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The role of branched-chain amino acids in the development and progression of insulin resistance and type 2 diabetes

Dissertation

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Summary

Increased levels of branched-chain amino acids (BCAA) associate with insulin resistance and type 2 diabetes (T2D), which could result from dietary habits, gut microbiome composition or altered cellular energy metabolism. We hypothesized that reduced dietary intake of BCAA improves whole body insulin sensitivity and hyperinsulinemia in patients with T2D.

In a randomized, placebo-controlled, double-blinded cross over trial, 12 metabolically well-controlled patients with T2D received an isocaloric diet (protein: 1 g/kg body weight), containing either the complete amino acid set ($BCAA^+$) or a 60% reduced amount of BCAA ($BCAA^-$) for one week each. Effects on glucose homeostasis were assessed from mixed meal tolerance tests (MMT) and hyperinsulinemic-euglycemic clamp tests (HEC), and pathways affecting insulin signaling were analyzed in skeletal muscle and adipose tissue biopsies. Gut microbiome composition was assessed by next generation sequencing.

After the $BCAA^-$ diet, MMT-derived insulin secretion was 28% lower compared to the $BCAA^+$ diet ($p<0.05$). After the $BCAA^-$ diet, MMT-derived insulin sensitivity (PREDIM, the validated predicted HEC-derived M-value from meal data) was 23% higher ($p<0.01$), whereas HEC-derived insulin sensitivity (M-value) remained unchanged. Respiratory control ratio was unchanged in skeletal muscle, but 1.7-fold higher in adipose tissue ($p<0.05$). The mechanistic target of rapamycin (mTOR) was downregulated by 13% in adipose tissue ($p<0.05$). $BCAA^-$ diet was further associated with a 40% increase of fecal Bacteroidetes and a 11% decrease of Firmicutes (both $p<0.05$).

In conclusion, a short-term dietary reduction of BCAA decreases insulin secretion and increases postprandial insulin sensitivity, which may relate to adipocyte mitochondrial efficiency and altered gut microbiome composition in patients with T2D.

Zusammenfassung

Erhöhte Serumspiegel verzweigtkettiger Aminosäuren (BCAA: Valin, Leucin, Isoleucin) assoziieren mit Insulinresistenz und Typ-2-Diabetes (T2D), was aus den Ernährungsgewohnheiten, der Zusammensetzung des Darmmikrobioms oder Veränderung des zellulären Energiestoffwechsels resultieren könnte. Wir prüften die Hypothese, dass eine diätetische BCAA-Reduktion die Insulinsensitivität verbessert und die Hyperinsulinämie bei Patienten mit T2D vermindert.

In einer randomisierten, Placebo-kontrollierten Crossover-Doppelblindstudie erhielten 12 Patienten (8 männlich, 4 weiblich, 54±4 Jahre, BMI 30,8±2,8 kg/m^2, HbA1c 6,6±0,9%/49±10 mmol/mol) eine einwöchige Diät mit allen Aminosäuren ($BCAA^+$) oder mit einem um 60%igen reduzierten Gehalt an BCAA ($BCAA^-$). Die Glukosehomöostase wurde durch Mixed-Meal-Toleranz-Tests (MMT) und hyperinsulinämisch-euglykämische Clamp-Tests (HEC) erfasst. In Muskel- und Fettgewebebiopsien wurde die Insulin-Signalübertragung mittels Western-Blots und die mitochondriale Effizienz mittels hochauflösender Respirometrie (Respiratory Control Ratio, RCR) bestimmt. Die Zusammensetzung des Darmmikrobioms wurde durch Next-Generation-Sequenzierung ermittelt.

Verglichen mit der $BCAA^+$-Diät, bewirkte die $BCAA^-$-Diät eine Abnahme der BCAA-Serumkonzentration um 17% ($p<0,01$). Die MMT-induzierte Insulinsekretion war 28% niedriger als bei der $BCAA^+$-Diät ($p<0,05$). Die Insulinsensitivität stieg im MMT (PREDIM) um 23% ($p<0,01$), blieb aber im HEC (M-Wert) unverändert. Nach $BCAA^-$-Diät war die Respiratorische Kontrolle (RCR) im Skelettmuskel unverändert, im Fettgewebe jedoch 1,7-fach höher ($p<0,05$). Die Phosphorylierung des Mechanistic Target of Rapamycin (mTOR) war nur im Fettgewebe um 13% niedriger ($p<0,05$). Die $BCAA^-$-Diät führte desweitern zu einer Zunahme fäkaler Bacteroidetes um 40% und einer Abnahme der Firmicutes um 11% (beides $p<0,05$).

Eine kurzfristige diätetische Reduktion verzweigtkettiger Aminosäuren senkt die Insulinsekretion und steigert die postprandiale Insulinsensitivität, die durch verbesserte mitochondriale Effizienz des Fettgewebes und veränderte Zusammensetzung des Darmmikrobioms bedingt sein könnte.

List of abbreviations:

AA, amino acids

ADA, American Diabetes Association

ALT, alanine aminotransferase

AMPK, adenosine-monophosphate kinase

AST, aspartate aminotransferase

AUC, area under the curve

BCAA, branched-chain amino acids

BCKA, branched-chain alpha-keto acid

BCKDH, branched-chain alpha-keto acid dehydrogenase complex

BMI, body mass index

BW, body weight

CSA, citrate synthase activity

DPP, diabetes prevention program

EGP, endogenous glucose production

FFA, free fatty acids

FGF21, fibroblast-growth factor 21

FPG, free plasma glucose

GGT, gamma-glutamyltransferase

HEC, hyperinsulinemic euglycemic clamp test

iAUC, incremental area under the curve

IDF, international diabetes federation

IFG, impaired fasting glucose

IGT, impaired glucose tolerance

ILE, isoleucine

IRS1, insulin receptor substrate 1

LEU, leucine

MMT, mixed-meal tolerance test

MSUD, maple syrup urine disease

mTOR, mechanistic target or rapamycin

NAFLD, non-alcoholic fatty liver disease

OGIS, oral glucose insulin sensitivity index

PAL, physical activity level

PCOS, polycystic ovary syndrome

PREDIM, predicted M-value

PUFA, polyunsaturated fatty acids

RCR, respiratory control ratio

SCFA, short-chain fatty acids

T2D, type 2 diabetes

VAL, valine

WAT, white adipose tissue

Inhaltsverzeichnis

1. Introduction

The growing diabetes epidemic is one of the major global health problems caused by population growth and ageing [1]. It resembles a paramount high-cost biomedical challenge for the industrialized world and places a tremendous financial burden on society for the patient care and treatment. Furthermore, the constant rise of obesity and T2D-associated co-morbidities such as hyperlipidemia, cardiovascular disease, kidney disease and non-alcoholic fatty liver disease (NAFLD) demonstrate the urgent necessity to identify and ultimately target the molecular mechanisms underlying their onset, manifestation and progression, to combat this epidemic using a holistic approach.

1.1. Type 2 diabetes mellitus

Worldwide, 425 million people have diabetes with more than 58 million in Europe and T2D cases are projected to rise between 10.7 million (+54%) and 12.3 million (+77%) in the total adult population in Germany by 2040 [2]. Globally, 1 out of 11 have diabetes and the number has quadrupled over the past three decades with diabetes mellitus being the ninth major cause of death [3]. Major driving factors of the global T2D epidemic are overweight and obesity, sedentary lifestyle and increased consumption of unhealthy diets. Progressive and effective strategies to prevent gestational diabetes mellitus and its manifestation in children and young adults are urgently needed. Of note, prevalence of childhood obesity in many countries is rising leading to increased numbers of T2D already in pediatric populations which results in complications in early adulthood [4]. Considering its increasing prevalence, childhood T2D may become a threatening public health problem [3].

T2D is defined by chronic hyperglycemia and characterized by inadequate beta-cell function and insulin resistance of insulin-sensitive target tissues such as skeletal muscle, adipose tissue and liver. The initial defect is most likely insulin resistance with subsequent compensatory increase of pancreatic insulin secretion, which at some point declines and hyperglycemia starts. According to the American Diabetes Association (ADA) diabetes is diagnosed as follows [5]:

Table 1: Criteria for diagnosis of diabetes according to the American Diabetes Association

Diagnostic criteria	**Diabetes mellitus**
Fasting plasma glucose*	≥126 mg/dl (7.0 mmol/l)
or	
HbA1c**	≥ 6.5 % (48 mmol/mol)
or	
Symptoms of hyperglycemia and a random plasma glucose***	≥ 200 mg/dl (11.1 mmol/l)
or	
2-h plasma glucose during an OGTT******	≥ 200 mg/dl (11.1 mmol/l)

* Fasting is defined as no caloric intake for at last 8 hours.

** The test should be performed in a laboratory using a method that is certified by the NGSP (National Glycohemoglobin Standardization Program) and standardized by the assay according to DCCT (Diabetes Control and Complications Trial).

*** The classic symptoms of hyperglycemia are defined as polyuria, polydipsia and unexplained weight loss.

**** The test should be performed as described by the World Health Organization, using a glucose load containing the equivalent of 75 g anhydrous glucose dissolved in water.

The onset of diabetes mellitus often occurs years before the actual diagnosis. Globally, 45.8% (or 174.8 million cases) of all adult diabetes cases were estimated to be undiagnosed [6]. People with undiagnosed and untreated diabetes mellitus are at a greater risk of complications compared to those receiving treatment. The state of pre-diabetes with only slightly elevated blood glucose levels (i.e. impaired fasting glucose (IFG) and/or impaired glucose tolerance (IGT)) may also precede T2D for years [7]. Furthermore, the development of diabetes in pre-diabetic individuals can be prevented or delayed by lifestyle intervention actually by dietary changes and increased physical activity [8].

Moreover, expenditure for medical needs of patients with diabetes mellitus is up to three times greater than for the general population without diabetes mellitus [9] and increasing costs

should be spent on prevention programs and raising awareness. An increase in the number of adults with diabetes by 20% in developed and 69% in developing countries has been predicted to occur between 2010 and 2030 [10]. This comprises not only a social, but also an economic burden, since increasing prevalence is closely related to rising direct and indirect healthcare costs, with cardiovascular complications, diabetes duration and insulin therapy correlating positively with increased costs [11].

1.2. Pathophysiology and major risk factors

When the feedback loops between insulin action and insulin secretion do not function properly, the insulin action on insulin-target tissues like skeletal muscle, adipose tissue and liver as well as beta-cell insulin secretion are affected, which leads to abnormally high blood glucose levels [12] [Figure 1]. In addition, beta-cell dysfunction results in reduced insulin release, which is insufficient to maintain normal glucose levels [13, 14]. Insulin resistance is due to alterations in interorgan communication by multiple metabolites serving as mediators. Some key players such as amino acids (AA), ketoacids and lipids regulate insulin sensitivity in skeletal muscle, liver and adipose tissue of humans [15]. Previous studies suggest that the differences in insulin sensitivity observed between the individuals with prediabetes and those with overt diabetes can be attributed at least in part to differences in obesity and abdominal fat [16]. Nevertheless, most but not all patients with T2D are overweight. Differences in insulin sensitivity between groups were largely explained by distinctions in overall and particularly abdominal visceral obesity indicative of a linkage between abdominal fat depots and glucose regulation in the fasting state, potentially mediated by adipokines. Since skeletal muscle is responsible for 70-80% of insulin-stimulated glucose uptake and adipose tissue for just 5-10% [17], it is skeletal muscle insulin resistance that is considered the critical pathological component of T2D and metabolic syndrome [18]. Initially, dysfunction of white adipose tissue and circulating metabolites modulate tissue communication and insulin signaling [19]. A normal protective response of the cell to excess nutrients under physiological conditions may be the acute insulin resistance as a strategy to prevent glucotoxicity or oxidative stress [20] and preserve glucose for tissues critically depending on this metabolite such as the brain [19]. Insulin signaling returns back to normal as soon as nutrient levels restore to normal. Chronic nutrient excess seems to cause less easily reversible changes that prevent normal glucose uptake, leading to hyperinsulinemia and hyperglycemia and at the same time to glucose deprivation in the tissue. More harmful changes such as oxidative stress,

inflammation, vascular/endothelial dysfunction could occur as secondary outcomes [21]. Over the past decades, advanced epidemiological research on the causes of T2D have clarified the interrelationship between the variety of risk factors for T2D development. They can be categorized in modifiable and non-modifiable. The most prominent non-modifiable are age and ethnicity [22]. Although genetic factors identify those at particularly high risk, epidemiological studies reveal that T2D can be prevented with lifestyle improvements [23].

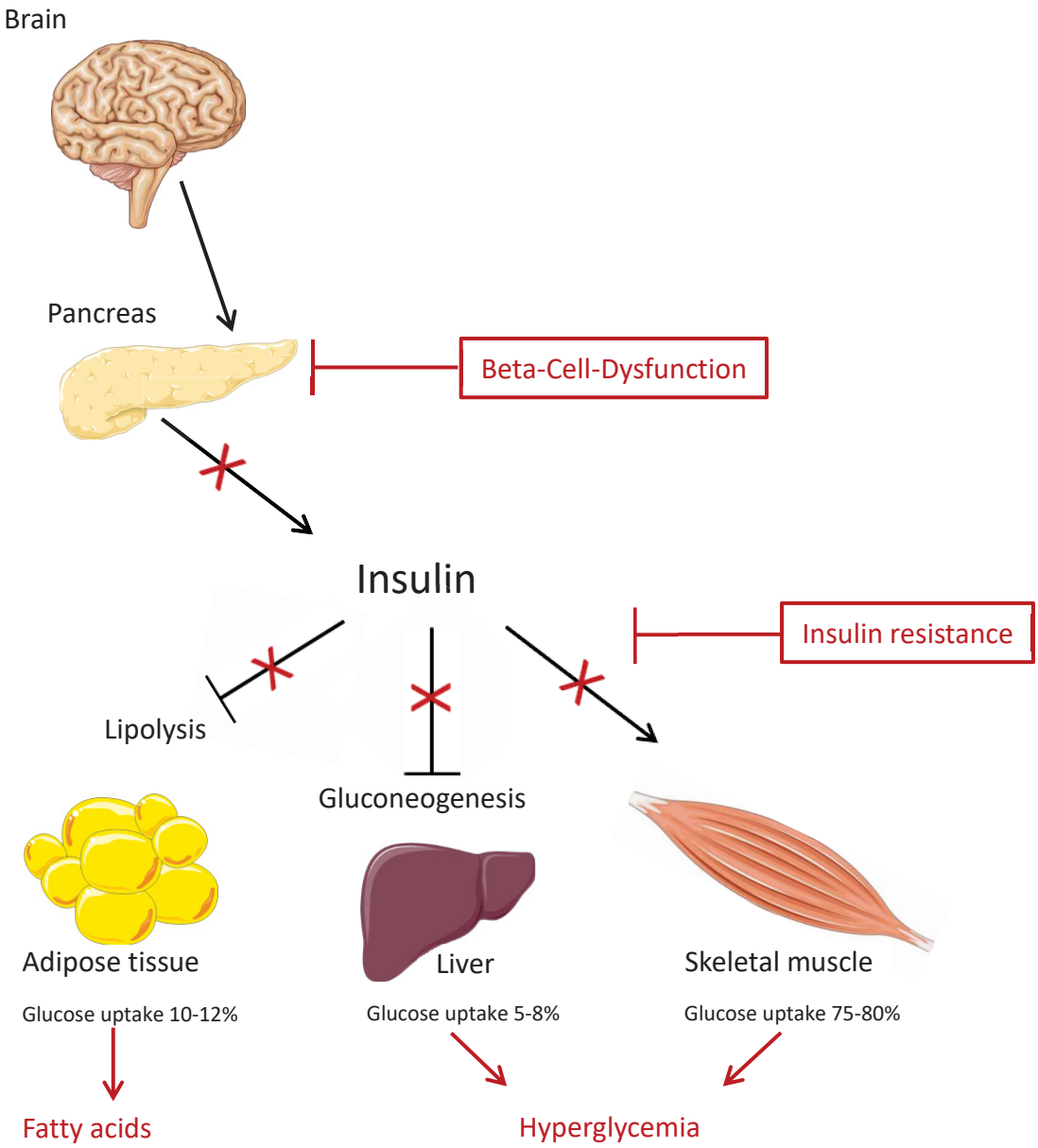


Figure 1. Fasting hyperglycemia in T2D. Beta-cells in the pancreas produce insulin, which reduces glucose output in the liver, supresses fatty acid release and increases glucose uptake by adipose tissue and skeletal muscle under physiological conditions. With beta-cell function impairment and progressive insulin resistance of liver, skeletal muscle or adipose tissue, excessive amount of glucose remains in circulation leading to hyperglycemia and increased circulating fatty acids.

1.2.1. Non-modifiable risk factors

The most important non-modifiable risk factor is age, with prevalence of T2D rising with age up to 8th decade among men and women [22]. Furthermore, genetic predisposition is another key non-modifiable risk factor, which plays an important role in the T2D and metabolic syndrome development [14]. The heterogeneous nature of T2D suggests that the focus should be predominantly placed on phenotypically homogenous subgroups of patients [24] as recently suggested [25]. Genetic factors play a major role in determining an individual's position along the population distribution of adiposity [26]. Thus, there are cases of T2D which can be prevented by maintaining normal body weight and leading a healthy lifestyle, but also some which are more difficult to respond or that remain insusceptible to change (non responders) [27]. Some studies provided compelling evidence for a genetic component in T2D [28] which might be the explanation for the non-responders' reactions [29]. Genome wide association studies have identified the polygenic nature of T2D [30]. Different ethnic groups as Hispanic and Asian Indians were proven to be at higher risk compared to Caucasians [31]. Females who had gestational diabetes defined as glucose intolerance with onset or first recognition during pregnancy and their infants are at a sevenfold higher risk for developing T2D later on [32, 33]. In utero exposure to maternal hyperglycemia is a strong risk factor for cardiometabolic diseases [34] due to diminished beta-cell function [31]. For instance, rates of T2D were dramatically increased over time in Pima Indian infant population in which incidence of gestational diabetes is very high [35]. Gestational diabetes is defined as any degree of impaired glucose tolerance with onset or first recognition during pregnancy [36]. It affects about 3-5% of all pregnancies [36]. Women with polycystic ovary syndrome (PCOS) comprise 10% of women in reproductive age and most of them are characterized by increased insulin resistance and impaired beta-cell function compared to age- and BMI-matched controls [37]. Around 30% of women with PCOS have an IGT and show accelerated progression to T2D, whereas 10% are already diagnosed with a T2D [37].

1.2.2. Modifiable risk factors

Cigarette smoking is a well-known risk factor in many metabolic diseases including a 45% higher risk of the development of T2D [38] but also passive exposure to smoking has also been associated with progression of insulin resistance [39]. Of note, smoking status was positively

associated with abdominal obesity in patients with T2D [40]. People who smoke are more often central obese and more hyperinsulinemic than nonsmokers [41]. They tend to be relatively insulin resistant and dyslipidemic, with evidence of endothelial dysfunction and at a higher risk for cardiovascular diseases compared to nonsmokers.

Over the past decades the role of alcohol in the etiology of T2D has been extensively discussed, as it has become one of the most prevalent lifestyle habits. There is growing consensus that moderate alcohol consumption is associated with a lower risk of T2D. The 20-year follow up Finnish twin study revealed that moderate alcohol consumption (5–29.9 g/day for men and 5–19.9 g/day for women) resulted in a reduced incidence of T2D compared to low consumption (<5 g/day) [42]. However, despite the positive association found between moderate alcohol consumption and insulin sensitivity [43], the message to the public should be cautiously communicated in the light of socioeconomic burden of binge drinking, chronic consumption or alcohol dependence and NAFLD. In a population-based prospective study, alcohol consumption was proven to strongly increase the risk of T2D by increasing insulin resistance, the most prominent in males with high genetic risk score for diabetes, [44]. This highlights the importance of refraining from excessive alcohol intake when making recommendations for healthy lifestyle habits to prevent diabetes

A further modifiable risk factor for T2D is therapy with drugs, such as glucocorticoids, antihypertensives (β-blockers, thiazide diuretics), immunosuppressive, atypical antipsychotic agents and drugs used for HIV-infection [45]. Finally, psychosocial stress and depression are also associated with an increased risk of T2D development by up to 37% [46].

Last but not least, physical activity plays a major role in the management of insulin resistance, prediabetes, gestational diabetes mellitus, T2D, and diabetes-related comorbidities and complications [47]. Regular exercise improves acute insulin action and helps to treat hyperglycemia, hyperlipidemia, hypertension, cardiovascular risk, quality of life and decreases mortality [48]. Both aerobic and resistance training have independently preventive effects on T2D development [49]. Higher levels of physical activity are associated with lower risk of T2D development. A program consisting of increased physical activity and moderate weight loss could decrease the risk of T2D development by about 60% [50]. The effect of exercise on lowering diabetes risk is explained by beneficial acute and chronic effects on insulin action and skeletal muscle insulin sensitivity and can be achieved by either aerobic or resistance training interventions [51]. Increased physical activity also provides additional effects on circulating lipids, blood pressure and mortality [50].

1.2.2.1. Overweight and obesity

The prevalence of T2D is increasing in parallel with the rising obesity incidence in industrialized countries [52]. In accordance, a previous study revealed that sustained weight loss resulted in sustained remission of T2D [53]. Elevated BMI (BMI≥25 kg/m^2) as a marker of overweight and adiposity, is the single most powerful risk factor for T2D [23]. In addition, waist-hip ratio predicts T2D risk independently of BMI [54] proving the importance of body fat distribution and visceral fat accumulation. In clinical practice, it is therefore important to monitor both BMI and waist circumference. Interestingly, visceral adiposity might be another link between obesity and insulin resistance [55]. Adiposity in childhood and young adulthood is associated with significantly higher risk of chronic diseases and T2D, which gradually occur in younger ages [56]. Understanding the T2D pathophysiology in youth, as well as evaluating the risk of complications and the psychosocial impact will enable the development of future research, treatment, and prevention approaches [57]. Furthermore, the positive correlation of beta-cell dysfunction with the severity of metabolic syndrome highlights the need to better understand the different stages of beta-cell dysfunction in the development of metabolic syndrome on the way to progress to T2D [58].

To refer to the binary epidemics of obesity and diabetes mellitus, we need to acknowledge the fundamental causes of both diseases with a focus on unhealthy diet and sedentary lifestyle.

It is estimated that 90% of T2D patients are obese, however only 20-25% of obese individuals develop a T2D. More important is the localization of obesity, with central obesity being tightly associated with insulin resistance, T2D and cardiovascular risk [59]. Waist circumference provides a measure for central adiposity with cut-off points ranging according to ethnicity. In this context, we should refer to the metabolic syndrome, as an important risk factor for cardiovascular events, T2D and all-cause mortality [60]. According to the current IDF definition, an individual suffers from the metabolic syndrome, if it suffers from central obesity (defined by waist circumference) and has 2 more of the following factors:

- Triglycerides (TGs) ≥150 mg /dl (1.7 mmol/l) or treatment for this lipid abnormality
- High-density lipoprotein cholesterol (HDL) < 40 mg/dl (1.03 mmol/l) in males
  or < 50 mg/dl (1.29 mmol/l) in females,
  or treatment for this lipid abnormality
- Systolic blood pressure ≥ 130 mm Hg or diastolic blood pressure ≥ 85 mm Hg, or treatment for previously diagnosed hypertension
- Dysglycemia defined as fasting blood glucose ≥ 100 mg/dl (5.6 mmol/l) or T2D.

According to these criteria, the metabolic syndrome is not only a cluster of risk factors for the development of T2D, but an important component of the disease itself. Obesity is mainly the result of increased food intake and decreased physical activity, although genetic predisposition is also involved in its development. As far as diet is concerned, foods rich in saturated fats, red and processed meat, as well as sugar-sweetened beverages are associated with increased diabetes risk, whereas a high intake of vegetables, coffee and fiber-rich foods decreases the risk of diabetes development and is associated with improved glycemic control in patients with established T2D [61, 62]. NAFLD also closely associates with obesity, and has been called the liver manifestation of the metabolic syndrome [63], cardiovascular disease, T2D and its complications [64].

1.2.3. Diet and lifestyle factors

At a global level, the T2D epidemic has been also interpreted a result of urbanization and environmental transition, including work pattern transformations from largely or exclusively physical work to predominantly sedentary occupations, growing cybernation and mechanization, and automated transportation [61]. Increased processed food consumption and nutrition transition is another driving force in international obesity in overweight [65]. Dietary intake of high-caloric high-fat diets and sedentary lifestyle lead to increased storage of triglycerides not only in adipose tissue but also ectopically in other tissues [66]. Intracellular lipid contents in skeletal muscle and liver have been related to insulin resistance and inflammatory processes. Furthermore, diets characterized by high glycemic index or high glycemic loads are associated with increased risk for T2D [67]. Milestone clinical trials such as the Diabetes Prevention Program (DPP) and the Finnish Diabetes Prevention Study (DPS) showed that lifestyle interventions including increased physical activity and adopting a healthy diet prevent or delay the development of T2D [8, 68]. Nevertheless, if lifestyle changes are not successful, pharmacological treatment such as metformin which still represents the first-choice drug in T2D, shall be introduced. The DPP compared the effectiveness between lifestyle intervention and metformin in preventing the onset of T2D. The lifestyle intervention involved a low-calorie, low-fat diet and a moderate physical activity for at least 150 min per week for patients at high risk for the development of T2D with mean age of 51 years and mean body mass index (BMI) of 34 kg/m^2 in order to achieve and maintain a weight reduction of 7% [68]. This study reported a reduction of T2D risk by 58% and 31% in the lifestyle intervention and

metformin groups, respectively and although it was not primarily designed to reduce weight, it delivered a solid evidence that weight reduction is the dominant predictor for reduced T2D risk. The T2D onset can be delayed by at least 4 years by reducing body weight by 4-7% and sustaining the reduction. It is however of particular interest to set the 'turning point' for insulin resistance to be reversed by lifestyle intervention only. From the Look Ahead trial an evidence was provided that a weight loss of 7-8.6% for one year is clinically significant and improves insulin sensitivity [69]. However, it was recently demonstrated that ~7% of body weight within a short period of time (2 weeks), does not translate into immediate improvement of muscle insulin resistance [70]. The burning question is at what point and maintained for what duration a multifactorial lifestyle intervention modifying obesity, physical inactivity, smoking, blood pressure and dyslipidemia is insufficient to preserve the beta-cell in the progression of the metabolic disorder. A holistic approach is needed to address the deteriorating beta-cell function, therefore it should be indirectly targeted through managing pre-diabetes and impaired fasting glucose as well as impaired glucose tolerance, in particular. Self-management education and patient-centered care are the cornerstones of T2D management in addition to lifestyle strategies with individualization of glycemic goals [71]. Individuals with T2D and their families usually share a common lifestyle that, not only predisposes the non-T2D members to developing metabolic disorder, but also increases their collective risk for cardiovascular disease. The collective nutritional behaviour of patients and their families should be assessed to make the adoption of a healthy lifestyle easily conceivable. Diabetes requires lifelong adjustments to lifestyle and pharmacotherapy; thus, in order to achieve glycaemic and other therapeutic targets, active participation and commitment of the individual is essential. These provide also expected short-term benefits [69] such as improved well-being that increases self-efficiency and personal motivation. Long-term benefits include late onset in the ones without diabetes [72] as well as reduction of microvascular complications development risk and improvement of quality of life in the ones with manifested T2D. Lifestyle interventions are cost-effective and delaying the onset of diabetes as proven for a duration over 10 years, from a payer perspective, compared with placebo. Investment in lifestyle for diabetes prevention in high-risk adults provides good value for the money spent [73]. The difficulty of implementing lifestyle interventions though is the challenge to apply them to real-life settings [71]. However, in contrast to any pharmacotherapy, in which side effects occur, such as weight gain, hypoglycemia, gastrointestinal discomfort, and fluid retention lifestyle interventions and change in nutrition are universal. Poor adherence may thus limit the effectiveness of this strategy and the key health achievements in people with T2D.

The focus on a “healthy” diet has been identified as a cornerstone of researchers and policymakers to prevent the onset of T2D. A rising interest has emerged on positive effects of vegetarian and vegan diets on the prevention of chronic conditions including obesity and cardiovascular diseases. Previous works reveal that plant-based diets, especially when rich in high-quality plant foods not including sweetened food and beverages, are associated with substantially lower risk of developing T2D [74]. Alongside overall diet quality, a few dietary highlights such as Mediterranean, low glycemic index, moderately low carbohydrate, and vegetarian diets can be adapted to personal and cultural food preferences and appropriate calorie needs to control body weight and to prevent and manage overt diabetes [61]. On the other hand, calorie restriction independent of the intake of fiber, coffee or meat failed to improve beta-cell function [22]. However, this might be due to the fact that dietary effects on reversing reduced beta-cell function are limited at the background of chronic prevalent insulin resistance characterized by hyperinsulinemia and hyperglycemia. This is why the necessity of adopting a healthy diet early on even before the diagnosis of T2D is essential. Dietary recommendations typically promote diets rich in fruits, vegetables, nuts, whole grains and low in refined grains, red or processed meat [61]. The quality of dietary fats and carbohydrates consumed is more crucial than the quantity of these macronutrients and changing the macronutrient composition of the diet while keeping the total number of calories constant is an intriguing alternative that may be more sustainable. An overview of different kinds of diets on glycemic control is given in Table 2.

Of note, acute dietary fat intake initiates alterations in energy metabolism and increases skeletal muscle insulin resistance in healthy adults [75]. In accordance, a low-fat vegan diet improves glycemic control and blood lipids in T2D compared to a diet based on American Diabetes Association (ADA) guidelines [76]. The difference between animal and plant fat on the risk of T2D has been discussed intensively with an emphasis on the levels of triglycerides, phospholipids and cholesterol. Recent studies indicated no association between total fat intake and risk of T2D, but origins of different fatty acids seem to play a role with a positive influence of plant-derived fatty acids on lowering the risk for T2D [77]. Of note, quality of dietary fat was proven to play an important role in comorbidities of T2D as higher intake of polyunsaturated fatty acids (PUFAs) reduces total mortality and cardiovascular disease mortality [78].

Also, the protein source is of importance. T2D risk is associated with higher red and processed meat consumption [79]. Furthermore, high total and animal protein intake was associated with a modest elevated risk of T2D [80]. Protein restricted diets have been shown to significantly

improve the longevity of animals [81, 82]. Epidemiological studies in humans suggest that high protein intake associates with increased mortality, whereas lower protein intake is associated with decreased mortality [83]. Individuals on high protein diets are more likely to develop metabolic diseases such as T2D and obesity [84].

Taken together, dietary behaviour and choice of nutrients are most often personal and it is more realistic for a dietary alteration to be individualized rather than to be applied as a universal approach. Identification of dietary patterns is important for glycemic management and management of insulin resistance in patients with T2D.

Table 2

Randomized controlled nutritional interventions on glycemic control in adult patients with T2D with duration 6 m - 1 y and clearly defined macronutrient composition

Ref.	Participants/conditions	Diet	Carbs	Protein	Fat	Duration	IS Test	Outcomes
[86]	severely obese N=51, 39% with T2D	low carb con	37% 51%	22% 16%	41% 33%	6 m	ISI	↓ BW, ↓ TG, ↓ HbA1C, ↓ FPG, IS n.d. in T2D, ↑ IS in non-T2D after low carb diet
[87]	obese N=109, 83% with T2D	low carb con	120 g 230 g	73 g 74 g	93 g 69 g	1 y	QUICKI index	~ BW, ↓ TG, ↓ HbA1C, ↑ HDL, ↑ IS in T2D after low carb diet
[88]	obese N=50 with T2D	low carb	13%	28%	59%	6 m	n.d.	↓ BW, ↓ HbA1C, ↑ HDL
[89]	N=156 with T2D	low carb	40%	20%	40%	1 y	n.d.	~ BW, ~ HbA1C
[90]	N=127 with T2D	low carb con diet	45% 57%	18% 16%	33% 26%	1 y	n.d.	↓ BW, ↓ HbA1C, ↓ LDL
[91]	N=105 with T2D	low carb con diet	20-25 g		 25%	1 y	n.d.	↑ HDL
[92]	overweight N=45 with T2D	low carb low fat	50-60 g 190 g	50-55 g 73-80 g	50-60 g 35-40 g	1 y	n.d.	↔
[93]	obese N=77 with T2D	low carb con diet	35 g 40 g	20 g 23 g	40 g 34 g	1 y		↔
[94]	overweight N=74 with T2D	vegetarian con diet	60% 50%	15% 20%	25% <30%	6 mo	HEC	↓ BW, ↑ IS in T2D after vegetarian diet
[95]	postmenopausal women N=245 with T2D	mediterranean diet vs con				6 mo	n.d.	↓ HbA1C, ↓ BMI
[96]	high-risk cardiovascular patients N=819 with T2D	mediterranean diet + olive oil vs mediterranean				1 y	n.d.	↓ TG in the + nuts group

		diet + nuts						
[97]	overweight women N=215 with T2D	mediterranean low in red meat con low in red meat	<50%		<30%	1 y	n.d.	reduction of oral antidiabetic drugs
[98]	overweight adults N=118 with T2D	mediterranean con diet	50-55% 60-70%	15-20% 15-20%	30% <7%	1 y	n.d.	↓ TG
[99]	obese N=38 with T2D	high-protein low-protein	40% 55%	30%+21 g 15%+7 g	30% 30%	1 y	n.d.	↔
[100]	overweight/obese N=99 with T2D	high-protein con diet	45% 48%	26.5% 19%	31% 32%	1 y	n.d.	↔
[101]	overweight/obese N=95 with T2D	high-protein con diet	54% 46%	15% 15%	28% 38%	1 y	n.d.	↔
[102]	overweight/obese N=227 with T2D	low carb low fat con diet	45% 60% 55%	25% 20% 30%	30% 20% 15%	1 y		↓ HbA1C, ↓ FPG, ↓ BW in all groups
[103]	obese N=115	low carb, high-unsaturated fat high carb low-saturated fat	14% 53%	28% 17%	58 30%	13 m	n.d.	↓ HbA1C, ↓ FPG, ↓ BW in all groups

BW, body weight; TG, triglycerides; con, control; carb, carbohydrates; HEC, hyperinsulinemic euglycemic clamp test; FPG, free plasma glucose; HDL, high density lipoprotein; LDL, low density lipoprotein; carb, carbohydrate; IS, insulin sensitivity; m, months; y, years; n.d., not done; QUICKI, quantitative insulin sensitivity check index; PUFA, polyunsaturated fatty acids.

1.3. Branched-chain amino acids (BCAA)

Among the proteinogenic AA, there are the three AA, valine, leucine and isoleucine, which have aliphatic chains with a branch in the end and therefore named BCAA [Figure 2]. These BCAA are also termed essential, as they cannot be synthesized by the human body and must be obtained from food sources. Nutrients with highest content of BCAA are meat, fish and dairy products. The BCAA content of mixed protein sources is approximately 20% [106]. In addition to building proteins, their numerous metabolic functions have been investigated [107]. Valine, leucine and isoleucine play important metabolic roles – enhance and promote protein synthesis, signaling pathways and glucose metabolism, activate a nutrient-sensitive, mTOR-mediated metabolism of glucose, lipid, and protein synthesis, intestinal health, and immunity through phosphoinositide 3-kinase/protein kinase B/mammalian target of rapamycin (PI3K/AKT/mTOR) signal pathway [108]. A large proportion of BCAA from dietary sources is absorbed from the intestines, bypasses the liver, reaches to the peripheral tissues [109] and is predominantly metabolized by skeletal muscle. Of the three BCAA, leucine is primarily responsible for the stimulation of protein synthesis which is mediated through the upregulation of mRNA translation [110]. BCAA further play physiologic roles in the immune system and brain function. In the brain they play a role in synthesis of proteins and neurotransmitters [111]. Under stress conditions such as surgery, trauma and starvation or during severe diseases such as fever, infections, liver cirrhosis, the requirement of BCAA is higher compared to that of other AA [112].

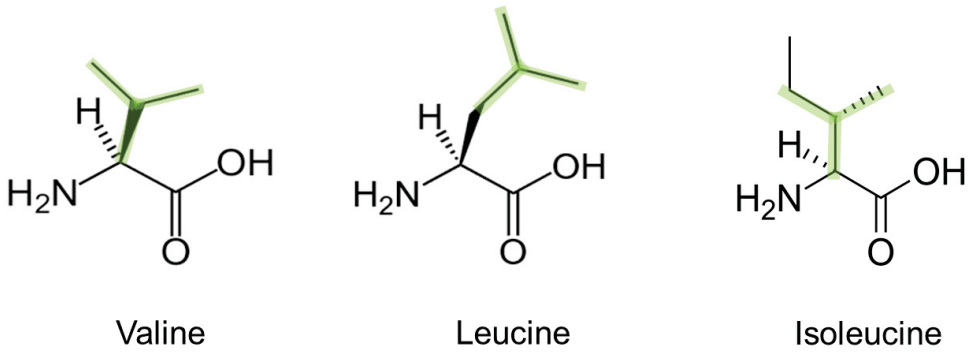


Figure 2. Branched-chain amino acids: valine, leucine and isoleucine.

BCAA are known for several health-promoting effects. They have been shown to enhance muscle protein synthesis and to be beneficial for patients with hepatic encephalopathy [113].

Drinking water supplemented with leucine resulted in doubled leucine plasma levels in high fat diet fed mice, leading to improvement of glucose homeostasis and higher insulin sensitivity, and an amelioration of hepatic steatosis and in adipose tissue inflammation without affecting food intake and weight gain [114]. Despite the effect of relieving hepatic encephalopathy symptoms, there is no evidence for improved mortality or overall quality of life.

1.3.1. Acquired BCAA deficiency

Undernutrition is common among aged individuals, due to multiple reasons that include reduced appetite and food intake, impaired nutrient absorption and other age-related medical, psychological and social changes. Particularly, protein-energy undernutrition leading to BCAA deficiency is associated with reduced strength, decreased bone mass, immune dysfunction, anemia, impaired cognitive function, long wound healing, delayed recovering from surgery and higher hospitalization rate and is a strong independent predictor of mortality in elderly people [115]. BCAA are decreased in patients with liver disease, such as hepatitis, hepatic coma, cirrhosis, extrahepatic biliary atresia or portacaval shunt; aromatic AA such as tyrosine, tryptophan and phenylalanine, as well as methionine-are increased in these conditions.

In contrast to the potential health-promoting BCAA effects under conditions of negative energy balance, chronic increased BCAA levels are observed in obesity-associated conditions as T2D, insulin resistance and cardiovascular conditions. BCAA have gained additional importance with the arising promising predictive role of metabolomics for the development of T2D and other cardiometabolic diseases [116]. In addition, BCAA are clearly a biomarker of cardiometabolic disease phenotypes [116]. In cardiomyocytes, glucose suppresses BCAA catabolism by inhibiting the expression of the Kruppel-like factor 15 (KLF15) [117]. In result, BCAA accumulate and subsequently activate the mechanistic target of rapamycin (mTORC1) to enhance protein synthesis and cardiac hypertrophy as shown in animal studies. Defect of BCAA degradation promotes heart failure progression and is associated with cardiovascular diseases [118]. Defects in BCAA transportation and metabolism is associated with autism [119]. Increased BCAA levels have been detected in early-stage pancreatic cancers [120].

1.3.2. Inborn errors of BCAA metabolism

In Maple Syrup Urine Disease (MSUD), an inborn deficiency of the intermediate metabolite of BCAA catabolism branched-chain alpha-keto acid (BCKA) accumulates in different tissues,

predominantly in the brain due to a genetic defect in the branched-chain alpha-keto acid dehydrogenase complex (BCKDH) leading to oxidative stress and suppressed mitochondrial respiration [121]. BCAA share the first enzymatic steps in their degradation pathways, including a reversible transamination followed by an irreversible oxidative decarboxylation to coenzyme-A derivatives [122] and the defect in MSUD leads to significant elevation of all three BCAA and the respective ketoacids. Children with MSUD present with poor feeding and irritability, which if left undiagnosed and untreated, may progress to lethargy, coma and death [123].

1.3.3. BCAA in T2D

1.3.3.1. Observational studies

Back in the 1970s, Felig et al. reported associations between elevated BCAA levels and impaired insulin signaling in obese versus lean individuals and positively correlated with fasting insulin levels [124]. The sources of increased BCAA in circulation are unknown, but contributors include increased protein intake, high protein turnover or defects in degradation pathways. The interplay between plasma AA and glucose homeostasis is multiplex and the various effects on insulin secretion, glucose production peripheral glucose disposal have been extensively studied [125, 126]. The link between BCAA gained importance the 21st century by metabolomics methods that have confirmed a strong association between the HOMA-IR and circulating BCAA concentrations [127, 128]. In T2D, BCAA levels are currently considered a predictive marker for disease development [129, 130] and genomic variants that increased BCAA levels were associated with T2D in a Mendelian randomization study [131]. There has been evidence provided for decreased expression of BCAA catabolic enzymes in adipose tissue in obese humans and animals, which might be the explanation for the increased levels of circulating BCAA in these subjects [132, 133]. The close link between BCAA and insulin resistance is supported also by the substantial reduction in circulating BCAA levels in patients undergoing Roux-en-Y gastric bypass surgery, resulting in improved insulin sensitivity and glucose homeostasis [134]. Metabolomic analyses revealed that BCAA seem to be the most robust marker of insulin resistance in both plasma [135] and urine [136].

1.3.3.2. Dietary interventions

An area of controversial ongoing investigation is the key question whether BCAAs actively modulate or passively reflect insulin sensitivity [133, 137]. Interestingly, BCAA

supplementation alone is insufficient to induce insulin resistance in regular chow-fed rats, but contributes to insulin resistance in high-fat-fed rats [137]. In addition, BCAA-restricted diets were shown to improve glucose tolerance in animal models [138] [Table 3]. There was a distinctive metabolic 'signature' related to BCAA metabolism and obesity. In animals the supplementation of a high-fat diet with BCAA (HF/BCAA) managed to reduce the food intake and body weight, but causes insulin resistance to the same degree as of animals fed on a HF diet only but with higher body weight [128]. However, the inverse relationship between insulin sensitivity and BCAA doesn't prove an effect-cause relationship, as elevated BCAA levels could be merely the consequence of insulin resistance. So far there has been no solid evidence provided by what mechanism elevated BCAA levels affect insulin resistance and T2D, but the role of adipose tissue has emerged [139]. In humans, there has been an inverse relationship registered between insulin sensitivity and BCAA [Table 4], it was not clear however how BCAA restriction affects metabolic health in humans and by which mechanisms.

Table 3. Interventional studies with modifications of BCAA intake in animals

Ref.	Animals	Diet	Duration of intervention	Methods	Δ BCAA	IR
[128]	male wistar rats N = 5-9/group	SC HF BCAA-HF SC-BCAA	13 weeks	IPGTT p-AKT levels in skeletal muscle & liver Glu- & ITT Immunoblot Indirect calorimetry	+ 80-150% : Leu/Ile + 48-109% : Val	IR (HF+BCAA) = IR (HF); ↑ IR (HF+BCAA)
[140]	male ob/ob (fatty/ lean 13 wk-old N=8/group) mice; male zucker rats (fatty and lean 13 wk-old) N = 10/ group	60% HF	11 weeks	HPLC, spectrophotometry western blot, ELISA		
[141]	male ob/ob mice, N=6/ group; fa/fa obese zucker rats, N=10; fa/fa lean N=10	10, 45, 60% HF	12 weeks	Gene expression analysis; western blot	35-50%	↑ BCAA → ↑ IR
[142]	male C57BL/6 J mice; N=5-6/group	+/- BCAA	1 week	GTT, ITT, HOMA-IR western blot	-100%	↓ BCAA → ↓ IR
[138]	male C57BL/6 J mice; N=9/group	+/- BCAA	3 weeks	GTT, IPTT	-66%	↓ BCAA → ↓ IR
[143]	male C57BL/6 J mice exlow BCAA diet	isocaloric exlow AA exlow BCAA	12 weeks	GTT, ITT	-67%	↓ BCAA → ↑ IS
[144]	C57BL/6 male and female mice N = 858 diets differing in content of protein (5%– 60%), fat (16%–75%), carbohydrate (16%–75%)		15 months	GTT, ITT	-	↑ protein (↑ BCAA) → ↓ IS

SC, standard chow; exlow, extra low; HF, high-fat; GLU, glucose; INS, insulin; ITT, insulin tolerance test; IPGTT, intraperitoneal tolerance test; HF, high fat; IR, insulin resistance; ILE, IS, insulin sensitivity; isoleucine; LEU, leucine; VAL, valine; NMR spectroscopy; nuclear magnetic resonance spectroscopy; GC-MS, gas-chromatography mass spectrometry, HPLC, high performance liquid chromatography.

Table 4. Cross sectional studies on the relationship between BCAA and insulin resistance in humans

Ref.	Humans/conditions	Methods	Δ BCAA	IR
[145]	N=30 LH N=10; MHO N=10; MUO N=10	MMT; metabolic profiling by GC-MS	MHO : LH Val: 29.3%, Ile: 25.9%, Leu: 25.4% MUO : LH Val: 35%, Ile: 40.1%, Leu: 29.1% MHO : MUO no difference	AUC (serum GLU + INS - MHO ≈ LH individuals, and significantly lower than MUO individuals ↑ BCAA → ↑ HOMA-IR
[125]	MH male N=7 Short-term plasma amino acid (AA) elevation	Clamp test 5.5 h in the presence of low (1,6 mmol/l) and increased (4,6 mmol/l) plasma AA concentrations	Ile 100%, Leu 110%, Val 160% BCAA 90%	Elevation of postprandial plasma AA → ↑IR, possible association ↑ BCAA → ↑ IR
[146]	N=500 weight loss maintenance	HOMA-IR; metabolic profiling by GC-MS	-	↑ BCAA → ↑ IR
[147]	N=1,680 healthy 911 female and 769 male Baseline and 6 yrs later	NMR spectroscopy HOMA-IR at baseline and after 6 years	-	Prospective ↑BCAA ~HOMA-IR ILE: most pronounced predictor of IR
[148]	N=182 healthy 118 female and 64 male	LCMS		↑ ILE → ↑ IR
[149]	N=263 non-obese males Asian-Indian and Chinese	HOMA-IR; metabolic profiling, GC-MS	-	↑ BCAA ~ ↑IR

LH, lean healthy; MHO, metabolically healthy obese; MH, metabolically healthy; MUO, metabolically unhealthy obese; GLU, glucose; INS, insulin; ITT, insulin tolerance test; IPTT, intraperitoneal tolerance test; HF, high fat; IR, insulin resistance; ILE, isoleucine; LEU, leucine; VAL, valine; NMR spectroscopy; nuclear magnetic resonance spectroscopy, GC-MS, gas-chromatography mass spectrometry; MMT, mixed-meal tolerance test

1.3.3.3. **Mechanistic studies**

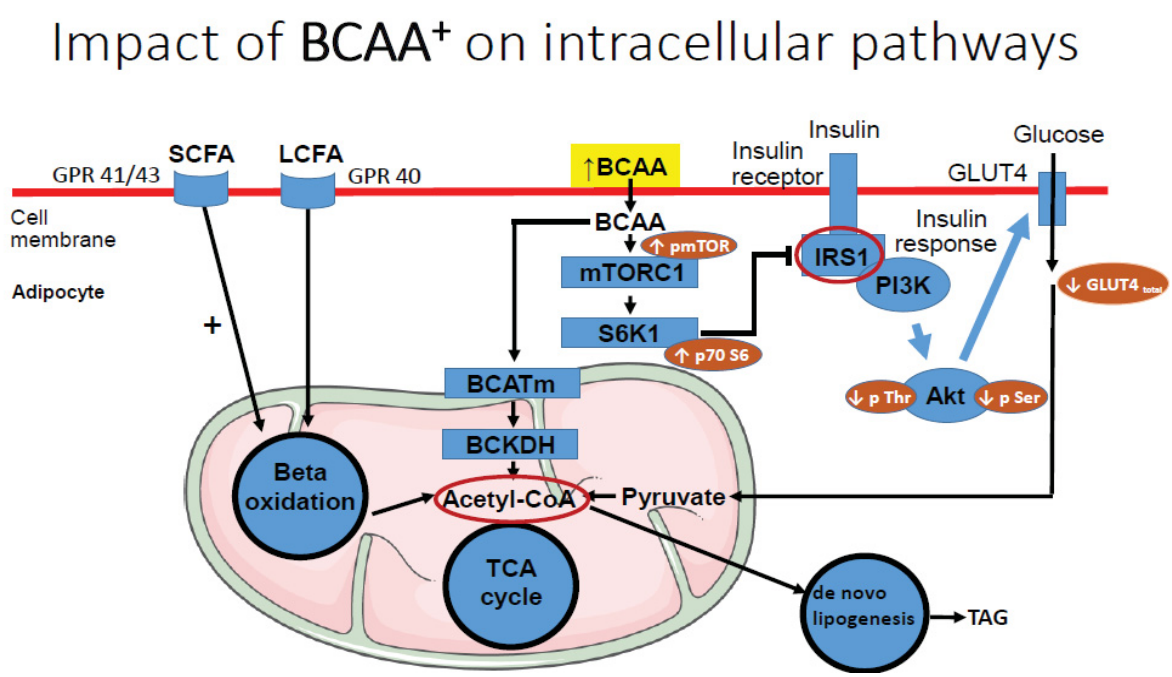
Two main mechanisms have been discussed, by which BCAAs may impair insulin action.

One mechanism involves the mechanistic target of rapamycin (mTOR) serine-threonine protein kinase expressed in various tissues and involved in numerous cellular functions. It consists of two complexes mTORC1 and mTORC2 whose regulation is activated postprandially and initiates anabolism and energy storage [150]. mTORC1 is highly sensitive to AA signaling and integrates signals from BCAA and glucose. As in particular leucine is suggested to be the main activator by translocating proteins and assuring their binding to the regulatory –associated protein on mTOR Raptor [151]. Endogenous signals such as insulin and hormones also lead to mTORC1 activation by phosphorylation of the insulin receptor (IRS1) and thereby lowers insulin sensitivity by activating a negative feedback loop. Of note, animals with deletion of mitochondrial BCAA transaminase (BCATm) exhibit strongly increased circulating BCAA concentrations but are protected from high-fat-diet-induced obesity and insulin resistance [140], meaning BCAA-mediated effects such as mTOR activation alone are insufficient to produce insulin resistance. The mTOR coordinates protein synthesis, mitochondrial activity and proliferation [152]. In the cell, mitochondria act as the conductors of metabolic signals and energy homeostasis [153]. At the background of insulin resistance mitochondrial flexibility is impaired implying a perturbed mitochondrial function [154]. Impairment of mitochondrial function and/or morphological features of mitochondria are referred to as ‘mitochondrial dysfunction’ [155]. Moreover, the interaction between mitochondria and insulin sensitivity is bidirectional and varies depending on tissue [156]. Animal experiments and studies in cultivated cells provided evidence for a persistent activation of the mTOR pathway by BCAA, proving that these AA do not only ‘report’ insulin resistance but also contribute to the disease development [128]. Previous findings indicate a mechanism suggesting a contribution of BCAA metabolism to the development of insulin resistance and ultimately T2D [157, 158] . This assumption has been based on previous studies involving infusion of AA mixtures causing elevation of circulating AA levels to up to 7-fold and causing decreased glucose uptake while increasing hepatic gluconeogenesis [159]. A schematic overview of the mTOR insulin signaling pathway is shown in Figures 3A und 3B at the presence of different amounts of peripheral BCAA.

In the second proposed mechanism for BCAA-induced insulin resistance, BCAAs themselves are not the culprit but rather their degradation products, propionyl CoA, succinyl CoA and/ or branched-chain ketoacids. Increased production of toxic mitochondrial BCAA catabolites as a

result of elevated BCAA turnover, could impair mitochondrial oxidative metabolism [137] and induce mitochondrial dysfunction.

A.



B.

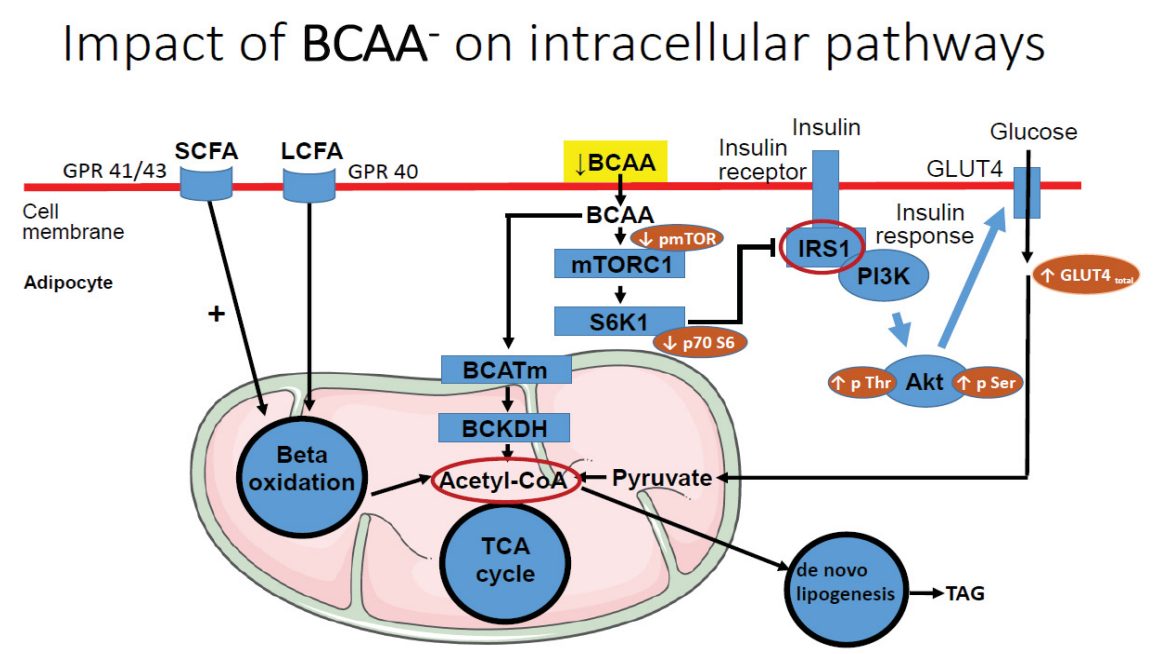


Figure 3. Effects of branched-chain amino acids on the mTor pathway and insulin signaling. A. Under conditions of elevated circulating BCAA, mTORC1 is activated which leads to inhibition of insulin signaling. B. Under conditions of decreased circulating BCAA, mTORC1 activity is decreased and as a result insulin signaling is enhanced. $BCAA^{+}$, high circulating BCAA levels, $BCAA^{-}$, decreased circulating BCAA levels, GPR, G protein-coupled receptor, GLUT4, glucose-transporter type 4, SCFA, short-chain fatty acids, LCFA, long-chain fatty acids, GPR 40, free fatty acid receptor 1, GPR 41/43, G-protein coupled receptor, IRS1, insulin receptor substrate 1, PI3K, phosphoinositide 3-kinases, Akt, protein kinase B, mTORC1, mechanistic target of rapamycin, S6K1, ribosomal protein s6 kinase, BCATm, mitochondrial branched-chain amino acid transaminase, BCKDH, branched-chain alpha-keto acid dehydrogenase complex , TCA, tricarboxylic acid cycle, TAG, triglycerides, p Thr, phosphorylated threonine. p Ser, phosphorylated serine.

2. Aims and hypotheses

Based on previous epidemiological studies reporting a strong association between elevated BCAA and insulin resistance, the present study (Clinicaltrials.gov registration number: NCT03261362 consistent with the Declaration of Helsinki and approved by the local ethics board of Heinrich Heine University, Düsseldorf, Germany registration number 4813R) aimed to examine the role of dietary BCAA intake for glucose metabolism in patients with overt T2D.

This study therefore tested the following hypotheses that a BCAA-reduced diet

i. improves whole body insulin sensitivity,
ii. decreases insulin secretion and/or
iii. alters human microbiome composition.

To this end, we designed a randomized, placebo-controlled, double-blinded, cross-over study in 12 (8 male/4 female) patients with T2D.

3. Publication

Karusheva Y, Kössler T, Strassburger K, Markgraf D, Jelenik T, Mastrototaro L, Simon MC, Zaharia OP, Bódis K, Baerenz F, Schmoll D, Burkart V, Müssig K, Szendroedi J, Roden M. 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 cross-over trial. *Am J Clin Nutr.* 2019 110 (5): 1098-1107

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

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ABSTRACT

Background: Epidemiological studies have shown that increased circulating branched-chain amino acids (BCAAs) are associated with insulin resistance and type 2 diabetes (T2D). This may result from altered energy metabolism or dietary habits.

Objective: We hypothesized that a lower intake of BCAAs improves tissue-specific insulin sensitivity.

Methods: This randomized, placebo-controlled, double-blinded, crossover trial examined well-controlled T2D patients receiving isocaloric diets (protein: 1 g/kg body weight) for 4 wk. Protein requirements were covered by commercially available food supplemented ≤60% by an AA mixture either containing all AAs or lacking BCAAs. The dietary intervention ensured sufficient BCAA supply above the recommended minimum daily intake. The patients underwent the mixed meal tolerance test (MMT), hyperinsulinemic-euglycemic clamps (HECs), and skeletal muscle and white adipose tissue biopsies to assess insulin signaling.

Results: After the BCAA$^-$ diet, BCAAs were reduced by 17% during fasting ($P < 0.001$), by 13% during HEC ($P < 0.01$), and by 62% during the MMT ($P < 0.001$). Under clamp conditions, whole-body and hepatic insulin sensitivity did not differ between diets. After the BCAA$^-$ diet, however, the oral glucose sensitivity index was 24% ($P < 0.01$) and circulating fibroblast-growth factor 21 was 21% higher ($P < 0.05$), whereas meal-derived insulin secretion was 28% lower ($P < 0.05$). Adipose tissue expression of the mechanistic target of rapamycin was 13% lower, whereas the mitochondrial respiratory control ratio was 1.7-fold higher (both $P < 0.05$). The fecal microbiome was enriched in Bacteroidetes but depleted of Firmicutes.

Conclusions: Short-term dietary reduction of BCAAs decreases postprandial insulin secretion and improves white adipose tissue metabolism and gut microbiome composition. Longer-term studies will be needed to evaluate the safety and metabolic efficacy in diabetes patients. This trial was registered at clinicaltrials.gov as NCT03261362. *Am J Clin Nutr* 2019;00:1–10.

Some data were presented as an abstract/poster at the 78th American Diabetes Association, the 79th American Diabetes Association, and the 54th European Association for the Study of Diabetes Annual Meeting in 2018 as well as an oral presentation at the 53rd German Diabetes Association Annual Meeting in 2018 and a poster presentation at the 54th German Diabetes Association Annual Meeting in 2019.

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Supplemental Figures 1 and 2 and Supplemental Table 1 are available from the "Supplementary data" link in the online posting of the article and from the same link in the online table of contents at https://academic.oup.com/ajcn/.

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Abbreviations used: AA, amino acid; BCAA, branched-chain amino acid; BW, body weight; CRC, Clinical Research Center; EGP, endogenous glucose production; fccp, carbonyl cyanide p-[trifluoromethoxyl]-phenyl-hydrozone; FFA, free fatty acid; FGF21, fibroblast-growth factor 21; HEC, hyperinsulinemic-euglycemic clamp; HRP, horseradish peroxidase; iAUC, incremental AUC; LCR, leak control ratio; M/I, HEC-derived *M* value adjusted for prevalent insulin concentrations during steady state; MMT, mixed meal tolerance test; mTOR, mechanistic target of rapamycin; OGIS, oral glucose sensitivity index; p70S6K, ribosomal protein S6 kinase; PAL, physical activity level; PREDIM, PREDIcted M; RCR, respiratory control ratio; SDS, sodium dodecyl sulfate; TG, triglyceride; T2D, type 2 diabetes; WAT, white adipose tissue.

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Keywords: branched-chain amino acids, patients with type 2 diabetes, diet, insulin sensitivity, insulin secretion, mitochondrial function, gut microbiome

Introduction

Branched-chain amino acids (BCAAs) account for ~20% of total protein intake, are important for protein and glucose metabolism, and are associated with insulin resistance in obesity and type 2 diabetes (T2D) (1–3). Their increased plasma concentrations predict impaired insulin signaling (4) and the development of T2D in prospective studies (5–7). Short-term intravenous infusion of amino acids (AAs) not only increases insulin secretion, but also induces insulin resistance in humans, likely due to activation of the mechanistic target of rapamycin (mTOR) complex 1/ribosomal protein S6 kinase (p70S6K) pathway (8–11).

Increased BCAA concentrations result from perturbed proteolysis in skeletal muscle (12), adipose tissue (13), and liver (14). Because insulin inhibits protein breakdown, insulin resistance may therefore also contribute to higher fasting BCAAs (15, 16). Finally, the microbiota can also alter protein degradation and circulating BCAA concentrations (17). Animal models of obesity exhibit less intestinal Bacteroidetes and correspondingly more Firmicutes (18). However, data on the impact of dietary BCAA modification on gut microbiome composition in humans are lacking.

Overnutrition could also affect circulating BCAA concentrations. Whereas diets enriched in either plant or animal protein rather increased peripheral insulin sensitivity (19), dietary supplementation with essential AAs decreased glucose infusion rates necessary during euglycemic clamp studies at different degrees of insulinemia (20). Interestingly, ingestion of BCAA-enriched whey protein increased postprandial insulinemia without reduction of glycemia, suggesting decreased insulin sensitivity (21). Moreover, acute whey protein supplementation improved postprandial glycemia in lean, overweight and obese, and T2D patients (22).

These findings raise the question of whether dietary reduction of BCAAs would improve insulin sensitivity. In humans, 1 previous study on dietary protein reduction showed lower serum BCAAs (23), but did not report metabolic effects. In mice, the dietary omission of leucine indeed improved glycemic control (11).

Thus, this study tested the hypothesis that a reduction of dietary BCAAs increases whole-body insulin sensitivity. To this end, we examined the effects of a controlled isocaloric dietary reduction in BCAAs ($BCAA^-$) on insulin sensitivity and secretion in patients with T2D using the 2-step hyperinsulinemic-euglycemic clamp (HEC) test with stable isotope dilution and the mixed meal tolerance test (MMT). On a cellular level, we assessed insulin signaling pathways and mitochondrial function in skeletal muscle and white adipose tissue (WAT), as well as intestinal microbiome composition. The primary outcome of this study was whole-body insulin sensitivity in response to the HEC, whereas the secondary outcome measures were insulin secretion, insulin signaling, and microbiome composition.

Methods

Patients

All participants ($n = 12$) gave written informed consent before inclusion in the study (NCT03261362), consistent with the Declaration of Helsinki and approved by the local ethics board of Heinrich Heine University, Düsseldorf, Germany. Inclusion criteria comprised 40–60 y of age; BMI 28–35 kg/m^2; T2D treated with lifestyle modification, metformin, or other oral glucose-lowering medication; and known disease duration of ≤5 y. Exclusion criteria were smoking; acute or chronic diseases including cancer; medications affecting the immune system; antibiotics; regular endurance training >1 h/wk; insulin; thiazolidinediones; glycated hemoglobin > 9.5% (80 mmol/mol); and a diabetes type other than T2D.

Study design

In a crossover double-blinded design, all participants, recruited between May 2016 and June 2017, were randomly allocated to a dietary intervention beginning either with the complete set of AAs ($BCAA^+$) or with a BCAA-reduced diet ($BCAA^-$) (**Supplemental Figure 1**). For this task, a reproducible code from the program package in SAS version 9.3 (SAS Institute Inc.) was used. The given number of analyzed participants allows detecting large effect sizes (Cohen's $d = 1$) of measures of whole-body insulin sensitivity with a power ≥80% (24) and an α error rate <5%.

At all visits, the study participants arrived at the Clinical Research Center (CRC) in the morning after 10 h of overnight fasting (**Supplemental Figure 2**). They were instructed to refrain from any form of exercise for 3 d before the analyses. Patients withdrew their oral glucose-lowering medication for ≥3 d before all measurements to exclude its acute effects on glucose metabolism (25). Patients participated in a 4-wk isocaloric dietary intervention with 55% carbohydrates, 30% fat, and 15% protein uptake. The protein intake was kept constant at 1 g/kg body weight (BW) for the entire period of the study. During weeks 1 and 3, the protein intake was covered by commercially available regular foods, whereas in weeks 2 and 4 ~60% of the protein intake was covered by an AA-powder either containing all AAs (K-AM, Nutricia Metabolics) or lacking BCAAs (ILV-AM3, Nutricia Metabolics) dissolved in c.200 mL water at room temperature; the rest was covered by commercially available regular foods. Powders were indistinguishably packed and labeled by an external pharmacist to ensure blinding of participants, care providers, and persons involved in the assessment of outcomes. The individual daily calorie intake was calculated using the basal metabolic rate according to the Harris–Benedict formula—for males: basal metabolic rate (in kcal) = 66.5 + (13.8 × BW in kg) + (5.0 × height in cm) − (6.8 × age in y) × physical activity level (PAL) 1.4; and for females: basal metabolic rate (in kcal) = 655.1 + (9.6 × BW in kg) + (1.9 × height in cm) − (4.7 × age in y) × PAL 1.4 (26, 27).

Monitoring of diet and exercise behavior

An experienced dietitian designed detailed individually tailored nutritional protocols and supervised participants' dietary

behavior. Dietary protocols were analyzed using the Prodi system [Prodi 6.3.0.1 (Nbase 3.60), Nutri-Science GmbH]. The participants documented any deviation from these protocols, which were adapted by nutritional advice. The intensive monitoring ensured constant protein and correspondingly average BCAA intake throughout the study period. BW changes had to be <5%. The patients' compliance was checked from serum AA concentrations, measured in the fasted state at each visit, and from urinary excretion of riboflavin (vitamin B-2), which had been added to the AA powders as a dosage of 300 mg/d. Urine samples were collected thrice—at baseline and at the end of each of the 2 intervention weeks with/without AA supplementation—over 24 h during the intervention to measure the concentration of riboflavin by a fluorimetric assay (28) corrected for individual creatinine concentrations. An exercise physiologist supervised the physical activity behavior of participants. For monitoring, 3-axial acceleration sensors (move II, Movisens GmbH) were attached to the participant's waist above the right anterior axillary line according to the manufacturer's recommendations and worn throughout the intervention. Steps and energy expenditure were calculated with the Movisens DataAnalyzer software (29).

MMT

To assess the acute effects of 1 MMT on top of 1 wk exposure to a modified BCAA intake on insulin secretion, an MMT was performed at the end of each intervention week (weeks 2 and 4). These findings were referred to as "meal-induced." After 10 h overnight fasting, participants ingested a standardized liquid meal (Duocal, Nutricia Metabolics), either containing the complete set of AAs or being BCAA-free, within 2 min starting at zero time. The meal size was adapted to the patients' individual energy requirements and corresponded to 25% of their estimated daily energy requirement (30). Blood samples were taken at minutes −10, −1, +10, +20, +30, +60, +90, +120, +180, and +240 for measurements of glucose, insulin, C-peptide, free fatty acids (FFAs), and triglycerides (TGs) to calculate incremental AUCs (iAUCs), using the trapezoidal rule after subtracting the basal (fasting) values (31). The oral glucose sensitivity index (OGIS) was calculated from the MMT as described previously (32). The PREDIcted M (PREDIM) index was computed from the OGIS and MMT data and allows for nominal comparison with the HEC-derived *M* value as described previously (33).

Tissue biopsies

The biopsies were obtained at the end of each intervention period at the end of the MMT. For skeletal muscle biopsies, the region above the vastus lateralis muscle was anaesthetized by subcutaneous injection of 15 mL 2% lidocaine. Thereafter, ~70–200 mg tissue was obtained using a modified Bergström needle with suction as described (8). Adipose tissue biopsies were obtained in the paraumbilical region at the level of the rectus abdominis muscle as described (34).

High-resolution respirometry

Ex vivo analysis of mitochondrial oxidative capacity was performed on permeabilized muscle fibers and isolated mitochondria in a 2-chamber oxygraph (Oroboros Instruments) as described previously (21). Maximal fatty acid oxidative capacity (state 3) was measured using either octanoyl-carnitine (50 μmol/L) and ADP (1 mmol/L) to assess β-oxidation–linked respiration, or pyruvate (10 mmol/L), glutamate (10 mmol/L), ADP (1 mmol/L), and succinate (10 mmol/L) to assess tricarboxylic acid cycle–linked respiration. Cytochrome C (10 μmol/L) was added to test the integrity of the outer mitochondrial membrane. Respiration due to proton leak and not coupled to ATP synthesis (state 4o) was measured after addition of oligomycin. Finally, the maximal uncoupled respiration capacity of the electron transport chain (state u) was assessed by incremental titration with carbonyl cyanide p-[trifluoromethoxyl]-phenyl-hydrozone (fccp) (0.1 mmol/L per step) and nonmitochondrial respiration by adding 2.5 μM antimycin A. The respiratory control ratio (RCR) and the leak control ratio (LCR), markers of mitochondrial coupling and efficiency, respectively, were calculated as the ratios of state 3:state 4o and state 4o:state u respiration, respectively. A high RCR and low LCR indicate tight coupling and high efficiency of mitochondrial function. Oxygen consumption was normalized to adipose tissue wet weight or to mitochondrial density assessed from a citrate synthase activity assay (35).

Two-step HEC test

Patients arrived at the CRC at 0650 on the day of the clamp test. A primed-continuous infusion {3.6 mg/kg [(free plasma glucose in mg/dL)/90]} of D-[6,6-$^2H_2$] glucose (99% enriched, Cambridge Isotope Laboratories) was started at 0700. At 0855, the somatostatin infusion (0.1 μg · kg BW^{-1} · min^{-1}) was commenced, simultaneously with infusion of 20 mU · min^{-1} · m^{-2} (low-dose for 2 h, low clamp), followed by 40 mU · min^{-1} · m^{-2} (high-dose for 2 h, high clamp) of short-acting human insulin (Insuman Rapid, Sanofi-Aventis) (36). Plasma glucose was measured every 5 min and kept constant by a variable intravenous glucose infusion (20% glucose, enriched in D-[6,6-$^2H_2$] glucose). Insulin-stimulated whole-body glucose disposal (*M* value: expressed as mg · kg BW^{-1} · min^{-1}) was calculated as described (37). M/I was calculated as the HEC-derived *M* value adjusted for the prevailing insulin concentrations during steady-state conditions. For measuring endogenous glucose production (EGP), participants received a 20-min priming bolus [0.36 mg · kg BW^{-1} · min^{-1} · fasting plasma glucose (mg/dL)] of D-[6,6-$^2H_2$] glucose (99% enriched in ^{2}H glucose; Cambridge Isotope Laboratories) at −240 min, followed by a continuous infusion (0.036 mg · kg BW^{-1} · min^{-1}) (25).

Laboratory analyses

For analysis of AA concentrations, serum samples were processed using the Phenomenex EZ:faast AA analysis kit (Phenomenex) for GC-MS with norvalin and an isotopically labeled AA mixture (Cambridge Isotope Laboratories) as internal

standards (38, 39). AAs were analyzed on a Hewlett Packard 6890 gas chromatograph interfaced to a Hewlett Packard 5975 mass selective detector (Agilent Technologies). For the analysis of arginine, serum samples were treated with arginase (Creative Enzymes) for 20 min at 37°C to convert arginine to ornithine. Ornithine was then quantified after sample processing as described above. Arginine concentration was calculated as the difference of ornithine concentrations before and after arginase treatment. The CVs for individual AAs ranged from 1.4% to 5.1%.

Total LDL cholesterol, HDL cholesterol, TGs, and FFAs as well as transaminases were measured on a Cobas c311 analyzer (Roche Diagnostics) (25). Plasma fibroblast growth factor 21 (FGF21) concentrations were measured with the Human FGF21 Quantikine ELISA [R&D Systems (Bio-techne)] as described previously (40).

Fecal microbiome composition

Stool samples were collected by the participants on the last day of each intervention week and stored at −80°C. Total genomic DNA was extracted from 120 mg fecal material using the QIAcube with QIAamp DNA mini kit (Qiagen) according to the manufacturer's instructions (41). Next-generation sequencing was performed as described previously (42).

Western blot

Proteins were extracted from ∼30 mg frozen tissue (skeletal muscle or WAT) and homogenized in 300 μL lysis buffer (25 mM Tris-HCl), 1 mM EDTA, 150 mM NaCl, and 0.20% NP-40 with protease (cOmplete Tablets, EASYpack, Roche Diagnostics) and phosphatase (PhosSTOP, EASYpack, Roche Diagnostics) inhibitors. Samples were shaken 3 times for 1 min at 20 Hz in a Tissue Lyzer and centrifuged (16,000 × *g* for 15 min at 4°C) to pellet insolubilized material, such as DNA, nuclei, and unbroken cellular membranes. The concentration of the extracted proteins was determined in the supernatant using the Bradford Assay (Quick Start Bradford, Biorad) (43). Aliquots of 30 μg total proteins were diluted 6 times with the loading buffer [0.35 M Tris-HCl at pH of 6.8, 10% sodium dodecyl sulfate (SDS), 30% glycerol, 0.6 M dithiothreitol, 0.175 mM Bromophenol Blue] and then loaded onto an SDS-polyacrylamide gradient gel (4–20% Mini-PROTEAN TGX Precast Protein Gels, Biorad). After electrophoresis, a semidry blotting to a polyvinylidene difluoride membrane was performed at 8 mA/cm^2 for 1 h. After blocking the membranes for 2 h at room temperature using the blocking solution (5% milk in Tris-buffered saline-Tween), the membranes were incubated with the primary antibodies diluted in blocking solution in combination with the respective horseradish peroxidase (HRP)-conjugated secondary antibodies: anti-rabbit 1:2500 for all the primary antibodies. The membranes were finally coated with Immobilon Western Chemiluminescent HRP Substrate (Millipore) and the proteins were detected using a Bio-Rad ChemiDoc MP Imaging System in combination with the software ImageLab 6.0.1 (Bio Rad Laboratories) for densitometric analysis.

Primary antibodies were all purchased from Cell Signaling Technology: phospho-AKT (Thr308) (9275) [pAKT (Thr308)]; phospho-AKT (Ser473) (9271) [pAKT (Ser473)]; phospho-p70S6K (Thr389) (70-kDa p70S6K) (9205) [p-p70S6K (Thr389)]; phosphor-p70S6K (Thr421/Ser424) (70-kDa p70S6K) (9204) [p-p70S6K (Thr421/Ser424)]; phospho-mTOR (Ser2481) (2974) [pmTOR (Ser2481)]; and GAPDH (2118) as the housekeeping protein. Data are expressed in arbitrary units and normalized to the housekeeping protein.

TABLE 1 Study participants' anthropometric and metabolic characteristics[1]

Variables	Values
n (men/women)	12 (8/4)
Age, y	54 ± 4
BMI, kg/m^2	30.8 ± 2.8
HbA1c, mmol/mol	49 ± 10
HbA1c, %	6.6 ± 0.9
Fasting blood glucose, mg/dL	118 ± 8
Triglycerides, mg/dL	273 ± 245
Total cholesterol, mg/dL	224 ± 135
LDL cholesterol, mg/dL	145 ± 32
HDL cholesterol, mg/dL	45 ± 11
ALT, U/l	38 ± 14
AST, U/l	28 ± 6
GGT, U/l	48 ± 8
Total BCAAs, μmol/L	531 ± 98
Isoleucine, μmol/L	87 ± 25
Leucine, μmol/L	159 ± 35
Valine, μmol/L	285 ± 41

[1]Values are mean ± SD unless otherwise indicated. ALT, alanine aminotransferase; AST, aspartate aminotransferase; BCAA, branched-chain amino acid; GGT, γ-glutamyltransferase; HbA1c, glycated hemoglobin.

Statistical analyses

Data are presented as means ± SDs or percentages. Because time sequence effects may cause systematic variations in the outcomes of crossover studies, differences between treatment effects were tested using the classical crossover test, which compares the intraindividual period differences of the outcome between the sequence groups (24). Variables with skewed distributions were ln-transformed before analyses to approximate normality. *P* values <5% were considered statistically significant effects. All statistical analyses were performed using SAS version 9.4 (SAS Institute).

Results

Patients' characteristics

All patients had near-normal glycemic control (**Table 1**) under treatment with lifestyle modification ($n = 6$) or metformin ($n = 6$). BW decreased similarly, by 1.7 ± 1.1 kg in the group which first received the BCAA$^+$ diet and by 1.2 ± 0.8 kg in the group which first received the BCAA$^-$ diet, at the end of the whole intervention period relative to BW at baseline. The change in BW relative to baseline after the dietary intervention was <2% (both $P < 0.05$) in both groups and not different between the groups. Total energy expenditure (2621 ± 516 kcal/d under BCAA$^+$ compared with 2576 ± 483 kcal/d under BCAA$^-$)

and steps (6340 ± 3897/d under BCAA$^+$ compared with 5646 ± 2811/d under BCAA$^-$) were similar in all participants during both diets (both $P > 0.05$).

Fasting BCAA concentrations and diet adherence during the intervention

The 60% reduction of dietary BCAA intake (BCAA$^-$) resulted in a 17% decrease of total circulating BCAA concentrations from 507 ± 90 to 422 ± 56 μmol/L ($P < 0.001$) under fasting conditions. Serum concentrations of valine, leucine, and isoleucine decreased by 22% from 276 ± 50 to 214 ± 28 μmol/L ($P < 0.001$), 11% from 155 ± 28 to 139 ± 19 μmol/L ($P < 0.05$), and 9% from 76 ± 14 to 69 ± 12 μmol/L ($P < 0.05$), respectively, whereas those of non-BCAAs increased by 10% from 2706 ± 217 to 2982 ± 163 μmol/L ($P < 0.01$) (**Supplemental Table 1**). At the end of weeks 1 and 3, when participants did not receive AA mixtures, serum concentrations of all BCAAs, non-BCAAs, and total AAs were comparable.

Adherence to diets was demonstrated by a 33.4-fold increase of urinary concentration of riboflavin after the BCAA$^+$ diet (from 1.3 ± 1.2 to 43.4 ± 15.8 mg/24 h) and a 48.8-fold increase after the BCAA$^-$ diet (from 1.3 ± 1.2 to 62.1 ± 31.2 mg/24 h) (both $P < 0.05$). There were no differences in urinary concentrations of riboflavin between the 2 diets ($P > 0.05$).

Meal-induced effects of BCAA reduction during the MMT

During the MMT, serum BCAAs decreased by 62% from 158,484 ± 24,410 μmol · L^{-1} · 4 h^{-1} after BCAA$^+$ to 61,864 ± 9386 μmol · L^{-1} · 4 h^{-1} ($P < 0.001$) after BCAA$^-$ diet (**Figure 1**A, B). Serum valine, leucine, and isoleucine were reduced by 54% from 75,689 ± 11,562 to 35,195 ± 5138 μmol · L^{-1} · 4 h^{-1} ($P < 0.001$), 71% from 48,848 ± 7938 to 14,931 ± 2610 μmol · L^{-1} · 4 h^{-1} ($P < 0.001$), and 71% from 26,229 ± 4229 to 7699 ± 1524 μmol · L^{-1} · 4 h^{-1} ($P < 0.001$), respectively. Blood glucose concentrations were similar after both dietary interventions (Figure 1C, D). Furthermore, there were no changes in maximal concentrations of blood glucose during the MMT. Incremental insulin release (iAUC) was lower after 1 wk of BCAA$^-$ compared with BCAA$^+$ diet (21 ± 11 compared with 29 ± 19 mU · mL^{-1} · 4 h^{-1}, $P < 0.05$) (Figure 1E, F). In parallel, incremental C-peptide release was lower after BCAA$^-$ diet (2.5 ± 0.8 compared with 2.8 ± 0.9 μg · mL^{-1} · 4 h^{-1}, $P < 0.05$) (Figure 1G, H). Accordingly, incremental release of insulin and C-peptide was reduced by 28% and 11%, respectively (both $P < 0.05$). The time course of serum FFA concentrations was similar after both diets (Figure 1I, J).

Assessment of postprandial insulin sensitivity during the MMT revealed that under conditions of reduced BCAA concentrations, OGIS was 24% higher (increased from 279 ± 94 mL · min^{-1} · m^{-2} after BCAA$^+$ to 346 ± 91 mL · min^{-1} · m^{-2} after BCAA$^-$, $P < 0.01$) (**Figure 2**A) and PREDIM was 27% higher (increased from 2.6 ± 0.9 mg · kg^{-1} · min^{-1} after BCAA$^+$ to 3.3 ± 1.3 mg · kg^{-1} · min^{-1} after BCAA$^-$, $P < 0.01$) (Figure 2B).

Changes in HEC during 2–4 wk of BCAA reduction

During the HEC steady state, circulating BCAA concentrations were only 13% lower after the BCAA$^-$ (307 ± 57 μmol/L) than after the BCAA$^+$ diet (352 ± 85 μmol/L) ($P < 0.01$); specifically, valine concentrations were reduced by 15% from 206 ± 48 to 175 ± 35 μmol/L ($P < 0.001$), leucine concentrations were reduced by 8% from 101 ± 25 to 93 ± 18 μmol/L ($P < 0.05$), and isoleucine concentrations were reduced by 14% from 44 ± 13 to 38 ± 9 μmol/L ($P < 0.05$).

Whole-body (M/I), hepatic (insulin-mediated EGP suppression), and adipose-tissue insulin sensitivity (insulin-mediated FFA suppression) remained unchanged after 1 wk of the BCAA$^-$ or BCAA$^+$ diet (**Table 2**).

The BCAA$^-$ diet increased fasting FGF21 concentrations in serum by 21% (from 323 ± 55 to 405 ± 68 pg/mL, $P < 0.05$) relative to the BCAA$^+$ diet.

Insulin signaling and mitochondrial function in skeletal muscle and WAT

At 240 min of the MMT, Ser473- and Thr308-phosphorylation of AKT as well as phosphorylation of mTOR p70S6K in skeletal muscle were not different between the 2 dietary interventions (data not shown). Also, skeletal muscle oxidative capacity was similar after both diets (data not shown).

On the other hand, adipose tissue pAKT (Ser473) and pAKT (Thr308) decreased by 61% ($P < 0.05$) and 64% ($P < 0.01$), respectively, after BCAA$^-$ (**Figure 3**A, B). Also, pmTOR (Ser2481) decreased by 38% ($P < 0.05$) (Figure 3C). The BCAA$^-$ diet resulted in increased RCR by 67% (Figure 3D) and unchanged LCR (data not shown), whereas oxidation capacity after exposure to oligomycin, fccp, and antimycin A decreased (Figure 3E).

Composition of fecal microbiota

Next-generation sequencing revealed an 11% lower abundance of Firmicutes after BCAA$^-$ dietary intervention compared with BCAA$^+$, whereas the abundance of Bacteroidetes was 40% higher (both $P < 0.05$, **Figure 4**) in stool samples collected at the end of each intervention period.

Discussion

A short-term dietary reduction of BCAAs in patients with T2D *1*) decreased insulin secretion, *2*) increased postprandial insulin sensitivity, *3*) stimulated mitochondrial efficiency in adipose tissue, and *4*) altered gut microbiome composition in favor of Bacteroidetes.

The acute effects of BCAA reduction during the MMT reflect the endocrine response of the pancreas to the absence of the physiological AA stimulus. Leucine is a prominent allosteric activator of insulin secretion from the pancreatic β-cell (44) and all BCAAs are potent insulin-secretion stimulators (45). Interestingly, the lower insulin secretion did not result in higher blood glucose concentrations during the MMT, suggesting improved insulin sensitivity. Indeed, postprandial insulin sensitivity as calculated from the OGIS and PREDIM, which have been validated and correlate with clamp-derived

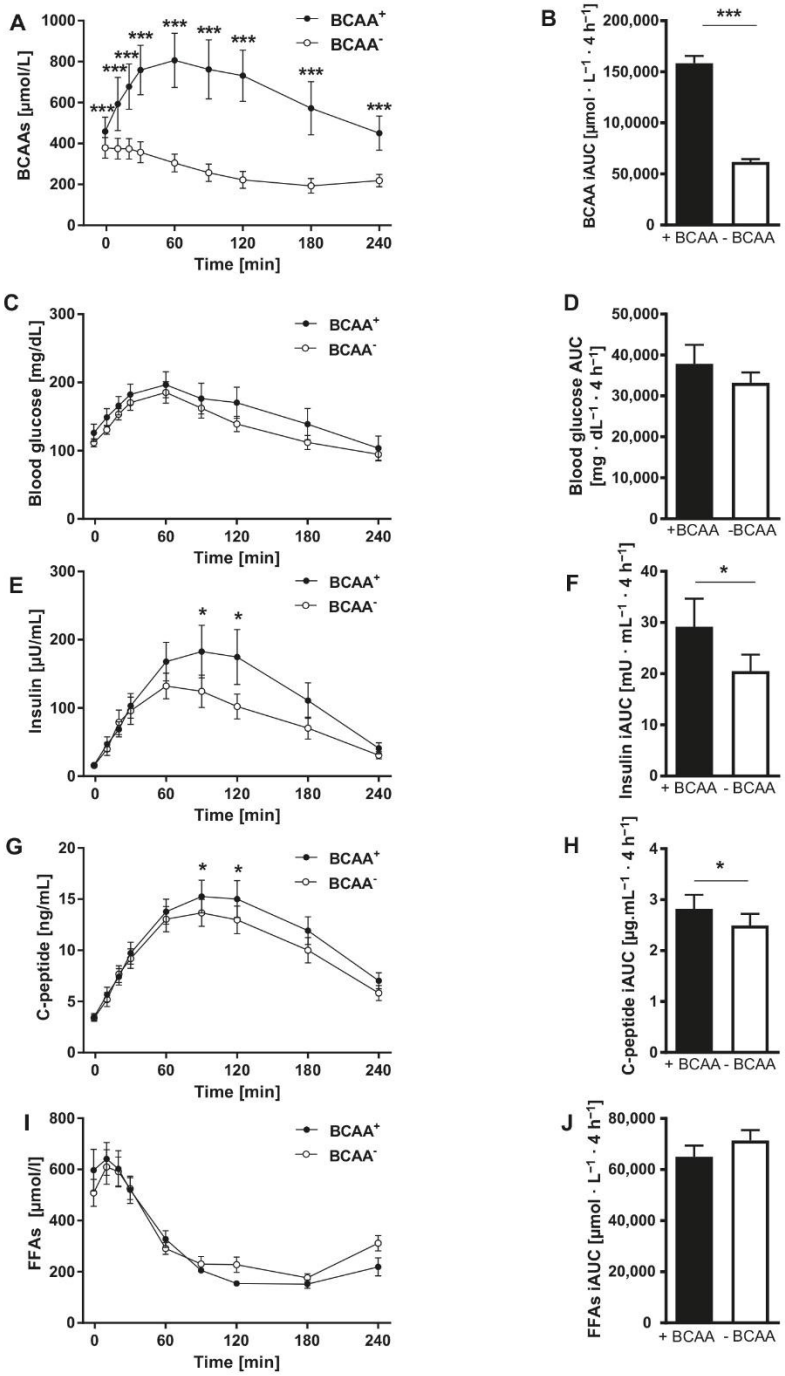

FIGURE 1 Insulin secretion assessed by mixed meal tolerance test. (A) Concentrations of BCAAs, (B) iAUC for BCAAs, (C) blood glucose, (D) AUC for blood glucose, (E) insulin, (F) iAUC for insulin, (G) C-peptide, (H) iAUC for C-peptide, (I) FFAs, and (J) iAUC for FFAs at the end of each intervention week. Differences between treatment effects were tested using the classical crossover test, which compares the intraindividual period differences of the outcome between the sequence groups. Values are mean ± SEM. $^{*}P < 0.05$, $^{***}P < 0.01$ compared to the corresponding $BCAA^{-}$ values, $n = 12$. BCAA, branched-chain amino acid; FFA, free fatty acid; iAUC, incremental AUC.

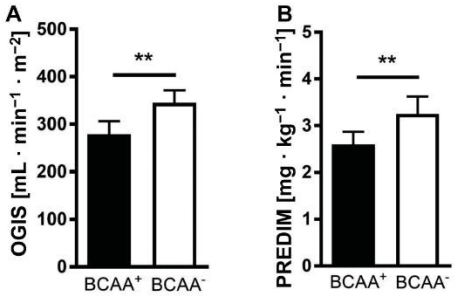

FIGURE 2 OGIS (A) and PREDIM (B) at the end of both interventions. Differences between treatment effects were tested using the classical crossover test, which compares the intraindividual period differences of the outcome between the sequence groups. Values are mean ± SEM. $^{**}P < 0.01$, $n = 12$. BCAA, branched-chain amino acid; OGIS, oral glucose sensitivity index; PREDIM, PREDIcted M.

measures of whole-body insulin sensitivity (33), was >20% higher than after the BCAA$^+$ diet. This improvement could have resulted from increased insulin-mediated glucose disposal, which mainly occurs in skeletal muscle under these conditions (46). Alternatively, EGP could have been decreased under BCAA-depleted conditions owing to lower substrate supply for hepatic gluconeogenesis (47). Improvements in OGIS and PREDIM were registered in insulin-resistant patients in the face of decreased peripheral insulin concentrations. Of note, the lower degree of insulinemia would rather favor glycogenolysis and thereby stimulation of EGP (47). The reduced insulin and C-peptide secretion upon acute stimulation after the BCAA$^-$ diet is in line with a previous finding of decreased ex vivo glucose-stimulated insulin secretion in isolated pancreatic islets from mice (48).

Interestingly, insulin-stimulated peripheral glucose disposal was not different between the 2 dietary interventions under HEC conditions, when skeletal muscle is responsible for the majority of glucose disposal (49). Of note, hepatic and adipose tissue insulin sensitivity were also comparable during HEC. Also of note, the experimental setup created conditions of dynamic changes during the MMT compared with constant concentrations of hormones and metabolites during the clamp. This includes different degrees of splanchnic compared with peripheral insulinemia, which markedly affect hepatic glucose turnover (50). Nevertheless, the most obvious explanation for the difference between the MMT and HEC resides in the different degree of BCAA reduction. The 60% decrease in total serum BCAA concentrations during the MMT was associated with higher postprandial insulin sensitivity. In contrast, the minor reduction of circulating BCAA concentrations during HEC did not correlate with insulin sensitivity. Insulin decreases the appearance and increases the uptake of AAs in the periphery (51). Consequently, the reduction of BCAA concentrations during the HEC steady state under hyperinsulinemia was, as expected, lower than during the MMT. It is conceivable that the dietary reduction of BCAAs by 60% does not suffice to improve the insulin resistance of these patients with overt T2D. These data also suggest that the acute effect of BCAA reduction observed during the MMT does not persist during the course of the dietary intervention. Thus, another possible reason for the lack of an effect on insulin sensitivity could be the short intervention period. But even a modulation of BCAA intake for 1 mo failed to affect whole-body insulin sensitivity as assessed from HEC (52). Because BCAAs are essential and are ubiquitously present in regular foods, it was not possible to further reduce their dietary intake. BCAA degradation may also stimulate fatty acid synthesis and induce insulin resistance in skeletal muscle or WAT (53); however, measuring BCAA catabolic products was beyond the scope of this study.

The tissue-specific contributions to improved postprandial insulin sensitivity were examined in biopsies from skeletal muscle and adipose tissue taken at 4 h after the start of the MMT. In skeletal muscle, there were no differences in the phosphorylation of AKT or mTOR/p70S6K. This may be due to the relatively small differences in circulating insulin and BCAA concentrations at the end of the MMT and the transient nature of insulin on its cellular signaling pathways (54). Although skeletal muscle strongly relies on mitochondrial oxidative phosphorylation and decreased oxidative capacity can be a major contributor to the development of insulin resistance (55), there were also no differences in ex vivo mitochondrial function.

Previous studies suggested that adipose tissue can also metabolize substantial amounts of BCAAs (56) and may be a prominent site to store excess BCAAs as lipids (4). Indeed, the reduction of

TABLE 2 Results from whole-body insulin sensitivity testing by 2-step HEC test[1]

	Low clamp			High clamp			ΔL vs. ΔH[2]
	BCAA$^+$	BCAA$^-$	*P* value	BCAA$^+$	BCAA$^-$	*P* value	*P* value
BCAAs, μmol/L	n.d.	n.d.	—	346.6 ± 80.2	312.1 ± 67.3	<0.001	—
Glucose, mg/dL	91.9 ± 2.2	91.6 ± 2.3	0.78	90.7 ± 1.8	91.4 ± 2.4	0.41	0.17
Insulin, μU/mL	25.5 ± 5.9	26.3 ± 7.0	0.43	54.2 ± 10.4*	52.2 ± 11.4$^\#$	0.46	0.31
M/I	0.04 ± 0.03	0.04 ± 0.03	0.98	0.07 ± 0.04*	0.06 ± 0.04$^\#$	0.21	0.17
EGP suppression, %	29.2 ± 16.6	49.4 ± 10.6	0.97	83.1 ± 18.0*	86.8 ± 11.4$^\#$	0.36	0.17
FFA suppression, %	65.7 ± 19.5	64.6 ± 14.8	0.84	85.1 ± 11.0*	86.6 ± 6.2$^\#$	0.66	0.07

[1] Values are mean ± SD, $n = 12$. All parameters were measured during the HEC steady state. $^*P < 0.05$ between variables derived after BCAA$^+$ intervention under low and high clamp conditions; $^\#P < 0.05$ between variables derived after BCAA$^-$ intervention under low and high clamp conditions. BCAA, branched-chain amino acid; EGP, endogenous glucose production; FFA, free fatty acid; HEC, hyperinsulinemic-euglycemic clamp; M/I, HEC-derived *M* value adjusted for prevalent insulin concentrations during steady state; n.d., not done; ΔH, changes of parameters during high clamp; ΔL, changes of parameters during low clamp.

[2] *P* values of comparisons between ΔL and ΔH.

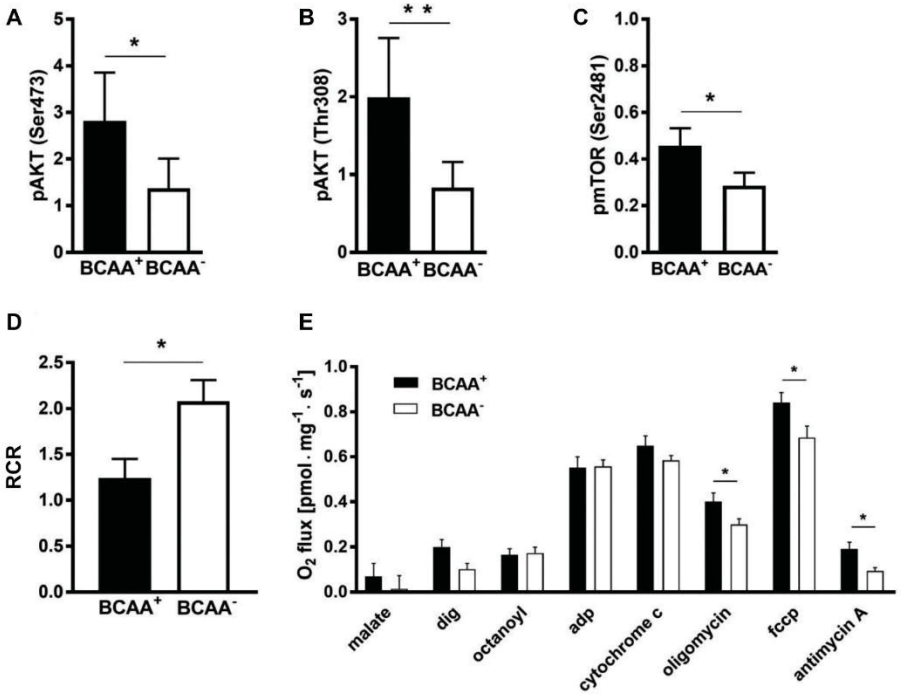

FIGURE 3 Insulin signaling and mitochondrial oxidative efficiency/capacity in white adipose tissue biopsy samples. (A) Phosphorylation of protein kinase B (AKT) at serine 473, (B) phosphorylation of AKT at threonine 308, (C) pmTOR at serine 2481, (D) RCR (state 3:state 4o), and (E) β-oxidation–linked respiration in adipose tissue. Differences between treatment effects were tested using the classical crossover test, which compares the intraindividual period differences of the outcome between the sequence groups. Values are mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$, $n = 12$. BCAA, branched-chain amino acid; fccp, carbonyl cyanide p-[trifluoromethoxyl]-phenyl-hydrozone; pmTOR, phosphorylation of mechanistic target of rapamycin; RCR, respiratory control ratio.

BCAA intake resulted in lower mTOR phosphorylation. Reduced AKT activity in WAT, however, might result from decreased peripheral insulinemia. Meal-induced effects of reduced BCAA intake were also detected in WAT energy metabolism, resulting in higher RCR and lower oxidative capacity. A high RCR in adipose tissue indicates a higher efficiency of mitochondrial function under conditions of BCAA reduction. The difference in RCR can be explained mainly by a difference in the proton leak and, to a certain extent, by altered substrate oxidation (57). Substrates contributing electrons to the ubiquinone pool such as succinyl-CoA under reduced-BCAA conditions possibly alter proton translocation stoichiometry and proton leak compared with predominantly NADH-linked substrates during high-BCAA conditions.

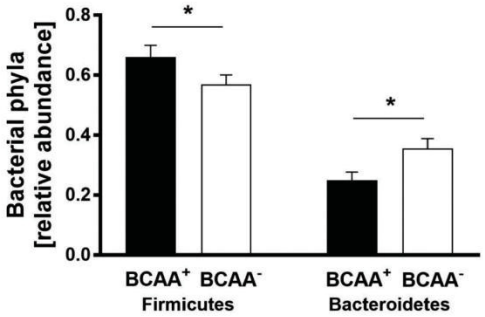

FIGURE 4 Alterations in microbiota: abundance of fecal Firmicutes and Bacteroidetes. Values are mean ± SEM. Differences between treatment effects were tested using the classical crossover test, which compares the intraindividual period differences of the outcome between the sequence groups. $^{*}P < 0.05$, $n = 10$.

Furthermore, the hepatokine FGF21 could contribute to the interplay between BCAAs and altered adipose tissue energy metabolism. In line with previous findings (23), plasma FGF21 concentrations increased after 1 wk of the BCAA^{-} diet. The insulin-sensitizing hormone FGF21 is considered a metabolic signal of dietary protein restriction (58) and a marker of improvement of metabolic health (48) by enhancing glucose uptake in adipose tissue. Thus, the increase in FGF21 after BCAA reduction may support increased mitochondrial efficiency in adipose tissue. This possibly results in subsequent activation of the FGF21–AMPK (5'-adenosine monophosphate-activated protein kinase) pathway. In line with this, increased FGF21 concentrations after deprivation of the single BCAA leucine have been reported previously (59).

Reduced BCAA intake also affected the gut microbiome, with increased Bacteroidetes and decreased Firmicutes phyla. In contrast, long-term protein-rich diet revealed a correlation with

increased abundance of Bacteroidetes (60). In addition, short-term dietary changes have been proven to alter the human gut microbiome (61). The altered composition of gut bacteria at the end of only 1 wk of BCAA-reduced food intake might be the link to decreased insulin secretion. Dietary modifications may affect gut microbiome composition including bacterial species producing SCFAs such as acetate, propionate, and butyrate, which contribute to the regulation of glucose homeostasis (62). Precisely, acetate modulates insulin secretion (63) and increased concentrations of acetate and butyrate have been found in parts of the distal guts of obese mice, which grants the microbiome an independent role in the development of obesity (64). However, gut acetate and butyrate were not measured in this study.

The present study benefits from the supervised dietary intervention, the comparison of meal-induced effects with effects during 2–4 wk treatment, and the comprehensive phenotyping of the patients. On the other hand, this study does not allow us to draw conclusions as to dose–effect relations and chronic effects of dietary BCAA depletion.

In conclusion, short-term dietary reduction of BCAAs acutely decreases meal-induced insulin secretion, and improves postprandial insulin sensitivity and the mitochondrial efficiency of WAT. Dietary BCAA reduction for 1 wk does not affect whole-body insulin sensitivity, but increases circulating FGF21 concentrations and the abundance of intestinal Bacteroidetes.

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The authors' responsibilities were as follows—MR and DS: had the idea for this study; MR, YK, JS, M-CS, and VB: designed the research; YK and TK: conducted the research, YK: enrolled participants, assigned participants to interventions, analyzed the data, and wrote the paper; VB, KM, JS, and MR: contributed to the discussion; GP and AT: analyzed the data with mathematical modeling; VB, KM, JS, MR, KB, and O-PZ: reviewed and edited the paper; TJ and LM: collected the data; DP and MW: provided essential materials; DM and FB: performed laboratory analyses; KS: performed statistical analyses and generated the allocation sequence; MR and JS: are the guarantors of this work and, as such, had full access to all the data in the study and take responsibility for the integrity of the data and the accuracy of the data analysis; and all authors: read and approved the final manuscript. DS and FB are employees of Sanofi-Aventis Deutschland, a pharmaceutical company. None of the other authors reported a conflict of interest related to the study.

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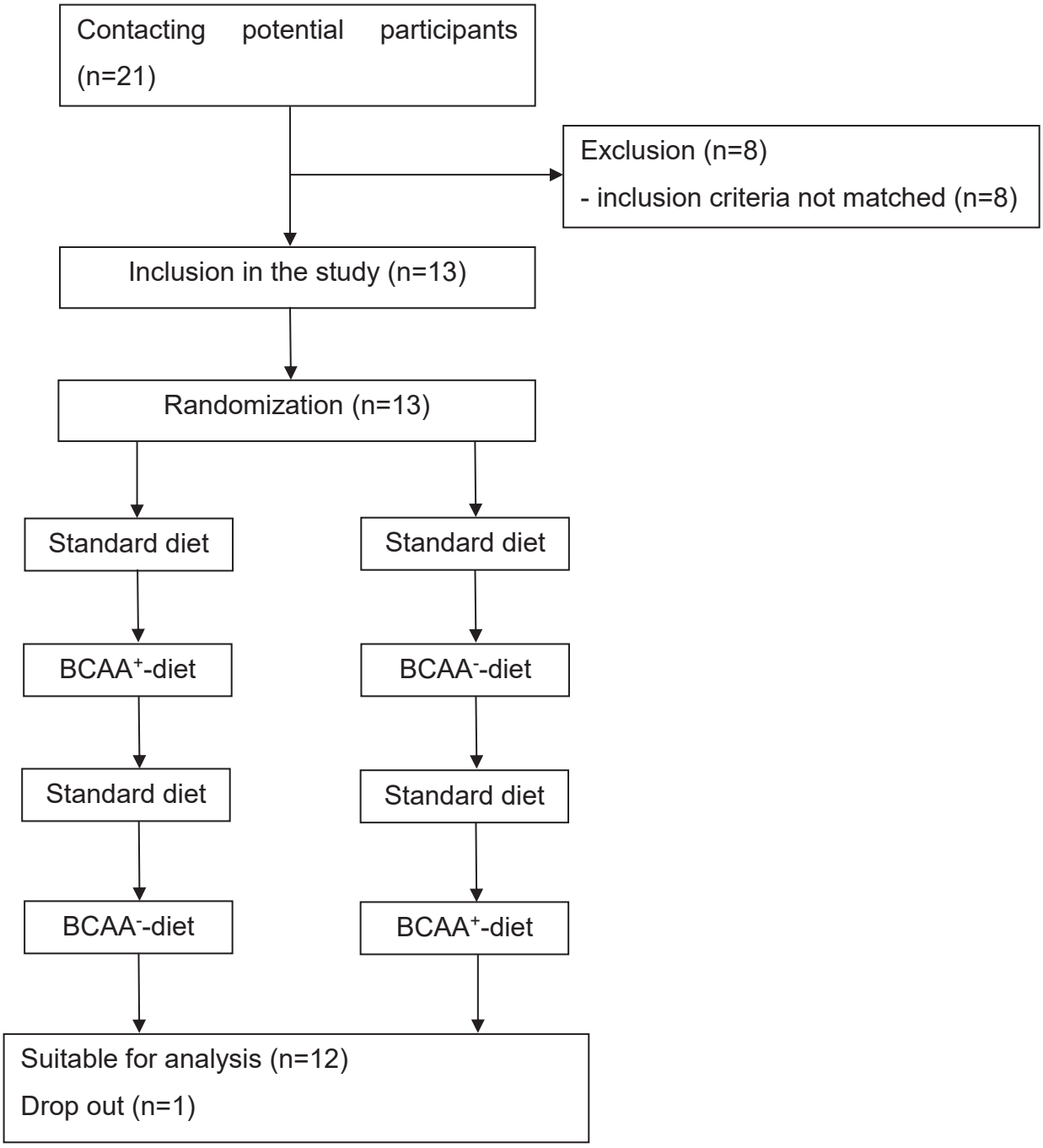
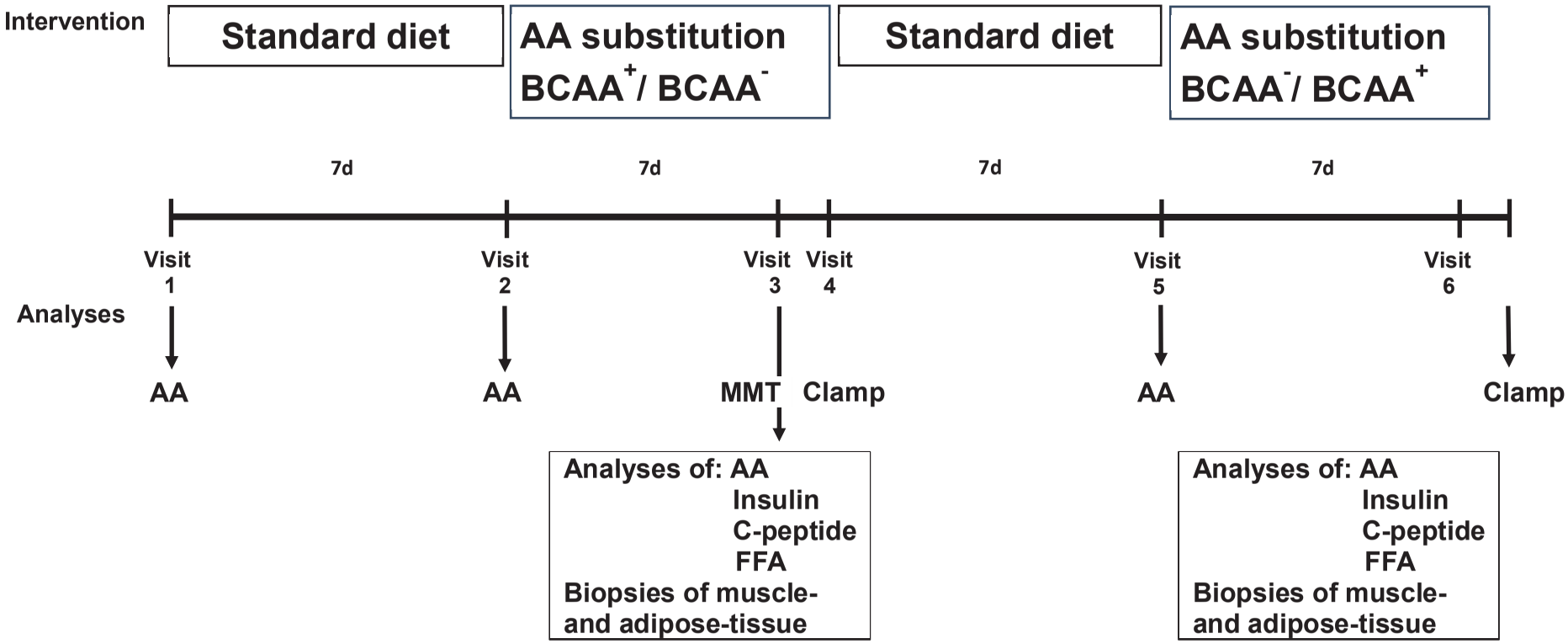
Supplemental Figure S1. Flow diagram of participants' recruitment



Supplemental Figure 2



Supplemental Table 1

µmol/l	ABA	ALA	ARG	ASN	ASP	GLU	GLN	GLY	HIS	HYP	ILE	LEU	LYS	MET	ORN	PHE	PRO	SER	THR	TRP	TYR	VAL
Baseline	22±8	475±85	150±27	42±6	17±6	176±70	433±56	213±31	83±10	8±3	87±25	159±35	193±30	22±5	58±13	68±9	213±49	108±21	118±21	67±7	80±17	285±41
BCAA$^+$	19±5	462±86	142±51	45±5	31±11	203±76	443±86	247±38	82±9	6±1	76±14	155±28	191±22	23±5	80±19	79±9	241±55	132±21	127±16	68±10	84±19	276±50
BCAA$^-$	30±6	523±85	146±47	46±6	36±9	227±60	465±58	287±33	84±6	6±1	69±12	139±19	203±18	21±2	95±18	76±8	290±69	142±20	155±23	62±7	88±24	214±28
***P* BL/BCAA$^+$**	-	-	-	-	<0.01	<0.01	-	<0.01	-	<0.05	-	-	-	-	<0.05	<0.01	<0.05	<0.05	-	-	-	-
***P* BL/BCAA$^-$**	<0.05	<0.05	-	-	<0.01	<0.01	<0.05	<0.01	-	-	<0.05	<0.05	-	-	<0.01	<0.05	<0.01	<0.001	<0.001	<0.05	-	<0.001
P	<0.001	<0.001	-	-	-	-	-	<0.01	-	-	<0.05	<0.05	-	-	<0.01	-	<0.01	-	<0.01	<0.01	-	<0.001
BCAA$^+$/BCAA$^-$																						

Amino acid concentrations. Amino acid concentrations prior to study enrolment (baseline) and at the end of 1-week dietary intervention with the complete set of amino acids (BCAA$^+$) or a 60%-reduction of BCAA (BCAA$^-$); BL (baseline). ABA (a-aminobutyric acid), ALA (alanine), ARG (arginine), ASN (asparagine), ASP (aspartic acid), GLU (glutamic acid), GLN (glutamine), GLY (glycine), HIS (histidine), HYP (4-hydroxyproline), ILE (isoleucine), LEU (leucine), LYS (lysine), MET (methionine), ORN (ornithine), PHE (phenylalanine), PRO (proline), SER (serine), THR (threonine), TRP (tryptophan), TYR (tyrosine), VAL (valine). Data are shown as mean±SD. (n=12).

4. Discussion

This study contributes to elucidating the role of the BCAA, valine, leucine and isoleucine, during the development and progression of insulin resistance. We used a randomized placebo-controlled double-blinded cross-over study to assess the effects of a diet low in BCAA in intensively phenotyped overweight patients with T2D. As main results, the study found that dietary reduction of BCAA intake for one week in persons with T2D (i) decreased postprandial insulin secretion, (ii) stimulated mitochondrial efficiency in adipose tissue and (iii) altered gut microbiome composition.

4.1. Effects of reduced BCAA intake on insulin secretion

In T2D, beta-cell dysfunction occurs before hyperglycemia develops [160] and supersedes the disease outbreak. High levels of glucose, lipids and inflammatory factors act as harmful agents to induce inadequate glucose sensing to stimulate insulin secretion. Glucose can have a dual effect as a beta-cell mass extender in order to satisfy higher insulin demands and together with AA are well known insulin secretory stimulators [107]. A reduced early insulin secretory response to oral glucose load, limited ability of the beta-cell to compensate for the degree of insulin resistance, decreased glucose-sensing resources of the beta-cell, and shifts to the right in the dose-response curves relating glucose and insulin secretion, are among the secretory defects which are indicative of a progressive insensitivity of the beta-cell to glucose [161].
To assess the acute effects of BCAA reduction on the beta-cell in the presented work, we applied a MMT with reduced BCAA content and evaluated the acute endocrine response of the pancreas to a less intense physiological AA stimulus. Of note, the lower insulin secretion rates did not result in higher blood glucose levels during the MMT implying improved insulin sensitivity. Indeed, postprandial insulin sensitivity as calculated from the oral glucose sensitivity index (OGIS) and the PREDIcted M-value (PREDIM index), which have been validated and correlate with clamp-derived measures of whole-body insulin sensitivity [162], were more than 20% higher than after $BCAA^+$ diet. This phenomenon could have resulted from increased insulin-mediated glucose disposal, which takes place predominantly in skeletal muscle under these conditions. Interestingly, data from HEC showed no improvement of skeletal muscle insulin sensitivity. This controversial finding could be explained by the different extent of reduction of

circulating BCAA levels. There was a postprandial reduction by 60% during MMT versus 13% under steady state conditions during HEC. There has been previous evidence, that hyperaminoacidemia could promote development of T2D through hyperinsulinemia, which ultimately leads to beta-cell exhaustion in the long term [130]. Alternatively, endogenous glucose production (EGP) could have been decreased under BCAA-depleted conditions due to lower substrate supply for hepatic gluconeogenesis [159]. The BCAA- diet for the duration of seven days improved postprandial glucose disposal in patients with T2D, which can be of high clinical significance. On the one side, decreasing postprandial endogenous insulin secretion could help to preserve beta-cell insulin reserve, thereby potentially reducing the patient's dependency on exogenous insulin supplementation [163]. On the other hand, the administration of a complete set of AA leads to increased insulin levels and thereby to an improvement of glucose homeostasis [164]. In addition, it results in aminoacidemia which stimulates protein synthesis and inhibits proteolysis[164].

4.2. Effects of reduced BCAA intake on insulin sensitivity

This study also explored the effects of a modification of a single nutrient factor on the insulin sensitivity of peripheral tissues. Others have shown a quantitative relationship between the BCAA cluster and insulin resistance as assessed by HOMA-IR in Asian-Indian and Chinese males [149]. In our study BCAA modification did not affect insulin-stimulated peripheral glucose disposal levels under HEC conditions after normalization for the prevalent insulin levels during steady state of the HEC [165]. Interestingly, hepatic and adipose tissue insulin sensitivity were also comparable during HEC. The contrasting outcomes of whole-body insulin sensitivity from HEC and MMT may be due to different experimental conditions in the two setups. Whereas during HEC, constant levels of hormones and metabolites are present, MMT measurements are performed under dynamic conditions during a postprandial period.

These aspects of the test conditions may result in different degrees of splanchnic versus peripheral insulinemia, which can markedly affect hepatic glucose turnover [166]. Nevertheless, the most obvious explanation for the difference between MMT and HEC resides in the different degree of BCAA reduction. The 60%-decrease in total serum BCAA levels during MMT associated with higher postprandial insulin sensitivity. In contrast, the minor reduction of circulating BCAA levels during HEC did not induce change of insulin sensitivity. Insulin decreases the appearance and increases the uptake of AA in the periphery [167]. Consequently, reduction of BCAA levels during steady state of HEC under hyperinsulinemia

was as expected lower than during MMT. It is conceivable that the dietary reduction of BCAA by 60% does not suffice to improve the insulin resistance of these patients with overt T2D. These data also suggest that the acute effect of BCAA reduction observed during MMT does not persist during the course of the dietary intervention. On the other hand, the lower insulinemia as observed during MMT would rather favor glycogenolysis and thereby stimulation of EGP [159]. However, no change of EGP was registered. Our results suggest that the improvement of insulin sensitivity is solely a short-term reaction but fails at achieving a permanent effect probably due to the magnitude of BCAA reduction. Thus, another possible reason for the lack of an effect on insulin sensitivity could be the short intervention period. But even a modulation of BCAA intake for one month failed to affect whole-body insulin sensitivity as assessed from HEC [168]. Since BCAA are essential and are omnipresent in commercially available foods, it was not possible to further reduce their dietary intake. BCAA degradation may also stimulate fatty acid synthesis and induce insulin resistance in skeletal muscle or white adipose tissue (WAT) via BCAA catabolic products or adipokines such as leptin and adiponectin or pro-inflammatory factors [132]. This will need to be addressed in future studies.

4.3. Effects of reduced BCAA intake on insulin signaling

Insulin is produced and secreted from the pancreatic beta-cells as a result of nutrient influx [169]. Both insulin resistance and beta-cell dysfunction influence each other and presumably synergistically exacerbate diabetes. At the background of systemic insulin resistance, insulin signaling within the glucose sensitive tissues is defective and therefore hyperglycemia perseveres [170]. Preserving beta-cell function and insulin signaling in beta-cells and in the glucose recipient tissues would maintain glucose homeostasis. In the presented work we assessed the activated mTORC1 complex. In addition, defective BCAA oxidative metabolism might occur in obesity, leading to a further accumulation of BCAAs and toxic intermediates but the presented work did not assess the metabolites of BCAA degradation.

The tissue-specific contributions to improved postprandial insulin sensitivity, were examined in biopsies from skeletal muscle and adipose tissue taken at 4 h after the start of MMT. In skeletal muscle, there were no differences the mTOR/p70S6K signaling pathway between $BCAA^+$ and $BCAA^-$ diets. This may be due to the relatively low levels of circulating insulin and BCAA at the end of the MMT and the weak stimulation of the insulin signaling pathway [171]. Although skeletal muscle strongly relies on mitochondrial oxidative phosphorylation and decreased oxidative capacity can be major contributor to the development of insulin resistance

[172], there were also no differences in ex-vivo mitochondrial function. The effects of insulin however, are reduced in patients with T2D [173] and since insulin secretion was reduced, possible effects on insulin signaling might have been diminished. Of note, skeletal muscle insulin resistance has been considered to be one of the earliest signs in the pathogenesis of metabolic syndrome [174] and therefore might need a much longer period of exposure to reduced BCAA to improve. Despite significantly reduced BCAA levels in the periphery under all conditions – fasted, steady state of HEC and during MMT, whole body insulin sensitivity remained unchanged, which was supported by lacking changes of insulin signaling in skeletal muscle.

Previous studies suggested that also adipose tissue can metabolize substantial amounts of BCAA [175] and may be a primary storage place of excess BCAA as lipids [176]. Indeed, the reduction of BCAA intake resulted in lower mTOR phosphorylation. Reduced AKT activity in WAT, however, might result from decreased peripheral insulinemia. Postprandial effects of BCAA reduced intake detected in WAT energy metabolism included an increased respiratory control ratio (RCR) and decreased oxidative capacity. A high RCR in adipose tissue indicates a higher efficiency of mitochondrial function under BCAA reduction. Furthermore, the hepatokine, fibroblast-growth factor 21 (FGF21), which is maximally elevated under conditions of reduced protein supply [178], could contribute to the interplay between BCAA and altered adipose tissue energy metabolism. As shown in previous studies AA deprivation [179, 180] increased plasma FGF21 concentrations after one week of the $BCAA^-$ diet. The insulin-sensitizing hormone FGF21 is considered a metabolic signal of dietary protein restriction [181] and BCAA-restriction in particular [143] and a marker of improvement of metabolic health [138] by enhancing glucose uptake in adipose tissue. This possibly results in subsequent activation of the FGF21-AMPK pathway. In line, increased FGF21 levels after deprivation of the single BCAA leucine have been previously reported [183] and FGF21 is sensitive to nutrient deficiency and maximum serum levels of this hepatokine are found during low-protein high carbohydrate intake [184].

4.4. Effects of reduced BCAA intake on gut microbiome composition

Several studies have shown that lean and overweight humans and rodents may present altered composition of their intestinal flora [185]. Previous publications describing gut microbiota transplantations after adherence of the donors to different diets showed that gut microbiota is easily modified by dietary [186], caloric intake [187] and age. The gut microbiota produces a

large number of enzymes which can extract energy from the host's diet and deposits in fat stores [188], but can also be pre-determined at infancy age [189], so dietary composition is clearly an important factor in regulating microbiota composition. Metagenomic analyses of lean animals and humans showed that almost all the bacteria present in the distal gut belong to one of the bacterial phyla Bacteroidetes or Firmicutes [190, 191]. Most studies reported that in diet induced obesity mice (DIO) and in obese humans, Firmicutes prevail [192]. One week of reduced BCAA intake resulted in altering the gut microbiome composition, with increasing the Bacteroidetes and decreasing the Firmicutes phyla. In contrast, long-term protein-rich diet revealed a correlation with increased abundance of Bacteroidetes [193]. It is possible that environmental factors such as diet, lifestyle, medication use or hygiene have a high impact on the microbiota composition in obese [185]. It is however relevant that individual microbiome composition is dynamic and changes in age. In addition, short-term dietary changes have been proven to alter the human gut microbiome [194]. The altered composition of gut bacteria at the end of only one week of BCAA-reduced food intake might be the link to decreased insulin secretion [185]. Previous studies have shown that differences in glucose-stimulated insulin secretion between different mouse strains were reduced through microbiota transfer [195]. Dietary modifications may affect gut microbiome composition including bacterial species producing short-chain fatty acids (SCFA) such as acetate, propionate und butyrate, which contribute to the regulation of glucose homeostasis [196]. Precisely, acetate modulates insulin secretion [197] and increased levels of acetate and butyrate have been found in parts of the distal guts of obese mice which grants the microbiome an independent role in the developments of obesity [198]. In this study acetate and butyrate were not measured. High-fat diet modulates microbiota and induces modifications in the intestinal barrier associated with an increase in absorption and circulation of lipopolysaccharides and BCAA and a reduction in acetate, propionate, and butyrate and secondary bile acids [185]. The results of the present study provide reports changes in microbiota after only 1 week of reduced BCAA intake reach beyond investigations of microbiota composition in obese patients. In fact, this describes the time course of changes. As there might be a cause-effect relationship between the microbiota and insulin resistance from previous studies [198], these data support the role of BCAA as relative nutrients connected with impaired glucose tolerance and a modifiable tool in its prevention. The potential causal and personalized role of human microbiota in the development of metabolic disorders should be further elucidated.

The present study benefits from the supervised dietary intervention, the comparison of meal-induced versus effects during 2-4 weeks treatment and the comprehensive phenotyping of the

patients. Further strengths include the cross-over experimental design and the detailed assessment of a single beta-cell-function-component by mathematical modeling of MMT-derived variables.

Possible limitations of this study include the relatively short intervention period of only one week, which does not allow to draw conclusions on chronic effects of dietary BCAA reduction and the relatively small sample size. Furthermore, the study does not examine the dose-effect relationships. In addition, only patients with disease duration of <5 years taking oral hypoglycemic medication were included which does not allow to draw conclusions on the effects in longer disease duration and in insulin dependent T2D.

5. Conclusions

In conclusion, short-term dietary reduction of BCAA acutely decreases meal-induced insulin secretion, improves postprandial insulin sensitivity and mitochondrial efficiency of WAT in humans with T2D. Dietary BCAA reduction for one week does not affect whole-body insulin sensitivity, but increases circulating FGF21 levels and the abundance of intestinal Bacteroidetes. This proof-of-concept study can serve as the basis for future trials on dose-effect relationships between BCAA or metabolites and insulin secretion and sensitivity in T2D as well for designing novel lifestyle modifications aiming of prevention or treatment of T2D. Last but not least, the strategy of moderate reduction of BCAA intake should be examined in larger trials to evaluate its feasibility and efficacy in human metabolic diseases. In the future, the finding of this thesis contributes to opening new therapeutic avenues for treating obesity and insulin resistance and its comorbidities.

6. References

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