particularly addressed when comparing groups with different expected insulin sensitivity.

Original works of DeFronzo and others measured glucose concentration in arterial blood [31,36]. Others, to simplify the protocol, avoided the arterial cannulation and sampled either capillary or arterialized venous blood. The latter is achieved using hand-heating techniques with the aim to heat the arm to 55-60 °C. As the heat induces vasodilation, it shortens perfusion time and oxygen/glucose extraction and glucose concentrations in the vein equals those in the artery. This technique has been widely used and accepted [31,49]. From our own experience we gradually figured-out that arterialization may vary greatly even when using the same technique. Therefore, we suggest to measure saturation of oxygen in the venous samples to confirm adequate arterialization.

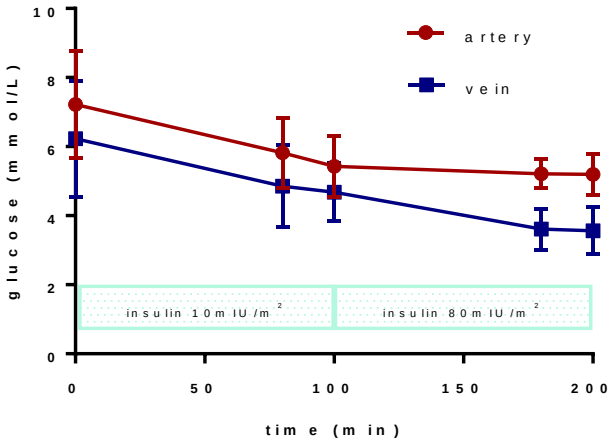


Figure 6 Differences of glucose concentration across forearm, i.e. in radial artery and brachial vein. 12 subjects with pancreatic cancer were subjected to two-step hyperinsulinemic euglycemic clamp. Arterial (radial artery) and venous (brachial vein) blood was sampled. Own observation (pilot data from PAMIR study, Czech ministry of Health *NV19-01-00101*).

Endogenous insulin release suppression. The insulin infusion is administered so that it produces hyperisnulinemic plateau. In the original experiments the dose was 1 mIU/kg/min (it equals 4.2 IU/hrs in a 70 kg person) and this produced a plateau roughly 100 µM above fasting levels, a plateau that correspond roughly to postprandial levels. When increasing the dose so that it produces insulinaemias above 500 µIU/ml physiological clearance of insulin via receptor binding is exceeded and there will be continuous rise in insulin levels [50] albeit glucose disposal is already at its maximum. Insulinaemias obtained from clamp with identical insulin dose vary among studies and even among subjects under very same conditions (see Figure 9). Indeed,

it depends on clearance capacity, metabolic conditions and on capability of exogenous insulin to supress endogenous production [50]. Here, it is one of the assumptions of the clamp physiological model, that endogenous insulin secretion is suppressed during hyperinsulinaemia and that insulinaemias in steady state correspond to insulin infused and therefore insulin infusion is the sole determinant of glucose infusion rate. It was shown that this assumption may not be valid, namely in insulin resistant subjects [51]. In obese insulin resistant subjects it has been already suggested that incompetence of the feedback loop suppression (insulinaemia – glycaemia) contributes to hyperinsulinaemias in metabolic syndrome [52,53].

To overcome this obstacle M-value is normalized to steady state insulinaemias (producing M/I index). Unfortunately based on our experience, as steady state insulinaemias show high interindividual variability, this normalization usually increases greatly M-value scattering and variability, therefore decreasing statistical power in small scale physiological studies.

Though decrease in circulating C-peptide as a parameter of sufficient suppression of endogenous insulin production is generally accepted, there are few primary sources available for comparison [51–53]. As we do routinely measure C-peptide suppression in the steady state we investigated into this research question. We conducted a retrospective analysis of C-peptide changes throughout the clamps performed at our facility from 2015 – 2017. At that time, we analysed 115 clamps (same design, same conditions/facility, C-peptide measurement). Results are summarized in Figures 7 – 9.

To conclude there is a huge variability in ssC-peptide suppression, this change does not correlate with prevailing insulin levels and those who do not supress C-peptide are more insulin resistant (as depicted on Figure 8). The results suggest that as with increasing insulin resistance beta cells become resistant too, resistant to insulin mediated suppression of endogenous insulin release. These results should stimulate further research into how beta cells become their self insulin resistant.

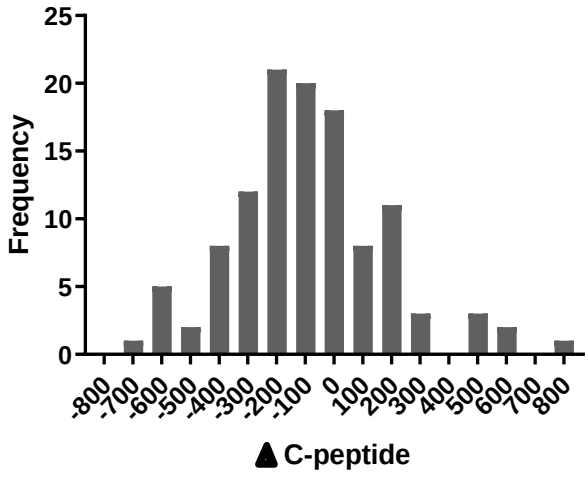


Figure 7 Analysis of 115 consecutive hyperinsulinemic glucose clamps (insulin dose 40 mIU/m^2) performed at our facility in 2015 – 2017. Only healthy (age 31.4 ± 3.7 years), non-obese (BMI 23.8 ± 3.1), non-diabetic subjects' data were analysed. Here we show frequency distribution of changes in C-peptide (steady state – baseline).

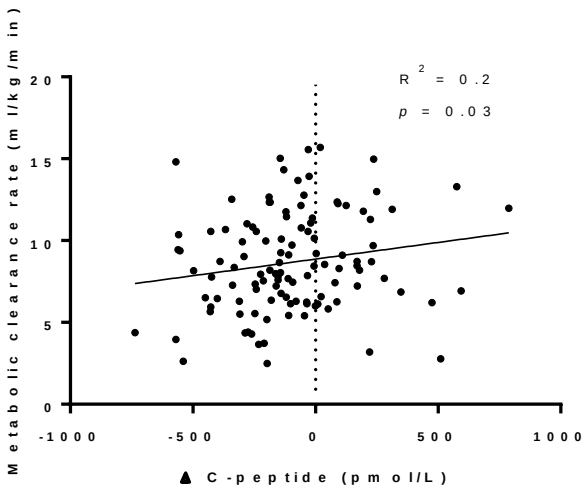


Figure 8 Analysis of 115 consecutive hyperinsulinemic glucose clamps (insulin dose 40 mIU/m^2) performed at our facility in 2015 – 2017. Only healthy (age 31.4 ± 3.7 years), non-obese (BMI 23.8 ± 3.1), non-diabetic subjects' data were analysed. Here we show that C-peptide change (steady state – fasting C-peptide; ΔC-peptide) correlates significantly but poorly with MCR.

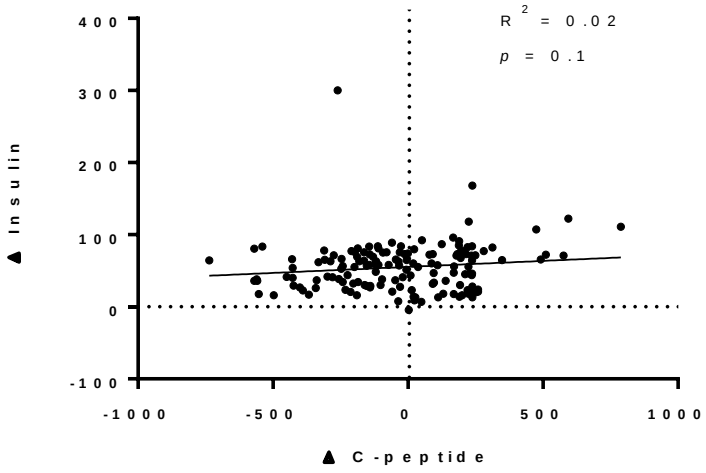


Figure 9 Analysis of 115 consecutive hyperinsulinemic glucose clamps (insulin dose 40 mIU/m^2) performed at our facility in 2015 – 2017. Only healthy (age 31.4 ± 3.7 years), non-obese (BMI 23.8 ± 3.1), non-diabetic subjects' data were analysed. Here we show that C-peptide change (steady state – fasting C-peptide; ΔC-peptide) does not correlate with Insulin change (steady state – fasting Insulin; ΔInsulin).

Hepatic glucose output suppression. In metabolically healthy persons steady state hyperinsulinaemia was repeatedly shown to inhibit completely hepatic glucose production (HGO, hepatic glucose output). Yet, this assumption may not be valid in insulin resistant subjects and increased glucose output in basal as well as stimulated state has been acknowledged as one of the drivers of diabetes [24], referred as hepatic insulin resistance. In case glucose output is not zero during steady state, it adds glucose to the glucose space and GIR does not equal R_d. If not accounted for HGO leads to underestimating M-value during the clamp. Therefore, assessing HGO is warranted in any case and especially when comparing metabolically heterogenous groups (for example obese vs. lean) [31]. In practice the most common approach to assess HGO are isotope dilution techniques. This requires substantially longer protocol with

additional cannulation and isotope compound administration making this procedure suitable just for small scale deep physiology studies. Details of these procedures go beyond the extent of this test and we refer to respective literature [54]. We have gained experience with using deuterated glucose (D-[6,6-$^2H_2$]) for HGO assessment at our facility. This procedure requires 120 min of stabilization period with continuous isotope infusion. This leads to stable tracer/tracee ratio that reflects the amount of endogenous glucose input to the glucose space. With known infusion rate and measured (using mass spectrometry) tracer/tracee ratio it is possible to calculate basal hepatic glucose output. Afterward the clamp is performed with isotope enriched glucose infusion and again with the same principle the decline in HGO can be calculated. Detail of the protocol are depicted in Figure 10 (own observation).

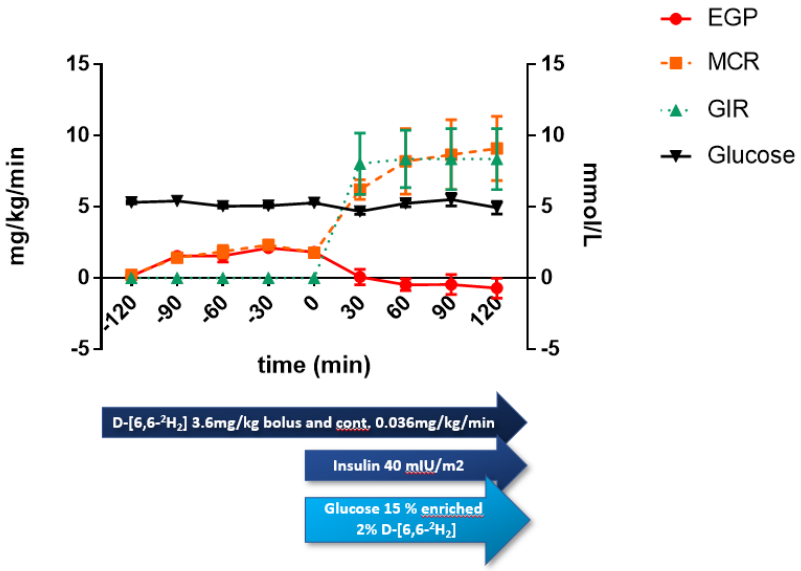


Figure 10 Hyperinsulinemic euglycemic clamp with D-[6,6-$^2H_2$] glucose to assess hepatic glucose output and its suppressibility by hyperinsulinaemia. Three healthy young (age<50, F=1) subjects were subjected to primed D2 glucose infusion to reach stable tracer/tracee ratio (fasting hepatic glucose output). After 120 min primed 40 mIU/m^2 insulin infusion was initiated, glucose was maintained ~5 mM with using variable 15% glucose infusion enriched with D2 glucose (suppressibility of hepatic glucose output). EGP, endogenous glucose production as per isotope dilution (mg/kg/min); MCR, metabolic clearance rate of glucose (mg/kg/min); GIR, glucose infusion rate (mg/kg/min); Glucose, glycaemia in mmol/L. Author 's own observation.

Safety considerations. There are some safety considerations of HEC. If we disregard technical problems with cannulation, hypoglycaemia and hypokalaemia are of a concern. *Hypoglycaemia* and/or rapid drop in glycaemia levels should avoided as it is associated with a contraregulatory response and posthypoglycemic insulin resistance that blurs interpretation of results [55]. We should also make sure that once we remove the catheter after the termination of the clamp procedure, glycaemia is stable. Depending on the dose, this interval may be 60-120 min and subjects are advocated to oral carbohydrate intake. Insulin stimulates cellular potassium uptake similarly in a dose-dependent manner. *Hypokalaemia* with its potential arrhythmogenic risk must be expected and avoided. For that reason, we give simultaneous potassium infusion throughout the HEC study. The dose used in our facility is 10 mmol of potassium (7,45% KCl) per 50 g of glucose infusion. It is advisable to check for potassium levels more often when implementing a new protocol.

Generally, when performed by an experienced operator the clamp procedure is very safe. There have been more than 300 clamp studies performed at our facility in recent years with different protocols. Based on this experience, we had no severe hypoglycaemia, 3 cases of significant glycaemic drop/hypoglycaemia with mild or no symptoms that nevertheless required discarding of the results and repeating the examination, and no hypokalaemia. We experienced 1 paravasal application of insulin and we were unable to cannulate any vein for samples/infusion in 2 patients.

Clamp studies are usually combined with other techniques to get deeper metabolic insight in substrate fluxes. It can be combined with indirect calorimetry to assess proportion of glucose oxidation vs. non-oxidative metabolism and metabolic flexibility [56], with isotopes to assess hepatic glucose output or lipolysis inhibition [57], with forearm/leg catheterization to assess regional glucose uptake, hepatic vein catheterization, plethysmography, PET scan, NMRI spectroscopy, microdialysis [31] and/or with adipose tissue [42] and skeletal muscle biopsy [58] to assess at the tissue level the impacts of insulin. To conclude, paraphrasing Pacini and Mari [31], HEC is widely used and accepted method for assessment insulin stimulated glucose disposal at the whole-body level and glucose infusion rate at a defined insulinemic plateau adjusted for weight (FFM etc.) is termed M-value and regarded as a marker of insulin sensitivity.

1.3. INSULIN RESISTANCE

Insulin resistance (IR) is mechanistically defined as a state where increased amount of insulin is needed to elicit quantitatively normal response of a tissue [31,59]. With respect to the above mentioned gluco-regulatory loop principles, we define it as a rightward shift of dose-response curves for insulin mediated suppression of glucose production and insulin stimulated glucose disposal (see Figure 2).

Insulin shows various metabolic and proliferative effects almost in every tissue. Considering close relation among insulin function and glycaemia and historical clinical contexts, decreased response to insulin was defined and researched primarily with respect to its glucoregulatory function. Krebs was studying substrate competition for the respiration between glucose, amino acids, aceto-acetate and palmitate as early as in the thirties of 20th century [60]. Randle did further elaborate glucose-fatty acids cycle in the 60th. Principle of cycle that bears his name is that higher availability of acetyl-CoA in mitochondria inhibits pyruvate dehydrogenase complex, phosphofructokinase and hexokinase, all upstream key regulatory enzymes of glycolysis. Leading to decreased glucose oxidation and uptake [61]. The mechanism that allows for glucose sparing during starvation. On the other hand, T2DM is characterised by increased circulating FFA. It was therefore postulated that elevated FFA inhibits in this state skeletal muscle glucose uptake [49]. Since then many defects at postreceptor insulin signalling and glycolysis that relate to higher lipid availability have been described [61]. Similarly substrate competition has been verified between amino acids and glucose [62,63]. Description of further consequences will follow.

Insulin resistance may well be perceived as a phenomenon that relates to many physiological and pathological states, though the major focus nowadays is on the IR in metabolic syndrome

and diabetes. Notwithstanding, IR takes various forms depending on the environmental context and it may well be that it relates also to historical, cultural and social ones. IR is an evolutionary preserved response that can be tracked back to, at least, chordates and maybe even earlier (once a molecule similar to insulin and its effects depending on the signal appeared). This leads to several questions. Has the physiology, i.e. substrate redistribution, remained the same while its relevance in different social – environmental conditions has changed? Are there different „physiologies", clinical scenarios? Do we have the appropriate methods for description of the phenomenon? In the further reading I will try to summarize physiological states that share this common phenomenon of insulin resistance.

1.3.1. Insulin resistance as a metabolic adaptation for starving

Here we review the history of starvation diabetes and how the insulin resistance phenomenon relates to substrate redistribution as an adaptation response for caloric restriction.

As early as in the half of nineteenth century Claude Bernard described glucogenic capabilities of fasted liver and observed glycosuria after refeeding of otherwise healthy but starved rabbit [64]. The phenomena that would become later known as starvation diabetes. In 1915 Benedict showed metabolic changes associated with a prolonged fasting, reduced substrate oxidation and reduced nitrogen loss [65], as he stated: "When the demands are not met, body reserves must be drawn upon." These seminal works paved the way for accepting substrate redistribution and energy sparing as a physiological response to fasting. Concerning glucose metabolism, it was later shown by Unger et al. that the adaptation is orchestrated by decreased insulin/glucagon ratio [66] and low insulin secretion had been perceived as a major contribution to starvation diabetes since then. Decline in glycaemia after an overnight fast, inhibits secretion of insulin. In this humoral milieu hepatocyte switches to catabolism and degrades stored glycogen to release glucose to the systemic circulation. As the stores are about to be depleted, three carbon molecules (namely carbon skeleton of glucogenic AA, glycerol, lactate) are used for gluconeogenesis. After 60 hours of fasting glycogen stores are depleted and all glucose appearance comes from visceral gluconeogenesis [67]. With the development of clamp techniques it became possible to study peripheral insulin sensitivity and a major decline in glucose disposal after 60-h fast was observed [68]. Later Nilsson showed that splanchnic glucose uptake remains unaltered during fasting and that skeletal muscle is responsible for the whole body insulin resistance [67]. Indeed, during fasting skeletal muscle relies on FA oxidation and spares glucose to be oxidized in glucose dependent tissues (i.e. brain and erythrocytes) or used for biosynthetic purposes. This shift is possible only when lipolysis in AT is deblocked with increased flux of FA to the liver. Our own observations are depicted in Figure 11.

Of note, it was shown, that during short term starvation (i.e. up to 60 hours) BCAA and BCKA levels in circulation rise. Some suggest that after it peaks in 48-72 hours BCAA consequently decline [69,70] though the data about the decline are not unanimous [71]. Decreased proteosynthesis / increased proteolysis of fibrillar proteins lead to a higher protein turn-over and higher

availability of BCAA in the skeletal muscle as well as in the circulation. Increased activity of BCAT in the starved skeletal muscle has been shown [72]. BCAA are deaminated in the skeletal muscle to provide nitrogen source for glutamine and alanine synthesis. ALA/GLN serve consequently as precursors for liver (and to a smaller extent extrahepatic) gluconeogenesis. Similar inter-organ flux as in Cori cycle [73]. BCKA are used as a fuel or an anaplerotic substrate in the extramuscular tissues.

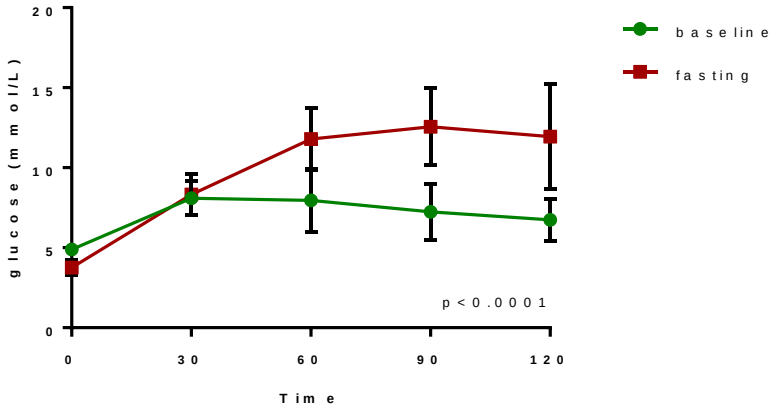


Figure 11 To prove the presence of glucose intolerance associated with fasting. Twenty healthy young women (BMI 20-30) were subjected to 60-h fasting. Before and after the intervention 75 g glucose OGTT was performed. *p*-value for time vs. group interaction per two-way ANOVA. Data from our group's ongoing trial, ClinicalTrials.gov NCT04260542. De Novo Lipogenesis and Insulin Sensitivity in Obese (DELISA).

Once the physiological response of redistributive IR is modulated during fasting by other phenomena that are themselves associated with the same phenomenon. One of the key pathophysiological changes observed in patients with T2DM is resistance to effects of insulin in major metabolic organs. Paradoxically, in the time before the discovery of insulin, starvation was a possible supportive treatment for diabetes, despite its drastic side effects. Frederick Allen and Elliot Joslin were responsible for expansion of this starvation diet in England at the beginning of the 20th century [74]. Following the discovery of insulin, this treatment modality rightly fell into oblivion, but later fasting would be shown associate with improved glucose tolerance in obese and diabetic patients [75,76]. While lean healthy subjects reduce glucose disposal during fasting, in patients with diabetes, this is not even observed, glucose disposal insulin sensitivity was even shown even increase [56]. In line, promising results come from growing evidence on intermittent fasting or severe caloric restriction showing amelioration of HbA1c and body weight [77]. In spite of that, there still remain some safety concerns as well individual predictors of the effect that could justify the drastic caloric restriction or fasting.

Higher physiological question could be though posed. Do these changes reflect loss of dynamic capability of substrate redistribution in T2DM and therefore metabolic inflexibility or should they be perceived as a trend towards improvement of the glucoregulatory system *in toto*? This remains to be disputed and studied.

1.3.2. Insulin resistance as a metabolic adaptation to the inflammation

Here we review how inflammatory response relates to insulin sensitivity and show how the metabolic response leads to profound derangements of normal substrate handling.

Once accepting that insulin resistance in fasting is an adaptive response the same premise should apply for stress (i.e. trauma, illness) situation. As per example we will describe metabolic changes associated with critical illness. With a sense of some simplification we take this model to describe extreme changes associated with a cytokine storm. Supposing that continuum exists in between low grade and high-grade inflammatory response and the same physiological changes can be extrapolated to advanced chronic diseases (such as cardiac cachexia, COPD etc.) and cancer cachexia.

Yet again, the first observation of hyperglycaemia accompanying hemorrhagic shock, was described by anyone other than Claude Bernard [78]. However, not before the development of intensive care methods in the end of 20th century, the phenomenon of stress hyperglycaemia gained critical appraisal in the literature. There is a well-documented association between the degree of stress hyperglycaemia and many clinical outcomes [79]. Many facilities were adopting protocols of controlling hyperglycaemia, especially after results of Leuven Intensive Insulin Therapy Trial showing a potential benefit of tight glucose control [80]. Since then many others researched into the question and repeatedly proved tight control as inefficient or even potentially harmful to patients [81]. Indeed, association was here confounded with causation and tight glucose control should de-adopted though it may bring some benefit when parenteral nutrition is delivered [82]. Course of insulin sensitivity over critical illness is depicted in Figure 12.

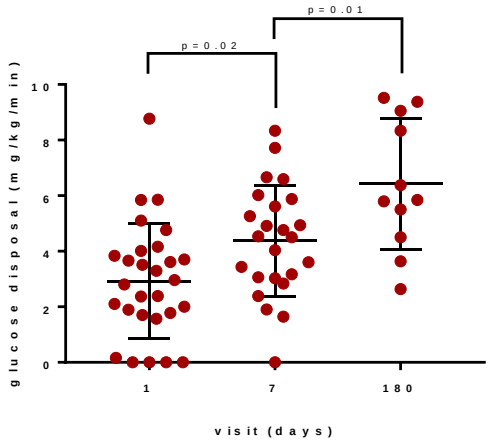


Figure 12 To substantiate the claim of stress insulin resistance. Critically ill subjects requiring intubation were examined at the time of admission to ICU (day 1, 30 subjects), at day 7 (24 subjects) and after 180 days post-ICU follow up (11 subjects). Glucose clamp (120 mIU of insulin) was performed to assess glucose disposal. Data from ClinicalTrials.gov, NCT02864745 [83].

Indeed, stress hyperglycaemia is an extreme expression of the whole-body substrate redistribution and insulin resistance and can be perceived yet again as an evolutionary well-preserved response to ensure survival in all or nothing mode. The complex response is mediated by a synergic activity of sympathoadrenal axis (catecholamines), hypothalamo-pituitary axis

(cortisol) and proinflammatory cytokines (namely IL-1, IL-6 and TNF alpha). The state is characterised by increased hepatic glucose output, decreased skeletal muscle glucose disposal and increased lipolysis.

The logic of the response is such that glucose is spared for central organs (brain, immune system) as a readily available fuel that can be utilized even when oxygen supply is scarce. Besides, of importance is glucose sparing for biosynthetic purposes [84]. Immune system activation, clonal proliferation of lymphocytes, synthesis of acute phase proteins, these all are extremely energy and building blocks demanding processes. Monocyte-macrophage system that plays pivotal role in cytokine secretion, chemoattraction and antigen presentation is heavily dependent on glucose delivery. Both for energy and for NADPH repletion. NADPH is reduced in the pentophospate pathway (PPP) of glucose and allows for regulation oxidative burst of macrophages and neutrophils. Another important product of PPP is ribose, five carbon sugar a precursor for ribonucleic acid. When interstitial space is expanded as in the case of capillary leak syndrome, the diffusion distance from intravascular to intracellular compartment increases. As glucose transport depends on the concentration gradient, the initial concentration in the capillaries must be greater to overcome the microvascular defect [79].

Obviously, increased glucose turn-over must be kept high over the course of the stress response and there is no such shift towards protein sparing effect of ketone bodies utilization as in simple starvation. Glucogenic AA remain the major fuel for gluconeogenesis that could eventually lead to a severe muscle wasting, a common feature in critical illness survivors.

There are many unanswered yet important questions that warrant further research. What is the role of early exogenous supply of glucose/parenteral nutrition? Should we limit stress hyperglycaemia using exogenous insulin and at which level? When is the time chronic glucotoxicity becomes an important issue?

1.3.3. Insulin resistance as the reflection of pathological substrate redistribution in the metabolic syndrome and diabetes

Here we review how the same phenomena observed in physiological substrate distribution relate to metabolic syndrome and diabetes development. We try to summarize concisely current understanding of the pathophysiology.

Once an adaptive phenomenon, insulin resistance becomes a pivotal mechanism in type 2 diabetes evolution. Could IR be perceived as a maladaptation to changing evolutionary conditions? Could IR be once a normal adaptive mechanism that becomes a self-perpetuating pathology because of the duration of its activation? Or is it that simple that physiological models used to define IR focus on surface phenomena and are not able to distinguish between different states? Though pathophysiology of insulin resistance syndrome propagation and progression has been thoroughly examined and well described today, we find much less certainty in the mechanism of its initiation.

The debate about initiation of diabetes is ongoing and no clear unifying mechanism has not been elucidated yet [85]. Here we do not intend to review all proposed mechanisms of IR initiation but provide reader a simplified view that will allow for further reading.

Genetic background is among suggested players. It has been studied in depth and many candidate genes have been identified [86]. Indeed, a thrifty genotype/phenotype, the ability to effectively store surplus of energy, had once been an evolutionary advantage. In a modern and rapidly changing world these dispositions become deleterious. Though important, genetic background remains only a predisposing factor and the major driver of metabolic syndrome incidence is the environmental one.

There is a lot of association data that identifies potential risk factors of IR/T2DM development such as overfeeding, sleep deprivation, physical inactivity, chronic stress, endocrine disruptors etc. Nevertheless, the causality is based upon treatment efficacy in interventional studies. Here the overall agreement remains that "hypothesis-driven research is needed to define direct causal relationships between specific environmental factors and pathophysiologies leading to diabetes." [85].

In a logical sequence to the previous chapters, one pathogenetic mechanism stands out above the others and deserves attention: the inflammation. It has been established for a period of time that diabetes is associated with a low-grade inflammation.

As early as the turn of the 19th and 20th centuries, the hypoglycemic effect of aspirin was first described. In the middle of the 20th century, this knowledge received further attention, but it was not before the beginning of the new millennium that the definitive molecular target NF-κB axis were identified [87]. In parallel many observational data have shown association between various acute phase reactants and diabetes as rev. in [88]. The inflammation predicts incident diabetes [89] and may be reverted by life-style or pharmacological intervention [90]. All these emerging evidence eventually paved the way to a proof-of-concept study published in 1993 that showed that neutralizing of a proinflammatory cytokine TNFα in rodent models of obesity leads to an increase of peripheral glucose uptake [91]. Consequent research identified many circulating compounds (leptin, resistin, MCP1, IL-6 and others) produced by adipose tissue that can eventually modify glucose handling in other metabolically active tissues. To what extent the production of these is limited to local macrophages remains to be elucidated.

Molecular targets of IR related cytokines are surface proteins that recognize foreign substances on target tissues (adipocyte, hepatocyte, macrophages). These are families of TLR (toll-like-receptors), RAGE (receptors of advance glycation end products), TNF receptor and IL-6 receptor. Postreceptor effectors associated with IR and obesity are both JNK (c-Jun N-terminal kinase) and IKKβ/NF-κB. JNK kinases are a group of stress-responsive enzymes that relate to many biological functions. NF-κB is the transcription factor activating inflammation and cellular growth. NF-κB is bound to its inhibitory proteins (IκB) and remains inactive in the cytoplasm. Various stimuli (including TNF-α and IL-1) can activate IκB kinase β (IKKβ) eventually leading to NF-κB release [92]. It was shown on different models that inhibition of these pathways ameliorates insulin resistance, as rev. by Rehman [93].

Development of IR is related to function of major glucoregulatory organs, namely adipose tissue, skeletal muscle and the liver. Skeletal muscle is responsible for a major portion of insulin stimulated glucose disposal and in a postabsorptive state it provides glucoplastic AA for gluconeogenesis. Adipose tissue is a major buffer of the postprandial nutrient load and its plasticity decides how many substrates are available to other tissues. Besides it is a major endocrine organ secreting adipokines effecting on other tissues and influencing food intake. Hepatocyte is an energy factory of the organism, gluconeogenesis and ketogenesis in a post-prandial state take place here. Hepatocyte disposes of glycogen that is readily available in a postabsorptive state for peripheral tissues.

Adipose tissue is a first buffer for the postprandial charge of substrates (i.e. FA, glucose and AA). Its storage capacity, i.e. plasticity, therefore determines the availability of FA for other organs. Adipocyte can accumulate a maximum of about 0.8 μg of lipids. Hyperplastic adipocytes become insulin resistant, failing in the anti-lipolytic action of insulin [94,95] with FA overflow to systemic circulation even in an postabsorptive state [96]. Iozzo was the one to postulate that impaired subcutaneous AT (SAT) plasticity, i.e. incapability of newly differentiated adipocytes to store triglycerides, may be the key defect in the whole-body IR development. Overflow of triglycerides from SAT to circulation leads to an immediate ectopic deposition of lipids in extradipose tissue, for instance skeletal muscle, epicardium, pancreas [96]. The concept of overflowing sink implicates that there exist a "personal fat threshold" for ectopic fat accumulation [97]. Visceral fat (VAT) could be perceived among the first ectopic depos, as it is just VAT that predicts development of metabolic complications [98]. VAT is drained by portal circulation, therefore loss of suppressibility of lipolysis there leads to an increased flux of FA and pro-inflammatory adipocytokines to the liver with consequences discussed below. Increased and sustained availability of FA leads to lipotoxic derangements in metabolic organs: induce hepatic gluconeogenesis [57], decrease skeletal muscle glucose disposal [99] and interfere with secretory response of β-cells [100].

Moreover, insulin resistant adipocyte changes its secretory profile in favour of pro-inflammatory adipocytokines [95], as AT is infiltrated by macrophages [101]. These metabolically activated macrophages directs their phenotype and secretom towards inflammation and lysosomal clearance of dead adipocytes (having some of the properties of both M1 and M2 phenotype), conferring both detrimental and beneficial role [102]. Contrary circulating monocytes-macrophages gain M2 like phenotype characterized by increased rates of glycolysis, persistent mTOR activation and failure to activate Akt upon insulin stimulation, i.e. they become insulin resistant themselves. Uptake of glucose is maintained through increased expression of GLUT1 and GLUT3. Interestingly same phenotype of macrophages is present in atherosclerotic plaques and cancer microenvironment [103].

AT also disposes postprandial glucose load. It accounts for up to 5-20 % of the whole body uptake [96,104]. The adipocyte is capable of exposing GLUT-4 on its surface upon insulin stimulation and direct the disposed glucose to de novo lipogenesis. The effect that is impaired in an insulin resistant states [105], and an experimental deletion of GLUT-4 leads to IR [106].

Among AA that are buffered by AT, branched chain amino acids are the most important. BCAA constitute a significant portion (up to 30 %) of acyl-CoAs feeding lipogenesis [107,108]. Therefore,

AT plays important role in IR associated BCAA metabolomic signature. This will be discussed in details in respective chapters.

Skeletal muscle is an organ responsible for up to 85 % of whole-body insulin stimulated glucose disposal. Failure of SM to uptake postprandial glucose load makes it the major contributor to glucose intolerance. Lean T2DM patients have an approximately 50 % reduction in insulin-stimulated skeletal muscle glucose disposal compared to controls with normal glucose tolerance, this corresponds to defects in insulin signaling at the level of glucose transport, IRS-1 phosphorylation, and signal transduction. Both non-oxidative utilization (mostly glycogen synthesis) and oxidative utilization decrease [24].

SM is a fairly flexible organ in terms of substrate preference, myocytes can utilize both glucose and FA or other ketoplastic precursors. In line with the pioneering work on substrate competition, it has been postulated that important factors influencing insulin sensitivity in SM are plasma levels of FFA and the associated ectopic intramyocellular lipid deposition (IMCL), or deposition of oxidation intermediates respectively [61].

First studies in the field aimed to show a causal relationship between dietary fat intake, FFA levels and IR development. It was repeatedly shown that artificial elevation of FA using IV lipid emulsion leads to decline of glucose disposal [99].

Transport of FA into the myocyte is highly regulated process. Several transport mechanisms are involved: fatty acid binding protein (FABP), fatty acid translocase (CD36) and a group of fatty acid transport proteins [109]. The most studied from the group being FA translocase with a fate similar to GLUT4 transporter. Upon insulin stimulation the transporter is exposed at the sarcoplasmic membrane and facilitates influx of FA into the cell. There is however a certain competition in between translocase and GLUT4 in an insulinized muscle [110]. Intracellular fate of FA is either β-oxidation or any synthetic pathway. Besides SM is capable of lipid storage even under physiological conditions. It was shown that after endurance training IMCL are deposed in an intermyofibrillar space, in a close contact with mitochondria. Once termed "athletic paradox" [111] was these depos composed mainly of TAG were shown to be readily available sources of FA for aerobic work [112,113]. Contrary IMCL disposed mainly in a subsarcolemmal space and containing ceramides (CER) and diglycerides (DAG) are associated with IR and metabolic disturbances [112,114,115]. Indeed, CER and DAG are metabolically active intermediates with many signalling functions. DAG inhibits phosphorylation of IRS and PKC translocation, CER inhibits Akt kinase. Anyhow, identification of a target molecules remains unresolved as there exists more than 60 thousands of sphingomyelins (and ceramides among them) in a cell [116] .

β-oxidation takes place exclusively in mitochondria. Decrease in mitochondrial oxidative capacity is among studied culprits of SM IR, lower mitochondrial abundance and oxidative capacity was repeatedly shown in T2DM [117,118] and the idea of mitochondrial dysfunction linking lipotoxicity and IR has been debated over 40 years [119–122] .

The term mitochondrial dysfunction is nevertheless used for description of many parameters. With relation to insulin function aerobic phosphorylation and substrate oxidation is the function of interest, In real experiments mRNA gene expression for mitochondrial bioge-

nesis (PGC1α and others), proteomic analysis, enzyme analysis of mitochondrial complexes (i.e. citrate synthase), changes in mitochondrial density/morphology and/or direct measuring of substrate oxidation, is used [122,123].

Lower mitochondrial capacity for oxidation of substrates is associated with degradation intermediates accumulation (namely derivates of FA) and loss of insulin function. This mechanism could also be perceived as an adaptive mechanism defending mitochondria from energy stress. Leak of electrons from respiratory chain running at low speed induces superoxide and other radical oxygen species that that leads to mitophagy resulting in lower mitochondrial density [124]. The question whether mitochondrial dysfunction is the primary defect or a consequence is far from being solved [122].

Liver is the organ integrating intermediary metabolism. The brain is dependent on a continuous supply of glucose that can reach up to 50 % of the whole-body glucose turn-over, representing roughly 75-150 g per day. Though the brain may adapt to ketone bodies oxidation and spare glucogenic substrates after a long-term fasting [125], shorter periods of fasting are associated with mobilisation of endogenous glucose precursors [24]. There is up to 600 g of glycogen disposed in the liver, a readily available source of glucose. Besides when insulin action decline, gluconeogenesis accelerates. Major substrates are glycerol (hand in hand with accelerated lipolysis), glucoplastic amino acids and lactate.

Hepatic glucose output (HGO, product of glycogenolysis and gluconeogenesis assessed by isotope dilution techniques, see respective chapter) equals 2 g/kg/min in persons with normal insulin sensitivity. HGO becomes higher in patents with T2DM, approximately 2.5 g/kg/min. This represents an overnight excess appearance of 30 g of glucose that impacts on morning glycaemia [24]. With fasting insulinaemias several times higher when compared to healthy and lean counterparts this means that hepatocyte is resistant to effects of insulin, the phenomena is referred as the hepatic (central) IR. Not only the fasting HGO is increased, but analogically the hepatocyte is resistant to postprandial (insulin mediated) suppression of HGO [126].

Several mechanisms are related to the hepatic IR development. 1/ Increased glucagon secretion and loss of α-cells suppressibility by hyperglycaemia [127], 2/ increased circulating FFA that activate phosphoenolpyruvate carboxykinase and pyruvate carboxylase, the key enzymes of gluconeogenesis (referred as a lipotoxic effect) and 3/ increased circulating glucagon levels and hyperglycaemia activate glucose-6-phosphatase in the hepatocyte and lead to an increased glucose efflux (referred as a glucotoxic effect) [24].

DeFronzo was among those proposing a hypothesis of natural history of diabetes that is widely accepted today [24]. Overlapping etiological mechanisms converge on long-term decrease in insulin sensitivity. The tissue effects of insulin are maintained at the cost of an increase in β-cell insulin secretion. Normal glucose tolerance remains in this phase though the IR might have already peaked at its maximum (see Figure 13).

Would there be no major lifestyle change, β-cells are eventually unable to further augment their secretion and gradually wear off. The decline in relatively sufficient insulin response marks the onset of glucose intolerance. Postprandial glycaemia rises initially, followed by fasting glycaemia and overt diabetes eventually develops. This theory has been based upon

seminal works on prospective cohorts [128–130]. Obesity itself is associated with 29 % decline in insulin sensitivity. Once an obese maximally insulin resistant patient progresses to impaired glucose tolerance (IGT), there is yet an additional decline of 28 % (i.e. 57 % sensitivity decline from the baseline). Further IGT progression towards diabetes is limited to β-cell failure and IR does not worsen any more [128]. Indeed, β-cells' failure determines the future course of the disease.

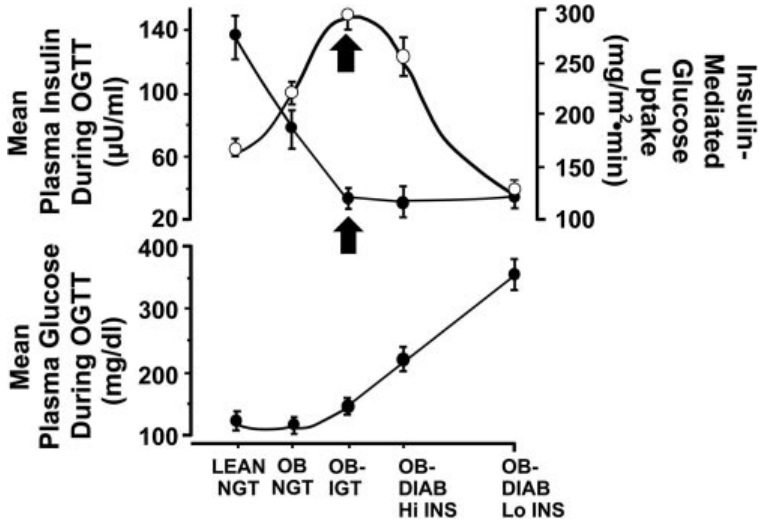


Figure 13 Natural history of type 2 diabetes. Lean NGT: lean subjects with normal glucose tolerance; OB NGT: obese with normal glucose tolerance; OB IGT: obese with an impaired glucose tolerance; OB DIAB Hi INS: obese subjects with diabetes and preserved insulin response; OB DIAB Lo INS: obese subjects with diabetes and no residual insulin response. From [24].

Though a body evidence suggests that there is a continuum between obesity – impaired glucose tolerance – T2DM, and that IR is the primary defect followed by β-cell failure [131], this mechanistic theory cannot be accepted without limits. Known paradox exists that 20 % obese patients does not have insulin resistance and metabolic derangements, on the other 18 % of patients with T2DM are lean [96].

β-cell dysfunction. Insulin is produced and secreted from β-cells. There is about 1000 of these per one Langerhans' islet. And there is about a million of islets dispersed in body and tail of pancreas with a total weight about 0.9 g [132]. β-cells secrete approximately 20-40 units of insulin a day in a healthy lean person, an amount that can be augmented almost ten times when needed.

As stated above, in a flexible system, any decrease in insulin sensitivity is followed by an increase in insulin secretion to maintain euglycaemia. Insulin secretion reaches its peak when there is maximal IR without IGT. As the demand on insulin secretion rises, β-cell eventually becomes exhausted and fail to compensate. Overt diabetes develops. In spite of the progressive decline in secreted insulin and C-peptide, both parameters show supraphysiological concentrations and cannot be solely used for determining of residual insulin function. That is best expressed using dynamic indices, for instance a change in insulinaemia plotted against a change in glycaemia (after a meal or glucose ingestion), so called insulinogenic index (ΔI/ΔG). We are able to test

this parameter using oral or IV glucose (oral or IV glucose tolerance test) and after some amino acids with secretagogic effects (i.e. arginine). Acute insulin response (AIR) measured this way adjusted to IR represents the best a secretory capacity of β-cells, unrelated to absolute insulinaemias [31]. The relationship between AIR and IR is hyperbolic and is referred to as disposition index (see above and Figure 3) [28].

It has been established based on longitudinal data that when hyperglycaemia manifests, secretory response of β-cells has been already reduced down to 50 % [24]. Aetiology of progressive β-cell failure in the course of IR development is not known in details but it is clearly multifactorial. Easy theory that long-lasting high demands on β-cells' secretory response lead to a gradual fatigue and extinction, is easy to understand but not completely based on evidence.

The factors associated in the literature with β-cell failure are many. Glucotoxicity, lipotoxicity, genetic background, islet amyloid deposition or enteroinsular defects were all suggested [133,134]**.** There has been three major, not mutually exclusive, mechanisms involved [135]. 1/ *Reduced β-cell mass* was repeatedly identified in autopsied samples [136]. Lower regenerative potential or genetically determined lower number of β-cells may be among predisposing factors of T2DM development [135]. On the other hand, surgical removal of the functional β-cell mass (as per hemiduodenopancreatectomy) does not lead to diabetes in a short-term run though insulin response may be decreased and glucose tolerance impaired [137,138]. 2/ Persistent high glucose stimulation may lead to metabolic stress of β-cell mass with ROS generation and endoplasmic reticulum stress. As a consequence of high secretory demand, ER protein folding machinery exceeds its capacity and unfolded proteins accumulate in the ER. β-cells are among cell type the most prone to the accumulation as they work in just-in-time mode, reaching its secretory capacity limits when stimulated [139]. The demand overload leads to the secretory dysfunction and *β-cell exhaustion*, i.e. histologically normal cells unable to secrete sufficient amount of insulin [135]. Mitochondrial oxidation is inherently associated with ROS genesis. Metabolic overload with persistent demand on the oxidative machinery eventually leads to excessive generation of ROS that shut-down mitochondria and inhibit insulin secretion [140]. 3/ β-cells are terminally differentiated cells that may under certain circumstances undergo dedifferentiation or transdifferentiation into other cell subtype, losing their phenotype properties. Glucotoxicity is among proposed mechanisms [141] Loss of β-cell identity means loss of function without losing the mass [142]. 4/ β-cell harbour insulin receptors on the cell membrane and insular autocrine and paracrine regulation has been described. When there is an effect there may be resistance – *β-cell insulin resistance* [143]. Insulin shows mainly proliferative and antiapoptotic effects [144]. Proliferative activity of insulin signalling cascade in the β-cell is crucial to maintain a compensatory increase in insulin response induced by IR at the periphery. It was shown that β-cell IRS-2 knocked-out mice develop a progressive β-cell failure [145]. Hyperinsulinism that accompanies IR states leads to β-cell insulin receptors down-regulation, perpetuating resistance to insulin effects. Therefore, increased insulinaemia is needed to inhibit insulin secretion.

1.4. BRANCHED CHAIN AMINO ACIDS

Branched chain amino acids (BCAA) are among substrates that has been constantly connected with insulin resistant states in the literature. These are valine, leucine, isoleucine (see Figure 14). BCAA are essential amino acids as they cannot be synthetized in the human body and has to be secured from dietary sources. Not only individual BCAA share a common structural resemblance but their circulating levels are synchronized in many clinical states, physical activity, fasting [146] and diabetes [147]. They are mostly abundant in the skeletal muscle representing up to 40 % portion of AA [148].

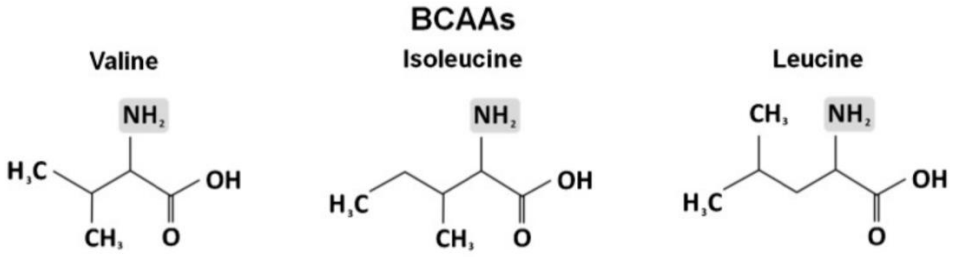


Figure 14 Branched chain amino acids. Adapted from [149].

1.4.1. BCAA as nutrients and signalling molecules

Here we explain how BCAA are resorbed and distributed, followed by description of their consequent intracellular fate. We specially emphasise how BCAA relate to major metabolic pathways.

1.4.1.1. Resorption, distribution and metabolism of BCAA

After ingestion of complex polypeptides containing BCAA are hydrolysed in the gastrointestinal tract to form di/tripeptides of BCAA. Hydrogen/protein co-transporters (PepT1, T2, SLC15A1, A2) are consequently subjected to resorption in the small intestine. Individual BCAA may be also exchanged for another AA over apical membrane of brush border using specific AA transporters (SLC3A1, SLC7A9), analogically bidirectional exchange transport is functioning on the basolateral membrane (SLC3A2) [150].

BCAA are not primarily resorbed from colon, where are more likely to be subjected to bacterial fermentation [151]. Up to 20 % of ingested BCAA may be sequestered in the gut (for more details see Chapter 2.2.). BCAA circulate in plasma as free AA and are uptaken practically to all tissues using specific carriers.

BCAA are unique among other AA as they do not undergo neither intestinal nor liver metabolism as neither of these tissues contains first key deamination enzyme (see below), therefore their systemic circulating concentrations reflects the administered and resorbed dose [152,153]. This fact predispose BCAA to be „nutrient-sensing“ signals for many target tissues [154].

1.4.1.2. Intracellular pathways

Intracellular fates of BCAA are many. Schematically they can be used as 1/ direct building blocks or nitrogen donors for proteosynthesis. 2/ They serve as an energy substrate. They can be degraded down to the final glucogenic (propionyl and succinyl-CoA) and ketogenic (acetyl-coA and acetoacetate) products and oxidized. 3/ The serve as nutritional signals via mTOR activation. Intracellular pathways of BCAA are summarized in Figure 15.

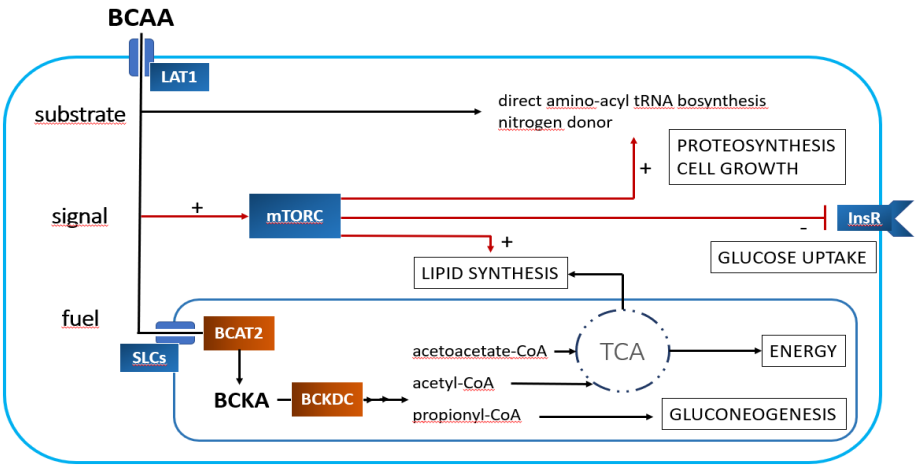


Figure 15 Schematic overview of major intracellular BCAA metabolic and signalling pathways. Adapted from [155–157].

Majority of BCAA is oxidized as fuel, their nitrogen residue is eventually used for structural functions. Even though the catabolic pathways are somewhat unique for each BCAA, two first key enzymatic steps are shared. The first is transamination catalysed by BCAA amino transferase (BCAT), where deamination occurs and amino-residue is transferred to α-keto-glutarate to eventually form glutamate/glutamine (see Figure 16).

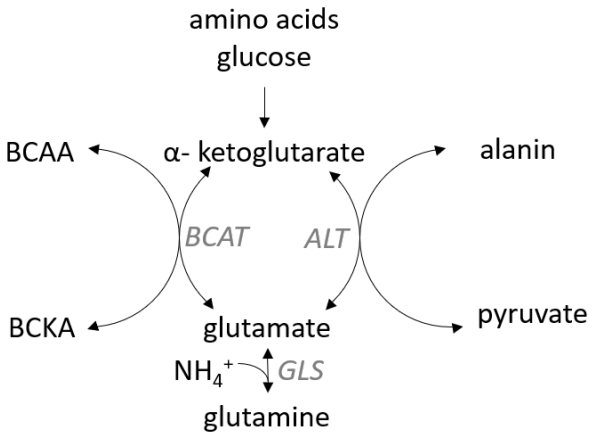


Figure 16 Schematic overview of an interaction of major transamination pathways.

There exist both mitochondrial and cytoplasmic isoform of BCAT and their distribution differs tissue to tissue. Product of the reaction is keto-analogue of respective BCAA, i.e. BCKA (α-keto-isokaproate, α-keto-β-metylvalerate or keto-isovalerate). BCAT reaction is reversible and runs near equilibrium is that the direction if the reaction depends on availability of BCKA and nitrogen donors. Moreover, there exists an interorgan flux of branched carbon skeleton

to prevent a loss of these essential compounds [158]. Further step towards eventual oxidation is an oxidative decarboxylation catalysed by BC α-keto-dehydrogenase complex (BCKDH). BCKDH is a multimeric complex located on the inner surface of the inner mitochondrial membrane, it is a part of decarboxylation complex family together with pyruvate dehydrogenase and α-ketoglutarate dehydrogenase. The oxidative decarboxylation is an ireversible reaction that is highly regulated and coupled with NADH reduction. Products of decarboxylation are isovaleryl-CoA, isobutyryl-CoA and α-methyl-butyryl-CoA. These substrates consequently undergo a series of dehydrogenase mitochondrial reactions to eventually form degradation end-products acetoacetate, acetyl-CoA a propionyl-CoA. Of note, at each catabolic step downstream from BCKDH the oxidation intermediates may be used for anaplerosis, synthesis of FA or cholesterol [147].

Signal effects of BCAA are mediated mainly by mTOR complex (mammalian/mechanistic target of rapamycin). This is an evolutionary very conserved nutrient sensing serine-threonine kinase with two major complexes: mTORC1 that is responsible for cell's anabolism – catabolism switch via control of metabolic state and mTORC2 which downstream targets control cell survival, proliferation and cytoskeleton dynamics. Of note, AKT kinase is an integrating node in between these two [159]. As summarized on Figure 17 the complex is an integrating system where many metabolic signals both nutritional and hormonal converges. BCAA acts on mTORC1 independently of insulin [160] and activates its downstream mediators. Major phosphorylation targets of mTORC are p70S6 serine kinase 1 (S6K1) and 4EBP1 (binding protein 1 for eukaryotic initiation factor 4E) [154] that serve as regulators of transcriptional activity leading to many anabolic processes including protein synthesis, autophagy inhibition and cell growth. Of note, phosphorylated S6K1 inactivates insulin receptor substrate (IRS) and downregulates insulin cascade activity, decreasing insulin dependent substrate uptake in the time of their abundance [161].

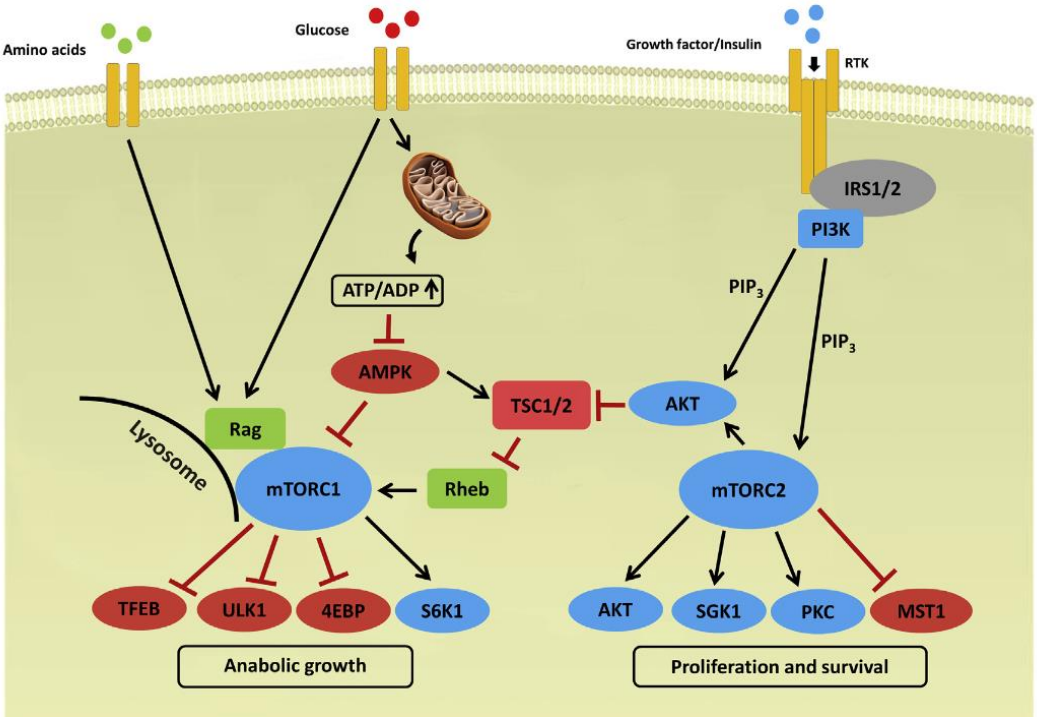


Figure 17 mTOR signalling pathways. Adapted from [159]

BCAT a BCKDH isoenzymes distribution shows important tissue specific differences [147]. Therefore, the extent of resorption and further subcellular fate of BCAA is different between tissues. At the whole-body level, the major site BCAA disposal is the *skeletal muscle* that disposes up to 50 % of dietary BCAA. As BCAT enzyme is predominantly expressed in the skeletal muscle, majority of deamination occurs here [162]. BCKA are to a greater extent released form skeletal muscle (see Figure 18) as well as glutamine and alanine formed in trans-amination reaction or they are oxidized in situ. *Liver* is on the other hand a major site of BCKA decarboxylation. BCKA produced in abundance in skeletal muscle are further metabolized here to provide both glucoplastic and ketoplastic catabolites [157]. *Adipose tissue* is an important buffer of postprandial substrate load. It uptakes excess of BCAA that is not utilized elsewhere (i.e. in skeletal muscle) and the carbon residue is used for de novo lipogenesis. Up to 30 % of lipogenic acetyl-CoA originates from BCAA [163]. BCAA were also shown to act on pancreatic beta cells. Several plausible mechanisms were suggested. Both direct fuelling insulin synthesis and secretion, stimulating glutamate dehydrogenase and glutaminolysis, stimulating protein synthesis via mTOR [164] and indirect via stimulation of incretin release [165]. BCAA also act as signals on *central nervous system*. BCAA cross the hematoencephalic barrier using a shared carrier system with aromatic amino acids (AAA). Both BCAA and AAA availability (i.e. plasma concentrations) modulate neurotransmitter synthesis (namely dopamine, noradrenaline and serotonin). There has been a long lasting clinical debate over supplementation of BCAA in patients with hepatic encephalopathy that is partially caused by increased flux of AAA into the brain [166,167]. mTOR regulates expression of neuromediators proopiomelanocortin (POMC) and cocain-amphetamin regulated transcript (CART) in hypothalamic arcuate nuclei. Both these mediators show anorexigenic effects. Central application of leucine to rat brain decrease phosphorylation of mTOR with consequent anorexigenic behaviour. These effects were reverted by administration of rapamycin [168]. However, oral administration has given unequivocal results so far [154].

1.4.2. Circulating BCAA and insulin resistance

Here we provide the evidence that circulating BCAA mirrors decline in insulin sensitivity, show biological effects of BCAA and factors contributing to their rate of appearance and disappearance. Lastly, we provide a reader with the logic for further text structuring.

The association between elevated circulating BCAA and insulin resistance has been described for more than fifty years [62], but it has received a significant scientific acknowledgement just recently [169,170]. BCAA correlate with insulin resistance indices, a correlation that becomes more significant with adiposity [170]. Peripheral BCAA levels predict a risk of incident diabetes up to twelve years before its manifestation [171]. In line, lifestyle intervention [169] as well as bariatric surgery [172] that reverts metabolic syndrome decreases BCAA too. In recent years quite a few very important review papers addressed this complex association [155–157,173]. Despite such a large body of evidence, important questions has not been fully answered and the debate is ongoing. Does the elevation of BCAA have a causal role in the insulin resistance development or is it merely an epiphenomenon of a diseased state? Is there a causality in between BCAA – IR and what is the direction?

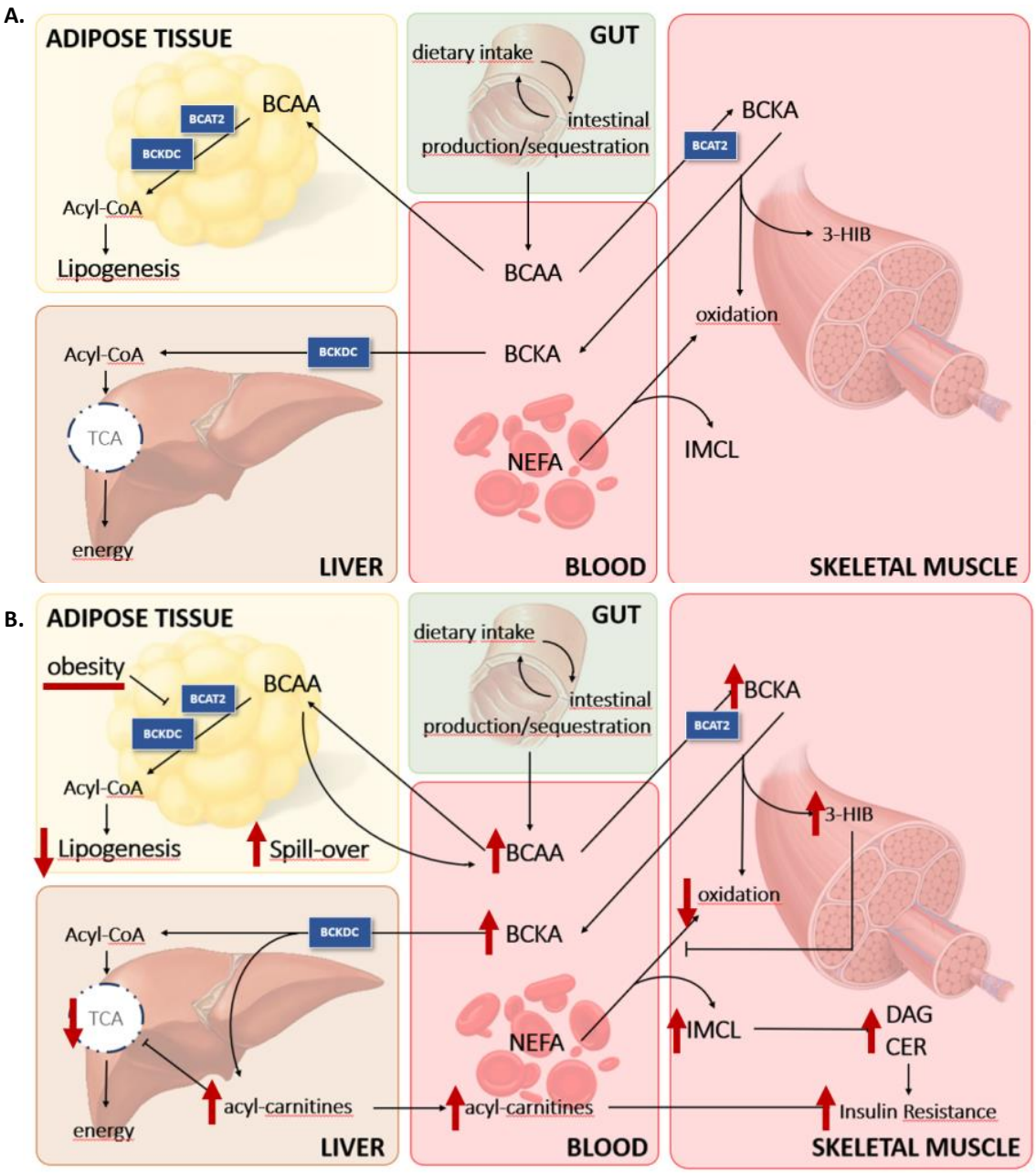


Figure 18 Interorgan BCAA metabolic cross-talk in healthy (A) and metabolically challenged (B) state. Adapted from [155–157]

1.4.2.1. Biologically plausible effects of BCAA inducing insulin resistance

As summarized earlier, BCAA impacts on many intracellular functions. There has been two major hypothesis how BCAA could induce insulin resistance as summarized by Lynch and Adams [156]. First one is linked to mTOR activation and the second one to BCAA catabolic intermediates.

Persistent mTOR activation. Activation of mTOR complex 1 and SK61 lead to serine phosphorylation of insulin receptor substrate (IRS1 and IRS2) thereby suppressing insulin signalling [161,174]. This may be the case of permanent hyperinsulinaemia or hyperaminoacidemia associated with obesity. It was shown *in vitro* that BCAA induce mTOR signalling in skeletal

muscle reflected by decreased glucose uptake [175]. Even though plausible, this theory remains controversial. mTOR signalling is involved in a proteosynthetic response to exercise [176], known to ameliorate insulin sensitivity in long-term. There are a few interventional studies with BCAA supplementation showing insulin sensitising effects despite increased mTOR signalling [177]. Moreover, it is not clear whether small differences in plasma BCAA observed in insulin resistant states are sufficient to trigger phosphorylation of IRS [178]. And lastly, mTOR activation in adipose tissue is perceived as protective being associated with an increase in fat cell mass [179,180].

Concerning beta cells, in acute settings BCAA stimulate insulin secretion and de novo synthesis [164,165]. It is plausible that the acute stimulation of mTOR in beta cells is associated with improved function but in long-term run the substrate overload may act conversely as chronic exposure to nutrient overload was repeatedly shown to cause beta cell dysfunction [159]. Interestingly, rapamycin (inhibitor of mTOR) mirrors the effects of fasting, inducing glucose intolerance without insulin resistance, and extends lifespan in various models rev. by Blagosklonny [181].

BCAA dysmetabolism. Another mechanism suggested in BCAA associated IR is an accumulation of intermediates of BCAA incomplete catabolism [156]. These intermediates feed into other metabolic pathways, namely TCA shifting substrate – product equilibria of reactions. This effects is also referred as anaplerotic stress [182]. In this theory, it is not BCAA *per se* that causes insulin resistance but its derivates. As reviewed by Lynch and Adams, it was shown in various cell and animal models that several BCKA and/or alpha ketoisocaproate alone cause mitochondrial dysfunction, inactivation of pyruvate dehydrogenase in the liver and heart. Circulating BCKA are also the toxic product in patients with maple urine syrup disease (for details see Box 1).

Box 1

Maple syrup urine disease (MSUD)

Maple syrup urine disease is an inborn disorder of BCAA catabolic pathway, described first in 1954 by Menkes et al. [183] that ranks among organic acidurias. The name is derived from maple syrup odour of urine of its classical form. It is caused by the mutation in genes encoding subunits of BCKDH, the enzyme that irreversibly catalyses BCKA decarboxylation. Loss of function leads to accumulation of BCKA (ketoisocapreic – KIC, ketoisovaleric-KIV and ketomethylvaleric acid-KMV) in the tissues, most prominently in the brain and skeletal muscle. There are several variants of the disease related to the BCKDH residual activity and clinical presentation. Three major organs involved in MSUD are brain, skeletal muscle and liver. In the classical form the presentation is encephalopathy, liver failure and ketoacidosis. In the central nervous system BCAA feed nitrogen into transamination reactions and shift in BCAA/BCKA ratio impacts on glutamate synthesis. Moreover KIC was shown to be neurotoxic and responsible for encephalopathic syndrome in MSUD [184]. Skeletal muscle involvement comprises of myofibrillar lesions and mitochondrial dysfunction [185].

BCKA are further metabolized by BCKDH and multiple downstream matrix-mitochondrial enzymes leading eventually to acyl-CoA. These are either oxidized, used for keto/gluconeogenesis or metabolized to acyl-carnitines and transported out of mitochondria and cell. It has been established based on clinical empirical experience that acylcarnitines are abundant in various organic acidurias and that their levels correlate with functional impairment, namely

mitochondrial dysfunction. As several BCAA derived acylcarnitines are elevated in circulation of T2DM patients, it was suggested that this effect may link elevated BCAA with mitochondrial dysfunction in metabolic organs. Though it is not clear whether relatively small increase in acyl-carnitine concertation seen in T2DM is alone sufficient to trigger insulin resistance [178]. Another intermediate associated with insulin resistance is 3-hydroxy-isobutyrte (3HIB). 3HIB is a product of valine degradation that is under PGC1 alpha regulation and it functions in paracrine manner to promote fatty acid uptake (via fatty acid transport protein expression) in the skeletal muscle [186]. It was repeatedly shown to be elevated in T2DM patients' circulation [157,187] and in animal models the intervention with 3HIB caused skeletal muscle insulin resistance via fatty acid uptake, their incomplete oxidation and diglyceride/ceramide deposition [186].

Another player of dysmetabolic theory is adipose tissue. AT is perceived as a major buffer of postprandial nutrient load and indeed BCAA constitute a significant portion of acyl-CoAs feeding lipogenesis [107,108]. A marked decrease of BCAT and BCKDH expression is present in various models of insulin resistance/obesity [156,188]. In line, obese women were found to have lower expression on BCAA catabolising enzymes (VCAT, BCKDH) in visceral fat, both at the mRNA level and protein levels [189]. So, it is plausible that decreased buffer capacity of AT for BCAA derivates' storage leads to their spill-over to systemic circulation. This defect has been already explored as a potential treatment target using a suppressor of BCKA dehydrogenase [190].

1.4.2.2. Factors contributing to BCAA plasma rate of appearance and disappearance

At this point, having presented hypothesis on BCAA tissue effects, it is important to summarize what are the contributors to circulating plasma BCAA. Rate of appearance (R_a) is determined by dietary intake, resorption and tissue protein degradation, rate of disappearance (R_d) by protein synthesis, excretion and catabolism/oxidation [178,191]. R_a/R_d mismatch leading to elevated circulating BCAA could be eventually caused by shift in any of these.

Factors increasing R_a. As per increased dietary intake, BCAA intake does not contribute to fasting BCAA levels [123,156] though it may modulate other dependent factors. It was shown that dietary protein composition has the major impact on BCAA concentrations [192,193]. This dietary hypothesis linking BCAA and insulin resistance was studied in-depth by our team, we provide more details in Chapter 2.1. Resorption and intestinal availability of BCAA is also determined by gut microbiota. It is well established that gut-derived metabolites influence the host. BCAA rank among these „postbiotics" and it was suggested that BCAA biosynthetic potential may contribute greatly to circulating BCAA levels [151,191]. There is our ongoing interest in this area and more details are provided in Chapter 2.2. As for the protein turn-over, dietary BCAA were shown repeatedly to stimulate protein synthesis (via mTOR) without influencing protein degradation. The other way around is valid for insulin that inhibits protein degradation without having effect of protein synthesis, so called specific effect of insulin on protein breakdown [194]. Therefore loss of insulin action could lead to disinhibition of protein breakdown and increased R_a of BCAA [195]. In depth studies of this hypothesis are provided in Chapter 2.3.

Showing that hyperinsulinaemia may be insufficient to block skeletal muscle protein breakdown in insulin resistant states.

Factors decreasing R_d. Decreased protein synthesis, decreased oxidation and increased excretion were all suggested as player in elevated BCAA levels [156]. Decreased catabolism and subsequent oxidation in tissues is tightly associated with activity of the key decarboxylation enzyme BCKDH. And it was shown that gene expression of that BCKDH is down-regulated in AT of IR subjects [189,196]. Adipose tissue may play a pivotal role in the whole body BCAA metabolism. When its storage capacity is exceeded, BCKDH slows down and there is BCAA spill over to systemic circulation [197]. BCAA per se were traditionally thought to stimulate protein synthesis [198], whereas the effect of insulin on protein synthesis was less pronounced in adult humans. Even though this anabolic postulate was recently disputed [199] BCAA remain major building blocks for skeletal muscle and there is a postprandial flux of BCAA into the skeletal muscle [200] increasing their R_d. It is conceivable that decreased protein synthesis may also be linked to elevated BCAA.

The logic of the text composition that follows mirrors our research scope in recent years. Obviously, there will be some outdated initial premises as well as hypotheses that were worth studying back that time and has been denied since then. Using several physiological models, we have tried to disentangle relationship between several factors modulating BCAA R_a/R_d associated with insulin resistance.

1/ First, we researched into the ***dietary hypothesis*** of BCAA-IR effects, i.e. dietary intake of BCAA is causally related to development of IR. For that we validated a model population vegan vs. omnivore and we showed that in low-BCAA intake group insulin sensitivity may be worsened by BCAA supplementation. VEGGIE-1 and VEGGIE-2 studies [123,201].

2/ Based on these results we hypothesized that dietary BCAA impact also on insulin secretion. We showed BCAA insulinogenic effects that are mediated by incretin system. VAMPIR study [165].

3/ We extended the use of vegan vs. omnivore model to include also metabolically challenged subjects to assess to which extent gut microbiome modulates intestinal availability of BCAA. This potential ***postbiotic hypothesis*** has been explored in an ongoing TRIEMA trial (NCT03710850).

4/ Lastly, we seek to find how IR in cancer related inflammatory response modulate BCAA handling. To assess specifically skeletal muscle contribution to circulating BCAA we implemented a complex physiological model using forearm substrate fluxes in pancreatic cancer patients. The ***distribution hypothesis*** was explored in the model, i.e. excess R_d of BCAA relates to loss suppressibility of BCAA skeletal muscle efflux in hyperinsulinemic conditions. PAMIR study, ongoing (Czech Ministry of Health NV19-01-00101).

2. EXPERIMENTAL WORK

2.1. EXPLORING ROLE OF DIETARY BRANCHED CHAIN AMINO ACIDS ON INSULIN FUNCTION

Number of observational studies indicates that elevated circulating levels of BCAAs are associated with glucose metabolism dysregulation. This association has been known for more than 30 years [62] and was recently reconfirmed showing that elevated fasting levels of BCAA can predict the development of T2DM already 12 years before its manifestation [171]. Indeed, as suggested in human acute interventional trials, intravenous administration of AA/BCAA is capable of modulating glucose uptake by SM [161,202,203]. Nevertheless, data on long-term BCAA supplementation has been inconclusive and yield conflicting results. Chronic exposition of BCAA in a rodent model was studied by Newgard et al. Rodents fed on high-fat diet supplemented with BCAA developed insulin resistance [174]. In line, diet low in BCAA could improve metabolic status in animals [204] as well as humans with diabetes [205]. On the other hand, improvement of glucose uptake after long term high-BCAA protein supplementation in insulin-resistant humans was repeatedly shown [206,207].

Data on the effect of chronic supplementation of healthy humans with BCAA are still limited making it impossible to clarify whether increased dietary BCAA itself are sufficient to trigger IR related diseases or whether the perturbations in BCAA levels are a mere epiphenomenon of a metabolically challenged state.

Aim of the project VEGGIE (Research Programme MSM 0021620814 and NT 14416) was to analyse the effects of dietary BCAA on insulin measures in healthy humans. For that purpose, we established vegan vs. omnivore model population and we subjected the model to long term BCAA supplementation.

2.1.1. Physiological model vegan vs. omnivore and its validation

There are a number of candidate environmental factors that may impact on development of insulin resistance and T2DM eventually. The composition of the diet is among them. In this respect, populations on diets that exclude certain common food sources and thus modify the nutrient intake that is common in the general population deserve attention. Among these diets are those with the exclusion of meat and meat products, including fish (vegetarian or more precisely lacto-ovo vegetarian diet) or with the exclusion of all animal-based products, i.e. milk and dairy products and eggs in addition to meat (vegan diet).

Box 2 Dietary patterns

Omnivorous diets. Diet without any restrictions, with meat and dairy products on daily basis.
Vegetarian, lacto-ovo vegetarian diet. Diet with restriction of meat and meat products, with milk and eggs on regular basis.
Vegan diet. Diet restricting any animal products (meat, milk and dairy products, eggs).

Vegan diet was shown to be of lower protein content [208] and lacks main dietary sources of BCAA [209]. Though various plant-based protein sources contain sufficient amount of BCAA per gram of protein (BCAA/AA ratio), the overall protein content and its bioavailability is lower when compared to animal-based sources [210]. It is conceivable that in absolute numbers the overall BCAA intake is lower on vegans when compared to omnivores. At the same time, strong body of epidemiological evidence suggests that vegans have more favourable parameters of glucose tolerance, lipid profile and lower insulin resistance (IR) compared to their counterparts without food restriction [211–213]. Prevalence of T2DM is lower in vegan populations as well [214,215] and several interventional trials showed that administration of vegetarian diet in T2DM patients improves diabetes compensation, lipid profile and lowers IR [216,217]. Thus we hypothesized that vegans could represent a unique model to analyse the impact of BCAA on the initial steps of IR development [123,201].

2.1.1.1. Higher insulin sensitivity in vegans is not associated with higher mitochondrial density and IMCL content – VEGGIE-1 study

Insulin function in the skeletal muscle is a key mechanism of postprandial glucose disposal and its deterioration may be the first step leading eventually to insulin resistance syndrome. Current epidemiological evidence suggests better metabolic health in vegan population. That comprises of insulin sensitivity measured by HOMA indices. Though HOMA index is easy to obtain, the interdependence of both variables used for calculation (i.e. fasting glycaemia and insulinaemia) makes it less reliable in a small-scale studies. Moreover, fasting values relates more to hepatic glucose production than SM glucose disposal. The gold standard of SM insulin sensitivity assessment is hyperinsulinaemia clamp that has never been performed beforehand. Insulin sensitivity in SM closely relates to IMCL deposition and mitochondrial function, both these has never been studied in vegans.

In the validation study (VEGGIE-1, Research Programme MSM 21620814, 2012) we explored whether epidemiological evidence translates to basic physiology of SM insuiln sensitivity and mitochondrial function in young and healthy subjects. In a small scale advanced physiological study we aimed verify that vegans have higher glucose disposal in a hyperinsulinemic clamp, whether it relates to SM mitochondrial density and intramyocellular lipid content and what is the succession of the phenomena. As per secondary outcomes endothelial function and nutritional status were assessed.

Eleven vegans and ten comparable (age, sex, BMI, physical activity, macronutrient intake) omnivorous controls without any previous health related issues were recruited. Sample characteristics are summarized in Table 4. After an overnight fast volunteers were subjected to 2 hours hyperinsulinemic (insulin dose 1 mIU/kg body weight) euglycemic clamp to assess insulin stimulated glucose disposal. Skeletal muscle biopsy was performed form m. vastus lateralis using Bergström technique as described [58]. IMCL were assessed using coupled gas chromatography [218] and mitochondrial density was assessed using citrate synthase (CS) activity and relative amount of mitochondrial DNA (semiquantitative PCR) [201].

	Vegan (n=11) mean ± SD	**Omnivore (n=10)** mean ± SD	***p*-value**
Age (years)	30.30 ± 3.93	27.12 ± 1.10	**0.023***
Height (m)	1.75 ± 0.09	1.77 ± 0.07	0.665
Weight (kg)	68.63 ± 14.02	73.15 ± 12.82	0.451
BMI ($kg.m^{-2}$)	22.15 ± 3.02	23.24 ± 2.89	0.412
WHR	0.86 ± 0.10	0.89 ± 0.14	0.583
Duration of veganism (years)	8.05 ± 3.83		
Physical activity – Baecke score	9.89 ± 1.42	8.88 ± 1.12	0.090
Bioimpedance			
Fat mass (kg)	9.22 ± 6.03	11.98 ± 8.39	0.419
Fat free mass (kg)	57.56 ± 13.38	62.01 ± 11.12	0.443
Visceral fat – US (cm)	4.11 ± 2.28	2.96 ± 1.1	0.181
Subcutaneous fat – US (cm)	1.33 ± 0.63	2.09 ± 1.32	0.134
Macronutrient intake			
Total energy (kcal)	2097.67 ± 531.2	2023.00 ± 563	0.771
Lipids (%)	36.13 ± 7.65	42.31 ± 8.49	0.116
Saccharides (%)	48.90 ± 10.57	40.39 ± 7.51	0.057
Proteins (g)	74.78 ± 22.96	81.10 ± 19.63	0.526
Proteins (%)	14.96 ± 5.08	17.31 ± 5.23	0.336

Table 4 Baseline characteristics of 11 vegans vs. 10 omnivorous controls. Data are presented as means ± SD. * $p<0.05$, significance for *t*-test. BMI, body mass index; WHR, waist hip ratio; US, ultrasound.

Major results are summarized in Table 5. We concluded that vegans had higher insulin stimulated glucose uptake and no difference in IMCL, mtDNA and CS activity compared to their matched omnivorous counterparts. These findings suggest that decrease in whole-body glucose disposal precedes muscle lipid accumulation and mitochondrial bioenergetic failure in IR development. Therefore, IMCL accumulation and mitochondrial dysfunction could be more likely consequence or epiphenomena correlating with IR and playing role in its progression rather than initiation. Moreover, we verified that even in young subjects with normal glucose tolerance insulin sensitivity may be higher in relation to vegan diet. Finding that justifies using vegan vs. omnivore model population for further interventions.

	Vegan (n=11) mean ± SD	**Omnivore (n=10)** mean ± SD	***p*-value**
Fasting glucose ($mmol.\ l^{-1}$)	4.82 ± 0.65	5.46 ± 0.69	**0.040 ***
Fasting insulin ($mU.\ l^{-1}$)	2.39 ± 1.01	4.07 ± 2.29	**0.039 ***
HbA1c (%)	3.5 ± 0.31	3.45 ± 0.32	0.722
M-value ($mg.kg^{-1}.min^{-1}$)	8.18 ± 1.62	6.36 ± 1.74	**0.023 ***
M-$value_{corrected}$ ($mg.kg^{-1}.min^{-1}$)	8.11 ± 1.51	6.31 ± 1.57	**0.014 ***
MSM ($mg.kg^{-1}.min^{-1}$)	3.51 ± 0.65	2.44 ± 0.90	**0.006 ***
Steady state insulin ($mU.\ l^{-1}$)	31.98 ± 7.86	34.45 ± 8.46	0.497
Mitochondrial density			
Activity CS ($\mu mol.g^{-1}.min^{-1}$)	18.43 ± 5.05	18.16 ± 5.41	0.906
mtDNA	1.36 ± 0.31	1.13 ± 0.36	0.135
Muscle lipids (mg FA.g $muscle^{-1}$)			
Total IMCL	19.33 ± 11.53	27.81 ± 22.46	0.283
SFA	5.27 ± 2.32	8.07 ± 6.03	0.169
MUFA	7.04 ± 6.30	12.80 ± 13.95	0.231
PUFA	7.02 ± 3.22	6.94 ± 2.60	0.952
PDA (C15:0)	0.01 ± 0.02	0.06 ± 0.05	**0.009 ***
DHA (C22:6n3)	0.09 ± 0.05	0.17 ± 0.05	**0.003 ***

Table 5 Glucose homeostasis, mitochondrial density and IMCL results of 11 vegans vs. 10 omnivorous controls. Data are presented as means ± SD. * $p<0.05$, significance for *t*-test. Glucose disposal as per M-value from glucose clamp (40 mIU/m^2 insulin). MSM, M-value adjusted for total skeletal muscle mass.

2.1.2. Supplementation with BCAA decreases insulin sensitivity in low-BCAA intake population (VEGGIE-2 study)

2.1.2.1. Rationale and design

The interventional study VEGGIE-2 (IGA NT14416/2013, registered at ClinicalTrials.gov, NTC02684929) using vegan vs. omnivore was carried out to assess causality among BCAA intake and insulin sensitivity. We aimed to explore both increased dietary intake and defective metabolism in SM and AT. SM is a major site of BCAA disposal where they account for put 35 % of AA in fibrillar protein. Metabolism of BCAA in SM committing steps of BCAA metabolism on SM are BCAT and BCKDH enzymes that were both found to be down-regulated in IR subjects [196]. The same applies to AT disposal. In the AT excess of BCAA not used for proteosynthesis in extra adipose tissues is stored and AA derived carbon serves as a precursor for de novo lipogenesis [163]. And analogically, lower capacity of BCAA oxidation in AT has been proposed as a link between IR and higher circulating BCAA [197].

The primary outcomes of the study were change in glucose disposal, SM mitochondrial function and gene expression of BCAT/BCKDH in SM and AT upon long-term BCAA dietary intervention.

Design of the study is depicted in Figure 19. Eight vegans and 8 matched (age, anthropometry, VO_2max) omnivores (5 men and 3 women in each group) were intervened with 15 (women) or 20 (men) grams of BCAA (BCAA 4:1:1 Fair Power®, Survival Nutrition Supplements, Czech Rep.) daily for 3 months. Examinations were performed at a baseline, after the intervention and after a 6-month wash-out period. Examinations comprised of anthropometry, blood analyses, hyperinsulinemic euglycemic clamp (HEC, 1 mIU insulin/kg body weight), and arginine test (5 g of arginine chloride 21%, Ardeapharma, Ševětín, Czech Republic). Samples of sub-cutaneous abdominal adipose tissue (AT) and skeletal muscle (SM) obtained before and after the intervention were used for analyses (mRNA levels of selected metabolic markers, respiratory chain (RC) activity). We refer for details of the protocol to the original publication [42].

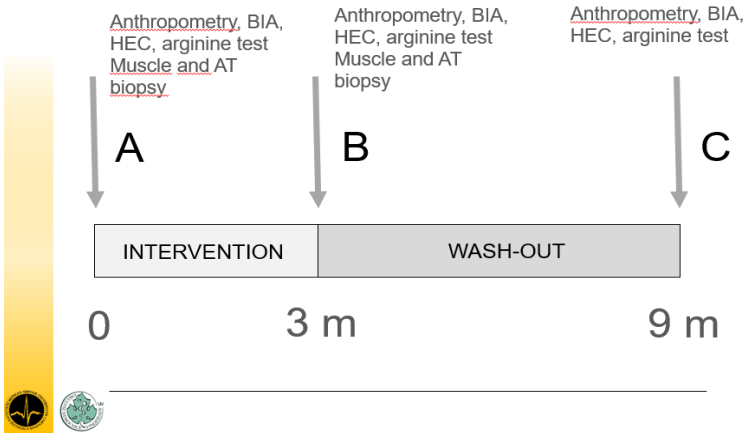


Figure 19 Design of VEGGIE-2 interventional trial [42]

As a part of the project, a method for the determination of BCAA using contactless conductivity capillary electrophoresis (CE) was developed and introduced into our experimental practice. This technology offers a number of advantages in the analysis of non-absorbent substances such as AA. It allows fast analysis of large numbers of samples, good separation from complex source matrices such as serum, relatively high detection sensitivity requiring only a minimal amount of sample for analysis and also lower chemical costs. Moreover, this method has a potential of point-of-care testing. For a detailed description of the development and implementation of the method, we refer to the original methodological papers [42,219,220]. For lipidomic analysis of blood and AT samples coupled gas chromatography was used with the protocol separately published [218].

2.1.2.2. Results and conclusions

The trial was completed without any unintended or adverse effects of the intervention, with 0 % drop-out. Omnivores had higher baseline dietary BCAA content (BCAA: vegans 10.33 ± 2.3 vs. omnivores 16.5 ± 3.2 g, p=0.03). Baseline and intervention/wash-out serum levels of BCAA are summarized in Table 6.

Parameter	Changes within group with 95% CI				Changes within group with 95% CI		
	Visit	Vegans	Omnivores	Difference [95% CI]	Visit	Vegans	Omnivores
Leucine (μg.ml^{-1})	0 m	108.1 ± 25.6	122.9 ± 13.8	-14.8 [-35.2;5.7]			
	3 m	125.5 ± 28.8	129.4 ± 31.3	-3.9 [-24.4;16.6]	3-0 m	**17.4 [4.6;30.1]**	6.5 [-6.2;19.2]
	9 m	104.4 ± 16.0	113.0 ± 9.4	-8.6 [-29.1;11.9]	9-3 m	**-21.1 [-33.9;-8.4]**	**-16.4 [-29.1;-3.6]**
Isoleucine (μg.ml^{-1})	0 m	53.5 ± 14.7	57.6 ± 7.7	-4.1 [-14.7;6.4]			
	3 m	50.1 ± 14.2	52.3 ± 14.4	-2.1 [-12.7;8.4]	3-0 m	-3.4 [-9.4;2.7]	-5.4 [-11.4;0.7]
	9 m	52.1 ± 8.1	55.0 ± 6.91	-2.9 [-13.4;7.7]	9-3 m	2.0 [-4.0;8.0]	2.8 [-3.3;8.8]
Valine (μg.ml^{-1})	0 m	181.5 ± 39.3	216.8 ± 26.2	-35.3 [-65.0;5.5]			
	3 m	180.8 ± 26.9	197.3 ± 46	-16.5 [-46.2;13.2]	3-0 m	-0.8 [-18.1;16.6]	**-19.5 [-36.9;-2.1]**
	9 m	179.9 ± 28.9	194.8 ± 20.3	-14.9 [-44.6;14.9]	9-3 m	-0.9 [-19.9;14.9]	-2.5 [-19.9;14.9]

Table 6 BCAA levels. 8 vegans and 8 omnivores were subjected to 3 months intervention with oral BCAA supplements. Multilevel regression analysis was used to assess statistical significance of differences in visits and between changes within each group; results are expressed with 95% CI, values in bold are statistically significant (p<0.05).

Glucose homeostasis. Results of intervention are presented in Table 7. Intervention resulted in marked decrease of glucose infusion rates (GIR) that returned to basal levels after the wash out in vegans, whereas no such change was observed in omnivores (p<0.001 for significant time-group interaction of GIR). GIR did not correlated with steady state insulin levels (R=0.05, p=0.73). AIR changes were in the same increasing direction in both groups but the differences reached statistical significance only in omnivores.

Parameter	Comparison between groups				Changes within group with 95% CI		
	Visit	Vegans	Omnivores	Difference [95% CI]	Visit	Vegans	Omnivores
GIR ($mg.kg^{-1}.min^{-1}$)	0 m	9.6 ± 2.4	7.1 ± 2.4	**2.5 [0.25; 5.0]**			
	3 m	8.0 ± 3.1	7.9 ± 2.6	0.1 [-2.4; 2.6]	3-0 m	**-1.64 [-2.5; -0.75]**	0.77 [-0.12;1.66]
	9 m	9.6 ± 2.5	8.1 ± 3.1	1.5 [-0.9; 4.0]	9-3 m	**1.65 [0.75; 2.54]**	0.2 [-0.68;1.1]
MCR ($ml.kg^{-1}.min^{-1}$)	0 m	11.22 ± 3.07	8.04 ± 2.7	**3.19 [0.55;5.82]**			
	3 m	8.04 ± 3.30	9.23 ± 2.63	-1.19 [-3.98;1.6]	3-0 m	**-3.18 [-4.27;-2.1]**	**1.19 [0.1;2.28]**
	9 m	10.62 ± 3.29	8.69 ± 4.00	1.95 [-1.38;5.28]	9-3 m	**2.58 [1.32;3.85]**	-0.56 [-1.82;0.71]
MCR/I ($ml.kg^{-1}.min^{-1}/mU.l^{-1}$)	0 m	0.151 ± 0.06	0.138 ± 0.06	0.13 [-0.06; 0.08]			
	3 m	0.117 ± 0.03	0.127 ± 0.05	0.01 [-0.08; 0.06]	3-0 m	-0.03 [-0.08;0.1]	0.01 [-0.06;0.03]
	9 m	0.218 ± 0.11	0.190 ± 0.11	0.03 [-0.04; 0.1]	9-3 m	**0.1 [0.04;0.16]**	**0.06 [0.01;0.12]**
Steady-state insulin ($mU\ l^{-1}$)	0 m	78.1 ± 19.6	64.5 ± 20.8	13.6 [-3.55;30.7]			
	3 m	68.4 ± 16.1	77.6 ± 19.1	-9.24 [-26.4;7.9]	3-0 m	-9.7 [-26.9;7.43]	13.1 [-4.0;30.3]
	9 m	54.4 ± 16.8	53.3 ± 19.4	1.14 [-16.0;18.3]	9-3 m	-14.0 [-31.1;3.2]	**-24.3 [-41.5;-7.2]**
AIR ($mIU.l^{-1}.min^{-1}$)	0 m	214.9 ± 73.6	171.0 ± 81.0	43.0 [-56.4;144.3]			
	3 m	240.9 ± 89.3	281.3 ± 201.9	-40.5 [-140.8;59.9]	3-0 m	25.9 [-55.7;107.6]	**110.3 [28.7;192.0]**
	9 m	236.5 ± 34.4	251.9 ± 99.8	-15.4 [-115.7;84.9]	9-3 m	-4.4 [-86.0;77.3]	-29.4 [-111.1;52.2]

Table 7 Glucose homeostasis parameters. 8 vegans and 8 omnivores were subjected to 3 months intervention with oral BCAA supplements. Multilevel regression analysis was used to assess statistical significance of differences in visits and between changes within each group; results are expressed with 95% CI. * Geometric mean with 95% CI for log normally distributed data. Values in bold are statistically significant (p<0.05).

AT gene expression. Groups differed in baseline comparison in BKDHA (vegans vs. omnivores: -0.009 [-0.017;-0.001] dCt, p=0.037) and CD3g (vegans vs. omnivores: -0.55 [-1.04;-0.49] dCt, p=0.03). The effects of intervention on the mRNA expression for individual genes in AT are shown in Figure 20. Significantly different expression was found in DGAT 2 and FASN only in omnivores. Changes in DGAT 2 in omnivores correlated with changes in other lipogenic genes: FASN (R 0.91, p=0.002), SCD-1 (R 0.85, p=0.008) and PPARγ (R 0.76, p=0.03) changes. No such changes were reached in vegan group where there was only significant decrease in SCD 5 after the intervention. In plasma lipid spectrum total NEFA were not different at baseline among groups and no change was observed in either group after the intervention: vegans baseline 162.6 ± 54 vs. intervention 170.5 ± 41.2 µg/ml, p=0.7; omnivores baseline 210 ± 62.4 vs. intervention 173.3 ± 54.5 µg/ml, p=0.2. No significant differences were found in triglyceride and total cholesterol levels in baseline comparisons and after intervention (data not shown).

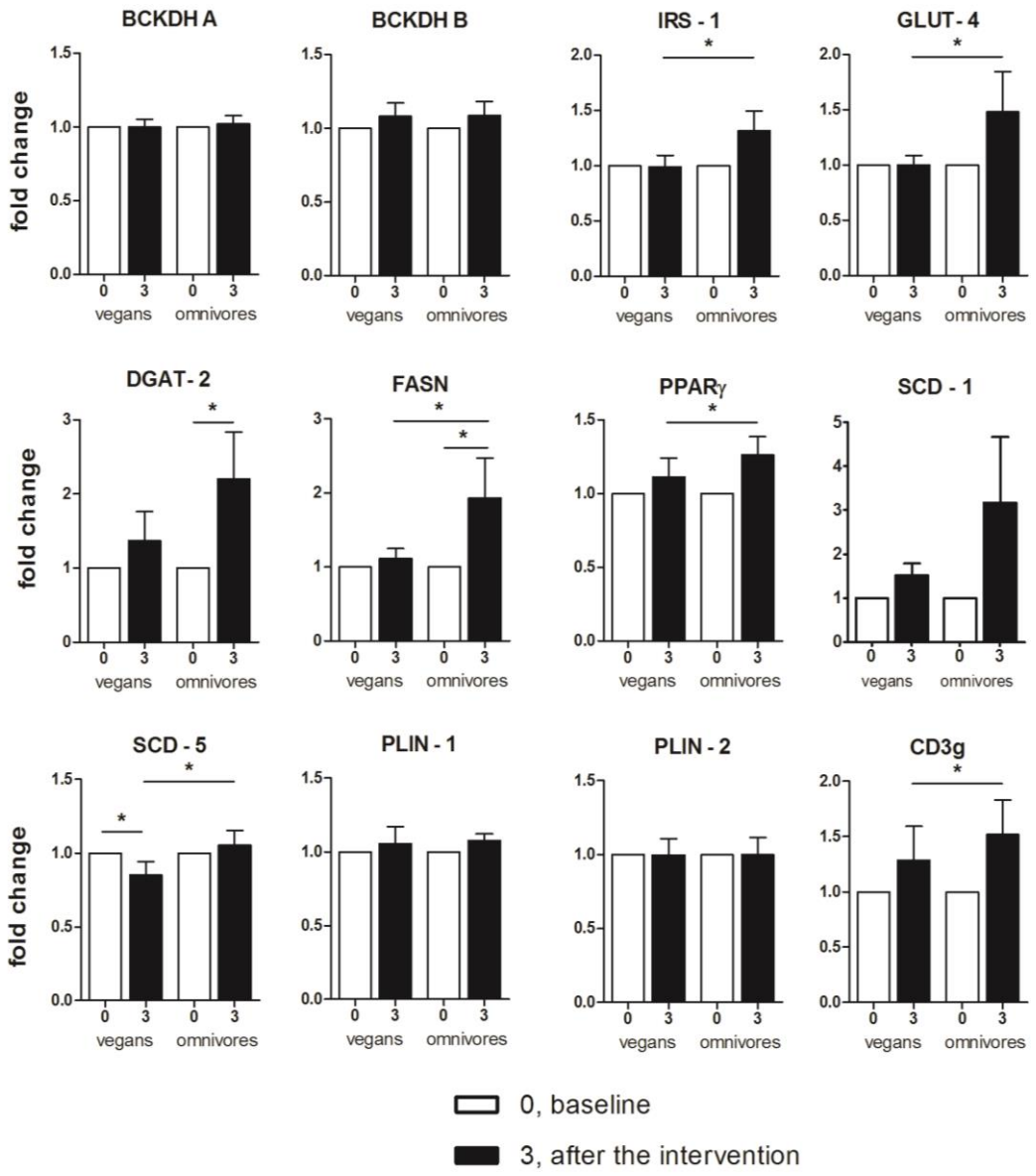


Figure 20 AT mRNA expression in subcutaneous AT. Changes in subcutaneous AT mRNA expression in 8 vegans and 8 omnivores subjected to 3 m intervention with BCAA supplementation. * p<0.05: omnivores baseline vs. intervention (multilevel regression).

SM characteristics, gene expression, mitochondrial activity. Significant increase was found in CII complex in the same direction in both groups though more pronounced in omnivores. Increase in CII complex activity correlated with changes in other complexes: CIII (R=0.75, p=0.0008) and CS (R=0.55, p=0.03) in the whole sample.

Results of mRNA expression of genes related to major skeletal muscle metabolic pathways are shown in Figure 21: glucose uptake (IRS-1, GLUT-4), BCAA metabolism (BCKDH A, BCKDH B), fatty acid oxidation (ACOX, CPT1b) and lipogenesis (PLIN2, PLIN5). No significant difference was found on baseline dCt. Significant differences were found in omnivores only, where IRS-1 expression was lower whereas GLUT-4 was higher after the intervention (with no correlation between the parameters, R 0.08, p=0.84).

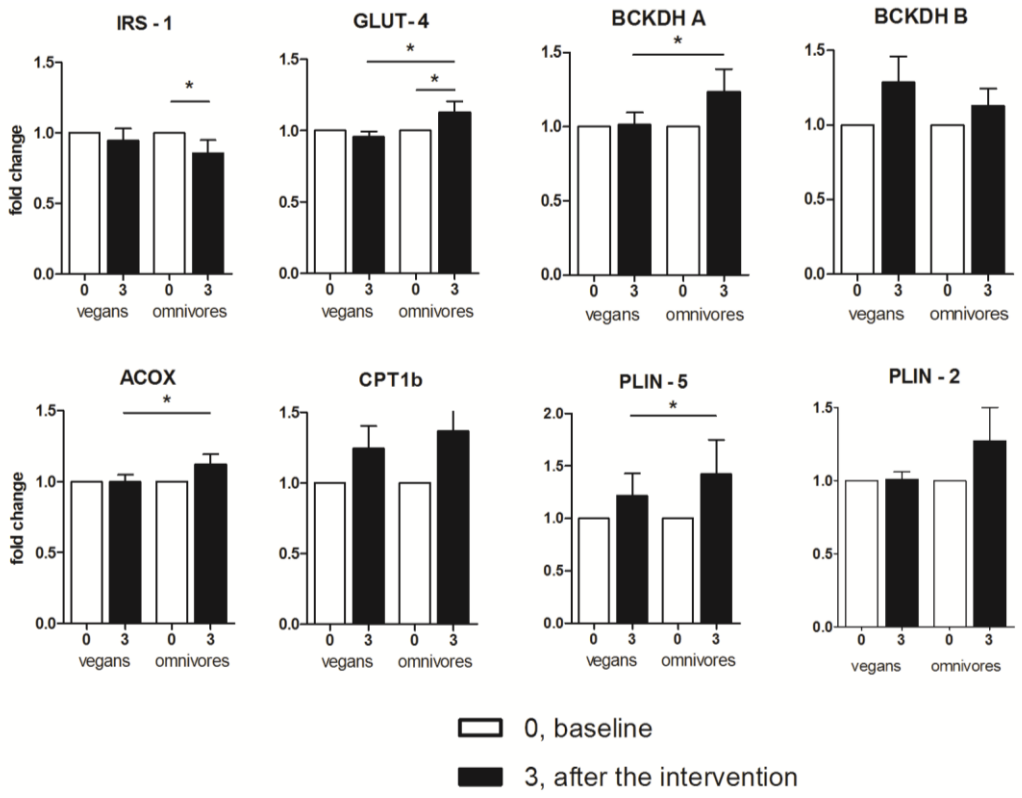


Figure 21 SM mRNA gene expression. Changes in skeletal muscle mRNA expression in 8 vegans and 8 omnivores subjected to 3 m intervention with BCAA supplementation. * p<0.05: omnivores baseline vs. intervention (multilevel regression).

Major finding of the VEGGIE-2 trial is that dietary BCAA lead to impairment of insulin sensitivity only in vegans, i.e. subjects with low basal amino acids/BCAA intake, who appear to be unable to induce compensatory changes within AT and SM upon BCAA challenge.

Despite only mild effect of BCAA supplementation on BCAA circulating levels, BCAA treatment resulted in significant decline of glucose disposal in vegans. Importantly, GD in vegans dropped to the level resembling omnivorous baseline and improved again after wash-out period. This observation supports the conclusion that BCAA could have a direct negative impact on IS in healthy humans. This was suggested already by previously published studies using IV infusion of AA mixtures including BCAA [161,203]. No such changes were observed in omnivores. Such group specific impact of long term BCAA supplementation on IS in two cohorts differing in their nutritional habits could indeed explain previous conflicting results on animals [174,177]. Also, contrary to our study focused on IS and metabolically healthy subjects, studies performed on IR subjects described an improvement of glucose uptake after long term high-BCAA protein supplementation in humans [206,207]. Thus, it seems that only IR subjects may benefit from BCAA supplementation. This may be related to insulinotropic effect of BCAA [221]. BCAA stimulate beta cells secretion affecting directly beta cell as well as via incretin effect [164,207]. Indeed, BCAA supplementation in T2DM patients increased AIR and this led to improved postprandial glycaemia compensation [207]. But in healthy individuals, the insulinotropic effect of BCAA may not necessarily be advantageous as hyperinsulinaemia can itself cause decreased insulin signalling and IS in liver and SM [222]. Accordingly, in the current study

we observed increased AIR after BCAA intervention only in omnivores who are less IS than vegans. These insulinogenic properties were further addressed in a VAMPIR trail (see below).

Increased RC complex activities were found in both groups, though more pronounced in omnivores. Significant increase was found in complex II activity (that correlated with complex III and CS activity). Valine and isoleucine are precursors for succinyl-CoA, a substrate for complex II so its abundance in mitochondria could be a possible explanation for the change after intervention. Nevertheless, both valine and leucine circulating levels were not increased.

We do not have an explanation based on our observations for differential metabolic response to BCAA supplementation in vegans and omnivores, but we hypothesize this could be due to different baseline AA and BCAA consumption. We hypothesize that in low AA intake group (i.e. vegans) dietary BCAA are primarily directed to SM where they are used in proteosynthesis. This could also explain the decrease of IS in vegans as it is known that leucine could blunt insulin action via mTORC1-SK61 stimulation that inhibits IRS-1 and IRS-2 in SM [223]. Contrary, in high AA intake group (i.e. omnivores) excess of dietary BCAA are buffered by AT where they are cleared from circulation via de novo lipogenesis as was already suggested. But since we assessed neither SM proteosynthesis nor mTORC1 activity we cannot make conclusion on the hypothesis.

2.1.3. Insulinogenic effects of branched chain amino acids are mediated by incretin axis, the VAMPIR study

Interestingly, one of the outcomes of long-term BCAA supplementation was a finding of an increase in insulin secretion, as per increase in an acute insulin response in arginine test [123]. To get deeper insight in BCAA insulinogenic properties a VAMPIR (**V**ětvené **Am**inokyseliny: **P**ostprandiální **I**nzulínová **R**esponse; BCAA postprandial insulin response, registered at www.clinicaltrials.gov, NTC02697305) study was prepared and conducted [165].

2.1.3.1. Rationale and study design

It has been known for more than 50 years that amino acids (AA) exert insulinotropic properties when given intravenously (IV) as well as orally [224,225]. Among dietary protein mixtures bovine whey protein is of special interest because of its unique insulinotropic properties [192,226,227]. Significant insulin response was shown both in acute settings as well as during long-term supplementation [192,226]. Of note, BCAA have been implicated as mediators of the insulinotropic effects of whey [228]. Our results from VEGGIE-2 study supported the direct role of long-term BCAA on insulin response [123]. As BCAA are not metabolized in their first pass through the liver, changes in plasma BCAA primarily reflect the rate of intestinal absorption and the dose administered [152]. Leucine serves as a particularly important postprandial nutrient sensing signal. There are several plausible mechanisms suggested that could explain direct leucine stimulated insulin release from beta cells [164].

Mechanism of BCAA stimulated insulin secretion that has not been addressed in depth in the literature is so called incretin effect, originally defined as a potentiated insulin response

after oral vs. IV administration of glucose [229]. It is mediated mainly by glucagon like peptide 1 (GLP-1) and glucose-dependent insulinotropic peptide (GIP). It has been shown that intra-duodenal [230] as well as oral administration [231] of an AA mixture elicits a greater insulin response compared with IV administration. Increase in incretin levels has already been shown after oral whey administration and AA mixtures [192,231–233] and *in vitro* GLP-1 response has been demonstrated in an intestinal cell line after incubation with BCAA [234]. It could be therefore hypothesized that incretin effect of oral BCAA contributes significantly to insulinotropic properties of whey and AA mixtures.

The rationale behind the VAMPIR study was to explore 1/ whether in acute settings, BCAA stimulate insulin response and 2/ whether it is mediated by incretin effect. Using an equivalent dose study as it was used in the original works on glucose incretin effect [229], an IV BCAA administration was compared to an oral BCAA, and to an oral placebo with regard to insulin, glucagon, glucose, GLP-1, and GIP responses. The oral placebo test was conducted to exclude non-BCAA stimulation of insulin secretion. For the details of the study protocol please see the original publication [165]. Eighteen subjects were examined on three different occasions. IV application of BCAA solution (Nutramin VLI 3%, Fresenius Kabi, KGaA, Germany; Leucine 43 %, Isoleucine 24 %, Valine 33 %), oral ingestion of BCAA (Reflex Nutrition, UK, Brighton; Leucine 50 %, Isoleucine 25 %, Valine 25 % in gelatine capsule) and placebo (methylcellulose in gelatine capsule) in an equivalent dose (30.7 ± 1.1 g) was conducted. Serum glucose, insulin, C-peptide, glucagon, GLP-1, GIP, valine, leucine and isoleucine concentrations were measured.

2.1.3.2. Results and conclusion

Results are summarized in Figures 22 – 24. Rise in serum BCAA was achieved in both BCAA tests, with iAUC being 2.1 time greater for IV BCAA compared with oral BCAA test (p<0.0001), see Figure 22. Oral and IV BCAA induced comparable insulin response that was greater than placebo (240 min insulin iAUC: oral 3.411 ± 577 vs. IV 2.361 ± 384 vs. placebo 961.2 ± 175 pmol/l, p=0.0006), Figure 23. Oral BCAA induced higher GLP-1 (p<0.0001) and GIP response (p<0.0001) compared with the IV or placebo, Figure 23. Glucose levels declined significantly (p<0.001) in the same pattern during both BCAA tests with no change in the placebo group, Figure 23.

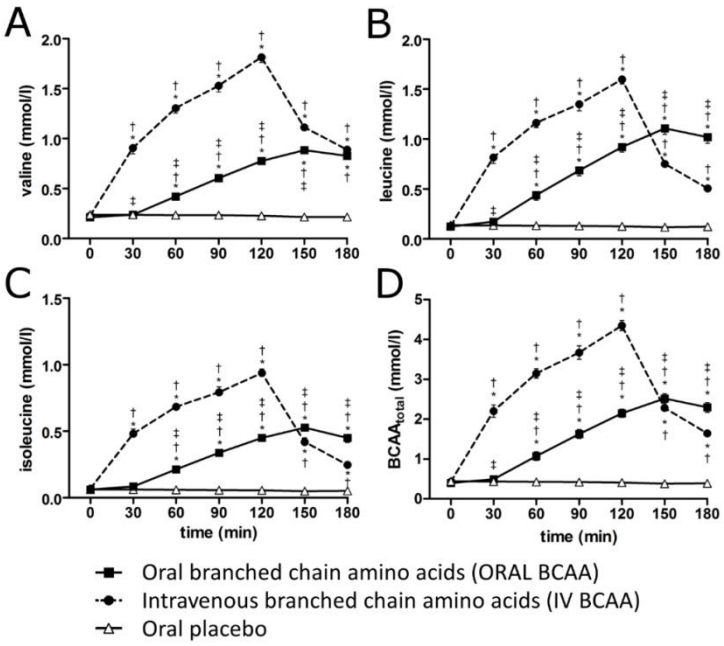


Figure 22 BCAA response. Mean (±SEM) serum valine (A), leucine (B), isoleucine (C) and total BCAA (D) changes in 18 participants administered 30.7 g of branched chain amino acids intravenously, orally or 30.7 g of placebo orally. Significant (p<0.05) treatment and time vs. treatment interaction effect was found for each BCAA. *p<0.05 for RM ANOVA comparing values to baseline; †p<0.05 for 2-way ANOVA compared with placebo; ‡p<0.05 for 2-way ANOVA comparing IV vs. oral BCAA.

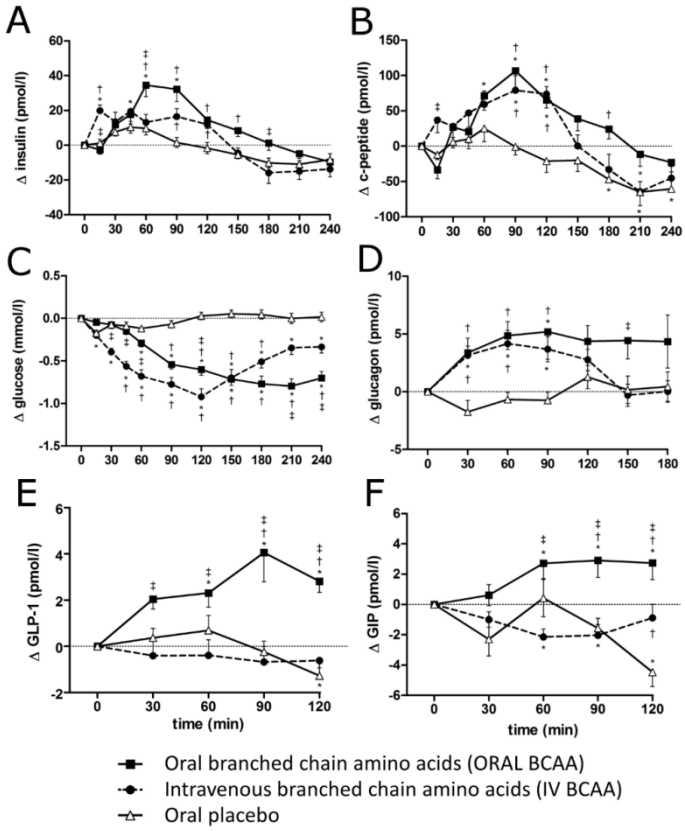


Figure 23 Mean (±SEM) incremental changes in serum insulin (A), serum C-peptide (B), plasma glucose (C), serum glucagon (D), plasma GLP-1 (E) and plasma GIP (F) in 18 participants administered 30.7 g of branched chain amino acids intravenously, orally or 30.7 g of placebo orally. Significant (p<0.05) treatment and time vs. treatment interaction effect was found for each parameter. *p<0.05 for RM ANOVA comparing values to baseline; †p<0.05 for 2-way ANOVA compared with placebo; ‡p<0.05 for 2-way ANOVA comparing IV vs. oral BCAA.

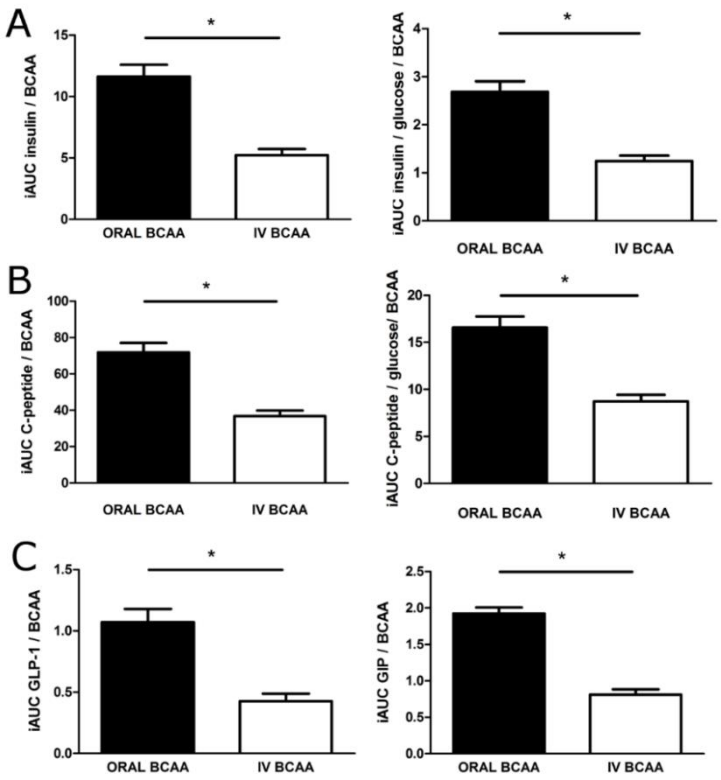


Figure 24 Insulinogenic responses to BCAA in 18 subjects administered 30.7 g of branched chain amino acids intravenously (IV BCAA) and orally (ORAL BCAA). Mean (±SEM) 120 min iAUC of insulin (A) C-peptide (B) and GLP-1 and GIP (C) adjusted for serum levels of BCAA and glucose respectively. Units: insulin (pmol/l), C-peptide (pmol/l), glucose (mmol/l), total BCAA (µmol/l). * p <0.0001 for Student´s t-test.

Major findings of the current study are that 1) equivalent dose of BCAA produced comparable insulin response when administered orally and IV, 2) equivalent dose of BCAA produced increased GLP-1 and GIP response when given orally than IV and 3) significantly lower BCAA levels were achieved after oral administration of BCAA.

We hypothesized that oral administration of BCAA would lead to increased insulin response when compared to IV. This incretin effect was already shown after AA mixture [230]. We found that BCAA induced significant insulin and C-peptide response in both IV and ORAL test. Contrary to what hypothesized we showed no difference in the insulin response between IV and ORAL BCAA test. In the current study we assessed incretin effect in an equivalent dose study and therefore circulating BCAA levels were not matched at any given time point. Given that BCAA and, in particular leucine, stimulate beta cells insulin secretion directly and the secretion response is dose-dependent [22] higher BCAA levels reached in the IV test (mean 11.6 times baseline) than in ORAL one (mean 6.5 times baseline) influenced our results. When adjusting insulin levels to prevailing BCAA levels (as shown in Figure 24) we confirmed that ORAL BCAA could induce much greater insulin responses when compared to IV BCAA at matched peripheral levels.

We showed significantly greater GLP-1 and GIP response in the ORAL BCAA test, and there was no significant incremental change observed in the IV and PLACEBO test. We showed both GLP-1 and GIP response after oral BCAA which is in contrast to previously published data [234,235].

Whether this is due to higher dose used in the current study or a special incretin stimulating effect of BCAA remains to be established. It has already been shown that incretin secretion from the bowel can be regulated by mechanisms other than direct nutrient stimulation. Increased GLP-1 secretion has been shown before an anticipated meal, the phenomena known as the cephalic phase of secretion [236]. Other nutrients and possibly any bolus that distends the small bowel can induce a GIP and GLP-1 response. In this regard water load has been shown to increase GLP-1 secretion [237]. This could be a major confounder when it comes to the incretin effect of BCAA. Therefore, we also included a placebo branch of the experiment. The placebo was prepared and administered under double-blinded conditions relative to the BCAA capsules. Additionally, the amount of water ingested and drinking time was also controlled in both ORAL BCAA and PLACEBO tests.

We found lower BCAA iAUCs after the oral ingestion of BCAA. Peripheral concentrations reached its peak not until 150 min after oral BCAA. Slower release of BCAA from the splanchnic bed to the systemic circulation could be caused either by delayed/decreased absorption and/or increased splanchnic catabolism. Previous studies found that gastric emptying is modulated by meal composition. Food rich in protein were shown to delay gastric emptying by a mechanism involving increased GLP-1 and glucagon secretion [238]. In line with this we found both glucagon and GLP-1 increase after oral BCAA. Another explanation for lower peripheral BCAA levels after oral load is increased intestinal catabolism of BCAA. This could relate to the role of BCAA as a metabolic substrate for both intestinal microbiota and intestinal mucosal cells [151]. Indeed splanchnic uptake of leucine was shown to be as high as 20-30 % [239] and catabolism of BCAA in intestinal cells links to incretin release [234]. We showed that IV and oral BCAA administration induced statistically significant decline in plasma glucose levels. Insulin independent hypoglycaemic effect of BCAA was already shown [240] and it was hypothesized that it is caused by inhibition of hepatic gluconeogenesis and increased muscle glucose uptake unopposed by increased glucagon secretion. Contrary to relatively low glucagon responses after BCAA previously described [193,241] we showed same increase in glucagon levels after ORAL and IV BCAA and unchanged insulin/glucagon ratio.

Incretin response decreases as glycaemic response and HbA1c in diabetic patients deteriorate [242]. Whether a decreased response would be observed also after BCAA in patients with T2DM remains to be established but diet composition modulation is a promising intervention to achieve better diabetes compensation. In this respect if T2DM patients consumed whey before meals better postprandial glycaemia control could be achieved [192,226] as well improved HbA1c through supplementation with an AA mixture [243]. Whether these effects could be attributed to incretin mediated delayed gastric emptying, decreased hepatic glucose production or increased glucose uptake remains to be evaluated. We conclude that BCAA effects on insulin response are partially incretin dependent and that modulation of BCAA content in a diet represents an interesting mechanism for better glycaemic control.

2.2. BRANCHED CHAIN AMINO ACIDS AS POSTBIOTICS MODULATING INSULIN SENSITIVITY

Reviewing a role of intestinal microbiota in a substrate handling. We show that diet ranks among major determinants of intestinal metabolic products and how this could relate to elevated circulating BCAA. Finally, we provide a reader with description of our physiological model to assess these relationships.

Gut microbiota is a complex of different microorganisms dwelling in the intestine. It colonizes intestinal surfaces in abundance reaching approximately 10^{14} bacteria of more than 500 species [244]. It has co-evolved with its human host to a form of a certain symbiosis. While the human provides a stable environment and substrates for growth, microbiota provide the host with services ranging from nutrient digestion and vitamin production to immune functions and defence against pathogen [245]. Microbiota is therefore sometimes referred as a separate organ in the body. Four major phyla colonizing the gut are Firmicutes, Bacteroidetes, Actinobacteria and Proteobacteria. The two first mentioned being the most abundant. Just as mutual interactions bring benefits, so they are complex and prone to disruptions eventually leading to a disease. Many chronic diseases such as those associated with insulin resistance, obesity, diabetes and cardiovascular diseases as well as cancer were shown to be associated with profound dysbiosis [246].

When exploring further into this emerging area of research we found out that among many biological compounds produced and/or sequestered by microbiota are also amino acids [151]. We have been (among others!) since then captured by the idea that gut microbiota may either modulate bioavailability or even itself produce BCAA that could impact on R_a of BCAA in the circulation. Therefore, bridging insulin resistance development and elevated BCAA.

Among major factors influencing gut microbiota is a diet. Indeed, just as we fertilize the gut garden, the flowers bloom. The diet creates a selective pressure to microbiota: as it provides substrates for growth it changes the metabolic end-products respectively. Though biologically plausible, the current evidence is far from being conclusive with a detailed mechanistic view still missing. Promising data form animal models [247] were not easily reproduced in humans. Dietary interventions in humans failed to find significant diet-specific effects [248] or demonstrated responses among a limited number of bacterial taxa [249]. Moreover, the effort to identify distinct microbiota signature related to a particular dietary pattern is complicated by high redundancy of key metabolic pathways among different bacterial taxa. Therefore, the key to the functional interrelationship may lie in microbiota's products. Metabolomic studies in gut microbiota-related research serve as a useful method for establishing biological links between microbiota and host organism and combined with metagenome data may provide even more complex tableau [250].

Box 3

Prebiotics are defined as substrates that are selectively utilized by host microorganisms conferring a health benefit. The benefit should be documented in target organism and may be of gastrointestinal, metabolic, cardiovascular, etc. nature. Majority of described probiotics are complex carbohydrates, polyphenols and polyunsaturated fatty acids [251].

Probiotics is a term used for microbiota that confer beneficiary effects on the host [251,252].

Postbiotics are metabolites of microbiota with a defined effect. Any factor resulting from a metabolic activity of a probiotic or any molecule capable of conferring beneficial to the host in a direct or indirect way [244,253].

2.2.1. How diet influences microbiota composition and its metabolic features

The major substrates for intestinal bacteria are organic polymers. Among them dietary fibre is the preferred one for the fermentation, but any polypeptides/proteins or fats that escape digestion and end up in colon may also be metabolized, though with lower energy yields than carbohydrates. After depolymerization, individual carbohydrate monomers undergo series of metabolic changes to end up as short chain fatty acids (SCFA), the details of these can be found elsewhere [245]. Overview is given in the Table 8. Out of these butyrate (C4) is the preferred substrate for colonocyte, while the others (acetate, C2 and propionate, C3) are drained to a larger extent to portal vein. Propionate is metabolized in the liver and thus is only present at low concentration in the circulation, whereas acetate is the most abundant SCFA in peripheral circulation [245]. All these SCFAs are host signal molecules influencing many physiological functions of the host. There is plethora of animal studies with SCFA colon delivery showing increased thermogenesis, weight gain resistance and improved glucose homeostasis [254]. In line, human trials have repeatedly shown that SCFAs are capable of improving insulin function measures and glucose homeostasis via multiple neuroendocrine and immune mechanisms (increased enteroinsular hormone release, increased satiety, increased intestinal gluconeogenesis etc.) [245,255,256].

SCFAs	Pathways/Reactions	Producers	References
Acetate	from pyruvate via acetyl-CoA	most of the enteric bacteria, e.g., *Akkermansia muciniphila*, *Bacteroides* spp., *Bifidobacterium* spp., *Prevotella* spp., *Ruminococcus* spp.	Louis et al., 2014; Rey et al., 2010
	Wood-Ljungdahl pathway	*Blautia hydrogenotrophica*, *Clostridium* spp., *Streptococcus* spp.	
Propionate	succinate pathway	*Bacteroides* spp., *Phascolarctobacterium succinatutens*, *Dialister* spp., *Veillonella* spp.	Louis et al., 2014; Scott et al., 2006
	acrylate pathway	*Megasphaera elsdenii*, *Coprococcus catus*	
	propanediol pathway	*Salmonella* spp., *Roseburia inulinivorans*, *Ruminococcus obeum*	
Butyrate	phosphotransbutyrylase/ butyrate kinase route	*Coprococcus comes*, *Coprococcus eutactus*	Duncan et al., 2002; Louis et al., 2014
	butyryl-CoA:acetate CoA-transferase route	*Anaerostipes* spp. (A, L), *Coprococcus catus* (A), *Eubacterium rectale* (A), *Eubacterium hallii* (A, L), *Faecalibacterium prausnitzii* (A), *Roseburia spp.* (A)	

A, acetate is the substrate for producing butyrate; L, lactate is the substrate for producing butyrate.

Table 8 Major SCFA end products with respective pathways/producers. From [245]

In addition to production of SCFA, commensal microbiota plays a pivotal role in the conversion of primary bile acids in the lower part of the small intestine and colon to generate secondary bile acids. Diet rich in fat leads to an increase in secretion of bile acids and modulate synthesis of their potentially toxic secondary counterparts. As secondary bile acids become abundant,

it leads to the shift towards microbiota tolerating them. Increased FA colonic availability with a higher abundance of secondary products and bile acid metabolites that can significantly influence the host metabolism via bile acid receptors signalling (farnesoid X receptor and G protein-coupled bile acid receptor 1). This was shown to be linked to a leaky gut and endotoxemia [257]. The state that is associated both with metabolic diseases and cancerogenesis [258].

The last macronutrient is protein. Type of protein ingested impacts on intestinal postbiotic production as amino acids are among important energy substrates. Sulphate reducing bacteria oxidize AA containing sulphur AA (i.e. methionine and cysteine) to produce hydrogen sulphite gas and this process is largely augmented by its increased consumption [259]. BCAA are discussed below.

Decomposition of macronutrients results in a multitude of metabolites that are active both locally in the gut and systemically in the host organism. Besides SCFAs that have been already discussed are branched-chain fatty acids (BCFA), alcohols, ammonia, certain amines, sulphur compounds, phenols, and indoles (derived from amino acids); glycerol and choline derivatives (obtained from the breakdown of lipids); and tertiary cycling of carbon dioxide and hydrogen [260]. Some species (i.e. Bacilli) possess branched chain dehydrogenase activity, therefore producing BC keto-analogues from BCAA for further oxidation or release BCFAs that could be used by colonocyte when butyrate is scarce. Intestinal BCFAs could be used as a biomarker of BCAA decomposition [260].

The hypothesis we refer to as postbiotic, i.e. that intestinal microbiota directly modulates AA/BCAA bioavailabity, hence contributing to BCAA R_a, has been recently proposed [261,262].

There were several findings in the research supporting this hypothesis. BCAAs co-vary tightly with fasting serum metabolites that are known to be of microbial origin. Metabolomic analysis showed a relationship between microbiota biosynthetic capacity and decreased transport of BCAA to the bacteria, circulating BCAA and insulin resistance associated pathways [261]. Faecal transplantation form IR subject to mice, eventually leads to an increase in circulating BCAA [263]. Prevotella Copri was the genus most tightly associated with BCAA and insulin resistance metabolomic signatures. Inoculation of mice with P. Copri both decreases glucose tolerance and increases circulating BCAA [261]. The effects may be however modulated by a diet as in high fibre intake arm glucose tolerance did not worsen [264].

Nevertheless, the evidence from mechanistic studies is still inconclusive in terms of intestinal transportation and relationship to the metabolic state of the host.

2.2.2. Physiological model TRIEMA

Ongoing TRIEMA project (TReatment of Insulin rEsistance by gut MicrobiotA modification), registered under NCT NCT03710850, sponsored by MH CZ no. 18-01-00040.

When studying relationship between diet and metabolic health, populations with extreme dietary patterns could represent a convenient model for hypothesis generating observational

data [201]. Vegans is a group that possesses several characteristics of interest when compared to omnivores: better metabolic health, different intestinal microbiome composition and high plant-based nutrients intake. Moreover it was shown, that upregulation of BCAA degradation in metatranscriptomic analysis in vegetarians' faecal samples could be linked to lower absorption and lower circulating BCAA in this group [255].

We hypothesise that high intake of dietary fibre changes microbiome composition with increased sequestration and lower absorption of BCAA. Decreased flux of BCAA leads to lower circulating levels and eventually better metabolic health.

As per our expertise we aimed to exploit our vegans vs. omnivore model population to assess to what extent intestinal microbiota contributes to circulating BCAA and insulin resistance, besides we extend the model population for metabolically challenged (obese and obese with pre/diabetes) subjects (Figure 25). Though we do not have all the results to make strong conclusions, we provide a reader with an overview of the physiological model we use to approach the problem and first preliminary data that are limited to comparison in between vegans vs. omnivores. To see detailed methodology, please see supplementary file (Procházková et al., 2020).

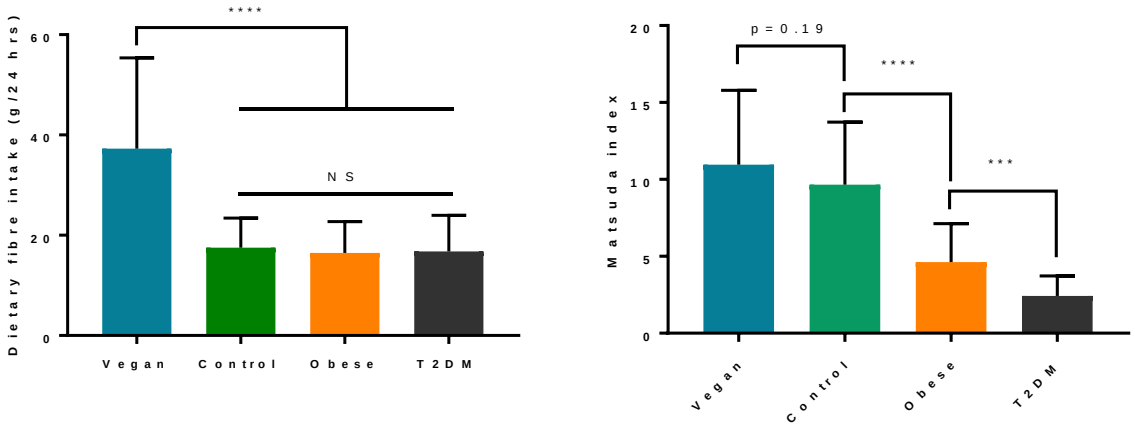


Figure 25 Cross-sectional comparison of dietary fibre intake (per 3-day prospective record) and Matsuda index of insulin sensitivity [29] in vegans (n=62), lean controls (n=33), obese (n=50) and obese with pre/diabetes (n=50). Clear gradient in insulin sensitivity is visible among these groups. Our own observations from an ongoing trial TRIEMA (18-01-00040, NCT03710850).

2.2.2.1. Rationale of the study design and results

Given the association between diet, gut microbiome/metabolome (MIME) and metabolic health, the core hypothesis of the observational TRIMA trial is that there are specific compositional and functional characteristics of MIME that link different eating habits to the metabolic phenotype, including elevated BCAA and insulin resistance. Therefore, we explored two metabolically healthy groups defined by diet composition, i.e. lean healthy vegans (VG) and lean healthy omnivores (O). We aimed to describe their faecal microbiome as well as their serum, urine and faecal metabolome and to identify key features associated with different eating habits as well as potential functional links among them.

Participants' enrolment and characteristics. Sixty-two self-reported vegans (VG: n=62, 6 ± 3.6 years on exclusive vegan diet) and omnivore (O: n=33, eating meat and dairy products on a daily basis) were enrolled and examined. Anthropometry, indirect calorimetry and fasting and OGTT blood sampling was performed. Each participant filled in a prospective dietary record. Baseline characteristics are summarized in Table 9.

Faecal sample collection and manipulation. Stool collected at home was immediately stored at -20 °C until transported in the frozen state to the laboratory. Once thawed, the samples were homogenized using stomacher (BioPro, Czech Republic); one aliquot was used for DNA extraction, one aliquot was used for dry mass estimation and the rest was aliquoted and stored at -50 °C. For metabolome analyses, the faecal samples were diluted with sterile water to 1 % dry mass equivalent.

Exploring gut microbiome. DNA from faecal samples was isolated by QIAmp PowerFecal DNA Kit (Qiagen), and the V1-V3 region of the bacterial 16S rRNA gene was amplified by polymerase chain reaction. Sequencing analysis was performed on MiSeq (Illumina, Hayward, CA, USA). The data were treated as compositional (proportions of total read count in each sample, nonrarefied), and prior to all statistical analyses were transformed using cantered log-ratio transformation (Aitchison).

In all the 90 samples we identified 62,683 ASVs and we detected 10 phyla, 19 classes, 24 orders, 44 families and 144 genera. The normalized α-diversity of the gut microbiota was estimated using indexes measuring richness (observed species), and evenness (Chao1, Shannon, Simpson, pielou_e). In all three parameters, the diversity was higher in omnivore compared with vegan group. At the phylum level, we identified 10 phyla dominated by *Firmicutes* and *Bacteroidetes* followed by much less abundant *Proteobacteria, Actinobacteria* and *Verrucomicrobia*. There was no significant difference between representtations of individual phyla. Multivariate statistics (PERMANOVA) revealed significant differences in β-diversity at the level of order (p=0.023), family (p=0.013) and genus (p=0.001) between vegan and omnivore cohort. Using univariate statistics, we found that 3 classes, 5 orders, 10 families and 46 genera varied significantly between groups (Table 10). At genus level, 17 genera were upregulated while 29 were downregulated in vegans compared with omnivores. Only four genera upregulated in vegans, *Ruminococcaceae UCG-014 and UCG-013, Lachnospira* and *Ruminiclostridium* belong to more abundant (0.1-1 %) genera present in majority of samples, i.e. core microbiome. In contrast, twelve of the upregulated taxa belong to very low abundant (<0.01 %) and rare genera and vegan diet affected rather their incidence rate than abundance. Within our data set, three genera were found only in vegans (*Alloprevotela, Erysipelotrichaceae UCG-004, Succinivibrio*). In contrast, most of the genera down-regulated in vegans belong to core microbiome, as 26 of them has higher than 75 % incidence at least in one group. The most abundant genera (>1 %) down-regulated in vegans were *Alistipes, Subdoligranulum, Blautia, Dorea*, unassigned bacteria from *Lachnospiraceae family* and *Collinsela*. Among others, vegan diet was associated with lower abundance of potential pathogens like *Escherichia-Shigella, Streptococus* or *Bilophila.*

General characteristics	Omnivore	Vegan	*p*-value
Sex [F/M]	17/16	25/37	
Height [m]	1.76 (0.14)	1.76 (0.15)	n.s.
Weight [kg]	73.0 (24.4)	67.9 (16.6)	n.s.
Age [years]	31.3 (11.2)	30.9 (10.5)	n.s.
BMI [kg/m^2]	22.8 (4.4)	21.6 (3.6)	n.s.
WHR	0.82 (0.10)	0.81 (0.08)	n.s.
Harris-Benedict equation	1 636 (510)	1 611 (350)	n.s.
RMR [kcal/day]	1 655 (556)	1 601 (331))	n.s.
Body composition			
Fat [kg]	13.9 (5.8)	11.6 (9.3)	n.s.
FFM [kg]	54.2 (23.4)	57.1 (19.3)	n.s.
TBW [kg]	39.7 (17.1)	41.8 (14.1)	n.s.
Macronutrients intake			
Total energy [kcal/day]	2 100 (683)	2 072 (706)	n.s.
Proteins [g]	81.0 (29.0)	69.0 (38.0)	0.02
Lipids [g]	83.0 (49.0)	70.0 (35.0)	0.03
Carbohydrates [g]	232 (98)	250 (105)	0.03
Dietary fiber [g]	18.0 (10.0)	33.0 (20.0)	<0.001
Glucose metabolism			
Fasting glucose [mmol/l]	4.78 (0.34)	4.69 (0.40)	0.10
2h OGTT glucose [mmol/l]	5.93 (1.43)	5.48 (1.27)	0.07
AUC for OGTT glucose [mmol/l.120min^{-1}]	255 (137)	184 (159)	n.s.
Insulin [mIU/l]	3.90 (2.70)	3.40 (1.70)	n.s.
AUC for OGTT insulin [mIU/l.120min^{-1}]	4416 (1938)	3143 (2603)	0.004
C-peptide [pmol/l]	232.0 (103.0)	229.0 (79.0)	n.s.
HbA1c [mmol/mol]	32.0 (2.5)	30.0 (4.0)	0.01
Matsuda index	10.18 (6.57)	9.87 (5.20)	n.s.
Lipid metabolism			
Total cholesterol [mmol/l]	4.32 (1.14)	3.31 (0.84)	<0.001
HDL-C [mmol/l]	1.70 (0.65)	1.39 (0.40)	<0.001
LDL-C [mmol/l]	2.35 (1.16)	1.71 (0.82)	<0.001
Triglyceride [mmol/l]	0.69 (0.52)	0.67 (0.41)	0.20
Intestinal permeability			
zonullin	25.5 (33.9)	18.1 (27.3)	0.03
Stool characteristics			
pH in faeces	7.3 (0.69)	6.9 (0.76)	0.005
dry mass (%)	25.1 (9.9)	20.3 (8.8)	0.002

Table 9 Baseline characteristics of vegan and omnivore in TRIEMA study. Data are given as medians and interquartile range (IQR). BMI – body mass index, WHR – waist-hip ratio, RMR – resting metabolic rate, FFM – fat-free mass, TBW – total body water, AUC – area under the curve during oral glucose tolerance test, HbA1c – glycated haemoglobin, HDL-C – high density lipoprotein-cholesterol, LDL-C – low density lipoprotein-cholesterol

We analysed ***faecal, serum and urine metabolome*** using three approaches, GC-MS, NMR and LCQ-TOF-MS. *Faecal metabolome* using GC-MS was enriched in 157 different VOCs, all of them were detected in all samples. Within this subset, 83 volatile organic compounds (VOCs) were of very low (<0.1 %) abundance, 58 VOCs represented 0.1-1 % of the total (low abundant), 13 VOCs belonged to the abundant compounds (1-5 %), and three (p-cresol, indole, scatole) were highly abundant (>5 %).

	Median abundance (%) (IQR)		incidence (%)		*p*-value
	VG	O	VG	O	
Lachnospira	0.79 (1.358)	0.40 (0.539)	100	100	0.003
Ruminiclostridium	0.43 (0.669)	0.29 (0.249)	95	97	0.008
Ruminococcaceae UCG-003	0.28 (0.333)	0.15 (0.253)	92	77	0.006
Ruminococcaceae UCG-014	0.79 (3.237)	0.29 (1.495)	78	60	0.011
Lachnospiraceae UCG-005	0.06 (0.239)	< 0.01 (0.008)	67	33	0.001
Lachnospiraceae UCG-010	< 0.01 (0.054)	< 0.01 (0.014)	50	30	0.010
Tyzzerella 3	< 0.01 (0.127)	< 0.01 (0.003)	45	27	0.016
Oxalobacter	< 0.01 (0.050)	< 0.01 (<0.001)	40	17	0.001
Lachnospiraceae UCG-003	< 0.01 (<0.001)	< 0.01 (<0.001)	22	13	0.031
Acetitomaculum	< 0.01 (<0.001)	< 0.01 (<0.001)	20	10	0.016
Mitsuokella	< 0.01 (<0.001)	< 0.01 (<0.001)	12	7	0.023
Prevotella 2	< 0.01 (<0.001)	< 0.01 (<0.001)	10	3	0.003
Erysipelotrichaceae UCG-004	< 0.01 (<0.001)	< 0.01 (<0.001)	8	0	0.002
Alloprevotella	< 0.01 (<0.001)	< 0.01 (<0.001)	8	0	0.010
Prevotellaceae; G_uncultured	< 0.01 (<0.001)	< 0.01 (<0.001)	7	3	0.029
Weissella	< 0.01 (<0.001)	< 0.01 (<0.001)	7	3	0.029
Succinivibrio	< 0.01 (<0.001)	< 0.01 (<0.001)	5	0	0.015
Alistipes	1.70 (3.080)	3.21 (3.390)	90	100	0.039
Subdoligranulum	1.66 (1.719)	2.35 (2.853)	97	100	0.047
Blautia	0.74 (0.822)	1.82 (1.101)	100	100	0.004
Lachnospiraceae; G_Unassigned	1.06 (1.212)	1.71 (1.708)	100	100	0.021
Lachnoclostridium	0.80 (1.005)	1.70 (1.323)	100	100	0.036
Collinsella	0.29 (0.616)	1.03 (2.063)	83	87	0.019
Fusicatenibacter	0.26 (0.475)	0.64 (0.715)	95	100	0.021
Dorea	0.27 (0.390)	0.58 (0.340)	92	100	0.002
F_Ruminococcaceae; G_uncultured	0.24 (0.269)	0.40 (0.459)	97	100	0.005
[Eubacterium] hallii group	0.15 (0.301)	0.39 (0.444)	92	97	0.003
Peptoclostridium	0.09 (0339)	0.33 (0.275)	83	97	0.011
Odoribacter	0.08 (0.311)	0.32 (0.311)	78	100	0.004
Ruminiclostridium 5	0.08 (0.115)	0.23 (0.236)	86	100	0.000
Erysipelotrichaceae UCG-003	0.09 (0.207)	0.22 (0.401)	93	100	0.006
Streptococcus	0.06 (0.144)	0.22 0.438)	77	97	0.000
Anaerotruncus	0.03 (0.152)	0.16 (0.439)	70	93	0.001
Ruminiclostridium 9	0.07 (0.098)	0.14 (0.293)	88	97	0.014
Escherichia-Shigella	< 0.01 (0.089)	0.13 (0.566)	50	80	0.001
[Ruminococcus] gauvreauii group	0.03 (0.074)	0.11 (0.202)	60	77	0.005
[Eubacterium] ventriosum group	0.05 (0.109)	0.09 (0.143)	75	90	0.041
Bilophila	0.04 (0.097)	0.08 (0.179)	75	87	0.049
Family XIII UCG-001	0.01 (0.032)	0.05 (0.079)	60	87	0.002
Coriobacteriaceae; G_Unassigned	< 0.01 (0.043)	0.05 (0.197)	40	70	0.006
Lachnospiraceae UCG-008	0.02 (0.026)	0.04 (0.044)	88	93	0.029
Family XIII AD3011 group	0.01 (0.026)	0.02 (0.058)	58	83	0.008
Actinomyces	< 0.01 (0.012)	0.01 (0.040)	35	60	0.029
Peptococcaceae; G_uncultured	< 0.01 (<0.001)	< 0.01 (0.031)	12	50	0.011

Table 10 *Gut microbiome: genus level.* Data are given in % as medians (IQR, interquartile range). Only results significant at P<0.05 and at false discovery rate <0.1 are shown. Bacterial species highlighted in grey are more abundant in vegans compared with omnivores. VG:O ratio was not calculated when the abundance of particular taxa in O was true zero.

Multivariate statistics (PERMANOVA) revealed significant differences in β-diversity (*p*=0.004). According to the univariate statistics, the concentration of 47 analytes differed significantly between VG and O, median abundance of 35 of them was >0.05 %. Only seven VOCs were more abundant in vegan than in omnivore stool samples. VOC pattern associated with vegan diet was characterized by lower concentration of amino acid fermentation products, i.e. p-cresol (median abundance 7.7 vs. 19.8 %), scatole (4.9 vs. 9.5 %), indole (0.4 vs. 5.9 %), methional (0.02 vs. 0.07 %). Various aromatic compounds (median abundance ranging from

1.5 to 0.09 %), primary alcohols (0.03 to 0.36 %) and medium-chain ketones or aldehydes (0.08 to 0.28 %) were lower in vegans as well. In contrast, butanoic acid (3.4 vs. 1.4 %), butanoic acid methyl ester (0.9 vs. 0.2%), methyl propionate (0.06 vs. 0.01 %) and 4-ethylphenol (0.04 vs. 0.01 %) were increased in vegans (Table 11).

		VG	O	VG:O ratio	*p*-value
AA-derivatives	p-Cresol	7.4 (14.8)	19.8 (14.2)	0.4	0.000
	Indole	4.9 (7.7)	9.5 (10.5)	0.5	0.000
	Scatole	0.4 (2.5)	5.85 (11.3)	0.1	0.001
	Methional	0.02 (0.07)	0.07 (0.14)	0.3	0.007
SCFA and SCFA-derivatives	Butanoic acid	3.4 (4.4)	1.4 (1.9)	2.4	0.034
	Butanoic acid. methyl ester	0.87 (3.05)	0.16 (0.62)	5.3	0.019
	Methyl propionate	0.06 (0.13)	0.01 (0.05)	4.8	0.047
MCFA	Nonanoic acid	0.05 (0.35)	0.03 (0.05)	1.6	0.022
	Octanoic acid, ethyl ester	0.04 (0.30)	0.00 (0.04)	0.0	0.041
aromatic compounds	Benzene acetaldehyde	0.64 (0.84)	1.10 (1.25)	0.6	0.001
	Benzene, 1,2,4-trimethyl-	0.80 (0.61)	1.03 (0.82)	0.8	0.020
	Benzene, 1,2,4-trimethyl-	0.30 (0.23)	0.40 (0.33)	0.8	0.017
	Benzene, 1-ethyl-2-methyl-	0.23 (0.19)	0.26 (0.26)	0.9	0.026
	Carvone	0.08 (0.56)	0.17 (1.01)	0.5	0.027
	Diallyl disulphide	0.03 (0.09)	0.07 (0.10)	0.4	0.003
	Phenol. 4-ethyl-	0.04 (0.35)	0.01 (0.04)	3.9	0.034
alcohols	1-Pentanol	0.19 (0.30)	0.28 (0.23)	0.7	0.007
	2-Tridecen-1-ol, (E)-	0.08 (0.09)	0.12 (0.35)	0.7	0.003
ketones	2-Nonanone	0.17 (0.17)	0.20 (0.20)	0.8	0.011
	2-Undecanone	0.20 (0.19)	0.36 (0.26)	0.6	0.000
	2-Tridecanone	0.20 (0.19)	0.32 (0.34)	0.6	0.000
	2-Tetradecanone	0.03 (0.06)	0.06 (0.08)	0.5	0.003
	2-Dodecanone	0.03 (0.04)	0.07 (0.09)	0.4	0.015
	unknown ketone	0.04 (0.05)	0.08 (0.11)	0.5	0.001
aldehydes	Dodecanal	0.12 (0.21)	0.33 (0.45)	0.4	0.000
	Penta decanal	0.06 (0.11)	0.07 (0.13)	0.9	0.014
	Hexadecanal	0.06 (0.08)	0.13 (0.15)	0.5	0.001
	2,4-Decadienal, (E,E)-	0.00 (0.02)	0.00 (0.03)	0.0	0.034
	unknown aldehyde	0.06 (0.09)	0.10 (0.12)	0.6	0.005
	unknown aldehyde	0.07 (0.15)	0.12 (0.19)	0.6	0.044
	unknown aldehyde	0.01 (0.03)	0.02 (0.04)	0.6	0.031
	unknown aldehyde	0.01 (0.01)	0.01 (0.02)	0.8	0.017
alkanes	Decane	0.17 (0.22)	0.19 (0.24)	0.9	0.013
	Dodecane	0.27 (0.29)	0.30 (0.14)	0.9	0.005
	Pentadecane	0.00 (0.00)	0.00 (0.05)	0.0	0.007
	unknown alkane	0.11 (0.17)	0.22 (0.22)	0.5	0.000
	2-pentyl thiophene	0.07 (0.09)	0.08 (0.07)	0.8	0.002
	beta-Ionone	0.01 (0.01)	0.01 (0.02)	0.6	0.029
	Ledane	0.02 (0.04)	0.00 (0.03)	0.0	0.018

Table 11 *Faecal metabolome: volatile organic compounds.* Data are given in % as medians (IQR, interquartile range). Only results significant at P<0.05 and at false discovery rate <0.1 are shown. AA, amino acids; SCFA, short-chain fatty acids; MCFA, medium-chain fatty acids. Metabolites highlighted in grey are more abundant in vegans compared with omnivores.

Multi-metabolite signature for better discrimination of vegans vs. omnivores included higher levels of 4-ethylphenol, as well as lower level of p-cresol and carvone. The sensitivity and specificity of this model was 90 % and 84 %, resp., for the optimal cut-point according to Youden index, AUROC curve was 93 %. The results were confirmed using NMR, we found 37 analytes in faeces. According to PERMANOVA analysis, the vegan and omnivore stool NMR

metabolome differed significantly (I=0.003) and univariate statistics revealed 13 metabolites discriminating between cohorts. Vegan profile was characterized by higher content of SCFA (butyrate, acetate), ethanol and four unidentified metabolites compared with omnivores while four amino acids (aspartate, methionine, leucine and tyrosine) and two unidentified metabolites were decreased (Table 12). Using LCQ-TOF-MS, we identified 11 bile acid species. PERMANOVA analysis did not prove significant differences between VG and O. According to the univariate statistics, only lithocholic acid (LCA) content was significantly lower in vegans. LCA was also the most abundant BA.

		VG	O	VG:O ratio	*p*-value
SCFA	butyrate J46	6.82 (4.22)	4.90 (4.41)	1.4	0.008
	acetate J32	45.5 (29.15)	37.9 (20.87)	1.2	0.025
	unidentified J13	0.03 (0.04)	0.03 (0.02)	1.2	0.002
	unidentified J19	0.53 (0.57)	0.31 (0.25)	1.7	0.010
	unidentified J48	0.03 (0.03)	0.02 (0.03)	1.3	0.010
	unidentified J14	0.13 (0.10)	0.08 (0.05)	1.6	0.013
alcohol	ethanol	2.45 (3.26)	1.57 (1.33)	1.6	0.033
AA	aspartate	0.12 (0.06)	0.14 (0.07)	0.8	0.001
	methionine	0.27 (0.14)	0.32 (0.14)	0.8	0.026
	leucine	0.48 (0.24)	0.52 (0.33)	0.9	0.034
	tyrosine	0.20 (0.11)	0.24 (0.10)	0.8	0.050
	unidentified J21	0.24 (0.18)	0.38 (0.38)	0.6	0.011
	unidentified J47	0.14 (0.11)	0.17 (0.08)	0.8	0.037

Table 12 *Faecal metabolome: NMR detected compounds* Data are given in % as (IQR, interquartile range). Only results significant at P<0.05 and at false discovery rate <0.1 are shown. AA, amino acids; SCFA, short-chain fatty acids. Metabolites highlighted in grey are more abundant in vegans compared with omnivores.

Serum metabolome was significantly different (PERMANOVA, p<0.001) between groups, and the difference was driven by thirty identified analytes. We further proceeded with an univariate statistics and identified 19 metabolites significantly differently abundant in O and VG cohorts, 13 of them being amino acids. Vegan-specific serum profile was characterized by the low content of essential branched-chain amino acids (BCAA) valine, leucine and isoleucine, BCAA-derivative ketoleucine and methyl-oxovalerate, aromatic AA tryptophane and two other amino acids (lysine, histidine). In contrast, glutamine, glycine, proline, threonine and asparagine were more abundant in vegans compared with LO. Two potential co-metabolites of host and bacterial metabolism, acetate and dimethyl sulfone, were increased in vegans as well as citrate and three unidentified compounds (Table 13). Multi-metabolite signature of vegans included higher levels of asparagine, glycine and dimethyl sulfone, as well as lower level of lysine. One of these signature compounds, dimethyl sulfone, may indicate the relationship between vegan microbiome and serum metabolome.

		VG	O	VG:O ratio	*p*-value
AA	lysine S20	0.31 (0.06)	0.37 (0.05)	0.8	0.000
	histidine S7	0.12 (0.02)	0.12 (0.03)	0.9	0.024
	phenylalanine S5	0.17 (0.02)	0.18 (0.02)	0.9	0.005
	glutamine S27	2.3 (0.3)	2.1 (0.2)	1.1	0.000
	glycine S17	0.70 (0.18)	0.49 (0.14)	1.4	0.000
	proline S33	0.65 (0.12)	0.61 (0.09)	1.1	0.010
	threonine S12	0.22 (0.04)	0.20 (0.03)	1.1	0.020
	asparagine S21	0.06 (0.02)	0.05 (0.01)	1.2	0.000
BCAA-derivative	leucine S48	0.81 (0.15)	0.90 (0.12)	0.9	0.000
	valine S45	0.87 (0.17)	1.02 (0.17)	0.8	0.000
	isoleucine S50	0.19 (0.04)	0.21 (0.03)	0.9	0.003
	2-oxoisocaproate S24	0.07 (0.02)	0.07 (0.01)	0.9	0.001
	methyl succinate S44	0.04 (0.02)	0.05 (0.02)	0.9	0.008
	citrate S25	0.09 (0.03)	0.08 (0.02)	1.1	0.017
	acetate S34	0.28 (0.10)	0.23 (0.05)	1.2	0.002
	dimethyl sulfone S18	0.18 (0.04)	0.16 (0.01)	1.2	0.001
	unknown S51	0.13 (0.04)	0.17 (0.06)	0.8	0.000
	unknown S42	0.08 (0.02)	0.09 (0.02)	0.9	0.001
	unknown S37	0.06 (0.01)	0.07 (0.01)	0.9	0.000

Table 13 *Serum metabolome: NMR detected compounds.* Data are given in % as (IQR, interquartile range). Only results significant at P<0.05 and at false discovery rate <0.1 are shown. AA, amino acids; BCAA, branched-chain amino acids.

In total, we identified 14 metabolites in urine, *urine metabolome* being significantly different between groups (PERMANOVA, *p*=0.001). Citrate and trigonelline (marker of soy consumption) were more abundant while methyl succinate (intermediate of BCAA metabolism) and 3-indoxylsulfate (intermediate of tryptophan metabolism) were less abundant in vegans compared with omnivores.

2.2.2.2. Discussion and conclusion

To conclude, the major finding of the observational TRIEMA results presented above is that better metabolic health of vegans relates to distinct faecal microbiome and metabolomic features and that insulin sensitivity relates both to circuiting and faecal BCAA, namely leucine.

Despite of widely accepted opinion that plant-based diets have beneficial effect on gut microbiome [245] the exact information how strict vegan diet modulate the gut microbiome in people living in westernized societies is scarce. We found on microbiome analysis only modest differences in its composition associated with either diet. Rather surprisingly, the alpha diversity was higher in omnivores compared to vegans what is in disagreement with previously published results that showed no difference [248] or higher diversity in vegans [265]. In accordance with some of the previously published studies, we observed a trend towards higher *Bacteroidetes* and lower *Firmicutes* in VG group albeit it did not reach statistical significance.

Although DNA based fingerprint procedures provide an information about the composition of microbial community, they do not reflect the metabolic activity of the populations [266]. To get better insight into metabolic performance of gut microbial communities in vegans and omnivores, we performed untargeted metabolome analysis. Multivariate statistical

approaches revealed the significant differences between vegans and omnivores in faecal, urine and serum metabolome. The striking characteristic of vegan faecal metabolome was the significantly lower content of several amino acids (of note leucine, aspartate, tyrosine, methionine) as well as amino acid fermentation products (p-cresol, indole, scatole and methional) and some phenolic compounds. On the other hand, butyrate, acetate and several SCFA-derived esters were elevated in vegans. The complex BAs profile in stool was not different between groups but LCA was significantly lower in vegans. LCA is exclusively microbial product; therefore, it indicates the differences in microbiome activity as well.

Most bacteria possess multiple metabolic programs that could be switched on and off according to the available substrates and environmental conditions. The composition of faecal metabolome indicates the shift from protein towards carbohydrate fermentation in vegans that may be a consequence of the different macronutrient composition of the diet. Vegan diet contains less total protein compared with omnivore diet and plant proteins are less digestible compared with those of the animal origin. The way the proteins are consequently fermented depends on its amino acid composition. Sulphur-containing amino acids are fermented to produce sulphur compounds while aromatic amino acids are fermented to p-cresol, phenolic compounds, scatole and indole. It was reported that plant proteins, particularly certain legumes, vegetables and soy protein contain less methionine and cysteine than meat protein [267]. In accordance, we found lower faecal content of several amino acids, including tyrosine and methionine, in the vegan group. Amino acids are less efficient as energy source for human gut microbes and therefore gut microbiota preferentially consume carbohydrates over proteins [260]. Contrary to protein dietary intake, the intake of fibre in vegans was twice as high as in omnivores. Dietary fibre is the major substrate for saccharolytic SCFA producers and indeed, we found significantly higher butyrate and acetate faecal content in vegans. This observation was confirmed by two independent analytical methods, i.e. GC-MS and NMR. Vegan faecal samples were also characterized by higher content of SCFA esters. De Preter described similar effect of dietary fibre inulin, i.e. increased production of SCFA esters, when incubating stool samples *in vitro* with inulin.

To our knowledge, we are the first who showed higher butyrate in vegan stool samples. The explanation may lay in the difficulties associated with the quantification of stool metabolites. Stool is extremely difficult material in terms of normalization of the variables to some independent measure. The obvious parameters like protein content or total DNA are unsuitable. The simplest parameter – weight – is disputable as well because the stool samples are highly variable in the water content. In our study the vegan samples have on average 5 % less dry mass to omnivores. Therefore, we normalized our measurements to dry mass in the original sample to make the measurements comparable.

The effect of different metabolic activity of gut bacteria in vegans and omnivores could be detected in serum metabolome. Vegans and omnivores significantly differed in 19 out 30 identified signals. Twelve of them were amino acids what may reflect a different dietary intake. However, seven metabolites are cometabolites formed both by the host and gut microbiota. This is the case of acetate, the most abundant SCFA formed in the gut, dimethyl sulfone, product of microbial metabolism of methionine and BCAA and their derivatives.

The major focus of the analysis was put on BCAA metabolism in relation to metabolic health. As summarized above there are more hypothesis on largely reproduced findings of elevated BCAA in insulin resistant states. We found in the current study that vegans' faecal metabolome is depleted from BCAA and its catabolites and that relates to lower circulating BCAA and better indices of insulin function. These findings are in line with previous data showing microbiota BCAA - biosynthetic and transport potential may relate to lower BCAA absorption [261] and that BCAA degradation pathways are up-regulated in combined vegetarian (vegans and vegetarians) when compared to omnivore gut microbiota [255]. The upregulated BCAA degradation and downregulated biosynthesis in the gut microbiota may contribute to the observed lower serum BCAA concentrations in vegans that in turn protects from anaplerotic stress associated with BCAA (for details see above). Thus, the reduction of circulating BCAA may represent one of the microbiome-related mechanism contributing to the health promoting effects of plant-based diet.

To sum up, our data show that long-term adherence to vegan diet causes only modest changes in gut microbiome but induce a significant alteration in gut, serum and urine metabolome indicating the switch from protein to carbohydrate fermentation in vegans. In line with expected availability of different types of substrates (fibre vs. protein), we found indices of lower amino acid and higher carbohydrate catabolism in vegans compared with omnivores. Consequently, vegan diet was associated with lower abundance of the potentially harmful and higher occurrence of the potentially beneficial metabolites. Lower circulating and faecal BCAA relate to better whole-body insulin measures. All these findings may impact on the improved metabolic health of vegans. Moreover, interventions aimed to shift in the gut metabolite profile may represent a promising treatment approach in metabolic syndrome.

For now, we are only half way there to get a complex tableau on BCAA-related metabolic signatures. Further research into our data is needed to infer on association and/or causality in between intestinal abundance of BCAA, circulating plasma BCAA and insulin measures impairment. Current gaps of knowledge that we plan to fill soon is dietary intake of individual AA and BCAA and detailed metatranscriptomic analysis of intestinal BCAA metabolism. These results could give further answers for elevated BCAA being either a mere epiphenomenon or a causal factor of a metabolically challenged state.

2.2.2.3. Further perspectives of TRIEMA project

The path of research further leads us towards dietary manipulation with different types of dietary fibre seeking to identify *ex ante* predictors of metabolic response by simple metabolomic analysis or a dynamic test.

This emerging area of research could further open a possibility to personalize dietary interventions in diabetes to maximize potential benefits in early stages of clinical evolution of the disease. Available clinical studies demonstrate that there is high variability in long-term glycaemic response upon supplementation. Here we hypothesize and would eventually prove that this variability depends on individual microbiome/metabolome patterns. We further aim to unravel physiology of the gut microbiota that would explain and even allow for prediction

of the response to dietary fibre in terms of glucose tolerance. The combination of metagenomics and NMR/MS metabolomics approaches enables us to search for complex signatures. We did implement a simple functional test, i.e. inulin test to explore kinetics of faecal butyrate production upon single administration of inulin bolus. We will study how acute augmentation of butyrate production links with long-term metabolic response (clinical trial TRIEMA, interventional arm). Further, the employment of the unique germ-free mice model will allow us to focus on the mechanisms underlying the outcomes observed in the human intervention study using responders/non-responders faecal transfers. After validation of our hypothesis with inulin supplementation (i.e. defining patterns associated with whole-body metabolic response), further steps towards other types of dietary fibre could follow. It could eventually open up a unique approach toward precision dietary medicine. Tailoring dietary intervention with fibre to the individual patient could potentially revolutionize first-line treatment in diabetes. Details of this interventional arm of TRIEMA project is beyond the scope of the current work and will be available in the press soon.

2.3. ELEVATED BRANCHED CHAIN AMINO ACIDS AS A REFLECTION OF SKELETAL MUSCLE INSULIN RESISTANCE

Here we review how BCAA R_a is influenced by inflammatory response. We show cancer cachexia, a multisystemic inflammatory syndrome, as a suitable physiological model to study loss of insulin mediated suppressibility of BCAA skeletal muscle efflux.

2.3.1. Cancer cachexia as a physiological model of inflammation driven insulin resistance

As we reviewed above, insulin resistance is a hallmark of inflammatory response. It represents an adaptive mechanism of substrate redistribution from major metabolic organs to immune system and the brain. Once an adaptive phenomenon, it may become detrimental when abused for securing substrates for growing cancer at the expanse of the host. Once the cancer orchestrates whole body substrate handling, a syndrome of cancer cachexia (CC) develops. CC is a systemic and complex syndrome resulting from fatal host metabolic reprogramming induced by tumour. It is characterized by fat and muscle depletion in spite of adequate nutrient supply, inflammation and metabolic derangements [268]. CC is not only a major mortality cause [269] but brings also a limiting condition for adjuvant therapy delivery as low lean body mass predicts dose-limiting toxicity of chemotherapy [270]. Currently there is no treatment available for CC except for supportive care and possible therapeutic targets remains to be discovered. Again, shall we consider cancer host being the ultimate source of substrates needed for growing tumour, then CC could be perceived as an extreme expression of cancer securing its fuel needs. For that purpose tumour tissue takes advantage of conserved host metabolic responses to stress stimuli, that leads to substrate shift from peripheral tissues to the tumour ensuring supply for its growth and expansion while causing a state of insulin resistance (IR) of the host [271].

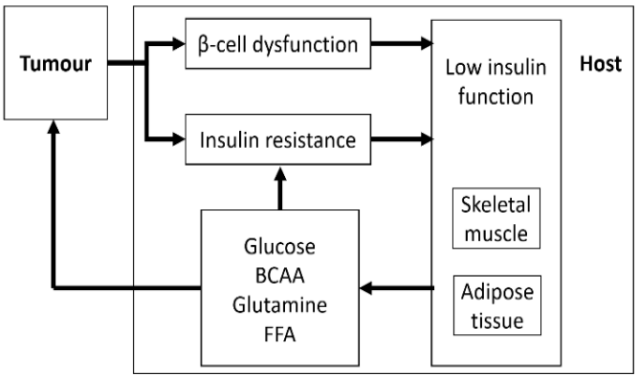


Figure 26: Proposed metabolic sequels of cancer cachexia. Tumour induces insulin resistance in host tissues. Lower insulin function is further aggravated by its absolute deficit due to decrease secretion from beta cells. An unopposed counter-regulatory drive leads to accelerated catabolism in peripheral tissues and efflux of major substrates that are needed for tumour growth. Substrates high turnover further decreases insulin function in the host compartment.

IR would reflect catabolic setting in the host peripheral tissues. Low insulin function would lead to impaired glucose tolerance, elevated plasma NEFA and higher efflux of free amino acids [272], mostly branched chain amino acids (BCAA) [273], from SM (see below for further details). Even though this host metabolic setting in CC is plausible, evidence remains contradictory. Several studies indicate decreased glucose disposal (GD) in CC [274–276], while others do not [277,278]. The discrepancy lies in the methodological problems in GD measurement in cancer as tumour itself drains significant amounts of glucose and contributes significantly to the whole-body GD (on expenses of skeletal muscle and AT). Insulin resistance causes compensatory increase of insulin secretion by beta cells. Long lasting demand for increased secretory response is associated with progressive beta cell failure. In fact, it has been acknowledged that beta cell dysfunction plays a central role in the development of T2DM. Based on similarities between T2DM and CC metabolic profile, it could be expected that beta cell failure may have an important role also in the development and progression of CC. The evidence, however, is inconsistent so far. Decreased insulin levels and lower insulin response to glucose was shown in colorectal and pancreatic cancer [278–280]. Nevertheless, none of the studies related insulin secretion to insulin sensitivity. This strongly limits interpretation of such data, because insulin release is dynamically adapted to changes in peripheral insulin sensitivity. Thus only an assessment of disposition index gives a valid picture of beta cell function [27].

The study of cancer cachexia is limited by several factors. While there is widely accepted physiological background as described above, there is much less agreement on diagnosis and clinical classification of CC. Yet stratification of patients with CC is crucial for setting up clinical trials. Major determinant of all available cachexia scoring systems is the involuntary weight loss [281–284]. Weight loss is a result of imbalance between energy intake and energy expenditure; caused either by increase in RMR (as a consequence of inflammatory response), decrease of energy intake (as a consequence of simple starvation) or both. To make it even more complex, reduction of energy intake leads to hypometabolic adaptation and a decrease in measured RMR. That is why patients with CC show a great variability of measured RMR [285]. Illustrated on our data in Figure 27. Yet RMR is not part of any CC scoring system, probably due to high demand on operation of indirect calorimetry. To diagnose hypermetabolism that

is theoretically a pivotal physiological mechanism of cancer wasting, one would need to match caloric intake and energy expenditure at every individual patient.

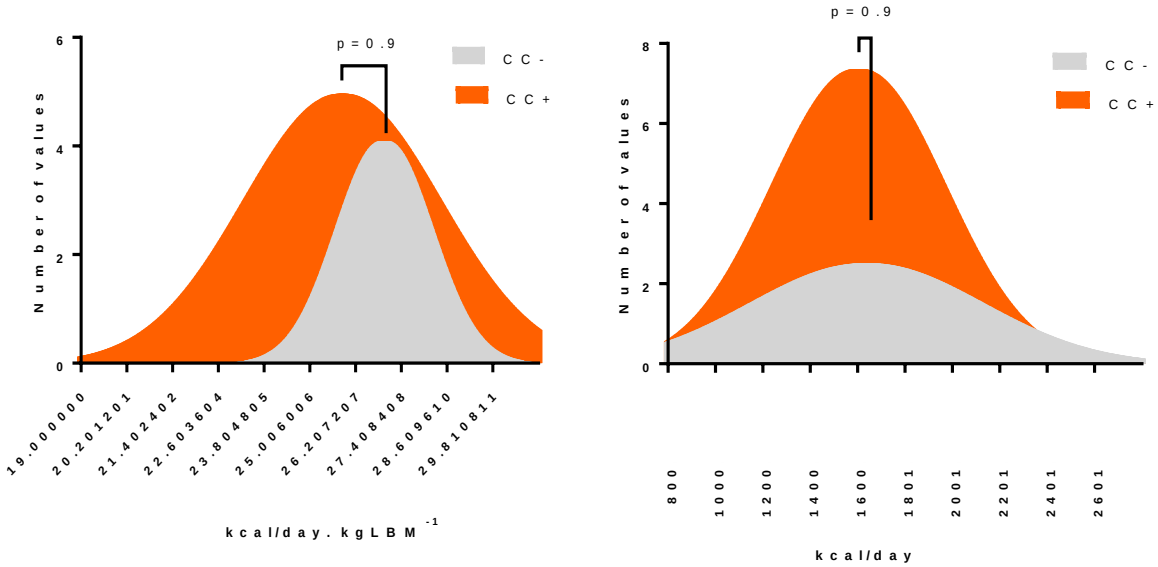


Figure 27 To demonstrate high variability of resting metabolic rate (range 80-150 % predicted RMR). Frequency distribution of measured RMR (Quark RMR, Cosmed, Italy) per kilogram of lean body mass in PDAC patients: 15 without cancer cachexia (CC-) and 33 with cancer cachexia (CC+).

2.3.2. Host amino acid metabolism disturbances

Changes in amino acid metabolism leading to progressive loss of body protein are among the major prognostic factors in cancer cachexia (CC). The unique reservoir of body protein is skeletal muscle, which accounts for up to 40 % of a healthy person's weight. The loss of muscle protein due associated with active tumour growth reflects the tumour's need for nitrogen and carbon supply.

In the skeletal muscle the inhibition of protesynthesis and activation of proteolysis in CC is driven mainly by inflammatory signals. TNF-α inhibits, via multiple mechanisms, mTOR the major proteoanabiolic regulator [286]. This is further augmented by decrease in IGF-1 [287] and insulin [288] signalization. Circulating TNF-α, IL-1, IL-6 a IFN-γ activates transcription factor NF-κB, that in turn downregulates expression of MyoD leading to inhibition of satellite cells differentiation and at the same time upregulates expression factors for ubiquitin proteasome system. In CC higher expression of skeletal muscle myostatin, myogenic proliferation inhibitor, was shown [289]. All these changes result in skeletal muscle myofibrillar degradation and higher availability of AA for anabolic tumour.

In general, circulating AA are decreasing in the course of the inflammatory response which is in contrast with the other substrates [290,291]. It was postulated that the decrease relates to higher disposal and consumption in the tissues. AA being oxidized, sources of gluconeogenic carbon and being precursors for acute phase reactants synthesis [291]. Though generally circulating AA levels decrease in the course of the critical illness, early supplementation with AA show now clear profit. It may actually be associated with worse outcomes [292]. Hypothesis

explaining this paradox is linked with signalling effects of AA in autophagy. This phylogenetically preserved process that can be tracked back to yeasts, consists of capability to degrade intracellular components so that substrates are available for critical cellular processes maintenance. Experimental knock-out of key autophagy related genes (Atg) precludes a transition from placentar to discontinuous intake of nutrients (fasting-postabsorption cycle): knock-out animals for the genes (namely Atg3, 5, 7, 9, 16L1) die soon after the birth [293]. Regulation of autophagy could be simplified to dichotomic response of mTOR (inhibition) vs. AMPK (activation). In this paradigm a decrease in circulating AA is an adaptive symptom of mTOR inhibition and down-regulation of proteosynthesis leading to autophagy response. In this paradigm anorexia and disease-related starvation can be also perceived as a mechanism leading in the end to autophagy and immune response stimulation. Indeed, autophagy plays a pivotal role in an immune response. Not only it enables substrates delivery to proliferating lymphocytes but also it represents a defence against intracellular pathogens. Activation of autophagy response leads to a recruitment of non-immune cells to present antigen epitopes, it regulates apoptosis and it is a back-up programme for cellular death [271]. While autophagy represents a protective mechanism against malignant transformation and propagation respectively, its role in a growing tumour is completely opposite. Up-regulation of autophagy has been described in Ras transformed cancers [294,295], where it is abused during the stages of oxygen or nutrients deprivation [296] to secure energy and free AA [297,298]. It was shown that autophagy determines also chemosensitivity of cancer [299].

The most abundant AA in extracellular space is *glutamine*. Its circulating levels are determined in an postabsrptive state mainly by efflux from tissues capable of *de novo* synthesis: skeletal muscle, lungs, liver and AT [286]. Plasmatic glutamine is readily used as an anaplerotic substrate in many fast-proliferating cancers [300], and dependence on glutamine delivery correlates with invasiveness in some [301]. Alpha keto-glutarate (or 2-oxo-glutarate), an intermediate in the TCA (aka Krebs') cycle, arising in glutaminolysis from glutamine, is either further oxidized or is subject to a reductive carboxylation to give isocitrate and eventually cytosolic acetyl-CoA. This in turn is a major direct precursor for *de novo* fatty acid synthesis [302]. In breast cancer the reductive carboxylation is induced mainly by hypoxia [303], in the early stages when the tumour does not have sufficient vascular infrastructure. The role of glutamine and glutamate independent on anaplerosis was shown also in glioblastoma [304]. Here glutamine synthetized from glutamate (glutamate synthase) becomes precursor for AMP and purines in poorly differentiated cancer cells. Of note, glutamine is a major energy substrate for lymphocytes and therefore in an inflammatory response its availability from peripheral tissues increases [305]. Utilizing glutamine aimed primarily for immune cells is yet another example of cancer abusing host physiological mechanisms to secure its own growth.

Branched chain amino acids have somewhat specific position among AA in an inflammation driven response. As already described in details in previous chapters, insulin resistance is associated with an increase BCAA R_a (proteolysis in skeletal muscle) and a decrease of BCAA R_d (inhibition of BCAA oxidation in SM and AT) [273]. Therefore, BCAA could be expected to increase also in the course of cancer cachexia. However, in spite of a constant presence of insulin resistance, the relationship between elevated BCAA and cancer cachexia is not straightforward.

Both absolute and relative increase in circulating BCAA was shown in tumours of pancreas and oesophagus [306,307], intriguingly different cancer types does not show constant data [273,308]. The interpretation is complicated by the fact that fasting itself leads to an increase in BCAA R_d and plasma elevation. Yet, not in all the studies dietary intake was monitored. So, the hypothesis that elevated circulating BCAA is generally associated with cancer cachexia is based on sporadic data and remains speculative. As the host metabolism cannot be studied independently from the cancer metabolism we decided in the research to focus on the specific tumour type. For several reasons (for details see below) we aimed to study pancreatic ductal adenocarcinoma (PDAC). This tumour, interestingly, was repeatedly shown to be associated with elevated circulating BCAA [306,309]. The attention has recently been drawn to BCAA metabolism in various types of cancer: breast cancer [310], myeloid leukaemia [311], glioblastoma [312], NSCLC [313] and PDAC [306,314]. Specifically in PDAC, BCAA uptake and transamination is diminished [313]. The reason for this effect is unknown but it could be hypothesized that channelling of alpha keto glutarate into the TCA cycle would inhibit transamination and uptake of BCAA in PDAC (depicted in Figure 28). Indeed, PDAC cancer cells has been shown to amplify glutaminolysis to support its growth [309,313]. Besides, glutamine is generally a major energy and nitrogen source for rapidly proliferating cancer cells and serves as an important carrier of nitrogen and carbon from peripheral tissues to the tumour [302]. Accordingly, uptake of glutamine by tumour correlates with cancer invasiveness [301]. Nevertheless, genetic and metabolic details of glutamine and BCAA metabolism in PDAC need to be revisited in order to clarify benefits of diminished BCAA metabolism, or its role in induction of CC. Since glutaminolysis is overamplified in PDAC cells compared to normal cells, possibly due to the expression of alternative enzyme isoforms, study of its role in the context of tumour growth and derangement of host metabolism remains to be clarified.

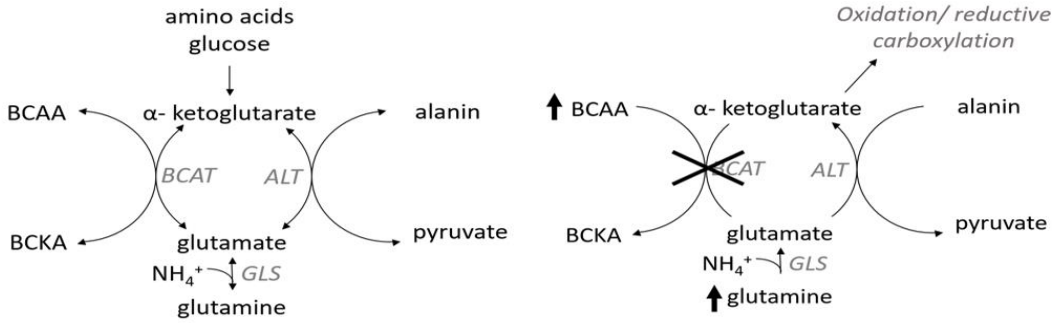


Figure 28 Interrelation between amino acid transamination reactions. Suggested channelling of alpha ketoglutarate into the TCA cycle would inhibit transamination and uptake of BCAA in PDAC.

2.3.3. Physiological model PAMIR

Ongoing PAMIR trial (PAncreatic cancer: Metabolic derangements associated with Insulin Resistance) trial supported by Czech Ministry of Health, no. NV19-01-00101 and EFSD mentorship programme supported by AstraZeneca.

Pancreatic cancer (PDAC, pancreatic ductal adenocarcinoma) incidence has been increasing continuously last decades and the Czech Republic is one of the leading countries within EU in the number of new cases. At the same time, one-year mortality of PDAC reaches almost

90 %. Indeed, PDAC is associated with an early development of cancer cachexia syndrome (CC) resulting in profound substrate handling derangements and adverse clinical outcome. Yet in vivo physiological studies in patients are limited by fast clinical course and many potential confounders such as co-morbidities and chemotherapy.

Here we propose a protocol to address skeletal muscle and adipose tissue insulin resistance (IR) and its association with BCAA metabolism in PDAC hosts. Aim of the pilot study was to assess feasibility of the design and reliability of the proposed physiological model to address whether decrease in an across forearm glucose uptake relates to loss of insulin mediated suppressibility of BCAA forearm efflux, and thus loss of insulin inhibitory function on skeletal muscle proteolysis (increasing BCAA R_a). Consequently, we aim to prove that BCAA R_d is limited by decrease oxidation/anaplerosis for de novo lipogenesis in adipose tissue. Targeting both poles of the distribution hypothesis. As per exploratory aims we also corroborated in tumour sampling and setting up of primary cultures to analyse cancer cells metabolism. We provide a reader with the PAMIR protocol overview and we present individual aims, hypothesis and preliminary results where available.

2.3.3.1. Design of the clinical trial

Cross-sectional study with longitudinal follow-up. Three experimental groups are being formed: *Group 1*, 20 patients diagnosed with PDAC (clinical stage 0-IIb), indicated for primary upfront surgical resection and having CC; *Group 2*, 20 patients diagnosed with PDAC (clinical stage 0-IIb) indicated for primary surgical resection without CC; *Group 3*, 20 control subjects (matched to group 2 in age, BMI, sex, weight-loss). Patients with resectable PDAC are recruited before being enrolled for surgery and any chemotherapy. CC is diagnosed using validated score [269,281]. In a fasting state indirect calorimetry and bioimpedance analysis is performed to assess substrate utilization and body composition. Radial artery and deep brachial vein are cannulated for AV samples. Hyperinsulinaemia (10 and 80 mIU/m^2) euglycemic clamp to assess whole body glucose disposal and across forearm glucose and BCAA uptake is conducted. Brachial artery flow is measured using doppler ultrasound. Peroperative samples of subcutaneous, visceral and peripancreatic fat tissue are obtained for further analysis (see below). For exploratory outcomes tumour tissue is sampled ad patients are enrolled for follow-up visit (the protocol is depicted in Figure 29). Novel method using counter-current electrophoresis with contactless conductivity detection for BCAA sampling use was implemented [219]. The method eventually allows for online point-of-care sampling.

On feasibility. From 24 patients assessed for eligibility over initial 10 months, ten patients with PDAC were recruited and enrolled, eight data-sets available for full data set analysis. Five patients being CC+ (F=2, aged ~76 years, BMI ~24 kg/m^2) vs. four CC- (F=1, aged ~63, BMI ~28 kg/m^2). Baseline characteristics of the sample are summarized in Table 14. CC+ had higher REE 30.5 vs. 24.9 kcal/day/kg FFM (p=0.03), other baseline characteristics were statistically comparable. Of note baseline arterial BCAA negatively correlate with glucose disposal (R -0.75, p=0.02), see Figure 30. Importantly, no adverse consequences of the protocol to patient outcomes were observed, all patients underwent surgery as scheduled.

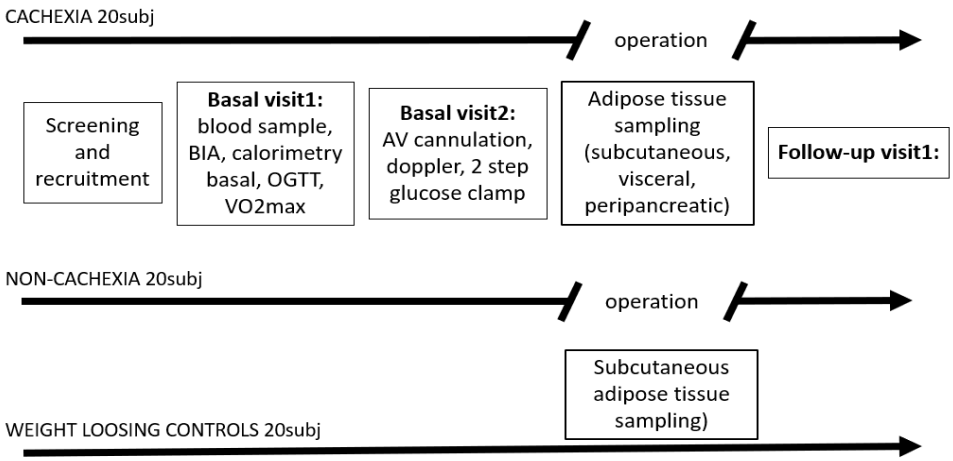


Figure 29 PAMIR clinical protocol overview. The aim is to enrol 40 patients with PDAC (n=20 for every group CC+/CC-) and 20 weight-losing controls. The controls would be recruited from patients undergoing abdominal surgery for non-cancer/non-inflammatory disease (i.e. cholecystolithiasis, benign pancreatic mass) or healthy weight-losing volunteers.

	CC+ (n=5)	CC- (n=5)	*p*-value
Age (years)	76.4 ± 6.0	63.3 ± 13.4	0.09
BMI (kg/m2)	23.9 ± 4.6	27.7 ± 2.5	0.13
Weight (kg)	64.5 ± 8.8	81.4 ± 15.2	0.15
Weight loss/6 months (%)	11 ± 7.0	9.8 ± 8.8	0.8
Lean body mass (kg)	51.6 ± 3.9	68.2 ± 12.6	0.15
REE (kcal/kg FFM/day)	30.5 ± 4.4	24.9 ± 1.2	0.03
CRP (g/L)	21.4 ± 18.6	5.14 ± 3.3	0.15
Alb (g/L)	39.4 ± 4.5	42.9 ± 1.6	0.2
Ca 19-9 (kU/L)	1497 (203; 4938)	1076 (26; 4195)	0.2
Baseline arterial glucose (mmol/L)	7.6 ± 1.8	6.1 ± 1.4	0.2
Steady state arterial glucose (mmol/L)	4.6 ± 0.5	4.6 ± 0.5	0.9
Glucose disposal (mg/kg/min)	5.9 ± 1.3	6.6 ± 2.1	0.9
Glucose disposal (mg/kgFFM/min)	17.1 ± 6.7	16.9 ± 6.7	0.7
Forearm glucose uptake (mg/min/forearm)	4.1 ± 3.1	17.2 ± 19.9	0.14
Arterial BCAA (µmol/L)	318.2 ± 75.0	335.4 ± 104.5	0.9
Forearm BCAA uptake (pmol/min/mL)	46.1 (-30; 167.7)	131 (-653; 1823)	0.25

Table 14 Ten patients with PDAC, 5 with cancer cachexia vs. 5 without cancer cachexia (control). Glucose disposal as per hyperinsulinemic (80 mIU/m^2 insulin) euglycemic clamp. Forearm uptake as per AV differences times plasma flow in mL/min during hyperinsulinemic steady state. Data are mean±SD or SD (min; max), Mann-Whitney test for differences, p<0.05 significant.

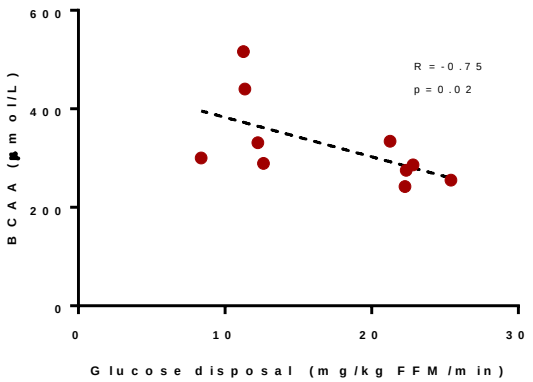


Figure 30 Ten patients with PDAC. Fasting arterial BCAA in the whole sample correlate negatively with glucose disposal; GD as per hyperinsulinemic (80 mIU/m^2 insulin) euglycemic clamp.

2.3.3.2. Skeletal muscle insulin function and BCAA turnover

First, we hypothesized that increased circulating BCAA in cancer cachexia are associated with negative AV forearm BCAA balance and loss of insulin-mediated suppressibility of BCAA forearm efflux. Baseline venous BCAA levels were comparable (CC+ 287.5 vs. CC- 308.5 µg/L), as there was no difference in across forearm BCAA uptake (CC+ 0 [30; 168] vs. CC- 143.6 [-226; 1823] pmol/min/forearm, p=0.4). Results are summarized in Table 15 and Figure 31. On the other hand, when examining BCAA peripheral levels changes during OGTT we found that levels are not suppressed to the same extent in CC+ group (see Figure 33).

Secondly, we aimed to verify that whole body and forearm insulin-stimulated glucose disposal is impaired in cancer cachexia. There was a trend towards decreased forearm glucose uptake in CC+ (CC+ 4.1 ± 3.1 vs. CC- 20.3 ± 21.5 mg/min/forearm, p=0.14) that was not reflected in the whole-body glucose disposal (CC+ 5.9 ± 1.4 vs. CC- 6.9 ± 2.2 mg/kg/min, p=0.68). Results again are summarized in Table 15 and Figure 31. Comparing whole-body vs. regional glucose disposal (see Figure 32) suggests that insulin stimulated glucose disposal in the skeletal muscle may be attenuated in patients with CC whereas at the whole-body level the tumour itself may dispose important amount of glucose.

Change (steady state – baseline)	Cachexia (n=5)	Control (n=5)	*p*-value
AV difference of glucose (mmol/L)	1.1 ± 0.7	1.3 ± 1.0	0.7
Forearm glucose uptake (mg/min/mL)	3.7 ± 2.7	15.9 ± 19.3	0.14
VA difference of BCAA (µmol/L)	-21.2 (-73; 10)	-15 (-43; 15)	0.9
Forearm BCAA uptake (pmol/min/mL)	204.3 (-79; 361)	679.7 (-435; 1691)	0.57

Table 15 relative changes (Δ) in substrate flow from baseline to hyperinsulinemic state. Ten patients with PDAC, 5 cancer cachexia vs. 5 without cancer cachexia (control). Comparisons in between Δ changes baseline vs. insulin stimulated (insulin dose 80 mIU/m^2 body surface) state. Data are mean±SD or SD (min; max), Mann-Whitney test for differences, p<0.05 significant.

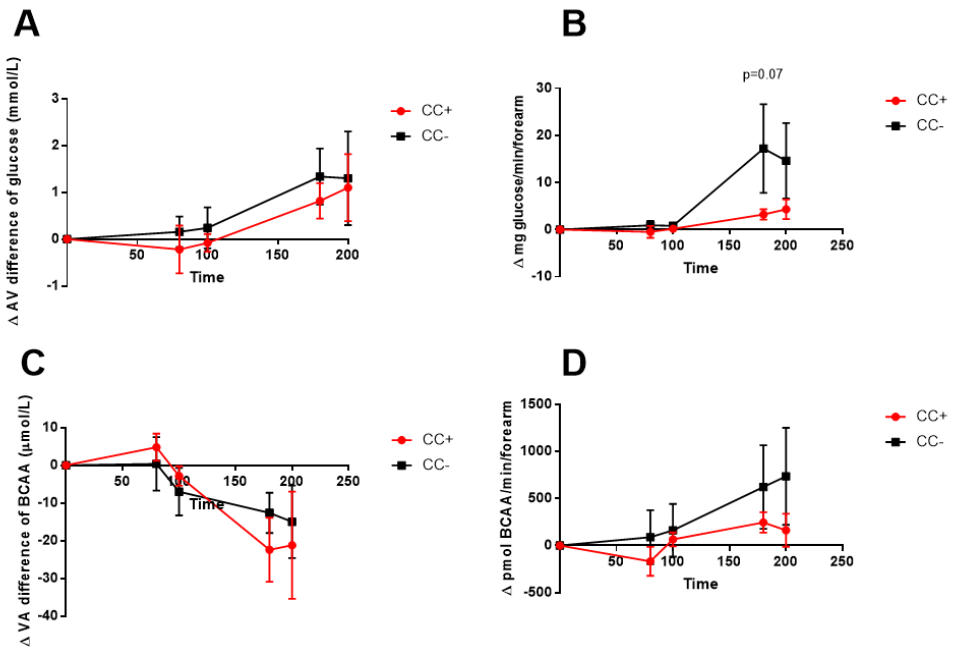


Figure 31 Changes in glucose and BCAA AV across forearm flux in 10 pts. with PDAC, 5 cancer cachexia (CC+) vs. 5 without cancer cachexia (CC-) during hyperinsulinemic (10 and 80 mIU/m^2) euglycemic clamp. A: changes in AV difference of glucose. B: forearm uptake of glucose. C: changes in AV difference if BCAA. D: forearm uptake of BCAA. Neither of changes reached significant time vs. group interaction in TW ANOVA. Data are mean±SEM.

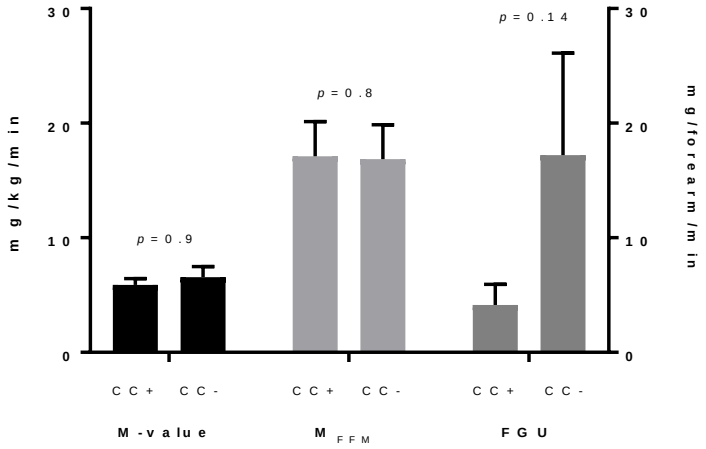


Figure 32 Comparison of three indices of insulin stimulated glucose disposal in 10 pts. with PDAC, 5 cancer cachexia (CC+) vs. 5 without cancer cachexia (CC-). M-value is space corrected glucose disposal in mg/kg/min, M_{FFM} is glucose disposal per kg fat free mass, FGU is forearm glucose uptake (mg/min/forearm). All derived from steady state (80 mIU/m^2 insulin) glucose clamp. Data are mean±SD, Mann-Whitney test for differences, p<0.05 significant.

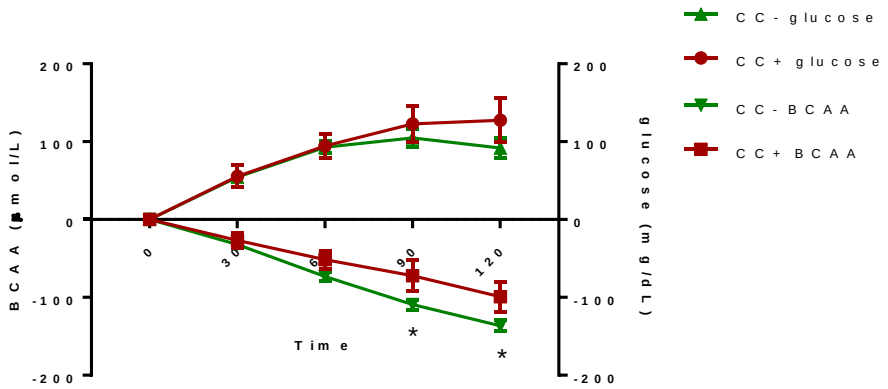


Figure 33 Increments in plasma glucose and suppression of BCAA levels during 75 g glucose OGTT in patients with pancreatic cancer having (CC+, n=6) or not (CC-, n=19) cancer cachexia. p<0.004 for time and group interaction in two-way ANOVA, * p<0.05 for Bonferroni post-test. Intermin analysis of the PAMIR project.

2.3.3.3. Lipolysis study

Dysregulation of the host lipid metabolism is tightly connected to CC development. Indeed, tumour needs fatty acids to build up membrane structures and accelerated fatty acid cycling was shown to support malignance [315]. Lipids can be provided by AT, which is one of the key players in substrate distribution and energy homeostasis. AT appears to be more sensitive to tumour-driven signals as the loss of AT occurs earlier than the loss of muscle [316]. Several pieces of evidence suggest that AT tissue wasting in CC is dependent mostly on exaggeration of stimulated AT lipolysis driven by the tumour [317]. First, isolated subcutaneous adipocytes from patients with CC or tumour bearing animals exert higher lipolytic response to catecholamines and natriuretic peptides due to higher expression and activity of HSL and ATGL [318]. This alteration of lipase expression is clearly driven by tumour-derived signals as the lipolysis rate was not increased in vitro differentiated adipocytes from CC patients cultivated in tumour free

environment [319]. Major signals driving the increase energy demand in CC are catecholamines, but lipolysis can also be activated by cortisol, natriuretic peptides, and proinflammatory cytokines, while insulin acts to inhibit lipolysis. Paradoxically, insulin induced lipogenesis, another pathway decisive of the amount of stored lipids within AT, does not appear to be decreased but rather increased by CC, which may (together with increased lipolysis) create a basis for a futile energy cycling [317,320].

Therapeutic blockade of AT lipolysis, which was also suggested as a possible treatment of IR, could also prevent AT wasting. Out of components used for IR treatment and/or affecting AT lipolysis metformin and beta blockers were of research interest for us. The effect of metformin on AT lipolysis remains rather controversial, as studies showing both inhibitory and stimulatory action of metformin on AT lipolysis have been published [321]. Importantly, metformin was found to decrease the tumour growth and aggressiveness and was already used in preclinical studies in certain type of cancers [322]. In addition, metformin activates AMP activated protein kinase (AMPK) and thus could block the CC associated AMPK inactivation linked with energy wasting in adipocytes in tumour bearing animals [323]. Contrary, beta blockers has been known to worsen glucose tolerance [324]. On the other BB antagonise higher catecholamine drive in CC and were already suggested as a possible therapeutic target to spare the host AT reserves [325].

Majority of data on the mechanisms of AT metabolism derangements in CC was until now based on the analysis of subcutaneous AT while tumours affect firstly the adjacent visceral (VAT) depots. Moreover, the regulation of lipolysis and lipogenesis differ in even healthy visceral AT when compared to subcutaneous AT, therefore the analysis of CC effect on VAT is a necessary step leading to deciphering the early phases of tumour driven reprogramming of host metabolism.

As per primary outcome (BCAA kinetics) we hypothesize that there is decreased capacity of SAT to utilize BCAA for DNL in cancer cachexia; leading to decrease in BCAA R_d and elevated circulating BCAA. To this end we plan use ^{13}C-leucine incorporation into triglycerides (measured by GCSM) to assess to which extent BCAA carbon skeleton is utilized for DNL in AT explants. The protocol is yet to be optimized. Besides, we plan to analyse mRNA level of key metabolic genes including BCAA by qRT-PCR. The genes that will be found to be differentially expressed between subjects with and without CC are presumably those that are the most important for tumour driven reprogramming.

The hypothesis related to secondary outcome (i.e. insulin function in AT) is that there is decreased insulin-mediated suppression SAT and visceral adipose tissue (VAT) lipolysis in cancer cachexia. And that metformin and beta-blockers can attenuate the pathologically raised lipolysis in cancer cachexia.

The treatment inhibiting fatty acid release from adipocytes could eventually limit tumour-induced AT wasting. As fatty acids are oxidized in CC affected adipocytes [326,327], switch to oxidation of other substrates as BCAA could be beneficial as it would spare FFA for the reesterification. Indeed, BCAA oxidation in adipocytes is decreased in IR individuals [188].

To this end we are collecting samples and analysing lipolytic and secretory activity of *ex vivo* AT explants are being washed to remove damaged cells, then divided into several 50-100 mg homogenous pieces, and cultivated in various conditions as described below.

For analysis of secreted molecules, AT explants in duplicate will be cultivated in Krebs Ringer buffer + 2 % BSA + 1 g -L glucose (KRB) for 4 hours in the presence of absence of 1 nM insulin and ^{13}C-leucine, then the conditioned media are centrifuged to remove any cellular components and frozen until further analysis of cytokine levels by Luminex technology. For lipolysis experiments, AT are being pre-treated for 4 hours with 500 µM metformin (MET), 1 µM metoprolol (BB) or DMSO and then stimulated to lipolysis by 0.1 µM isoproterenol (ISO) in the presence or absence of 0.1-100 nM insulin (INS) for 2 additional hours (in the presence of drugs used for pre-treatment). Conditioned media (CM) are used for the assessment of glycerol, an index of lipolysis, and secreted proteins, the tissue explants will be used for further analyses or assessment of protein content.

We also aim for application of media conditioned by both cancer cell lines and primary tumours on AT explants and differentiated adipocytes to mimic the tumour milieu that affects AT in vivo.

To summarize preliminary ***results***. Basal lipolysis in SAT/VAT was influenced neither by MET nor BB. In both SAT and VAT isoproterenol stimulated lipolysis was inhibited by BB (SAT BB ISO 147.9 ± 56.15 µM vs. SAT CO 270.5 ± 64.77 µM, *p*=0.0103; VAT BB ISO 97.93 ± 24.11 µM vs. VAT CO ISO 268.8 ± 99.56 µM, *p*=0,0042). Specifically, in SAT insulin mediated inhibition of lipolysis was potentiated by BB (SAT BB INS 0.1688 ± 0.1653 µM vs. SAT BB INS CO 0.4618 ± 0.2287 µM, *p*=0,0225). No such changes were observed in MET pre-treated explants (Figures 34 and 35).

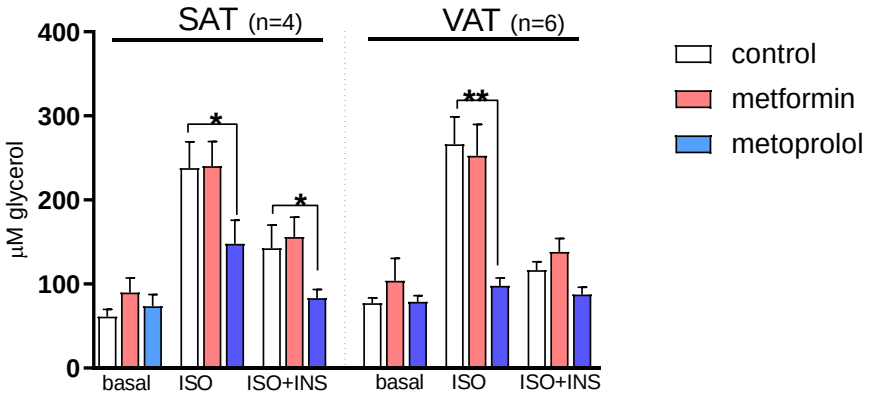


Figure 34 To show different metabolic characteristics of SAT and VAT. Ex vivo lipolysis in SAT and VAT in patients with PDAC: AT explants were pre-treated with vehicle or 1 µM metformin or 0.1 µM metoprolol for 1 hour and then the explants were exposed to 0.1 µM isoproterenol (ISO) in the presence/absence of 1 nM insulin (INS) for 2 hours.

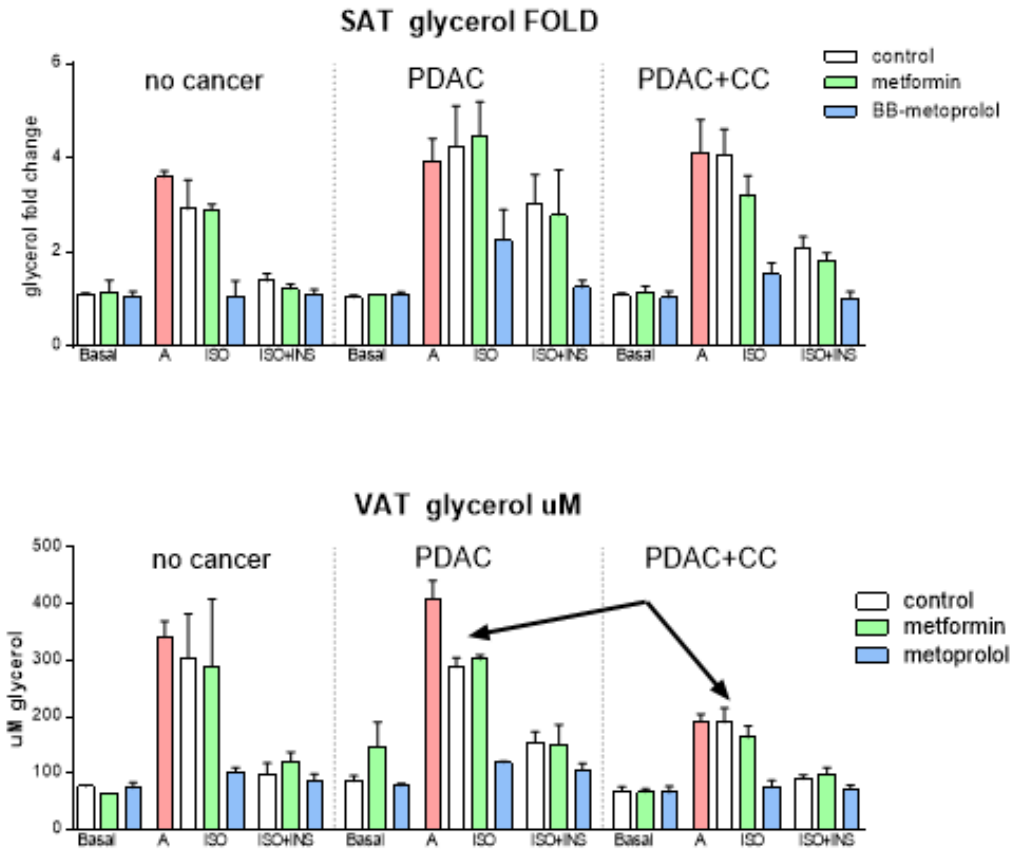


Figure 35 Comparison of VAT and SAT lipolytic properties in patients with PDAC (CC+ = 5, CC- = 4) and non-cancer controls (benign pancreatic mass, n = 3). Results suggesting lower stimulated lipolysis in VAT only in patients with PDAC having CC. No formal statistics performed.

2.3.3.4. Further perspectives of PAMIR project

Detailed metabolic phenotyping of the cancer host and host-tumour cross-talk would enable identification of specific metabolic signatures and oncometabolites that would reflect prevailing substrate dependence of the tumour at the whole-body level. This could justify targeted dietary, physical exercise and pharmacological treatment, i.e. approaches that deprive tumour growth and spare the host reserves. All these combined and/or used sequentially according to metabolic phenotype. Tumour is being sampled in the trial to set up short term *ex vivo* cultures that are optimal models to study metabolic arrangements specific to individual patients. Methodology of the tumour aim is beyond the scope of the works. In brief, we plan to employ metabolomics to analyse steady state levels of the metabolites as well as stable isotope tracing of BCAA, glutamine and glucose to quantify the respective metabolic fluxes in the organoids. Final metabolic phenotype is a result of the genetic and biochemical background, so expression of the involved genes would be considered. The functional analysis will be therefore accompanied with gene expression analysis, covering mitochondrial enzymes involved in BCAA/glutamine/alpha ketoglutarate-related metabolism. The results will be related to the results of whole-body phenotype, insulin resistance and presence/absence of CC. *Ex vivo* studies correlated with host metabolic changes would lead to further description of host – tumour cross talk, major whole-body substrate depen-

dence and targeted interventions with specific pharmacological inhibition of transport of major substrates that would enable identification of new therapeutic targets. *Ex vivo* tumour study would enable us to use modulation of BCAA/glutamine availability in a cancer cells culture medium as a proof-of-concept of glutaminolysis modulating BCAA utilization. These findings could translate fast to clinical practice in dietary supportive treatment of cancer patients. By modulating macroorganism's IR it would be possible to modulate also substrate availability for the tumour anabolic compartment. Novel treatment targets as well as translation of known therapeutic approaches from T2DM associated insulin resistance could be used to attenuate substrate depletion in AT and SM. The slower the CC wasting would be, the longer the anti-cancer therapy tolerance and the better the quality of life. Taken all together, results of the PAMIR project could yield unique information of the cancer host metabolism that could provide physiological basis for an emerging concept of cancer host targeted treatment. The results could have immediate impact on clinical practice and will allow for development of novel therapeutic approaches including pharmacological agents and nutritional intervention.

2.4. CONCLUSION

Insulin resistance, a decreased sensitivity and responsiveness of tissues to metabolic effects of insulin, is a complex phenomenon of substrate metabolism changes. As such it cannot be perceived as and limited to impairment in glucose homeostasis, though glucose tolerance is used for convenience reasons as a major physiological determinant for its description. Insulin resistance is a hallmark pathology in natural history of diabetes evolution, it has been well described at different tissue level and targeted by several treatment approaches including drugs. On the other hand, the very same phenomenon of insulin resistance is an adaptive response that has been evolutionarily well preserved. It can be tracked back to, at least, chordates and maybe even earlier. Once there was the insulin with its functions, there was signal-effect sensitivity. This paradox leads to several questions. Has the physiology, i.e. substrate redistribution, remained the same while its relevance in different environment/social conditions has changed? We live in a completely different environment, majority of world population is nowadays exposed to abundance of easily digestible hyperpalatable dietary substrates. Stress responses, once acute stimuli, becoming chronic and more frequent. Or, are there different clinical scenarios, disease and adaptation, and we lack appropriate physiological models for description of the phenomenon?

We tried to summarize current knowledge of insulin physiology related to fasting-refeeding cycle as well as related to inflammation response. Alas, what else happens in metabolic tissues when flooded by abundance of substrates then inflammatory response? And what else happens in muscles during physical exercise? We present physiological models to describe insulin resistance, we provide an overview of our own expertise in the field. We provide a reader with an insight in a very complex relationship between circulating BCAA and insulin function. After a background that introduces where BCAA are positioned in the intermediary metabolism, we showed in various model's mechanisms influencing BCAA R_a/R_d. We proposed

that elevated BCAA reflects insulin resistance and loss of insulin mediated suppression of proteolysis in major metabolic organs. And it is precisely elevated circulating BCAA that further perpetuate insulin resistance by mechanisms of anaplerotic stress. Though the hypothesis remains to be further verified, we show evidence and open ways how to prove it.

2.5. FUTURE PERSPECTIVES AND CLINICAL IMPLICATIONS

Insulin resistance associated diseases, namely diabetes and cancer, are global challenges. These non-communicable diseases together represent the major mortality and socio-economic burden. Environmental factors contribute significantly to this. The major challenge facing diabetes research today lies in redirecting efforts towards prevention and early treatment. For that purpose, we need to fully understand and identify these environmental risk factors. Additionally, there needs to be further study into the role of specific nutrients. We also need to find early markers that can indicate a patient is entering the "diabetes evolution pathway". Furthermore, methods to identify the prevailing pathophysiology (i.e., IR, beta cell dysfunction, metabolic flexibility, incretin action, etc.) as well as subsequent pathophysiology-based treatments would require considerably more investigation.

Signal role of nutrients, in the context namely BCAA, has been known for decades. BCAA are exclusive among other AA as they are not cleared primarily by the liver and they serve as important nutrient derived signals. We showed in a long-term clinical trial that supplementation with BCAA in lean and healthy volunteers may worsen insulin resistance when lipogenesis is not sufficiently upregulated [42]. On the other hand, we showed that supplementation with BCAA has insulinogenic potential. When exploring further we provided the evidence that these effect are mediated partially by incretins [165]. Beta cell failure to compensate for insulin resistance in metabolic organs is pathological *conditio sine qua non* of diabetes development. Dietary manipulation aimed to an increase insulin secretion may represent a promising approach to compensate glycaemias in patients with diabetes. On the other hand, increasing BCAA dietary content in healthy lean persons may bring a risk of insulin sensitivity deterioration.

Food-derived components mechanistically linked to diabetes development are yet to be described in details. The diet creates a selective pressure to intestinal microbiota: as it provides substrates for growth it changes the metabolic end-products respectively. These postbiotics in turn are among major signalling molecules for the host metabolism. Interventions aimed to shift the gut metabolite profile may represent a promising treatment approach in metabolic syndrome. In this regard we are awaiting our results on effects of long-term dietary inulin intervention on intestinal microbiome and its metabolic profile as well as the host metabolic health. Personalized dietary interventions according to individual fermentation capacity could be a key to the successful non-pharmacological intervention in new onset diabetes.

Insulin resistance phenomenon is yet again in the spotlight in the initiation and propagation of cancer. There is a bi-directional relationship between IR and cancer. IR is associated with an increased risk of several cancer types, whereas when cancer evolve, cancer cells take

advantage of this evolutionary well-preserved mechanisms of substrate redistribution away from metabolic organs to fuel rapidly proliferating immune cells. Metabolic reprogramming of the host metabolism gets significant scientific attention and it is conceivable that approaches depriving tumour growth and sparing the host reserves would eventually get among oncology treatment strategies. Taken all together, results of the project could yield unique information of the cancer host metabolism that could provide pathophysiological basis for an emerging concept of cancer host targeted treatment. The results could have immediate impact on clinical practice and will allow for development of novel therapeutic approaches including pharmacological agents and nutritional approaches.

Where else are we heading? Only recently, the importance of gut and even intra-tumour microbiome in the modulation of host immunity became apparent but the underlying mechanisms are far from being fully understood. Combing our expertise from microbiota and cancer related research could yield unique information about the crosstalk between host immune system and tumour-related microbiome and provide basis for an emerging concept of microbiome-based treatment.

Research on plant-based diets continues one way towards its safety profile [328,329] and the other towards intestinal health that has been briefly discussed above. Indeed, plant-based diets gain significant attention both for its health and environmental impacts. The nutritional epidemiology is based on cohort studies and setting-up one in the context of Czech Republic is a major challenge our group face today.

REFERENCES

1. Suh, S. & Kim, K. W. Diabetes and cancer: Cancer should be screened in routine diabetes assessment. *Diabetes and Metabolism Journal* **43**, 733–743 (2019).
2. Godsland, I. F., Lecamwasam, K. & Johnston, D. G. A systematic evaluation of the insulin resistance syndrome as an independent risk factor for cardiovascular disease mortality and derivation of a clinical index. *Metabolism* **60**, 1442–1448 (2011).
3. Zimmet, P., Alberti, K. G. & Shaw, J. Global and societal implications of the diabetes epidemic. *Nature* **414**, 782–787 (2001).
4. IDF Diabetes Atlas 9th Edition. IDF Diabetes Atlas 9th edition 2019. *2019* Available at: https://diabetesatlas.org/en/. (Accessed: 29th May 2020)
5. Benoit, S. R., Hora, I., Albright, A. L. & Gregg, E. W. New directions in incidence and prevalence of diagnosed diabetes in the USA. *BMJ Open Diabetes Res. Care* **7**, e000657 (2019).
6. Zvolsky, M. Činnost oboru diabetologie, péče o diabetiky v roce 2013. *ÚZIS* **02/15**, (2015).
7. Leal, J., Gray, A. M. & Clarke, P. M. Development of life-expectancy tables for people with type 2 diabetes. *Eur. Heart J.* **30**, 834–9 (2009).
8. Cosentino, F. *et al.* 2019 ESC Guidelines on diabetes, pre-diabetes, and cardiovascular diseases developed in collaboration with the EASD. *Eur. Heart J.* **41**, 255–323 (2020).
9. Karamanou, M. Milestones in the history of diabetes mellitus: The main contributors. *World J. Diabetes* **7**, 1 (2016).
10. Thorens, B. & Mueckler, M. Glucose transporters in the 21st Century. *Am. J. Physiol. Endocrinol. Metab.* **298**, E141-5 (2010).
11. Farfari, S., Schulz, V., Corkey, B. & Prentki, M. Glucose-Regulated Anaplerosis and Cataplerosis in Pancreatic $^{N}_{L}$-Cells Possible Implication of a Pyruvate/Citrate Shuttle in Insulin Secretion. *Diabetes* **49**, (2000).
12. Unger, R. H. Glucagon and the insulin: glucagon ratio in diabetes and other catabolic illnesses. *Diabetes* **20**, 834–838 (1971).
13. Djiogue, S. *et al.* Insulin resistance and cancer: the role of insulin and IGFs. *Endocr. Relat. Cancer* **20**, R1–R17 (2013).
14. Kahn, C. R. & Goldfine, A. B. Molecular determinants of insulin action. *J. Diabetes Complications* **7**, 92–105 (1993).
15. Turner, N. Mitochondrial Metabolism and Insulin Action. in *Type 2 Diabetes* (InTech, 2013). doi:10.5772/56449
16. Van Der Vusse, G. J. & Reneman, R. S. Lipid Metabolism in Muscle. in *Comprehensive Physiology* (John Wiley & Sons, Inc., 2011). doi:10.1002/cphy.cp120121
17. Bonadonna, R. C., Saccomani, M. P., Cobelli, C. & DeFronzo, R. A. Effect of insulin on system A amino acid transport in human skeletal muscle. *J. Clin. Invest.* **91**, 514–21 (1993).
18. Timmerman, K. L. *et al.* Insulin Stimulates Human Skeletal Muscle Protein Synthesis via an Indirect Mechanism Involving Endothelial-Dependent Vasodilation and Mammalian Target of Rapamycin Complex 1 Signaling. *J. Clin. Endocrinol. Metab.* **95**, 3848–3857 (2010).
19. Das, U. N. A defect in Δ6 and Δ5 desaturases may be a factor in the initiation and progression of insulin resistance, the metabolic syndrome and ischemic heart disease in South Asians. *Lipids Health Dis.* **9**, 130 (2010).
20. Murray, R. K. (Robert K. *Harper's illustrated biochemistry*. (McGraw-Hill Medical, 2012).
21. Joseph, S. E. *et al.* Renal glucose production compensates for the liver during the anhepatic phase of liver transplantation. *Diabetes* **49**, 450–456 (2000).
22. Yang, J. *et al.* Leucine regulation of glucokinase and ATP synthase sensitizes glucose-induced insulin secretion in pancreatic beta-cells. *Diabetes* **55**, 193–201 (2006).
23. DeFronzo, R. A., Ferrannini, E., Hendler, R., Felig, P. & Wahren, J. Regulation of Splanchnic and Peripheral Glucose Uptake by Insulin and Hyperglycemia in Man. *Diabetes* **32**, (1983).
24. Defronzo, R. A. Banting Lecture. From the triumvirate to the ominous octet: a new paradigm for the treatment of type 2 diabetes mellitus. *Diabetes* **58**, 773–95 (2009).
25. Porcellati, F., Lucidi, P., Bolli, G. B. & Fanelli, C. G. Thirty years of research on the dawn phenomenon: Lessons to optimize blood glucose control in diabetes. *Diabetes Care* **36**, 3860–3862 (2013).

26. Ahrén, B. & Pacini, G. Importance of quantifying insulin secretion in relation to insulin sensitivity to accurately assess beta cell function in clinical studies. *Eur. J. Endocrinol.* **150**, 97–104 (2004).

27. Ahren, B. & Pacini, G. Islet adaptation to insulin resistance: mechanisms and implications for intervention. *Diabetes, Obes. Metab.* **7**, 2–8 (2005).

28. Kahn, S. E. *et al.* Quantification of the relationship between insulin sensitivity and beta-cell function in human subjects. Evidence for a hyperbolic function. *Diabetes* **42**, 1663–1672 (1993).

29. Chen, T. *et al.* Glycemic variability in relation to oral disposition index in the subjects with different stages of glucose tolerance. *Diabetol. Metab. Syndr.* **5**, 38 (2013).

30. Himsworth, H. P. Management of diabetes mellitus. *Br. Med. J.* **2**, 188–190 (1936).

31. Pacini, G. & Mari, A. Methods for clinical assessment of insulin sensitivity and beta-cell function. *Best Pract. Res. Clin. Endocrinol. Metab.* **17**, 305–22 (2003).

32. Ekberg, K. *et al.* Contributions by kidney and liver to glucose production in the postabsorptive state and after 60 h of fasting. *Diabetes* **48**, 292–298 (1999).

33. Wallace, T. M., Levy, J. C. & Matthews, D. R. Use and abuse of HOMA modeling. *Diabetes Care* **27**, 1487–1495 (2004).

34. Hermans, M. P., Levy, J. C., Morris, R. J. & Turner, R. C. Comparison of insulin sensitivity tests across a range of glucose tolerance from normal to diabetes. *Diabetologia* **42**, 678–687 (1999).

35. Bequette, B. W. Glucose clamp algorithms and insulin time-action profiles. in *Journal of Diabetes Science and Technology* **3**, 1005–1013 (SAGE Publications Inc., 2009).

36. DeFronzo, R. A., Tobin, J. D. & Andres, R. Glucose clamp technique: a method for quantifying insulin secretion and resistance. *Am J Physiol* **237**, E214–E223 (1979).

37. Pozefsky, T., Andres, R., Coleman, D. & Swerdloff, R. S. Manual feedback technique for the control of blood glucose concentration. (1966).

38. Laakso, M., Edelman, S. V., Brechtel, G. & Baron, A. D. Decreased effect of insulin to stimulate skeletal muscle blood flow in obese man: A novel mechanism for insulin resistance. *J. Clin. Invest.* **85**, 1844–1852 (1990).

39. Le, D. S. N. T., Brookshire, T., Krakoff, J. & Bunt, J. C. Repeatability and reproducibility of the hyperinsulinemic-euglycemic clamp and the tracer dilution technique in a controlled inpatient setting. *Metabolism.* **58**, 304–310 (2009).

40. L. Bokemark, A. Frödén, S. Attvall,. The euglycemic hyperinsulinemic clamp examination: variability and reproducibility. *Scand. J. Clin. Lab. Invest.* **60**, 27–36 (2000).

41. Yki-Jarvinen, H., Young, A. A., Lamkin, C. & Foley, J. E. Kinetics of glucose disposal in whole body and across the forearm in man. *J. Clin. Invest.* **79**, 1713–1719 (1987).

42. Gojda, J. *et al.* Chronic dietary exposure to branched chain amino acids impairs glucose disposal in vegans but not in omnivores. *Eur. J. Clin. Nutr.* **71**, (2017).

43. Ferrannini, E. *et al.* Insulin resistance and hypersecretion in obesity. *J. Clin. Invest.* **100**, 1166–1173 (1997).

44. Pratipanawatr, W. *et al.* Skeletal Muscle Insulin Resistance in Normoglycemic Subjects with a Strong Family History of Type 2 Diabetes is Associated with Decreased Insulin-Stimulated Insulin Receptor Substrate-1 Tyrosine Phosphorylation. *Diabetes* **50**, 2572–2578 (2001).

45. Fink, R. I., Kolterman, O. G., Griffin, J. & Olefsky, J. M. Mechanisms of insulin resistance in aging. *J. Clin. Invest.* **71**, 1523–1535 (1983).

46. Doberne, L., Greenfield, M. S., Rosenthal, M., Widstrom, A. & Reaven, G. Effect of Variations in Basal Plasma Glucose Concentration on Glucose Utilization (M) and Metabolic Clearance (MCR) Rates During Insulin Clamp Studies in Patients with Non-insulin-dependent Diabetes Mellitus. *Diabetes* **31**, 396–400 (1982).

47. Bergman, R. N., Phillips, L. S. & Cobelli, C. Physiologic evaluation of factors controlling glucose tolerance in man: measurement of insulin sensitivity and beta-cell glucose sensitivity from the response to intravenous glucose. *J Clin Invest* **68**, 1456–1467 (1981).

48. Nauck, M. A., Blietz, R. W. & Qualmann, C. Comparison of hyperinsulinaemic clamp experiments using venous, 'arterialized' venous or capillary euglycaemia. *Clin. Physiol.* **16**, 589–602 (1996).

49. Ferrannini, E., Barrett, E. J., Bevilacqua, S. & DeFronzo, R. A. Effect of fatty acids on glucose production and utilization in man. *J. Clin. Invest.* **72**, 1737–47 (1983).

50. Bergman, R. N., Finegood, D. T. & Ader, M. Assessment of insulin sensitivity in vivo. *Endocr. Rev.* **6**, 45–86 (1985).

51. Kaga, H. *et al.* Higher C-Peptide Level During Glucose Clamp Is Associated With Muscle Insulin Resistance in Nonobese Japanese Men. *J. Endocr. Soc.* **3**, 1847–1857 (2019).

52. Elahi, D. *et al.* Feedback Inhibition of Insulin Secretion by Insulin: Relation to the Hyperinsulinemia of Obesity. *N. Engl. J. Med.* **306**, 1196–1202 (1982).

53. Muscelli, E. *et al.* Lack of insulin inhibition on insulin secretion in non-diabetic morbidly obese patients. *Int. J. Obes.* **25**, 798–804 (2001).

54. Wolfe, R. & Chinkes, D. *Isotope Tracers in Metabolic Research: Principles and Practice of Kinetic Analysis, 2nd Edition | Wiley*. (2004).

55. Lucidi, P. *et al.* Mechanisms of insulin resistance after insulin-induced hypoglycemia in humans: The role of lipolysis. *Diabetes* **59**, 1349–1357 (2010).

56. Duska, F., Andel, M., Kubena, A. & Macdonald, I. A. Effects of acute starvation on insulin resistance in obese patients with and without type 2 diabetes mellitus. *Clin. Nutr.* **24**, 1056–1064 (2005).

57. Boden, G., Chen, X., Ruiz, J., White, J. V & Rossetti, L. Mechanisms of fatty acid-induced inhibition of glucose uptake. *J. Clin. Invest.* **93**, 2438–46 (1994).

58. Gojda, J. *et al.* Role of percutaneous biopsy of skeletal muscle in diabetes research. Methodological summary | Postavení perkutánní biopsie kosterního svalu v diabetologickém výzkumu. Metodologický přehled. *Diabetol. Metab. Endokrinol. Vyziv.* **18**, (2015).

59. Kahn, C. R. *et al.* Insulin resistance, insulin insensitivity, and insulin unresponsiveness: a necessary distinction. *Metabolism.* **27**, 1893–902 (1978).

60. Williamson, J. R. & Krebs, H. A. Acetoacetate as fuel of respiration in the perfused rat heart. *Biochem. J.* **80**, 540–7 (1961).

61. Randle, P. J. Regulatory interactions between lipids and carbohydrates: the glucose fatty acid cycle after 35 years. *Diabetes. Metab. Rev.* **14**, 263–83 (1998).

62. Felig, P., Marliss, E. & Cahill GF, J. Plasma amino acid levels and insulin secretion in obesity. *N Engl J Med* **281**, 811–816 (1969).

63. Krebs, M. *et al.* Mechanism of amino acid-induced skeletal muscle insulin resistance in humans. *Diabetes* **51**, 599–605 (2002).

64. Bernard, C. Leçons sur les propriétés physiologiques et les altérations pathologiques des liquides de l'organisme. (1859).

65. Benedict, F., Goodall, H., Ash, J. & Langfeld, H. A study of prolonged fasting. (1915).

66. Unger, R. H., Eisentraut, A. M. & Madison, L. L. THE EFFECTS OF TOTAL STARVATION UPON THE LEVELS OF CIRCULATING GLUCAGON AND INSULIN IN MAN. *J Clin Invest* **42**, 1031–1039 (1963).

67. Nilsson, L. H., Fürst, P. & Hultman, E. Carbohydrate metabolism of the liver in normal man under varying dietary conditions. *Scand. J. Clin. Lab. Invest.* **32**, 331–337 (1973).

68. Newman, W. P. & Brodows, R. G. Insulin action during acute starvation: Evidence for selective insulin resistance in normal man. *Metabolism* **32**, 590–596 (1983).

69. Schauder, P., Herbertz, L. & Langenbeck, U. Serum branched chain amino and keto acid response to fasting in humans. *Metabolism* **34**, 58–61 (1985).

70. Nair, K. S., Woolf, P. D., Welle, S. L. & Matthews, D. E. Leucine, glucose, and energy metabolism after 3 days of fasting in healthy human subjects. *Am. J. Clin. Nutr.* **46**, 557–62 (1987).

71. Felig, P., Owen, O. E., Wahren, J. & Cahill, G. F. Amino acid metabolism during prolonged starvation. *J. Clin. Invest.* **48**, 584–594 (1969).

72. Adibi, S. A., Peterson, J. A. & Krzysik, B. A. Modulation of leucine transaminase activity by dietary means. *Am. J. Physiol.* **228**, 432–435 (1975).

73. Cori, C. F. & Cori, G. T. Carbohydrate metabolism. *Annu. Rev. Biochem.* **15**, 193–218 (1946).

74. ALLEN, F. M. PROLONGD FASTING IN DIABETES. *Am. J. Med. Sci.* **150**, 480–484 (1915).

75. Jackson, R. A. *et al.* Differences between metabolic responses to fasting in obese diabetic and obese nondiabetic subjects. *Diabetes* **20**, 214–227 (1971).

76. Belfiore, F., Iannello, S., Rabuazzo, A. M. & Campione, R. Metabolic effects of short-term fasting in obese hyperglycaemic humans and mice. *Int. J. Obes.* **11**, 631–640 (1987).

77. Evert, A. B. *et al.* Nutrition therapy for adults with diabetes or prediabetes: A consensus report. *Diabetes Care* **42**, 731–754 (2019).

78. Bernard, C., Bert, P., Dastre, A. & Vulpian, A. *Leçons sur les phénomènes de la vie communs aux animaux et aux végétaux ... Leçons sur les phénomènes de la vie communs aux animaux et aux végétaux ...* (2011). doi:10.5962/bhl.title.44802

79. Marik, P. E. & Bellomo, R. Stress hyperglycemia: An essential survival response! *Crit. Care* **17**, 305 (2013).

80. Van den Berghe, G. *et al.* Intensive Insulin Therapy in Critically Ill Patients. *N. Engl. J. Med.* **345**, 1359–1367 (2001).

81. Finfer, S. *et al.* Intensive versus Conventional Glucose Control in Critically Ill Patients. *N. Engl. J. Med.* **360**, 1283–1297 (2009).

82. Gunst, J., De Bruyn, A. & Van den Berghe, G. Glucose control in the ICU. *Curr. Opin. Anaesthesiol.* **32**, 156–162 (2019).

83. Waldauf, P. *et al.* Functional electrical stimulation-assisted cycle ergometry in the critically ill: Protocol for a randomized controlled trial. *Trials* **20**, (2019).

84. Soeters, M. R. & Soeters, P. B. The evolutionary benefit of insulin resistance. (2012). doi:10.1016/j.clnu.2012.05.011

85. Skyler, J. S. *et al.* Differentiation of diabetes by pathophysiology, natural history, and prognosis. *Diabetes* **66**, 241–255 (2017).

86. Abdullah, N., Attia, J., Oldmeadow, C., Scott, R. J. & Holliday, E. G. The architecture of risk for type 2 diabetes: understanding Asia in the context of global findings. *Int. J. Endocrinol.* **2014**, 593982 (2014).

87. Pollack, R. M., Donath, M. Y., LeRoith, D. & Leibowitz, G. Anti-inflammatory agents in the treatment of diabetes and its vascular complications. *Diabetes Care* **39**, S244–S252 (2016).

88. Shoelson, S. E., Lee, J. & Goldfine, A. B. Inflammation and insulin resistance. *Journal of Clinical Investigation* **116**, 1793–1801 (2006).

89. Duncan, B. B. *et al.* Low-grade systemic inflammation and the development of type 2 diabetes: The atherosclerosis risk in communities study. *Diabetes* **52**, 1799–1805 (2003).

90. Haffner, S. *et al.* Intensive lifestyle intervention or metformin on inflammation and coagulation in participants with impaired glucose tolerance. *Diabetes* **54**, 1566–1572 (2005).

91. Hotamisligil, G. S., Shargill, N. S. & Spiegelman, B. M. Adipose expression of tumor necrosis factor-α: Direct role in obesity-linked insulin resistance. *Science (80-.).* **259**, 87–91 (1993).

92. Yamamoto, Y., Yin, M.-J. & Gaynor, R. B. IκB Kinase α (IKKα) Regulation of IKKβ Kinase Activity. *Mol. Cell. Biol.* **20**, 3655–3666 (2000).

93. Rehman, K. & Akash, M. S. H. Mechanisms of inflammatory responses and development of insulin resistance: How are they interlinked? *J. Biomed. Sci.* **23**, 1–18 (2016).

94. Groop, L. C. *et al.* The role of free fatty acid metabolism in the pathogenesis of insulin resistance in obesity and noninsulin-dependent diabetes mellitus. *J. Clin. Endocrinol. Metab.* **72**, 96–107 (1991).

95. Bays, H. E. *et al.* Pathogenic potential of adipose tissue and metabolic consequences of adipocyte hypertrophy and increased visceral adiposity. *Expert Rev. Cardiovasc. Ther.* **6**, 343–368 (2008).

96. Iozzo, P. Viewpoints on the way to the consensus session: where does insulin resistance start? The adipose tissue. *Diabetes Care* **32 Suppl 2**, S168–S173 (2009).

97. Cuthbertson, D. J. *et al.* What have human experimental overfeeding studies taught us about adipose tissue expansion and susceptibility to obesity and metabolic complications? *International Journal of Obesity* **41**, 853–865 (2017).

98. Neeland, I. J. *et al.* Visceral and ectopic fat, atherosclerosis, and cardiometabolic disease: a position statement. *The Lancet Diabetes and Endocrinology* **7**, 715–725 (2019).

99. Roden, M. *et al.* Mechanism of free fatty acid-induced insulin resistance in humans. *J. Clin. Invest.* **97**, 2859–65 (1996).

100. Giacca, A., Xiao, C., Oprescu, A. I., Carpentier, A. C. & Lewis, G. F. Lipid-induced pancreatic β-cell dysfunction: focus on in vivo studies. *Am. J. Physiol. Endocrinol. Metab.* **300**, E255-62 (2011).

101. Cancello, R. *et al.* Reduction of macrophage infiltration and chemoattractant gene expression changes in white adipose tissue of morbidly obese subjects after surgery-induced weight loss. *Diabetes* **54**, 2277–86 (2005).

102. Coats, B. R. *et al.* Metabolically Activated Adipose Tissue Macrophages Perform Detrimental and Beneficial Functions during Diet-Induced Obesity. *Cell Rep.* **20**, 3149–3161 (2017).

103. Ieronymaki, E. *et al.* Insulin Resistance in Macrophages Alters Their Metabolism and Promotes an M2-Like Phenotype. *J. Immunol.* **202**, 1786–1797 (2019).

104. Brehm, A. & Roden, M. Glucose Clamp Techniques. in *Clinical Diabetes Research* 43–76 (John Wiley & Sons, Ltd, 2007). doi:10.1002/9780470513095.ch4

105. Moraes-Vieira, P. M., Saghatelian, A. & Kahn, B. B. GLUT4 expression in adipocytes regulates de novo lipogenesis and levels of a novel class of lipids with antidiabetic and anti-inflammatory effects. in *Diabetes* **65**, 1808–1815 (American Diabetes Association Inc., 2016).

106. Cahová, M., Vavrínková, H. & Kazdová, L. Glucose-fatty acid interaction in skeletal muscle and adipose tissue in insulin resistance. *Physiol. Res.* **56**, 1–15 (2007).

107. Green, C. R. *et al.* Branched-chain amino acid catabolism fuels adipocyte differentiation and lipogenesis. *Nat. Chem. Biol.* **12**, 15–21 (2015).

108. Crown, S. B., Marze, N. & Antoniewicz, M. R. Catabolism of Branched Chain Amino Acids Contributes Significantly to Synthesis of Odd-Chain and Even-Chain Fatty Acids in 3T3-L1 Adipocytes. *PLoS One* **10**, e0145850 (2015).

109. Kazantzis, M. & Stahl, A. Fatty acid transport proteins, implications in physiology and disease. *Biochim. Biophys. Acta - Mol. Cell Biol. Lipids* **1821**, 852–857 (2012).

110. Turner, N., Cooney, G. J., Kraegen, E. W. & Bruce, C. R. Fatty acid metabolism, energy expenditure and insulin resistance in muscle. *J. Endocrinol.* **220**, T61–T79 (2014).

111. Goodpaster, B. H., He, J., Watkins, S. & Kelley, D. E. Skeletal muscle lipid content and insulin resistance: evidence for a paradox in endurance-trained athletes. *J Clin Endocrinol Metab* **86**, 5755–5761 (2001).

112. Dubé, J. J. *et al.* Exercise-induced alterations in intramyocellular lipids and insulin resistance: the athlete's paradox revisited. *Am. J. Physiol. - Endocrinol. Metab.* **294**, (2008).

113. van Loon, L. J. C. *et al.* Intramyocellular lipid content in type 2 diabetes patients compared with overweight sedentary men and highly trained endurance athletes. *Am. J. Physiol. - Endocrinol. Metab.* **287**, (2004).

114. Shulman, G. I. Cellular mechanisms of insulin resistance. *J Clin Invest* **106**, 171–176 (2000).

115. Krssak, M. *et al.* Intramyocellular lipid concentrations are correlated with insulin sensitivity in humans: a 1H NMR spectroscopy study. *Diabetologia* **42**, 113–116 (1999).

116. Kitessa, S. & Abeywardena, M. Lipid-Induced Insulin Resistance in Skeletal Muscle: The Chase for the Culprit Goes from Total Intramuscular Fat to Lipid Intermediates, and Finally to Species of Lipid Intermediates. *Nutrients* **8**, 466 (2016).

117. He, J., Watkins, S. & Kelley, D. E. Skeletal muscle lipid content and oxidative enzyme activity in relation to muscle fiber type in type 2 diabetes and obesity. *Diabetes* **50**, 817–23 (2001).

118. Ritov, V. B. *et al.* Deficiency of electron transport chain in human skeletal muscle mitochondria in type 2 diabetes mellitus and obesity. *Am J Physiol Endocrinol Metab* **298**, E49–E58 (2010).

119. Yamada, T. *et al.* Two distinct patterns of glucose intolerance in icteric rats and rabbits. Relationship to impaired liver mitochondria function. *J. Lab. Clin. Med.* **86**, 38–45 (1975).

120. Patti, M.-E. & Corvera, S. The role of mitochondria in the pathogenesis of type 2 diabetes. *Endocr Rev* **31**, 364–395 (2010).

121. Dela, F. & Helge, J. W. Insulin resistance and mitochondrial function in skeletal muscle. *Int J Biochem Cell Biol* **45**, 11–15 (2013).

122. Montgomery, M. K. & Turner, N. Mitochondrial dysfunction and insulin resistance: an update. *Endocr. Connect.* **4**, R1–R15 (2015).

123. Gojda, J. *et al.* Chronic dietary exposure to branched chain amino acids impairs glucose disposal in vegans but not in omnivores. *Eur. J. Clin. Nutr.* (2017). doi:10.1038/ejcn.2016.274

124. Sarparanta, J., García-Macia, M. & Singh, R. Autophagy and mitochondria in obesity and type 2 diabetes. *Curr. Diabetes Rev.* (2016).

125. Owen, O. E. *et al.* Brain metabolism during fasting. *J. Clin. Invest.* **46**, 1589–1595 (1967).

126. Rizza, R. A. Pathogenesis of fasting and postprandial hyperglycemia in type 2 diabetes: implications for therapy. *Diabetes* **59**, 2697–707 (2010).

127. D'Alessio, D. The role of dysregulated glucagon secretion in type 2 diabetes. *Diabetes, Obes. Metab.* **13**, 126–132 (2011).

128. Jallut, D. *et al.* Impaired glucose tolerance and diabetes in obesity: a 6-year follow-up study of glucose metabolism. *Metabolism.* **39**, 1068–75 (1990).

129. Martin, B. C. *et al.* Role of glucose and insulin resistance in development of type 2 diabetes mellitus: results of a 25-year follow-up study. *Lancet* **340**, 925–929 (1992).

130. Saad, M. F. *et al.* SEQUENTIAL CHANGES IN SERUM INSULIN CONCENTRATION DURING DEVELOPMENT OF NON-INSULIN-DEPENDENT DIABETES. *Lancet* **333**, 1356–1359 (1989).

131. Weyer, C., Bogardus, C., Mott, D. M. & Pratley, R. E. The natural history of insulin secretory dysfunction and insulin resistance in the pathogenesis of type 2 diabetes mellitus. *J. Clin. Invest.* **104**, 787–94 (1999).

132. Ferrannini, E. The stunned β cell: A brief history. *Cell Metabolism* **11**, 349–352 (2010).

133. Halban, P. A. *et al.* β-Cell Failure in Type 2 Diabetes: Postulated Mechanisms and Prospects for Prevention and Treatment. *Diabetes Care* **37**, (2014).

134. Andel, M. *et al.* [Factors causing damage and destruction of beta-cells of the islets of Langerhans in the pancreas]. *Vnitr. Lek.* **60**, 684–690 (2014).

135. Swisa, A., Glaser, B. & Dor, Y. Metabolic stress and compromised identity of pancreatic beta cells. *Frontiers in Genetics* **8**, (2017).

136. Rahier, J., Guiot, Y., Goebbels, R. M., Sempoux, C. & Henquin, J. C. Pancreatic β-cell mass in European subjects with type 2 diabetes. in *Diabetes, Obesity and Metabolism* **10**, 32–42 (Blackwell Publishing Ltd, 2008).

137. Kendall, D. M., Goetz, F. C., Robertson, R. P., Sutherland, D. E. R. & Najarian, J. S. Effects of hemipancreatectomy on insulin secretion and glucose tolerance in healthy humans. *N. Engl. J. Med.* **322**, 898–903 (1990).

138. Seaquist, E. R. *et al.* Hyperproinsulinemia is associated with increased β cell demand after hemipancreatectomy in humans. *J. Clin. Invest.* **97**, 455–460 (1996).

139. Ghosh, R., Colon-Negron, K. & Papa, F. R. Endoplasmic reticulum stress, degeneration of pancreatic islet β-cells, and therapeutic modulation of the unfolded protein response in diabetes. *Molecular Metabolism* **27**, S60–S68 (2019).

140. Roma, L. P. & Jonas, J. C. Nutrient Metabolism, Subcellular Redox State, and Oxidative Stress in Pancreatic Islets and β-Cells. *Journal of Molecular Biology* **432**, 1461–1493 (2020).

141. Ebrahimi, A. G. *et al.* Beta cell identity changes with mild hyperglycemia: Implications for function, growth, and vulnerability. *Mol. Metab.* **35**, (2020).

142. Wysham, C. & Shubrook, J. Beta-cell failure in type 2 diabetes: mechanisms, markers, and clinical implications. *Postgrad. Med.* 1–11 (2020). doi:10.1080/00325481.2020.1771047

143. Rhodes, C. J., White, M. F., Leahy, J. L. & Kahn, S. E. Direct Autocrine Action of Insulin on -Cells: Does It Make Physiological Sense? *Diabetes* **62**, 2157–2163 (2013).

144. Rhodes, C. J., White, M. F., Leahy, J. L. & Kahn, S. E. Direct autocrine action of insulin on β-cells: Does it make physiological sense? *Diabetes* **62**, 2157–2163 (2013).

145. White, M. F. *et al.* Disruption of IRS-2 causes type 2 diabetes in mice. *Nature* **391**, 900–904 (1998).

146. Nair, K. S., Woolf, P. D., Welle, S. L. & Matthews, D. E. Leucine, glucose, and energy metabolism after 3 days of fasting in healthy human subjects. *Am. J. Clin. Nutr.* **46**, 557–562 (1987).

147. Cole, J. T. Metabolism of BCAAs. in *Branched Chain Amino Acids in Clinical Nutrition* 13–24 (Springer New York, 2015). doi:10.1007/978-1-4939-1923-9_2

148. Shimomura, Y., Murakami, T., Nakai, N., Nagasaki, M. & Harris, R. A. Exercise promotes BCAA catabolism: effects of BCAA supplementation on skeletal muscle during exercise. *J Nutr* **134**, 1583S-1587S (2004).

149. de Campos-Ferraz, P. L. *et al.* An overview of amines as nutritional supplements to counteract cancer cachexia. *J. Cachexia. Sarcopenia Muscle* **5**, 105–10 (2014).

150. Kohlmeier, M. *Nutrient Metabolism*. (Academic Press, 2015).

151. Neis, E. P. J. G., Dejong, C. H. C. & Rensen, S. S. The role of microbial amino acid metabolism in host metabolism. *Nutrients* **7**, 2930–46 (2015).

152. Wahren, J., Felig, P. & Hagenfeldt, L. Effect of protein ingestion on splanchnic and leg metabolism in normal man and in patients with diabetes mellitus. *J Clin Invest* **57**, 987–999 (1976).

153. Gojda, J. *et al.* Increased Incretin But Not Insulin Response after Oral versus Intravenous Branched Chain Amino Acids. *Ann. Nutr. Metab.* **70**, 293–302 (2017).

154. Pedroso, J. A. B., Zampieri, T. T. & Donato, J. Reviewing the Effects of l-Leucine Supplementation in the Regulation of Food Intake, Energy Balance, and Glucose Homeostasis. *Nutrients* **7**, 3914–3937 (2015).

155. Zhang, Z. Y., Monleon, D., Verhamme, P. & Staessen, J. A. Branched-chain amino acids as critical switches in health and disease. *Hypertension* **72**, 1012–1022 (2018).

156. Lynch, C. J. & Adams, S. H. Branched-chain amino acids in metabolic signalling and insulin resistance. *Nat. Rev. Endocrinol.* **10**, 723–36 (2014).

157. Shou, J., Chen, P. J. & Xiao, W. H. The effects of BCAAs on insulin resistance in athletes. *Journal of Nutritional Science and Vitaminology* **65**, 383–389 (2019).

158. Holeček, M. Branched-chain amino acids in health and disease: Metabolism, alterations in blood plasma, and as supplements. *Nutrition and Metabolism* **15**, (2018).

159. Ardestani, A., Lupse, B., Kido, Y., Leibowitz, G. & Maedler, K. mTORC1 Signaling: A Double-Edged Sword in Diabetic β Cells. *Cell Metabolism* **27**, 314–331 (2018).

160. Gran, P. & Cameron-Smith, D. The actions of exogenous leucine on mTOR signalling and amino acid transporters in human myotubes. *BMC Physiol.* **11**, 10 (2011).

161. Tremblay, F. *et al.* Overactivation of S6 kinase 1 as a cause of human insulin resistance during increased amino acid availability. *Diabetes* **54**, 2674–84 (2005).

162. Shimomura, Y. *et al.* Branched-chain amino acid catabolism in exercise and liver disease. *J. Nutr.* **136**, 250S–3S (2006).

163. Green, C. R. *et al.* Branched-chain amino acid catabolism fuels adipocyte differentiation and lipogenesis. *Nat. Chem. Biol.* **12**, 15–21 (2016).

164. Yang, J., Chi, Y., Burkhardt, B. R., Guan, Y. & Wolf, B. A. Leucine metabolism in regulation of insulin secretion from pancreatic beta cells. *Nutr Rev* **68**, 270–279 (2010).

165. Gojda, J. *et al.* Increased Incretin but Not Insulin Response after Oral versus Intravenous Branched Chain Amino Acids. *Ann. Nutr. Metab.* **70**, (2017).

166. Gluud, L. L. *et al.* Branched-chain amino acids for people with hepatic encephalopathy. *Cochrane Database of Systematic Reviews* **2017**, (2017).

167. Fischer, J. E. *et al.* The role of plasma amino acids in hepatic encephalopathy. *Surgery* **78**, 276–290 (1975).

168. Zampieri, T. T. *et al.* Oral Leucine Supplementation Is Sensed by the Brain but neither Reduces Food Intake nor Induces an Anorectic Pattern of Gene Expression in the Hypothalamus. *PLoS One* **8**, e84094 (2013).

169. Kamaura, M. *et al.* Lifestyle modification in metabolic syndrome and associated changes in plasma amino acid profiles. *Circ J* **74**, 2434–2440 (2010).

170. Würtz, P. *et al.* Metabolic signatures of insulin resistance in 7,098 young adults. *Diabetes* **61**, 1372–1380 (2012).

171. Wang, T. J. *et al.* Metabolite profiles and the risk of developing diabetes. *Nat Med* **17**, 448–453 (2011).

172. Lips, M. A. *et al.* Roux-en-Y gastric bypass surgery, but not calorie restriction, reduces plasma branched-chain amino acids in obese women independent of weight loss or the presence of type 2 diabetes. *Diabetes Care* **37**, 3150–3156 (2014).

173. Gannon, N. P., Schnuck, J. K. & Vaughan, R. A. BCAA Metabolism and Insulin Sensitivity – Dysregulated by Metabolic Status? *Molecular Nutrition and Food Research* **62**, (2018).

174. Newgard, C. B. *et al.* A branched-chain amino acid-related metabolic signature that differentiates obese and lean humans and contributes to insulin resistance. *Cell Metab* **9**, 311–326 (2009).

175. Yin, Q., Brameld, J. M., Parr, T. & Murton, A. J. Leucine and mTORc1 act independently to regulate 2-deoxyglucose uptake in L6 myotubes. *Amino Acids* **52**, 477–486 (2020).

176. Lane, M. T. *et al.* Endocrine responses and acute mTOR pathway phosphorylation to resistance exercise with leucine and whey. *Biol. Sport* **34**, 197–203 (2017).

177. Macotela, Y. *et al.* Dietary leucine--an environmental modifier of insulin resistance acting on multiple levels of metabolism. *PLoS One* **6**, e21187–e21187 (2011).

178. Lynch, C. J. & Adams, S. H. Branched-chain amino acids in metabolic signalling and insulin resistance. *Nature Reviews Endocrinology* **10**, 723–736 (2014).

179. Blanchard, P. G. *et al.* Major involvement of mTOR in the PPARγ-induced stimulation of adipose tissue lipid uptake and fat accretion. *J. Lipid Res.* **53**, 1117–1125 (2012).

180. Paolella, L. M. *et al.* mTORC1 restrains adipocyte lipolysis to prevent systemic hyperlipidemia. *Mol. Metab.* **32**, 136–147 (2020).

181. Blagosklonny, M. V. Fasting and rapamycin: diabetes versus benevolent glucose intolerance. *Cell Death and Disease* **10**, 1–10 (2019).

182. Biswas, D., Duffley, L. & Pulinilkunnil, T. Role of branched-chain amino acid–catabolizing enzymes in intertissue signaling, metabolic remodeling, and energy homeostasis. *FASEB J.* **33**, 8711–8731 (2019).

183. Menkes, J. H., Hurst, P. L. & Craig, J. M. A new syndrome: progressive familial infantile cerebral dysfunction associated with an unusual urinary substance. *Pediatrics* **14**, 462–7 (1954).

184. Blackburn, P. R. *et al.* Maple syrup urine disease: Mechanisms and management. *Application of Clinical Genetics* **10**, 57–66 (2017).

185. Sonnet, D. S. *et al.* Metformin inhibits Branched Chain Amino Acid (BCAA) derived ketoacidosis and promotes metabolic homeostasis in MSUD. *Sci. Rep.* **6**, (2016).

186. Jang, C. *et al.* A branched-chain amino acid metabolite drives vascular fatty acid transport and causes insulin resistance. *Nat. Med.* **22**, 421–426 (2016).

187. Andersson-Hall, U. *et al.* Higher concentrations of BCAAs and 3-HIB are associated with insulin resistance in the transition from gestational diabetes to type 2 diabetes. *J. Diabetes Res.* **2018**, 4207067 (2018).

188. Herman, M. A., She, P., Peroni, O. D., Lynch, C. J. & Kahn, B. B. Adipose tissue branched chain amino acid (BCAA) metabolism modulates circulating BCAA levels. *J. Biol. Chem.* **285**, 11348–56 (2010).

189. Boulet, M. M. *et al.* Alterations of plasma metabolite profiles related to adipose tissue distribution and cardiometabolic risk. *Am. J. Physiol. - Endocrinol. Metab.* **309**, E736–E746 (2015).

190. Zhou, M. *et al.* Targeting BCAA catabolism to treat obesity-associated insulin resistance. *Diabetes* **68**, 1730–1746 (2019).

191. Pedersen, H. K. *et al.* Human gut microbes impact host serum metabolome and insulin sensitivity. *Nature* **535**, 376–381 (2016).

192. Nilsson, M., Stenberg, M., Frid, A. H., Holst, J. J. & Björck, I. M. E. Glycemia and insulinemia in healthy subjects after lactose-equivalent meals of milk and other food proteins: the role of plasma amino acids and incretins. *Am J Clin Nutr* **80**, 1246–1253 (2004).

193. Calbet, J. A. L. & MacLean, D. A. Plasma glucagon and insulin responses depend on the rate of appearance of amino acids after ingestion of different protein solutions in humans. *J Nutr* **132**, 2174–2182 (2002).

194. Fryburg, D. A., Jahn, L. A., Hill, S. A., Oliveras, D. M. & Barrett, E. J. Insulin and insulin-like growth factor-I enhance human skeletal muscle protein anabolism during hyperaminoacidemia by different mechanisms. *J. Clin. Invest.* **96**, 1722–1729 (1995).

195. Everman, S. *et al.* Insulin does not stimulate muscle protein synthesis during increased plasma branched-chain amino acids alone but still decreases whole body proteolysis in humans. *Am. J. Physiol. - Endocrinol. Metab.* **311**, E671–E677 (2016).

196. Lu, J., Xie, G., Jia, W. & Jia, W. Insulin resistance and the metabolism of branched-chain amino acids. *Front Med* **7**, 53–59 (2013).

197. Herman, M. A., She, P., Peroni, O. D., Lynch, C. J. & Kahn, B. B. Adipose tissue branched chain amino acid (BCAA) metabolism modulates circulating BCAA levels. *J Biol Chem* **285**, 11348–11356 (2010).

198. Matthews, D. E. Observations of branched-chain amino acid administration in humans. *J. Nutr.* **135**, 1580S–4S (2005).

199. Wolfe, R. R. Branched-chain amino acids and muscle protein synthesis in humans: Myth or reality? *Journal of the International Society of Sports Nutrition* **14**, 30 (2017).

200. Gojda, J. *et al.* Lactate production without hypoxia in skeletal muscle during electrical cycling: Crossover study of femoral venous-arterial differences in healthy volunteers. *PLoS One* **14**, e0200228 (2019).

201. Gojda, J. *et al.* Higher insulin sensitivity in vegans is not associated with higher mitochondrial density. *Eur. J. Clin. Nutr.* 1–6 (2013). doi:10.1038/ejcn.2013.202

202. Everman, S., Mandarino, L. J., Carroll, C. C. & Katsanos, C. S. Effects of acute exposure to increased plasma branched-chain amino acid concentrations on insulin-mediated plasma glucose turnover in healthy young subjects. *PLoS One* **10**, e0120049 (2015).

203. Robinson, M. M. *et al.* High insulin combined with essential amino acids stimulates skeletal muscle mitochondrial protein synthesis while decreasing insulin sensitivity in healthy humans. *J. Clin. Endocrinol. Metab.* **99**, E2574-83 (2014).

204. Woo, S.-L. *et al.* Effects of branched-chain amino acids on glucose metabolism in obese, prediabetic men and women: a randomized, crossover study. *Am. J. Clin. Nutr.* **109**, 1569–1577 (2019).

205. Karusheva, Y. *et al.* Short-term dietary reduction of branched-chain amino acids reduces meal-induced insulin secretion and modifies microbiome composition in type 2 diabetes: a randomized controlled crossover trial. *Am. J. Clin. Nutr.* **110**, 1098–1107 (2019).

206. Ouellet, V., Marois, J., Weisnagel, S. J. & Jacques, H. Dietary cod protein improves insulin sensitivity in insulin-resistant men and women: a randomized controlled trial. *Diabetes Care* **30**, 2816–21 (2007).

207. Jakubowicz, D. *et al.* Incretin, insulinotropic and glucose-lowering effects of whey protein pre-load in type 2 diabetes: a randomised clinical trial. *Diabetologia* **57**, 1807–1811 (2014).

208. Clarys, P. *et al.* Comparison of nutritional quality of the vegan, vegetarian, semi-vegetarian, pesco-vegetarian and omnivorous diet. *Nutrients* **6**, 1318–32 (2014).

209. Souci, S. W., Fachmann, W. & Kraut, H. Food composition and nutrition tables. (2000).

210. WHO Technical Report Series 935. WHO Technical Report Series PROTEIN AND AMINO ACID REQUIREMENTS IN HUMAN NUTRITION Report of a Joint WHO/FAO/UNU Expert Consultation. *Rep. a Jt. WHO/FAO/UNU Expert Consult.* World Health Orgarnization, Geneva, Switzerland

211. Hua, N. W., Stoohs, R. A. & Facchini, F. S. Low iron status and enhanced insulin sensitivity in lacto-ovo vegetarians. *Br J Nutr* **86**, 515–519 (2001).

212. Goff, L. M., Bell, J. D., So, P.-W., Dornhorst, A. & Frost, G. S. Veganism and its relationship with insulin resistance and intramyocellular lipid. *Eur J Clin Nutr* **59**, 291–298 (2005).

213. Yang, S.-Y. *et al.* Relationship of carotid intima-media thickness and duration of vegetarian diet in Chinese male vegetarians. *Nutr Metab* **8**, 63 (2011).

214. Tonstad, S., Butler, T., Yan, R. & Fraser, G. E. Type of vegetarian diet, body weight, and prevalence of type 2 diabetes. *Diabetes Care* **32**, 791–796 (2009).

215. Aune, D., Ursin, G. & Veier?d, M. B. Meat consumption and the risk of type 2 diabetes: a systematic review and meta-analysis of cohort studies. *Diabetologia* **52**, 2277–2287 (2009).

216. Barnard, N. D. *et al.* A low-fat vegan diet and a conventional diabetes diet in the treatment of type 2 diabetes: a randomized, controlled, 74-wk clinical trial. *Am J Clin Nutr* **89**, 1588S-1596S (2009).

217. Kahleova, H. *et al.* Vegetarian diet improves insulin resistance and oxidative stress markers more than conventional diet in subjects with Type 2 diabetes. *Diabet Med* **28**, 549–559 (2011).

218. Jaček, M., Gojda, J., Dlouhý, P. & Tůma, P. The use of coupled gas chromatography columns for the determination of individual isomers of trans fatty acids in the adipose tissue of vegans. *Monatshefte fur Chemie* **150**, 1417–1424 (2019).

219. Tůma, P., Gojda, J., Sommerová, B. & Koval, D. Measuring venous-arterial differences of valine, isoleucine, leucine, alanine and glutamine in skeletal muscles using counter-current electrophoresis with contactless conductivity detection. *J. Electroanal. Chem.* **857**, 113772 (2020).

220. Tuma, P. & Gojda, J. Rapid determination of branched chain amino acids in human blood plasma by pressure-assisted capillary electrophoresis with contactless conductivity detection. *Electrophoresis* **36**, (2015).

221. van Loon, L. J., Saris, W. H., Verhagen, H. & Wagenmakers, A. J. Plasma insulin responses after ingestion of different amino acid or protein mixtures with carbohydrate. *Am J Clin Nutr* **72**, 96–105 (2000).

222. Ueno, M. *et al.* Regulation of insulin signalling by hyperinsulinaemia: role of IRS-1/2 serine phosphorylation and the mTOR/p70 S6K pathway. *Diabetologia* **48**, 506–518 (2005).

223. Krebs, M. *et al.* The Mammalian target of rapamycin pathway regulates nutrient-sensitive glucose uptake in man. *Diabetes* **56**, 1600–1607 (2007).

224. Berger, S. & Vongaraya, N. Insulin response to ingested protein in diabetes. *Diabetes* **15**, 303–306 (1966).

225. Floyd JC, J., Fajans, S. S., Conn, J. W., Knopf, R. F. & Rull, J. Stimulation of insulin secretion by amino acids. *J Clin Invest* **45**, 1487–1502 (1966).

226. Frid, A. H., Nilsson, M., Holst, J. J. & Björck, I. M. E. Effect of whey on blood glucose and insulin responses to composite breakfast and lunch meals in type 2 diabetic subjects. *Am J Clin Nutr* **82**, 69–75 (2005).

227. Pal, S. & Ellis, V. The acute effects of four protein meals on insulin, glucose, appetite and energy intake in lean men. *Br J Nutr* **104**, 1241–1248 (2010).

228. Nilsson, M., Holst, J. J. & Björck, I. M. Metabolic effects of amino acid mixtures and whey protein in healthy subjects: studies using glucose-equivalent drinks. *Am J Clin Nutr* **85**, 996–1004 (2007).

229. Elrick, H., Stimmler, L., Hlad, CJ, J. & Arai, Y. PLASMA INSULIN RESPONSE TO ORAL AND INTRAVENOUS GLUCOSE ADMINISTRATION. *J Clin Endocrinol Metab* **24**, 1076–1082 (1964).

230. Raptis, S. *et al.* Differences in insulin, growth hormone and pancreatic enzyme secretion after intravenous and intraduodenal administration of mixed amino acids in man. *N Engl J Med* **288**, 1199–1202 (1973).

231. Lindgren, O. *et al.* Incretin effect after oral amino acid ingestion in humans. *J. Clin. Endocrinol. Metab.* **100**, 1172–6 (2015).

232. Gunnerud, U., Holst, J. J., Östman, E. & Björck, I. The glycemic, insulinemic and plasma amino acid responses to equi-carbohydrate milk meals, a pilot- study of bovine and human milk. *Nutr J* **11**, 83 (2012).

233. Salehi, A. *et al.* The insulinogenic effect of whey protein is partially mediated by a direct effect of amino acids and GIP on $-cells. *Nutr Metab* **9**, 48 (2012).

234. Chen, Q. & Reimer, R. A. Dairy protein and leucine alter GLP-1 release and mRNA of genes involved in intestinal lipid metabolism in vitro. *Nutrition* **25**, 340–349 (2009).

235. Lindgren, O. *et al.* Incretin effect after oral amino acid ingestion in humans. *J. Clin. Endocrinol. Metab.* **100**, 1172–6 (2015).

236. Vahl, T. P., Drazen, D. L., Seeley, R. J., D'Alessio, D. A. & Woods, S. C. Meal-anticipatory glucagon-like peptide-1 secretion in rats. *Endocrinology* **151**, 569–575 (2010).

237. Marina, A. S., Kutina, A. V, Shakhmatova, E. I., Balbotkina, E. V & Natochin, Y. V. Stimulation of glucagon-like peptide-1 secretion by water loading in human. *Dokl Biol Sci* **459**, 323–325 (2014).

238. Kuwata, H. *et al.* Meal sequence and glucose excursion, gastric emptying and incretin secretion in type 2 diabetes: a randomised, controlled crossover, exploratory trial. *Diabetologia* **59**, 453–61 (2016).

239. Biolo, G. *et al.* Leucine and phenylalanine kinetics during mixed meal ingestion: a multiple tracer approach. *Am. J. Physiol. - Endocrinol. Metab.* **262**, (1992).

240. Doi, M., Yamaoka, I., Nakayama, M., Sugahara, K. & Yoshizawa, F. Hypoglycemic effect of isoleucine involves increased muscle glucose uptake and whole body glucose oxidation and decreased hepatic gluconeogenesis. *Am. J. Physiol. - Endocrinol. Metab.* **292**, (2007).

241. Rocha, D. M., Faloona, G. R. & Unger, R. H. Glucagon-stimulating activity of 20 amino acids in dogs. *J Clin Invest* **51**, 2346–2351 (1972).

242. Calanna, S. *et al.* Secretion of glucagon-like peptide-1 in patients with type 2 diabetes mellitus: systematic review and meta-analyses of clinical studies. *Diabetologia* **56**, 965–972 (2013).

243. Solerte, S. B. *et al.* Improvement of blood glucose control and insulin sensitivity during a long-term (60 weeks) randomized study with amino acid dietary supplements in elderly subjects with type 2 diabetes mellitus. *Am J Cardiol* **101**, 82E-88E (2008).

244. Malashree, L., Angadi, V., Yadav, K. S. & Prabha, R. "Postbiotics" - One Step Ahead of Probiotics. *Int. J. Curr. Microbiol. Appl. Sci.* **8**, 2049–2053 (2019).

245. Koh, A., De Vadder, F., Kovatcheva-Datchary, P. & Bäckhed, F. From dietary fiber to host physiology: Short-chain fatty acids as key bacterial metabolites. *Cell* **165**, 1332–1345 (2016).

246. Tilg, H. & Moschen, A. R. Microbiota and diabetes: An evolving relationship. *Gut* **63**, 1513–1521 (2014).

247. Turnbaugh, P. J. *et al.* The effect of diet on the human gut microbiome: A metagenomic analysis in humanized gnotobiotic mice. *Sci. Transl. Med.* **1**, (2009).

248. Wu, G. D. *et al.* Linking long-term dietary patterns with gut microbial enterotypes. *Science (80-.).* **334**, 105–108 (2011).

249. Duncan, S. H. *et al.* Reduced dietary intake of carbohydrates by obese subjects results in decreased concentrations of butyrate and butyrate-producing bacteria in feces. *Appl. Environ. Microbiol.* **73**, 1073–1078 (2007).

250. Palau-Rodriguez, M. *et al.* Metabolomic insights into the intricate gut microbial-host interaction in the development of obesity and type 2 diabetes. *Frontiers in Microbiology* **6**, (2015).

251. Gibson, G. R. *et al.* Expert consensus document: The International Scientific Association for Probiotics and Prebiotics (ISAPP) consensus statement on the definition and scope of prebiotics. *Nature Reviews Gastroenterology and Hepatology* **14**, 491–502 (2017).

252. Wang, Y. *et al.* Probiotic Supplements: Hope or Hype? *Front. Microbiol.* **11**, (2020).

253. Collado, M. C., Vinderola, G. & Salminen, S. Postbiotics: Facts and open questions. A position paper on the need for a consensus definition. *Benef. Microbes* **10**, 711–719 (2019).

254. De Vadder, F. *et al.* Microbiota-generated metabolites promote metabolic benefits via gut-brain neural circuits. *Cell* **156**, 84–96 (2014).

255. Wang, F. *et al.* Lower Circulating Branched-Chain Amino Acid Concentrations Among Vegetarians are Associated with Changes in Gut Microbial Composition and Function. *Mol. Nutr. Food Res.* **63**, (2019).

256. do Rosario, V. A., Fernandes, R. & de Trindade, E. B. S. M. Vegetarian diets and gut microbiota: Important shifts in markers of metabolism and cardiovascular disease. *Nutr. Rev.* **74**, 444–454 (2016).

257. Ou, J., DeLany, J. P., Zhang, M., Sharma, S. & O'Keefe, S. J. D. Association between low colonic short-chain fatty acids and high bile acids in high colon cancer risk populations. *Nutr. Cancer* **64**, 34–40 (2012).

258. Cani, P. D. *et al.* Changes in gut microbiota control metabolic endotoxemia-induced inflammation in high-fat diet-induced obesity and diabetes in mice. *Diabetes* **57**, 1470–1481 (2008).

259. Magee, E. A., Richardson, C. J., Hughes, R. & Cummings, J. H. Contribution of dietary protein to sulfide production in the large intestine: An in vitro and a controlled feeding study in humans. *Am. J. Clin. Nutr.* **72**, 1488–1494 (2000).

260. Oliphant, K. & Allen-Vercoe, E. Macronutrient metabolism by the human gut microbiome: Major fermentation by-products and their impact on host health. *Microbiome* **7**, (2019).

261. Pedersen, H. K. *et al.* Human gut microbes impact host serum metabolome and insulin sensitivity. *Nature* **535**, 376–381 (2016).

262. Dai, Z., Wu, Z., Hang, S., Zhu, W. & Wu, G. Amino acid metabolism in intestinal bacteria and its potential implications for mammalian reproduction. *Molecular Human Reproduction* **21**, 389–409 (2014).

263. Ridaura, V. K. *et al.* Gut microbiota from twins discordant for obesity modulate metabolism in mice. *Science (80-.).* **341**, (2013).

264. Kovatcheva-Datchary, P. *et al.* Dietary Fiber-Induced Improvement in Glucose Metabolism Is Associated with Increased Abundance of Prevotella. *Cell Metab.* **22**, 971–982 (2015).

265. Losasso, C. *et al.* Assessing the influence of vegan, vegetarian and omnivore oriented westernized dietary styles on human gut microbiota: A cross sectional study. *Front. Microbiol.* **9**, (2018).

266. Ferrocino, I. *et al.* Fecal microbiota in healthy subjects following omnivore, vegetarian and vegan diets: Culturable populations and rRNA DGGE profiling. *PLoS One* **10**, (2015).

267. Mariotti, F. & Gardner, C. D. Dietary protein and amino acids in vegetarian diets—A review. *Nutrients* **11**, (2019).

268. Porporato, P. E. Understanding cachexia as a cancer metabolism syndrome. *Oncogenesis* **5**, e200 (2016).

269. Fearon, K. *et al.* Definition and classification of cancer cachexia: an international consensus. *Lancet Oncol.* **12**, 489–495 (2011).

270. Ali, R. *et al.* Lean body mass as an independent determinant of dose-limiting toxicity and neuropathy in patients with colon cancer treated with FOLFOX regimens. *Cancer Med.* **5**, 607–16 (2016).

271. van Niekerk, G., Isaacs, A. W., Nell, T. & Engelbrecht, A.-M. Sickness-Associated Anorexia: Mother Nature's Idea of Immunonutrition? *Mediators Inflamm.* **2016**, 8071539 (2016).

272. Tai, E. S. *et al.* Insulin resistance is associated with a metabolic profile of altered protein metabolism in Chinese and Asian-Indian men. *Diabetologia* **53**, 757–767 (2010).

273. Bi, X. & Henry, C. J. Plasma-free amino acid profiles are predictors of cancer and diabetes development. *Nutr. Diabetes* **7**, e249 (2017).

274. Copeland, G. P., Leinster, S. J., Davis, J. C. & Hipkin, L. J. Insulin resistance in patients with colorectal cancer. *Br. J. Surg.* **74**, 1031–5 (1987).

275. Lundholm, K., Holm, G. & Scherstén, T. Insulin Resistance in Patients with Cancer. *Cancer Res.* **38**, (1978).

276. Hernandez, A. V *et al.* Association between insulin resistance and breast carcinoma: a systematic review and meta-analysis. *PLoS One* **9**, e99317 (2014).

277. Lowry, S. F., Foster, D. M., Norton, J. A., Berman, M. & Brennan, M. F. Glucose Disposal and Gluconeogenesis From Alanine in Tumor-Bearing Fischer Gluconeogenesis 344 Rats2. *JNCI J. Natl. Cancer Inst.* **66**, 653–658 (1981).

278. Holroyde, C. P., Skutches, C. L., Boden, G. & Reichard, G. A. Glucose metabolism in cachectic patients with colorectal cancer. *Cancer Res.* **44**, 5910–3 (1984).

279. Nakamori, S. *et al.* Increased blood proinsulin and decreased C-peptide levels in patients with pancreatic cancer. *Hepatogastroenterology.* **46**, 16–24 (1999).

280. Cersosimo, E. *et al.* Insulin secretion and action in patients with pancreatic cancer. *Cancer* **67**, 486–93 (1991).

281. Vanhoutte, G. *et al.* Cachexia in cancer: what is in the definition? *BMJ Open Gastroenterol.* **3**, (2016).

282. Penet, M.-F. & Bhujwalla, Z. M. Cancer cachexia, recent advances, and future directions. *Cancer J.* **21**, 117–22 (2015).

283. Evans, W. J. *et al.* Cachexia: A new definition. *Clin. Nutr.* **27**, 793–799 (2008).

284. Argilés, J. M. *et al.* Validation of the CAchexia SCOre (CASCO). Staging Cancer Patients: The Use of miniCASCO as a Simplified Tool. *Front. Physiol.* **8**, 92 (2017).

285. Knox, L. S. *et al.* Energy expenditure in malnourished cancer patients. *Ann. Surg.* **197**, 152–162 (1983).

286. Lukey, M. J., Katt, W. P. & Cerione, R. A. Targeting amino acid metabolism for cancer therapy. *Drug Discov. Today* **22**, 796–804 (2017).

287. Penna, F. *et al.* Muscle atrophy in experimental cancer cachexia: Is the IGF-1 signaling pathway involved? *Int. J. Cancer* **127**, 1706–1717 (2010).

288. Fernandes, L. C., Machado, U. F., Nogueira, C. R., Carpinelli, A. R. & Curi, R. Insulin secretion in Walker 256 tumor cachexia. *Am. J. Physiol.* **258**, E1033-6 (1990).

289. Gallot, Y. S. *et al.* Myostatin Gene Inactivation Prevents Skeletal Muscle Wasting in Cancer. *Cancer Res.* **74**, 7344–7356 (2014).

290. Pouw, E. M., Schols, A. M. W. J., Deutz, N. E. P. & Wouters, E. F. M. Plasma and Muscle Amino Acid Levels in Relation to Resting Energy Expenditure and Inflammation in Stable Chronic Obstructive Pulmonary Disease. *Am. J. Respir. Crit. Care Med.* **158**, 797–801 (1998).

291. Suliman, M. E. *et al.* Inflammation contributes to low plasma amino acid concentrations in patients with chronic kidney disease. *Am. J. Clin. Nutr.* **82**, 342–9 (2005).

292. Vanhorebeek, I. *et al.* Effect of early supplemental parenteral nutrition in the paediatric ICU: a preplanned observational study of post-randomisation treatments in the PEPaNIC trial. *Lancet Respir. Med.* **5**, 475–483 (2017).

293. Mizushima, N. & Levine, B. Autophagy in mammalian development and differentiation. *Nat. Cell Biol.* **12**, 823–30 (2010).

294. Guo, J. Y. *et al.* Autophagy suppresses progression of K-ras-induced lung tumors to oncocytomas and maintains lipid homeostasis. *Genes Dev.* **27**, 1447–1461 (2013).

295. Lock, R., Kenific, C. M., Leidal, A. M., Salas, E. & Debnath, J. Autophagy-dependent production of secreted factors facilitates oncogenic RAS-Driven invasion. *Cancer Discov.* **4**, 466–479 (2014).

296. Degenhardt, K. *et al.* Autophagy promotes tumor cell survival and restricts necrosis, inflammation, and tumorigenesis. *Cancer Cell* **10**, 51–64 (2006).

297. Sheen, J.-H., Zoncu, R., Kim, D. & Sabatini, D. M. Defective Regulation of Autophagy upon Leucine Deprivation Reveals a Targetable Liability of Human Melanoma Cells In Vitro and In Vivo. *Cancer Cell* **19**, 613–628 (2011).

298. Strohecker, A. M. *et al.* Autophagy Sustains Mitochondrial Glutamine Metabolism and Growth of BrafV600E-Driven Lung Tumors. *Cancer Discov.* **3**, 1272–1285 (2013).

299. Yang, X. *et al.* The role of autophagy induced by tumor microenvironment in different cells and stages of cancer. *Cell Biosci.* **5**, 14 (2015).

300. DeBerardinis, R. J. & Cheng, T. Q's next: the diverse functions of glutamine in metabolism, cell biology and cancer. *Oncogene* **29**, 313–24 (2010).

301. Yang, L. *et al.* Metabolic shifts toward glutamine regulate tumor growth, invasion and bioenergetics in ovarian cancer. *Mol. Syst. Biol.* **10**, 728 (2014).

302. Smolková, K. *et al.* Waves of gene regulation suppress and then restore oxidative phosphorylation in cancer cells. *Int. J. Biochem. Cell Biol.* **43**, 950–968 (2011).

303. Smolková, K., Dvořák, A., Zelenka, J., Vítek, L. & Ježek, P. Reductive carboxylation and 2-hydroxyglutarate formation by wild-type IDH2 in breast carcinoma cells. *Int. J. Biochem. Cell Biol.* **65**, 125–133 (2015).

304. Tardito, S. *et al.* Glutamine synthetase activity fuels nucleotide biosynthesis and supports growth of glutamine-restricted glioblastoma. *Nat. Cell Biol.* **17**, 1556–1568 (2015).

305. Newsholme, P. *et al.* Glutamine metabolism by lymphocytes, macrophages, and neutrophils: its importance in health and disease. *J. Nutr. Biochem.* **10**, 316–24 (1999).

306. Mayers, J. R. *et al.* Elevation of circulating branched-chain amino acids is an early event in human pancreatic adenocarcinoma development. *Nat. Med.* **20**, 1193–1198 (2014).

307. Norton, J. A., Gorschboth, C. M., Wesley, R. A., Burt, M. E. & Brennan, M. F. Fasting plasma amino acid levels in cancer patients. *Cancer* **56**, 1181–1186 (1985).

308. Lee, J.-C. *et al.* Plasma amino acid levels in patients with colorectal cancers and liver cirrhosis with hepatocellular carcinoma. *Hepatogastroenterology.* **50**, 1269–73 (2003).

309. Son, J. *et al.* Glutamine supports pancreatic cancer growth through a KRAS-regulated metabolic pathway. *Nature* **496**, 101–105 (2013).

310. Zhang, L. & Han, J. Branched-chain amino acid transaminase 1 (BCAT1) promotes the growth of breast cancer cells through improving mTOR-mediated mitochondrial biogenesis and function. *Biochem. Biophys. Res. Commun.* **486**, 224–231 (2017).

311. Hattori, A. *et al.* Cancer progression by reprogrammed BCAA metabolism in myeloid leukaemia. *Nature* **545**, 500–504 (2017).

312. Tönjes, M. *et al.* BCAT1 promotes cell proliferation through amino acid catabolism in gliomas carrying wild-type IDH1. *Nat. Med.* **19**, 901–908 (2013).

313. Mayers, J. R. *et al.* Tissue of origin dictates branched-chain amino acid metabolism in mutant Kras-driven cancers. *Science (80-.).* **353**, 1161–1165 (2016).

314. Dey, P. *et al.* Genomic deletion of malic enzyme 2 confers collateral lethality in pancreatic cancer. *Nature* **542**, 119–123 (2017).

315. Luo, X. *et al.* Emerging roles of lipid metabolism in cancer metastasis. *Mol. Cancer* **16**, 76 (2017).

316. Arner, P. & Langin, D. Lipolysis in lipid turnover, cancer cachexia, and obesity-induced insulin resistance. *Trends Endocrinol. Metab.* **25**, 255–262 (2014).

317. Rohm, M., Zeigerer, A., Machado, J. & Herzig, S. Energy metabolism in cachexia. *EMBO Rep.* **20**, (2019).

318. Das, S. K. *et al.* Adipose triglyceride lipase contributes to cancer-associated cachexia. *Science* **333**, 233–8 (2011).

319. Agustsson, T. *et al.* Mechanism of increased lipolysis in cancer cachexia. *Cancer Res.* **67**, 5531–7 (2007).

320. Rohm, M. *et al.* An AMP-activated protein kinase-stabilizing peptide ameliorates adipose tissue wasting in cancer cachexia in mice. *Nat. Med.* **22**, 1120–1130 (2016).

321. Ren, T. *et al.* Metformin reduces lipolysis in primary rat adipocytes stimulated by tumor necrosis factor-alpha or isoproterenol. *J. Mol. Endocrinol.* **37**, 175–83 (2006).

322. Schuler, K. M. *et al.* Antiproliferative and metabolic effects of metformin in a preoperative window clinical trial for endometrial cancer. *Cancer Med.* **4**, 161–73 (2015).

323. Figueras, M., Olivan, M., Busquets, S., López-Soriano, F. J. & Argilés, J. M. Effects of Eicosapentaenoic Acid (EPA) Treatment on Insulin Sensitivity in an Animal Model of Diabetes: Improvement of the Inflammatory Status. *Obesity* **19**, 362–369 (2011).

324. Carella, A. *et al.* Antihypertensive Treatment with Beta-Blockers in the Metabolic Syndrome: A Review. *Curr. Diabetes Rev.* **6**, 215–221 (2010).

325. Lainscak, M. & Laviano, A. ACT-ONE - ACTION at last on cancer cachexia by adapting a novel action beta-blocker. *Journal of Cachexia, Sarcopenia and Muscle* **7**, 400–402 (2016).

326. Tsoli, M., Swarbrick, M. M. & Robertson, G. R. Lipolytic and thermogenic depletion of adipose tissue in cancer cachexia. *Semin. Cell Dev. Biol.* **54**, 68–81 (2016).

327. Dahlman, I. *et al.* Adipose tissue pathways involved in weight loss of cancer cachexia. *Br. J. Cancer* **102**, 1541–1548 (2010).

328. Světnička, M., Selinger, E., Gojda, J. & El-Lababidi, E. Plant based diets: from toddler to adolescence. *Pediatr. pro praxi* **21**, 264–269 (2020).

329. Selinger, E., Kühn, T., Procházková, M., Anděl, M. & Gojda, J. Vitamin B12 deficiency is prevalent among czech vegans who do not use vitamin B12 supplements. *Nutrients* **11**, (2019).

PUBLICATIONS AND ABSTRACTS

Here is a list of publications and abstracts directly related to the presented work.

1. GOJDA J., TRNKA J., ANDĚL M.: Cellular mechanisms of muscle atrophy | Buněčné mechanizmy svalové atrofie. Diabetologie Metabolismus Endokrinologie Výživa. 2011:14(3). ISSN 12119326 12126853.

Review paper on mechanisms of skeletal muscle metabolism, mainly focused on atrophy.

2. GOJDA J., PATKOVÁ J., JAČEK M., POTOČKOVÁ J., TRNKA J., KRAML P., ANDĚL M.: Higher insulin sensitivity in vegans is not associated with higher mitochondrial density. European journal of clinical nutrition [online]. 2013(September), 1–6. ISSN 1476-5640. Dostupné z: doi:10.1038/ejcn.2013.202.

The study has validated use of vegan vs. omnivore model for deep physiologcal studies. We showed higher insulin sensitivity using clamp studies but normal mitochondrial respiration in skeletal muscle in vegans.

3. TUMA P., GOJDA J.: Rapid determination of branched chain amino acids in human blood plasma by pressure-assisted capillary electrophoresis with contactless conductivity detection. Electrophoresis [online]. 2015:36(16). ISSN 15222683 01730835. Dostupné z: doi:10.1002/elps.201400585. Impact factor 3.028

The study presenting a novel method for fast BCAA analysis in blood samples using pressure-assisted capillary electrophoresis with contactless conductivity detection.

4. GOJDA J., ROSSMEISSLOVÁ L., TŮMOVÁ J., KRAJČOVÁ A., ELKALAF M., ŽIAK J., JAČEK M., BALUŠÍKOVÁ K., DUŠKA F., TRNKA J., ANDĚL M.: Role of percutaneous biopsy of skeletal muscle in diabetes research. Methodological summary | Postavení perkutánní biopsie kosterního svalu v diabetologickém výzkumu. Metodologický přehled. Diabetologie Metabolismus Endokrinologie Výživa. 2015: 18(4). ISSN 12126853 12119326.

Methodological review summarizing our expertise in skeletal muscle biopsy. We present our experience, operating procedure and analysis on mitochondrial respiration and satellite cells cultivation.

5. GOJDA J., STRAKOVA R., ROSSMEISLOVA L., TUMOVA J., ELKALAF M., JACEK M., TUMA P., POTOCKOVA J., TRNKA J., STICH V., ANDEL M.: Chronic Dietary Exposure to Branched Chain AMINO ACIDS Causes Impaired Glucose Disposal and Higher Adipose Tissue Lipogenesis. Clinical Nutrition [online]. OR013. B.m.: Elsevier 2015: 9.34, S5 [vid. 2017-03-24]. ISSN 02615614. doi:10.1016/S0261-5614(15)30110-2. Awarded by Travel Grant ESPEN.

6. GOJDA J., ROSSMEISLOVÁ L., STRAKOVÁ R., TŮMOVÁ J., ELKALAF M., JAČEK M., TŮMA P., POTOČKOVÁ J., KRAUZOVÁ E., WALDAUF P., TRNKA J., ŠTICH V., ANDĚL M.: Chronic dietary exposure to branched chain amino acids impairs glucose disposal in vegans but not in omnivores. European Journal of Clinical Nutrition [online]. 2017. ISSN 0954-3007. Dostupné z: doi:10.1038/ejcn.2016.274.

The study further exploiting vegans vs. omniovore model for clinical trial with BCAA supplementation. We showed that in the low BCAA intake group (i.e. vegans) supplementation with BCAA deteriorates insulin sensitivity. The effect is associated with incapability of upregulation of lipogenesis in adipose tissue.

7. GOJDA J., STRAKOVA R., HAVLOVA A., POTOCKOVA J., TŮMA P., ANDĚL M.: Incretin effect of branched-chain amino acids. EASD Congress 2015a - ORAL 59.

8. GOJDA, J., R. STRAKOVÁ, A. PLÍHALOVÁ, P. TŮMA, J. POTOČKOVÁ, J. POLÁK a M. ANDĚL, 2017b. Increased Incretin but Not Insulin Response after Oral versus Intravenous Branched Chain Amino Acids. Annals of Nutrition and Metabolism [online]. 70(4). ISSN 14219697. Dostupné z: doi:10.1159/000475604

The studies on incretin effect of BCAA. We showed in a randomized placebo controlled acute trial that BCAA modulate acute insulinogenic response via GLP-1.

9. JAČEK M., GOJDA J., DLOUHÝ P. TŮMA P.: The use of coupled gas chromatography columns for the determination of individual isomers of trans fatty acids in the adipose tissue of vegans. Monatshefte fur Chemie [online]. 150(8). ISSN 00269247. Dostupné z: doi:10.1007/s00706-019-02481-9

The study on coupled gas chromatography columns presenting a novel technique for the determination of individual isomers of trans fatty acid.

10. TŮMA P., GOJDA J., SOMMEROVÁ B., KOVAL D.: Measuring venous-arterial differences of valine, isoleucine, leucine, alanine and glutamine in skeletal muscles using counter-current electrophoresis with contactless conductivity detection. Journal of Electroanalytical Chemistry [online]. B.m.: Elsevier B.V., 857, 113772. ISSN 15726657. Dostupné z: doi:10.1016/j.jelechem.2019.113772

The study gurther developing novel techniques in counter-current electrophoresis with contactless conductivity detection allowing for future online sampling.

11. KAMIŠOVÁ J., ROSSMEISLOVA L., POTOČKOVÁ J., PROCHÁZKOVÁ M., POLÁK J., VARALIOVÁ Z., HRUDKA J., ANDĚL M., GOJDA J.: Metoprolol but not metformin attenuates stimulated lipolysis in visceral adipose tissue in patients with pancreatic cancer. National Diabetology Congress, virtual, Luhačovice, 2020. Poster

First data presented as an abstract on the complex protocol studying metabolic derrangements associated with cancer cachexia. We focus on glucose and lipid metabolism of the cancer host and here we show that beta blocker metoprolole is capable of inhibition of stimulated lipolysis in visceral fat of patient with pancretaic cancer.

12. PROCHAZKOVA M., KUZMA M., PELANTOVA H., CAHOVA M., GOJDA J.: Metabolic health corresponds to a distinct microbiome and metabolome signature: Cross sectional comparison of lean vegans, lean omnivores, obese and prediabetics. ESPEN Virtual congress 2020. Poster

First data presented as an abstract on association between eating patterns, intestinal microbiome and metabolome and metabolic health. We showed distinct intestinal metabolites associated with metabolic health. Intestinal BCAA were among these metabolites.