Branching-out

Branching-out: The role of branched-chain amino acid catabolism in insulin resistance and type 2 diabetes

Froukje Vanweert

Branching-out: the role of branched-chain amino acid catabolism in insulin resistance and type 2 diabetes

Froukje Vanweert

2023

The research presented in this thesis was performed within NUTRIM School of Nutrition and Translational Research in Metabolism.

Printed by: Ipskamp Printing, Enschede
Lay-out by: Douwe Oppewal
Cover by: Dries Nulens, Studio Vanal
ISBN: 978-94-6473-283-2

Branching-out: the role of branched-chain amino acid catabolism in insulin resistance and type 2 diabetes

PROEFSCHRIFT

ter verkrijging van de graad van doctor aan de Universiteit Maastricht,
op gezag van de Rector Magnificus, Prof.dr. Pamela Habibović
volgens het besluit van het College van Decanen,
in het openbaar te verdedigen op

vrijdag 17 november 2023 om 13.00 uur

door

Froukje Vanweert

Promotores
Dr. E. Phielix
Prof. Dr. P. Schrauwen
Prof. Dr. M.K.C. Hesselink

Assessment Committee

Prof. Dr. B.E. de Galan	(voorzitter)
Prof. Dr. C. Matthys	(Katholieke Universiteit Leuven, Belgium)
Dr. R. Meex	
Prof. Dr. C. B. Newgard	(Duke University Medical Center, USA)
Prof. Dr. S.W.M. Olde Damink	

CONTENTS

Chapter 1	Introduction	7
Chapter 2	Role of branched-chain amino acid metabolism in the pathogenesis of obesity and type 2 diabetes-related metabolic disturbances	17
Chapter 3	Elevated plasma branched-chain amino acid levels correlate with type 2 diabetes-related metabolic disturbances	53
Chapter 4	The effect of physical activity level and exercise training on the association between plasma branched-chain amino acids and intrahepatic lipid content in participants with obesity	71
Chapter 5	A randomized placebo-controlled clinical trial for pharmacological activation of BCAA catabolism in patients with type 2 diabetes	97
Chapter 6	The molecular link between elevated branched-chain amino acid levels and insulin resistance: a novel in vitro tool to study insulin-stimulated glucose uptake in C2C12 cells	125
Chapter 7	The association between circulating branched-chain amino acids and the temporal risk of developing type 2 diabetes mellitus: a systematic review & meta-analysis	145
Chapter 8	General discussion and conclusion	177
Appendices	Impact	201
	Samenvatting	207
	Summary	213
	Dankwoord	217
	About the author	223
	List of publications	225

1

Introduction

OBESITY AND TYPE 2 DIABETES MELLITUS

Worldwide, the high prevalence of obesity has become a major problem (1). Since 1975, the prevalence of obesity has nearly tripled. In 2016, 39% of adults aged >18 years were overweight, and 13% were obese (2). The fundamental cause of obesity and overweight is an imbalance between energy intake and energy expenditure (2), mainly caused by increased consumption of energy-dense food and decreased physical activity. Changes in dietary and physical activity patterns, the *'Western lifestyle'*, are often the result of environmental and societal changes (2). Obesity is the main risk factor for several metabolic impairments leading to the development of hypertension, metabolic syndrome, non-alcoholic fatty liver (NAFL), type 2 diabetes (T2D) and eventually cardiovascular disease (3). Therefore, it is not surprising that the prevalence of diabetes follows the same raising trend as for prevalence of obesity.

The rising burden of diabetes forms a major public health problem with great impact on quality of life and extraordinary health care costs (4). Diabetes affects patients' functional capacities, leading to significant morbidity and mortality (5). Diabetes is a chronic, metabolic disease characterized by elevated levels of blood glucose. Type 1 diabetes is well known as juvenile diabetes and is a result of an dysfunctional insulin production by the pancreas, and therefore, requires daily administration of insulin (6). The majority, more than 95%, of people with diabetes have T2D, which develops usually in older individuals and is largely the result of excess body weight and physical inactivity (6). Worldwide, 4.3 million deaths per year can be attributed to the consequences of T2D, making it the ninth leading cause of mortality (7).

Insulin resistance is the biggest risk factor for the development of T2D, which is defined as a reduced response of target tissues, such as skeletal muscle and liver towards the action of insulin (8). As a result, pancreatic insulin secretion will rise in an attempt to compensate for insulin resistance, thereby maintaining normal glucose levels (normoglycaemia) (9). With the progression of T2D, the pancreatic b-cells fail to produces sufficient insulin to compensate fully for decreased insulin sensitivity of mainly muscle and liver (10). Insulin resistance of muscle results in decreased insulin-stimulated glucose uptake and insulin resistance of the liver leads to a blunted suppression of hepatic glucose output. Together, this drives hyperglycaemia. Chronic hyperglycaemia will eventually result in micro- and macrovascular dysfunction (11), and therefore, strategies to improve insulin resistance to normalize glycemia are key to prevent the progression of T2D and its related complications (12, 13).

One of the most important strategies to prevent the development of T2D, as well delay of T2D and diabetes-related complications is weight-loss (14). It has been shown in people with overweight and obesity that achieving and maintaining a minimum weight loss of 7-10% could reduce their diabetes risk (15). Weight loss can be achieved by lifestyle measures, such as reduced-calorie meal plan (15-17), and 150 min/week of moderate-intensity physical activity (17). Although these strategies seem very effective, compliance to these lifestyle changes on the long-term is difficult to maintain in the general population (18). As a result, additional pharmacotherapy is often needed.

Metformin is generally the first medication prescribed for T2D, unless there are contraindications, in combination with lifestyle modifications (12). Since T2D is a progressive disease, with an increase of co-morbidities over time requiring additional attention, monotherapy usually does not suffice to maintain normoglycemia after a few years, and combination therapy starts to become necessary (19). For second line drug treatment, several drug classes exist including sulfonylureas, thiazolidinediones, DPP-4 inhibitors, SGLT2-inhibitors and GLP-1 receptor agonists. Despite the whole gamut of several lifestyle and pharmacological therapies, hyperglycaemia stays difficult to manage with the progression of T2D, and therefore, development of novel treatment strategies is crucial.

Disturbances in glucose and fat metabolism have been implicated in mediating the severity of insulin resistance (20), although in the recent years, reports collectively show that amino acids, especially the branched-chain amino acids (BCAA) leucine, isoleucine and valine interfere with insulin resistance (21-29).

BCAA METABOLISM AND INSULIN RESISTANCE

Over the last decade, branched-chain amino acids (BCAA) catabolism has progressively been considered to have a role in the development of insulin resistance and T2D. In patients with T2D, BCAA levels are substantially elevated in plasma and tissues (21-24, 26, 30, 31), which associate with diabetes-related metabolic disturbances, such as insulin resistance (21-29), mitochondrial dysfunction function (32, 33) and high liver fat content (34-36).

Leucine, isoleucine and valine are grouped as BCAA because they share a structural feature with a branched-side chain and common initiation steps of their catabolism. Proper catabolism of BCAA metabolism is pivotal to normal physiology, including the regulation of substrate oxidation. Reports show that BCAA act as

signaling molecules regulating metabolism of glucose, lipid, and protein (37). In addition, BCAA metabolism plays a key role in interorgan metabolic crosstalk and, therefore, disturbances in BCAA metabolism may play a significant role in several metabolic diseases, including T2D (38). Moreover, elevated circulatory BCAA levels predict diabetes development (39). However, it is still unknown why BCAA levels are elevated and why they associate with insulin resistance.

BCAA levels in plasma can be regulated by several mechanisms, and therefore multifactorial causes could underlie the elevated BCAA plasma levels seen in patients with T2D, of which a dysfunctional breakdown of BCAA may be one of the underlying factors (26, 40). Several tissues including skeletal muscle, liver, adipose tissue and cardiac tissue, play an important role in BCAA catabolism (41, 42). Skeletal muscle, which accounts for ~80% of the insulin-stimulated glucose uptake (44), is also the main site of BCAA catabolism (43). Therefore, a defective BCAA catabolism might have detrimental consequences for peripheral insulin sensitivity. So far, the mechanisms underlying elevated plasma BCAA levels leading to insulin resistance are not entirely elucidated. Besides the skeletal muscle, the liver, adipose and cardiac tissue (42) are involved in BCAA catabolism too, and may also be impaired in patients with T2D (45, 46). Tissue-specific BCAA metabolism has been extensively investigated in rodent models, with only limited research being performed in humans.

TREATMENT STRATEGIES OF BCAA-INDUCED INSULIN RESISTANCE

Several rodent studies show the power of enhancing BCAA catabolism as potential strategy to improve insulin sensitivity, however, this concept has not been extensively investigated in humans since limited number of tools are available. Nevertheless, several pharmaceutical and alternative strategies have been shown to effectively modulate BCAA catabolism and lower systemic BCAA levels, which will be overviewed in this thesis.

It has been shown in several rodent models with insulin resistance that boosting BCAA catabolism via several pharmaceutical agents resulted in lower plasma BCAA levels and improved glucose metabolism and insulin sensitivity (47-50). Although the data in rodents are promising, this concept has never been translated to humans. A possible pharmaceutical way to promote BCAA catabolism is sodium-phenylbutyrate (NaPB), a drug normally used for the management of urea cycle disorders. NaPB can activate BCAA catabolism, resulting in lower BCAA levels in healthy individuals (51-53), and therefore, could be beneficial for patients featuring insulin resistance and type 2 diabetes.

Other pharmaceutical classes of drugs that could act on BCAA catabolism are 3,6-dichlorobenzo(b)thiopene-2-carboxylic acid (BT2), SGLT2-inhbitors, fibrates and, GLP-1/GIP agonists, however, their mechanisms are not completely understood in humans. Another approach to modulate BCAA catabolism and concentrations are alternative strategies including physical exercise (54, 55), cold acclimatisation (56) and diet (57-59). The abovementioned interventions are well-known to improve metabolic health, however, the impact of BCAA catabolism herein has not been investigated: its effect on modulating BCAA catabolism may form one of the underlying mechanisms.

To conclude, obtaining more insight into BCAA metabolism in the development of T2D in humans is of importance. Promising results from pre-clinical work emphasize the relevance of investigating the impact of modulating BCAA catabolism in patients with T2D. Together, these new physiological insights would indicate if targeting BCAA catabolism forms a novel therapeutic approach to improve metabolic health and treat patients with T2D.

THESIS OUTLINE

The aim of this thesis is to investigate the role of BCAA metabolism in T2D and more specifically if stimulating BCAA catabolism improves insulin sensitivity and metabolic health in patients with T2D.

In **chapter 2** several hypothesized mechanistic links between BCAA catabolism and insulin resistance and current available tools to modulate BCAA catabolism *in vivo* in rodents and humans are being reviewed. Furthermore, this review discusses whether enhancing BCAA catabolism may form a potential future treatment strategy to promote metabolic health in insulin resistance and T2D.

Chapter 3 aims to investigate if patients with T2D feature low BCAA oxidation rates *in vivo*, by measuring whole-body leucine oxidation rates in patients with T2D and age- and BMI-matched control participants. Furthermore, in this chapter we investigate if BCAA plasma levels associate with well-known type 2 diabetes-related metabolic disturbances. This will be investigated in first-degree relatives (FDR) of patients with T2D, patients with T2D and in a control group.

Furthermore, we investigate in **chapter 4** if plasma BCAA levels are elevated in people with NAFL as well. This chapter studies the association between BCAA plasma levels and liver fat content, and in addition, examines if this association is

affected by the level of physical activity. Furthermore, we investigate if increasing physical activity via an exercise training program affects plasma BCAA levels along with alterations in liver fat in patients with T2D and in people with NAFL.

In **chapter 5**, we investigate if promoting BCAA oxidation with NaPB in patients with T2D forms a potential treatment to improve glucose homeostasis. To this end, a 2-week treatment intervention with NaPB on insulin sensitivity as primary outcome measure, and other key metabolic health parameters is performed. In this study, NaPB is administered 'off-label' as add-on medication to patients with T2D as a tool to modulate BCAA catabolism to lower BCAA plasma levels.

In **chapter 6,** we aim to examine the role of BCAA on *in vitro* glucose uptake in skeletal muscle cells of mice and humans in more detail and study the hypothesis that elevation of BCAA blunts glucose uptake. We also aim to explore the molecular pathways involved.

Chapter 7 focusses on the relationship between BCAA and T2D development through case-control study data of people with overweight. In addition, we also aim to appraise whether this relationship exhibits any variation based on follow-time to diagnosis of T2D.

Finally, in **chapter 8**, we highlight the major findings and conclusions of the previous chapters described in this thesis, and discuss their relevance in a broader perspective. Recommendations for future research in the field of BCAA metabolism are discussed.

REFERENCES

1. Kahn SE, Hull RL, Utzschneider KM. Mechanisms linking obesity to insulin resistance and type 2 diabetes. Nature. 2006;444(7121):840-6.
2. (WHO) WHO. Obesity and overweight 2021 (Available from: https://www.who.int/news-room/fact-sheets/detail/obesity-and-overweight.
3. Rhee EJ. The Influence of Obesity and Metabolic Health on Vascular Health. Endocrinol Metab (Seoul). 2022;37(1):1-8.
4. Organization WH. Global Report on Diabetes. France; 2016.
5. Ramtahal R, Khan C, Maharaj-Khan K, Nallamothu S, Hinds A, Dhanoo A, et al. Prevalence of self-reported sleep duration and sleep habits in type 2 diabetes patients in South Trinidad. J Epidemiol Glob Health. 2015;5(4 Suppl 1):S35-43.
6. (WHO) WHO. Diabetes 2022 (Available from: https://www.who.int/health-topics/diabetes.
7. Khan MAB, Hashim MJ, King JK, Govender RD, Mustafa H, Al Kaabi J. Epidemiology of Type 2 Diabetes - Global Burden of Disease and Forecasted Trends. Journal of epidemiology and global health. 2020;10(1):107-11.
8. DeFronzo RA, Tripathy D. Skeletal muscle insulin resistance is the primary defect in type 2 diabetes. Diabetes Care. 2009;32 Suppl 2:S157-63.
9. Abdul-Ghani MA, DeFronzo RA. Pathogenesis of insulin resistance in skeletal muscle. J Biomed Biotechnol. 2010;2010:476279.
10. Kahn SE. The Importance of β-Cell Failure in the Development and Progression of Type 2 Diabetes. The Journal of Clinical Endocrinology & Metabolism. 2001;86(9):4047-58.
11. Deshpande AD, Harris-Hayes M, Schootman M. Epidemiology of diabetes and diabetes-related complications. Phys Ther. 2008;88(11):1254-64.
12. Committee ADAPP. 3. Prevention or Delay of Type 2 Diabetes and Associated Comorbidities: Standards of Medical Care in Diabetes—2022. Diabetes Care. 2021;45(Supplement_1):S39-S45.
13. Lindstrom J, Ilanne-Parikka P, Peltonen M, Aunola S, Eriksson JG, Hemio K, et al. Sustained reduction in the incidence of type 2 diabetes by lifestyle intervention: follow-up of the Finnish Diabetes Prevention Study. Lancet. 2006;368(9548):1673-9.
14. Nathan DM, Bennett PH, Crandall JP, Edelstein SL, Goldberg RB, Kahn SE, et al. Does diabetes prevention translate into reduced long-term vascular complications of diabetes? Diabetologia. 2019;62(8):1319-28.
15. Hamman RF, Wing RR, Edelstein SL, Lachin JM, Bray GA, Delahanty L, et al. Effect of weight loss with lifestyle intervention on risk of diabetes. Diabetes Care. 2006;29(9):2102-7.
16. Knowler WC, Barrett-Connor E, Fowler SE, Hamman RF, Lachin JM, Walker EA, et al. Reduction in the incidence of type 2 diabetes with lifestyle intervention or metformin. N Engl J Med. 2002;346(6):393-403.
17. Diabetes Prevention Program Research G. The Diabetes Prevention Program (DPP): description of lifestyle intervention. Diabetes care. 2002;25(12):2165-71.
18. Gibson AA, Sainsbury A. Strategies to Improve Adherence to Dietary Weight Loss Interventions in Research and Real-World Settings. Behav Sci (Basel). 2017;7(3).
19. Association AD. 9. Pharmacologic Approaches to Glycemic Treatment: Standards of Medical Care in Diabetes—2020. Diabetes Care. 2019;43(Supplement_1):S98-S110.
20. Szendroedi J, Phielix E, Roden M. The role of mitochondria in insulin resistance and type 2 diabetes mellitus. Nat Rev Endocrinol. 2011;8(2):92-103.

21. Huffman KM, Shah SH, Stevens RD, Bain JR, Muehlbauer M, Slentz CA, et al. Relationships Between Circulating Metabolic Intermediates and Insulin Action in Overweight to Obese, Inactive Men and Women. Diabetes Care. 2009;32(9):1678-83.
22. Newgard CB, An J, Bain JR, Muehlbauer MJ, Stevens RD, Lien LF, et al. A branched-chain amino acid-related metabolic signature that differentiates obese and lean humans and contributes to insulin resistance. Cell Metab. 2009;9(4):311-26.
23. Palmer ND, Stevens RD, Antinozzi PA, Anderson A, Bergman RN, Wagenknecht LE, et al. Metabolomic profile associated with insulin resistance and conversion to diabetes in the Insulin Resistance Atherosclerosis Study. J Clin Endocrinol Metab. 2015;100(3):E463-8.
24. Perng W, Gillman MW, Fleisch AF, Michalek RD, Watkins SM, Isganaitis E, et al. Metabolomic profiles and childhood obesity. Obesity (Silver Spring). 2014;22(12):2570-8.
25. Shah SH, Crosslin DR, Haynes CS, Nelson S, Turer CB, Stevens RD, et al. Branched-chain amino acid levels are associated with improvement in insulin resistance with weight loss. Diabetologia. 2012;55(2):321-30.
26. Tai ES, Tan ML, Stevens RD, Low YL, Muehlbauer MJ, Goh DL, et al. Insulin resistance is associated with a metabolic profile of altered protein metabolism in Chinese and Asian-Indian men. Diabetologia. 2010;53(4):757-67.
27. Walford GA, Ma Y, Clish C, Florez JC, Wang TJ, Gerszten RE, et al. Metabolite Profiles of Diabetes Incidence and Intervention Response in the Diabetes Prevention Program. Diabetes. 2016;65(5):1424-33.
28. Wurtz P, Soininen P, Kangas AJ, Ronnemaa T, Lehtimaki T, Kahonen M, et al. Branched-chain and aromatic amino acids are predictors of insulin resistance in young adults. Diabetes Care. 2013;36(3):648-55.
29. Vanweert F, de Ligt M, Hoeks J, Hesselink MKC, Schrauwen P, Phielix E. Elevated Plasma Branched-Chain Amino Acid Levels Correlate With Type 2 Diabetes-Related Metabolic Disturbances. J Clin Endocrinol Metab. 2021;106(4):e1827-e36.
30. Krebs M, Krssak M, Bernroider E, Anderwald C, Brehm A, Meyerspeer M, et al. Mechanism of amino acid-induced skeletal muscle insulin resistance in humans. Diabetes. 2002;51(3):599-605.
31. Xu F, Tavintharan S, Sum CF, Woon K, Lim SC, Ong CN. Metabolic signature shift in type 2 diabetes mellitus revealed by mass spectrometry-based metabolomics. J Clin Endocrinol Metab. 2013;98(6):E1060-5.
32. Koves TR, Ussher JR, Noland RC, Slentz D, Mosedale M, Ilkayeva O, et al. Mitochondrial overload and incomplete fatty acid oxidation contribute to skeletal muscle insulin resistance. Cell Metab. 2008;7(1):45-56.
33. Muoio DM, Newgard CB. Molecular and metabolic mechanisms of insulin resistance and beta-cell failure in type 2 diabetes. Nat Rev Mol Cell Bio. 2008;9(3):193-205.
34. Sliz E, Sebert S, Wurtz P, Kangas AJ, Soininen P, Lehtimaki T, et al. NAFLD risk alleles in PNPLA3, TM6SF2, GCKR and LYPLAL1 show divergent metabolic effects. Hum Mol Genet. 2018;27(12):2214-23.
35. Iwasa M, Ishihara T, Mifuji-Moroka R, Fujita N, Kobayashi Y, Hasegawa H, et al. Elevation of branched-chain amino acid levels in diabetes and NAFL and changes with antidiabetic drug treatment. Obes Res Clin Pract. 2015;9(3):293-7.
36. Kaikkonen JE, Wurtz P, Suomela E, Lehtovirta M, Kangas AJ, Jula A, et al. Metabolic profiling of fatty liver in young and middle-aged adults: Cross-sectional and prospective analyses of the Young Finns Study. Hepatology. 2017;65(2):491-500.
37. Nie C, He T, Zhang W, Zhang G, Ma X. Branched Chain Amino Acids: Beyond Nutrition Metabolism. Int J Mol Sci. 2018;19(4).
38. Gancheva S, Jelenik T, Alvarez-Hernandez E, Roden M. Interorgan Metabolic Crosstalk in Human Insulin Resistance. Physiol Rev. 2018;98(3):1371-415.

39. Wang TJ, Larson MG, Vasan RS, Cheng S, Rhee EP, McCabe E, et al. Metabolite profiles and the risk of developing diabetes. Nat Med. 2011;17(4):448-53.
40. Lynch CJ, Adams SH. Branched-chain amino acids in metabolic signalling and insulin resistance. Nat Rev Endocrinol. 2014;10(12):723-36.
41. Neinast M, Murashige D, Arany Z. Branched Chain Amino Acids. Annu Rev Physiol. 2019;81:139-64.
42. Herman MA, She P, Peroni OD, Lynch CJ, Kahn BB. Adipose tissue branched chain amino acid (BCAA) metabolism modulates circulating BCAA levels. J Biol Chem. 2010;285(15):11348-56.
43. Neinast MD, Jang C, Hui S, Murashige DS, Chu Q, Morscher RJ, et al. Quantitative Analysis of the Whole-Body Metabolic Fate of Branched-Chain Amino Acids. Cell Metab. 2019;29(2):417-29 e4.
44. Petersen KF, Dufour S, Savage DB, Bilz S, Solomon G, Yonemitsu S, et al. The role of skeletal muscle insulin resistance in the pathogenesis of the metabolic syndrome. Proc Natl Acad Sci U S A. 2007;104(31):12587-94.
45. Biswas D, Duffley L, Pulinilkunnil T. Role of branched-chain amino acid-catabolizing enzymes in intertissue signaling, metabolic remodeling, and energy homeostasis. Faseb J. 2019;33(8):8711-31.
46. She P, Van Horn C, Reid T, Hutson SM, Cooney RN, Lynch CJ. Obesity-related elevations in plasma leucine are associated with alterations in enzymes involved in branched-chain amino acid metabolism. Am J Physiol Endocrinol Metab. 2007;293(6):E1552-63.
47. Hu H, Li L, Wang C, He H, Mao K, Ma X, et al. 4-Phenylbutyric acid increases GLUT4 gene expression through suppression of HDAC5 but not endoplasmic reticulum stress. Cell Physiol Biochem. 2014;33(6):1899-910.
48. Crossland H, Smith K, Idris I, Phillips BE, Atherton PJ, Wilkinson DJ. Exploring mechanistic links between extracellular branched-chain amino acids and muscle insulin resistance: an in vitro approach. Am J Physiol Cell Physiol. 2020;319(6):C1151-C7.
49. Ozcan U, Yilmaz E, Ozcan L, Furuhashi M, Vaillancourt E, Smith RO, et al. Chemical chaperones reduce ER stress and restore glucose homeostasis in a mouse model of type 2 diabetes. Science. 2006;313(5790):1137-40.
50. White PJ, McGarrah RW, Grimsrud PA, Tso SC, Yang WH, Haldeman JM, et al. The BCKDH Kinase and Phosphatase Integrate BCAA and Lipid Metabolism via Regulation of ATP-Citrate Lyase. Cell Metabolism. 2018;27(6):1281-+.
51. Darmaun D, Welch S, Rini A, Sager BK, Altomare A, Haymond MW. Phenylbutyrate-induced glutamine depletion in humans: effect on leucine metabolism. Am J Physiol. 1998;274(5 Pt 1):E801-7.
52. Scaglia F, Carter S, O'Brien WE, Lee B. Effect of alternative pathway therapy on branched chain amino acid metabolism in urea cycle disorder patients. Mol Genet Metab. 2004;81 Suppl 1:S79-85.
53. Seminara J, Tuchman M, Krivitzky L, Krischer J, Lee HS, Lemons C, et al. Establishing a consortium for the study of rare diseases: The Urea Cycle Disorders Consortium. Mol Genet Metab. 2010;100 Suppl 1:S97-105.
54. Glynn EL, Piner LW, Huffman KM, Slentz CA, Elliot-Penry L, AbouAssi H, et al. Impact of combined resistance and aerobic exercise training on branched-chain amino acid turnover, glycine metabolism and insulin sensitivity in overweight humans. Diabetologia. 2015;58(10):2324-35.
55. Xiao W, Chen P, Liu X, Zhao L. The Impaired Function of Macrophages Induced by Strenuous Exercise Could Not Be Ameliorated by BCAA Supplementation. Nutrients. 2015;7(10):8645-56.
56. Yoneshiro T, Wang Q, Tajima K, Matsushita M, Maki H, Igarashi K, et al. BCAA catabolism in brown fat controls energy homeostasis through SLC25A44. Nature. 2019;572(7771):614-9.

57. Cummings NE, Williams EM, Kasza I, Konon EN, Schaid MD, Schmidt BA, et al. Restoration of metabolic health by decreased consumption of branched-chain amino acids. J Physiol. 2018;596(4):623-45.
58. White PJ, Lapworth AL, An J, Wang L, McGarrah RW, Stevens RD, et al. Branched-chain amino acid restriction in Zucker-fatty rats improves muscle insulin sensitivity by enhancing efficiency of fatty acid oxidation and acyl-glycine export. Mol Metab. 2016;5(7):538-51.
59. Fontana L, Cummings NE, Arriola Apelo SI, Neuman JC, Kasza I, Schmidt BA, et al. Decreased Consumption of Branched-Chain Amino Acids Improves Metabolic Health. Cell Rep. 2016;16(2):520-30.

2

Role of branched-chain amino acid metabolism in the pathogenesis of obesity and type 2 diabetes-related metabolic disturbances

Froukje Vanweert[1], Patrick Schrauwen[1], and Esther Phielix[1]

[1] Department of Nutrition and Movement Sciences, NUTRIM, School of Nutrition and Translational Research in Metabolism, Maastricht University Medical Center, Maastricht, the Netherlands

This chapter has been published in: Nutr Diabetes. 2022;12(1):35.

ABSTRACT

Branched-chain amino acid (BCAA) catabolism has been considered to have an emerging role in the pathogenesis of metabolic disturbances in obesity and type 2 diabetes (T2D). Several studies showed elevated plasma BCAA levels in humans with insulin resistance and patients with T2D, although the underlying reason is unknown. Dysfunctional BCAA catabolism could theoretically be an underlying factor. *In vitro* and animal work collectively show that modulation of the BCAA-catabolic pathway alters key metabolic processes affecting glucose homeostasis, although an integrated understanding of tissue-specific BCAA catabolism remains largely unknown, especially in humans. Proof-of-concept studies in rodents -and to a lesser extent in humans – strongly suggest that enhancing BCAA catabolism improves glucose homeostasis in metabolic disorders, such as obesity and T2D. In this review, we discuss several hypothesized mechanistic links between BCAA catabolism and insulin resistance and overview current available tools to modulate BCAA catabolism *in vivo*. Furthermore, this review considers whether enhancing BCAA catabolism forms a potential future treatment strategy to promote metabolic health in insulin resistance and T2D.

INTRODUCTION

Type 2 diabetes (T2D) is one of world's most prevalent diseases, and is related to the epidemic of obesity (1). Obesity can lead to the onset of T2D when pancreatic β-cells are no longer able to compensate higher insulin secretion for the reduced insulin sensitivity that often accompanies obesity (2). Over the last decade, branched-chain amino acids (BCAA) catabolism has increasingly been considered to have an emerging role in the development of insulin resistance in people with obesity and T2D. In these individuals, BCAA levels are considerably elevated in plasma and tissues (3-9). Furthermore, elevated BCAA levels in plasma strongly associate with insulin resistance in people with obesity and T2D (3, 4, 6-8, 10-13). Although it is still unknown why these BCAA levels are elevated and why they associate with insulin resistance, a dysfunctional BCAA catabolism may be one of the underlying factors. This review aims to provide insight into the mechanisms behind elevated plasma BCAA levels in people with obesity and/or T2D and its role in the pathogenesis of insulin resistance. Furthermore, this review will overview pharmaceutical and alternative lifestyle intervention strategies in order to lower plasma BCAA levels and its effects on metabolic health.

WHY INVESTIGATING BCAA LEVELS?

Leucine, isoleucine and valine are grouped together as BCAA because they share a structural feature with a branched-side chain and common initiation steps of catabolism (14).

In general, BCAA play several important metabolic and physiological roles, aside from being considered as substrates for synthesis of proteins. Reports show that BCAA act as signaling molecules regulating metabolism of glucose, lipid, and protein (15). In addition, BCAA levels play a key role in interorgan metabolic crosstalk and, therefore, dysregulation of BCAA catabolism may play a significant role in several metabolic diseases (16).

Several studies showed that plasma BCAA levels in overweight and obese humans with insulin resistance (3-7) and patients with T2D (8, 9) were elevated compared to healthy individuals. Recently, in an observational study, we confirmed this finding and showed that plasma BCAA levels were elevated in patients with T2D compared to age- and BMI-matched controls without having T2D (13). Some (17-19), but not all studies (20, 21) found elevated plasma BCAA levels to be associated with increased risk of T2D and suggest that BCAA levels in plasma may predict future diabetes (17).

It has repeatedly been reported that the accumulation of plasma BCAA levels strongly associate with insulin resistance in obesity and T2D (3, 4, 6-8, 10-13). Similarly, a short-term intravenous infusion with amino acids in young, human volunteers induced temporary insulin resistance (22). However, as a mixture of amino acids were infused, it cannot be deduced from this study whether the BCAA *per se* are responsible for the development of insulin resistance. So far, there are no reports investigating whether particularly a raise of BCAA plasma levels in humans induces insulin resistance. Therefore, the underlying mechanisms of elevated BCAA plasma levels on insulin-stimulated glucose uptake in humans remain largely unknown.

WHY ARE PLASMA BCAA LEVELS ELEVATED WITH INSULIN RESISTANCE?

BCAA homeostasis and levels in plasma are defined by BCAA appearance and disappearance, affected by several processes. Processes contributing to BCAA appearance in the blood include protein breakdown in tissues (a process which is inhibited by insulin), food intake and gut microbial synthesis. The major processes involved in disappearance of BCAA are protein synthesis, excretion and BCAA catabolism (4, 23). As a result, an interplay between these mechanisms defines the levels of BCAA in plasma, and therefore multifactorial causes could underlie the elevated BCAA plasma levels seen in people with insulin resistance and patients with T2D.

Effect of insulin on protein breakdown and BCAA catabolism

Insulin is known to be one of the most important regulators of carbohydrate, fat and protein metabolism. Protein metabolism, or more specifically, protein turnover, is defined by the balance between protein synthesis and protein breakdown (24). During periods of steady state, the rate of protein synthesis equals the rate of protein breakdown. Both insulin as well as BCAA concentrations affects protein turnover in muscle (25), adipose tissue (26) and liver (27).

The effect of insulin on leucine flux has been investigated in humans with use of an intravenous infusion of insulin combined with (1-^{13}C] or (1-^{14}C]-leucine tracer (28-30). An intravenous insulin infusion in people without diabetes provoked a decline in the leucine flux due to a reduction in protein breakdown, without an effect on protein synthesis (28-30). The activation of protein kinase B (Akt) in response to insulin by the insulin receptor (IRS-1) induces phosphorylation of the Forkhead box class (FOXO) transcription, and indirectly activate mTOR, which seems to be responsible for the inhibited muscle protein breakdown (31-35).

In humans with insulin resistance, the effect of insulin on reducing muscle protein breakdown is blunted causing increased muscle wasting (36), as is confirmed in rodent models (37-40). BCAA are reported to activate the mTOR pathway (41) and stimulate protein synthesis in muscle of humans. However, the inhibitory effect of insulin on protein breakdown occurs independently of the levels of circulating plasma BCAA (42-44). Normally, insulin's inhibitory action on protein breakdown in muscle tissue (45-47) results in lower amino acid concentrations in plasma (42, 48, 49), with the most marked decline seen for BCAA (50-53). The effect of insulin on BCAA plasma levels has been investigated for the first time in patients with type 1 diabetes (54, 55) and results showed that the withdrawal of insulin treatment was associated with a substantial increase in circulating BCAA concentrations, as confirmed by others (58, 59). We recently confirmed the strong insulin-suppressive effect on BCAA levels in plasma during a euglycemic hyperinsulinemic clamp in healthy, insulin sensitive people with obesity, however, this insulin-suppressive effect was blunted in people with obesity, diagnosed with non-alcoholic fatty liver (NAFL) and/or T2D (56). Also others found less efficient BCAA reduction upon insulin infusion in obese humans with insulin resistance (57-59). The suggestion that increased BCAA levels could merely be a consequence of impaired insulin action is in accordance with the results from a recent mendelian randomization study (60), showing that insulin resistance drives higher plasma BCAA levels (60, 61). In contrast, a large-scale human genetic study by Lotta et al. pointed towards a causal role of diminished BCAA catabolism underlying insulin resistance (62), which is described below.

Diet and microbiome

BCAA cannot be synthesized by humans and are therefore essential dietary components that must originate from ingested food (63). In addition, gut microbiota is able to produce and degrade BCAA (64).

Major dietary sources of BCAA include milk, red meat, poultry, and high fat dairy products (65, 66). BCAA make up almost 20% of dietary protein (63). Since the Western diet is characterized by high fat and protein intake (3), one could assume that dietary intake of protein may contribute to changes in plasma BCAA levels. Indeed, evidence suggests that consumption of dietary protein increases the risk of diabetes and insulin resistance (3, 66, 67). Newgard et al. (3) reported that individuals with obesity and insulin resistance consumed more protein compared to lean individuals. Since in the individuals with obesity and insulin resistance BCAA levels in plasma were increased, this data matches the assumption that higher protein intake leads to increase of BCAA in plasma (3). However, in these studies only intake of total protein had been assessed, and not the BCAA consumption. In

contrast, others found that BCAA levels were elevated in individuals with insulin resistance compared to healthy participants, despite equal rates of protein intake. Furthermore, a weak correlation was found between BCAA dietary intake and plasma BCAA levels (4, 19, 65). McCormack et al. found that plasma BCAA levels, but not dietary BCAA intake, was associated with obesity and insulin resistance (19).

Besides direct dietary intake, BCAA can also be metabolized by the gut microbiome (68-71). More specifically, a recent study by Pedersen et al. (70) showed that a gut microbiome having a higher potential for biosynthesis of BCAA and reduced number of inward bacterial transporters for these amino acids were associated with increased levels of BCAA in plasma (70).

Interestingly, increased potential for BCAA biosynthesis and reduced potential for bacterial BCAA uptake are both linked with insulin resistance (70). Above all, it has been reported that circulating BCAA levels were increased in mice following transplantation of stool derived from individuals with insulin resistance (64). This data indicates that microbiota indeed contributes to changes in BCAA plasma levels, in which altered gut microbiota could be another underlying cause of elevated BCAA levels in individuals with insulin resistance.

BCAA catabolism

BCAA catabolism in health

Catabolism of all three BCAA, leucine, isoleucine and valine, is located inside the mitochondria, in which the first two steps are common for all BCAA (Figure 1) (72, 73). The first reaction is the reversible transamination catalysed by the branched-chain amino acid aminotransferases (BCAT) to form branched-chain α-keto acids (BCKA): α-ketoisocaproate (α-KIC), α-keto-B-methylvalerate (α-KMV), and α-ketoisovalerate (α-KIV), respectively formed out of leucine, isoleucine and valine (74). The second step is the irreversible oxidative decarboxylation by the branched-chain α -keto acid dehydrogenase (BCKD) complex, the rate-limiting enzyme of this pathway (75). BCKD comprising three catalytic components (E1, E2 and E3) is regulated by a phosphorylation-dephosphorylation catalysing process, whereby a specific kinase (BCKDK) is responsible for inactivation and a phosphatase (PPM1K) for activation of this complex (76, 77), both regulated by nutrient status and BCAA levels itself (78-80). It has been reported that phosphorylation occurs in the E1 component of the BCKD complex, whereas dephosphorylation reaction interacts with both the E1 and E2 domain (77, 81-83). Ultimately, the CoA compounds formed by the BCKD-complex are further metabolized to acetyl-CoA and succinyl-CoA, which are incorporated into the tricarboxylic acid (TCA) cycle (84). TCA cycle

fuelling also occurs via the alanine cycle (or termed Cahill cycle), which is tightly linked to BCAA catabolism. The alanine cycle involves series of reactions in which amino groups and carbons from skeletal muscle are transported to the liver (85). In short, in skeletal muscle, the reaction of BCAA to BCKA yields glutamate which then combines with pyruvate to generate alanine (86). Alanine is released by skeletal muscle and taken up by the liver (87, 88), where it forms an important source for gluconeogenesis (89). The glucose produced by the liver is shuttled into the circulation, taken up by muscle cells (87), and consequently converted back to glutamate, entering the TCA cycle via -ketoglutarate (86).

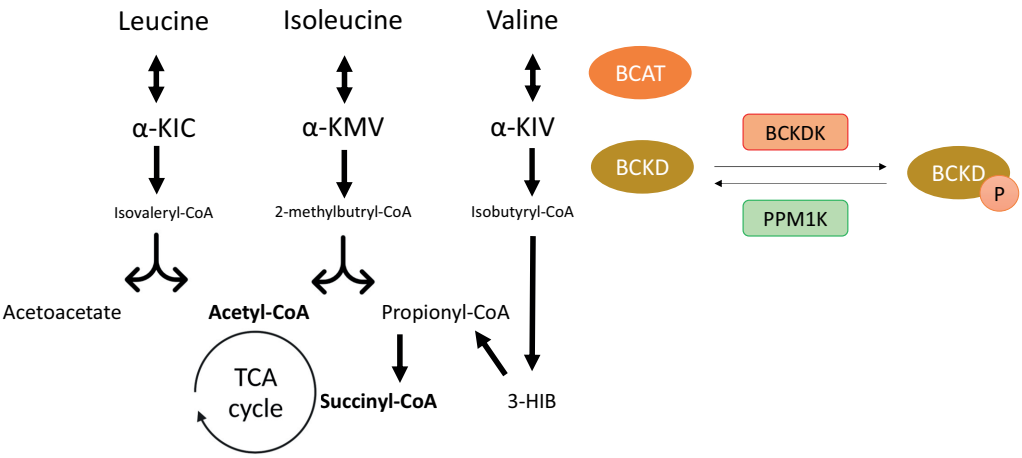


Figure 1. Schematic overview of BCAA catabolism.

BCAT: branched-chain amino acid transaminase; BCKD: branched-chain keto acid dehydrogenase; α-KIC: α-ketoisocaproate; α-KMV: α-keto-methylvalerate; α-KIV: α-ketoisovalerate; 3-HIB: 3-hydroxyisobutyrate; BCKDK: BCKDK kinase; PPM1K: BCKDK phosphatase. Adapted from Neinast et al.(73)

Tissue-specific BCAA metabolism has been investigated in rodent models. Neinast et al. investigated whole-body BCAA catabolism in mice using *in vivo* isotopic tracing and found that most tissues actively oxidize BCAA, with the largest contribution likely in skeletal muscle and liver (84). Other rodent studies showed that BCAT activity, the enzyme responsible for the BCAA transamination step, is relatively low in hepatocytes (90). Moreover, unlike other amino acids, BCAA circumvent first-pass metabolism in the liver (84), and are primarily transaminated to BCKA in extra-hepatic tissues since BCAT is mainly expressed in muscle, kidney and heart tissue in rodents (63, 91, 92). Next, BCKA are released back into the circulation and undergo oxidation by the BCKD complex in the liver (76).

Accordingly, it has been assumed that the liver of rodents has the highest BCKD activity (93), however, BCKD is also expressed in white adipose tissue (WAT) although to a lesser extent (75, 76).

Information on tissue-specific BCAA oxidation in humans is, however, very limited. In one study, enzymatic activities of BCAT and BCKD were evaluated in several human-derived tissues and showed large differences compared to the results observed in rodent tissues (76). Thus, Suryawan et al. (76) reported that both skeletal muscle and liver in humans are key tissues involved in BCAA catabolism and express BCAT and BCKD, with the highest expression in muscle, which was also found by others (85). Furthermore, human heart (94-96) and adipose tissue (75, 81, 97-102) depend on BCAA oxidative capacity as well.

BCAA catabolism in obesity and T2D

Since the first two steps of BCAA catabolism are common for all three BCAA, a reduced BCAA-catabolic flux in one of these steps forms a plausible explanation underlying the rise in plasma BCAA levels of obese insulin resistant individuals with and without T2D. Indeed, several studies points towards diminished or altered function of the key enzymes involved in BCAA catabolism (23, 75, 103-105). This has been confirmed in rodent studies showing that increased levels of BCAA in plasma are the result of reduced expression of BCAT (75, 106) or lower BCKD complex activity, via either increased expression of BCKDK (75, 84, 107, 108) or suppression of PPM1K (80, 103, 109, 110). Animal models of obesity and T2D as well show affected BCAA catabolism (75, 111, 112): tissue-specific expression of BCAA-catabolic enzymes are shown to be dysregulated (23, 27, 79, 108, 113-121) especially in adipose tissue (75, 122) and liver (75, 113). Moreover, decreased BCAA catabolism in WAT is assumed to be a contributor to increased plasma levels of BCAA as seen in obesity and insulin resistance (75, 81, 97-102, 104). The capacity of WAT to modulate circulating BCAA levels has been confirmed by Herman et al. (98), who demonstrated that transplantation of normal WAT into transgenic mice with defective peripheral BCAA catabolism reduced circulating BCAA levels.

Although only limited knowledge derives from human studies, collecting evidence supports the hypothesis that dysfunctional BCAA catabolism could underlie a rise in BCAA plasma levels. For instance, in patients with maple syrup urine disease (MSUD), an inborn error of metabolism caused by loss-of-function mutation in components of the BCKD complex (123-126) or its regulatory phosphatase, PPM1K (127), BCAA levels in plasma are found to be elevated. Others confirmed that altered activity of BCAT or the BCKD complex, at least in muscle and liver, plays a role in plasma BCAA levels (25, 62, 93, 117, 128, 129). Reduced expression levels of BCAT

were found in skeletal muscle of insulin resistant patients with T2D, which could explain the observed elevated BCAA plasma levels (117). Also expression of PPM1K in skeletal muscle of people with T2D failed to increase in contrast to healthy controls during in oral glucose challenge, which could indicate dysregulation of the BCAA pathway (62). Indeed, gene expression studies revealed downregulation in multiple steps of the BCAA-catabolic pathway in skeletal muscle of individuals with insulin resistance (25, 129) and patients with T2D (62). In addition, individuals with obesity and/or T2D were shown to have a marked decrease in BCKD protein content in liver biopsies when compared to the non-obese control group (93). In human liver cells, mutation or deletion of PPM1K resulted in elevated BCAA levels (128).

The BCAA-catabolic pathway has also been shown to be downregulated in WAT of people with obesity (99). The idea that BCKD in WAT contributes to changes in BCAA levels in humans is supported by the fact that BCAA levels in plasma significantly decreased after bariatric surgery (75, 130), while BCKD expression in WAT increased (75). Together, these results demonstrate the capacity of WAT to modulate circulating BCAA levels. WAT is, however, suggested to be responsible for less than 5% of whole-body BCAA oxidation (84), meaning that the increase in plasma BCAA levels must have additional origins (131).

Others have suggested that reduced BCAA oxidation in adipose tissue and liver may induce BCAA overflow to skeletal muscle, driving its BCAA oxidation there (23, 84, 131-133). Since skeletal muscle has a high capacity to oxidize BCAA, it could be postulated that muscle functions as the metabolic sink for impaired BCAA oxidation in adipose tissue and liver (132). Interestingly, a recent study using a heavy isotope steady-state infusion of BCAA, showed a shift in BCAA oxidation from adipose tissue and liver toward skeletal muscle in obese, insulin resistant mice (84), consistent with the finding that BCKD enzyme activity in liver and adipose tissue is downregulated in animals with obese/insulin-resistant or diabetic states (75, 103, 111, 112, 134-138). This was also confirmed by She et al. who found that BCKD activity was decreased in adipose tissue.

Recently, we reported that *in vivo* whole-body leucine oxidation rates were significantly lower in patients with T2D compared to control participants with similar age and BMI (13). Previously, no differences were reported between FDR and matched controls (139) nor between obese and control participants (140). As leucine, valine and isoleucine share the same oxidation route via the BCKD complex, one could assume that *in vivo* 1-^{13}C leucine tracer kinetics represent the total BCAA pool (141-143). Nevertheless, it would be of interest to measure the

oxidation rates of the three individual BCAA (i.e. with 1-^{13}C leucine, 1-^{13}C isoleucine, and 1-^{13}C isoleucine), which has never been investigated in humans. Furthermore, as BCAA and BCAA-derived catabolites has mostly been investigated in plasma, levels in human peripheral tissues would give more insight into tissue-specific BCAA catabolism. These considerations highlight the need for future research to investigate whether tissue-specific BCAA-catabolic defects occur in individuals with obesity, insulin-resistance or T2D individuals.

HOW DO PLASMA BCAA LEVELS LINK TO INSULIN RESISTANCE?

As already mentioned, several reports have been suggested that increased BCAA levels could merely be a consequence of impaired insulin (60, 61), however, evidence indicates that plasma BCAAs act as signaling molecules and contribute to the development of insulin resistance in humans (3, 5, 22, 25, 144-147). Several mechanisms have been hypothesized explaining how plasma BCAA levels contribute to insulin resistance, which are overviewed in Figure 2 and discussed in the following paragraphs.

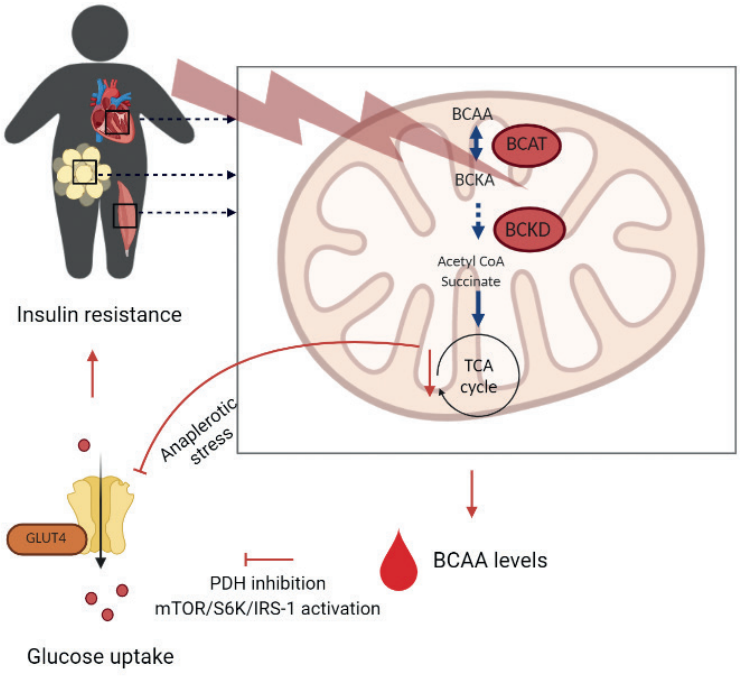


Figure 2. Schematic overview of mechanisms linking BCAA catabolism with insulin resistance.
BCAA: branched-chain amino acids; mTOR, mammalian target of rapamycin complex; S6K: ribosomal S6 kinase; IRS-1: insulin receptor substrate-1; PDH: pyruvate dehydrogenase complex; GLUT4: glucose transporter type 4

Dysfunctional mitochondrial BCAA catabolism

We as well as others have repeatedly reported that people with insulin resistance and patients with T2D feature low muscle mitochondrial oxidative capacity (13, 148, 149). The end products of BCAA catabolism inside the mitochondria, succinyl-CoA and acetyl-CoA, enter the TCA cycle and are important anaplerotic substrates fuelling the TCA cycle. Defects in BCAA-catabolic enzymes may cause so-called anaplerotic stress and underlie low mitochondrial respiratory rates resulting in disturbed glucose and fat oxidation seen in this population (25, 62), which has been supported by *in vitro* studies (150-153). In humans, it has been hypothesized that individuals with impaired or incomplete BCAA metabolism are susceptible to develop insulin resistance (23), in which anaplerotic stress originating from reduced BCAA-derived carbon flux to TCA cycle intermediates is an important underlying factor (23, 138, 154-158). Additional studies investigating this concept, are however warranted.

Dysfunctional mitochondrial BCAA catabolism may explain the accumulation of a number of BCAA-catabolic metabolites in plasma in insulin-resistant people with obesity or T2D, including BCAA-derived acylcarnitines (C3 and C5), 3-hydroxyisobutyrate (3-HIB) and 2-hydroxbutyric acid (2-HB) and 2-ketobutyric acid (2-KB) (3, 10, 25, 81, 133, 137, 159, 160), which can have toxic effects on cellular function. It has been shown that acylcarnitines can cause mitochondrial dysfunction (3, 23, 47, 133, 161-164). Furthermore, several studies link defective BCAA catabolism and consequently accumulation of toxic metabolites to increased lipotoxicity (109, 127, 128, 165, 166) and insulin resistance (3, 23, 47, 133, 161-164). 3-hydroxyisobutyrate (3-HIB), a catabolic intermediate of valine, can exit the mitochondrion via the covalent binding to CoA (146). Several reports have indicated an elevation of 3-HIB in plasma of people with insulin resistance (146, 167). In addition, comprehensive metabolic profiling found that 2-HB and 2-KB, both catabolites of methionine/threonine metabolism, are elevated in individuals with reduced insulin sensitivity (168). Moreover, in individuals with impaired glucose tolerance, plasma levels of 2-HB associate with hyperglycaemia and insulin sensitivity and are an early marker for insulin resistance and risk for future T2D (169-171). Interestingly, since 2-HB can be produced from and converted back into 2-KB, and 2-KB is an BCKD substrate, the increase in these metabolites may reflect impaired BCAA catabolism (172).

To summarize, dysfunctional mitochondrial BCAA catabolism in several tissues may cause anaplerotic stress thereby dysregulating glucose and fat oxidation (Figure 2). Accumulation of either toxic BCAA-intermediates may exacerbate mitochondrial dysfunction, linked to impaired glucose homeostasis and insulin resistance.

Elevated BCAA levels hamper insulin signaling pathways

mTOR/S6K pathway

Both insulin and BCAA are known to stimulate the activity of mammalian target of rapamycin (mTOR), although the mechanisms for their action is not completely understood (173). In normal conditions, insulin mediates phosphorylation of IRS-1, which in turn activates the phosphatidylinositol 3-kinase (PI3K)/Akt pathway (174). Akt regulates glucose transport via the phosphorylation of Akt substrate of 160 kDa (AS160) to trigger GLUT4 translocation from intracellular site to the surface of the cell (23, 175-177). In addition, Akt is able to activate mTOR via phosphorylation of tuberous sclerosis complex 1/2 (TSC 1/2) leading to degradation of Ras homolog enriched in brain (Rheb) (174), which alleviates the inhibition of mTOR (175). To summarize, insulin is able to activate mTOR via the PI3K-Akt signaling pathway (178).

It has been suggested that increased BCAA levels in plasma or tissue also activate the mTOR pathway, although independently of TSC regulation (179, 180). Elevated BCAA levels could lead to persistent activation of mTOR followed by serine phosphorylation of IRS-1 via S6 kinase (p70S6K). Phosphorylation of IRS-1 prevents further Akt-signaling leading to diminished glucose transport and consequently insulin resistance (181, 182). Therefore, chronic accumulation of plasma BCAA levels could impede with the insulin signaling via activation of the mTOR/p70S6K pathway (181-184) with leucine as most potent mTOR activator (180).

BCAA-induced activation of the mTOR/p70S6K pathway has been shown by multiple rodent studies (3, 133, 146, 181, 182, 185, 186) and cell culture experiments (187-189). In addition, *in vivo* and *in vitro* BCAA deprivation in mice reduced the activation of the mTOR pathway and increased pAkt in liver and muscle, resulting in improved insulin sensitivity (190-192). Interestingly, Newgard et al. reported that dietary BCAA-induced mTOR activation only occurred in the presence of a high fat load (3, 104). Moreover, mTOR-stimulated pAkt activation in muscle with the consequent development of insulin resistance, solely occurred when BCAA were supplemented in combination with a high-fat diet, and not upon BCAA supplementation combined with chow (3, 104). Overall, collecting data in preclinical models support the notion that elevated BCAA availability - especially under high fat conditions - plays a key role in the development of insulin resistance, mediated by downregulation of PI3K-Akt signaling pathway and hyperactivation of the mTOR/p70S6K pathway.

Evidence for a role of BCAA in mTOR signaling and insulin resistance in humans is scarce. A short-term infusion of a mixture of amino acids, including BCAA,

activated mTOR paralleled by reduced peripheral insulin sensitivity in humans (181, 184). In addition, Weickert et al. (193) showed that a 6-week high-protein diet enriched with leucine and isoleucine, induced insulin resistance with increased p70S6K levels observed in adipose tissue (193). Although these results show that BCAA-induced mTOR activation play a role in the development of insulin resistance in humans, normalized BCAA plasma levels which occurred after gastric bypass surgery, did not result in reduced mTOR activation (159), although insulin resistance improved substantially in these patients. The excessive weight loss in the latter study therefore seems to be the driving factor underlying improved insulin sensitivity, and not the change in BCAA plasma levels per se.

Inhibition of PDH

Pyruvate dehydrogenase complex (PDH) is the rate-limiting enzyme involved in glucose oxidation (194), linking glycolysis to the TCA cycle by transferring pyruvate into acetyl-coenzyme A (CoA) (94). A common manifestation in obese individuals with insulin resistance is the inability to shift from fatty acid oxidation in the fasted state to glucose oxidation in the fed state, also called metabolic inflexibility (195). This fatty acid-induced suppression of glucose oxidation as well glucose disposal can be explained by the model of Randle et al. (196): by-products of fatty acid oxidation, such as acetyl-CoA, NADH and ATP, act as potent allosteric inhibitors of glycolysis and PDH (197). Several studies in animals reported that accumulation of BCAA and its derived metabolites can also directly inhibit PDH activity, at least in liver (153, 198) and heart (94, 152, 199), resulting in a marked decrease in glucose uptake and oxidation. Moreover, animal studies show that dysfunctional BCAA oxidation result in accumulation of BCAA in cardiac tissue and forms a hallmark in cardiovascular disease (95, 200). A mouse model with impaired BCAA oxidation revealed that the chronic accumulation of BCAA in heart tissue suppressed glucose metabolism (94). More specifically, high levels of BCAA selectively disrupted mitochondrial pyruvate (end product of glucose oxidation) utilization through inhibition of PDH activity. It has long been established that PDH activity is a key determinant for insulin resistance of the heart (201, 202), in which BCAA may play a pivotal role. This link has not been investigated in humans, however, one study demonstrated that BCAA concentrations accumulate in failing heart tissue as a resultant of a coordinated decrease in BCAA oxidative genes (95), and was associated with impaired cardiac insulin signaling. However, whether BCAA-inhibited PDH activity played a role, was not investigated. In addition, one study showed that supplementing BCAA during exercise as well as during the recovery period resulted in increased plasma glucose levels due to reduced glucose uptake in the leg in the recovery period (203). The authors suggest that the oxidation of supplemented BCAA resulted in increased BCAA-oxidative

derived acetyl-CoA concentrations thereby inhibiting PDH activity, however, the elevated BCAA levels could as well be responsible for reduced pyruvate utilization.

Although there is evidence that elevated BCAA levels hamper insulin signaling pathways, it remains still unclear whether elevated BCAA levels are a cause or rather a consequence of insulin resistance. Future research, specifically cohort studies, could provide more information about causality between BCAA levels and insulin resistance.

EFFECTIVE STRATEGIES TO LOWER BCAA LEVELS

Pharmaceutical strategies

BT2

A compound called 3,6-dichlorobenzo(b)thiopene-2-carboxylic acid (BT2) is a small-molecule inhibitor of BCKDK and accelerates the BCAA-catabolic pathway via increased activation of the BCKD complex (Figure 3) (95, 204). Its working mechanism has been confirmed in obese and diabetes mice models, who report accelerated BCAA catabolism in skeletal muscle (84, 200), liver, heart and adipose tissue (105, 107). In these models, the administration of BT2 resulted in lower plasma BCAA levels, improved insulin sensitivity and hyperinsulinemia, and reduced hepatic fat levels (105, 107). Together, these results demonstrate that BT2 is effective to restore BCAA-catabolic activity in various tissues alleviating the BCAA-catabolic defect, and thus improving insulin sensitivity, irrespective of the site.

Furthermore, several studies administered BT2 in mice with heart failure (110, 205, 206), and collectively show that dysfunctional BCAA catabolism plays a pivotal role in the development of cardiac dysfunction. Results show that BT2-induced accelerated cardiac BCAA catabolism in failing hearts decreased cardiac BCAA levels, with beneficial effects on heart tissue remodeling, improved cardiac insulin sensitivity and function (110, 200, 205, 206). The mechanisms underlying the cardiometabolic protective effects observed in these studies remain to be elucidated, however, results point out that restoring dysfunctional BCAA catabolism optimizes substrate use and attenuates mitochondrial function (110, 205, 206). Interestingly, some studies show that the beneficial effects of BT2 on improved glucose metabolism were exerted by reduced mTOR activity and/or via a reduction in the formation of BCAA-derived toxic metabolites (110, 205). To conclude, BT2 is a pharmacological agent which directly modulate BCAA catabolism via activating BCKD activity. As BT2 is not suitable for human use, so far, effects of pharmacologically modulating BCAA catabolism on the human heart and other tissues, as well on glucose homeostasis has not been investigated in humans.

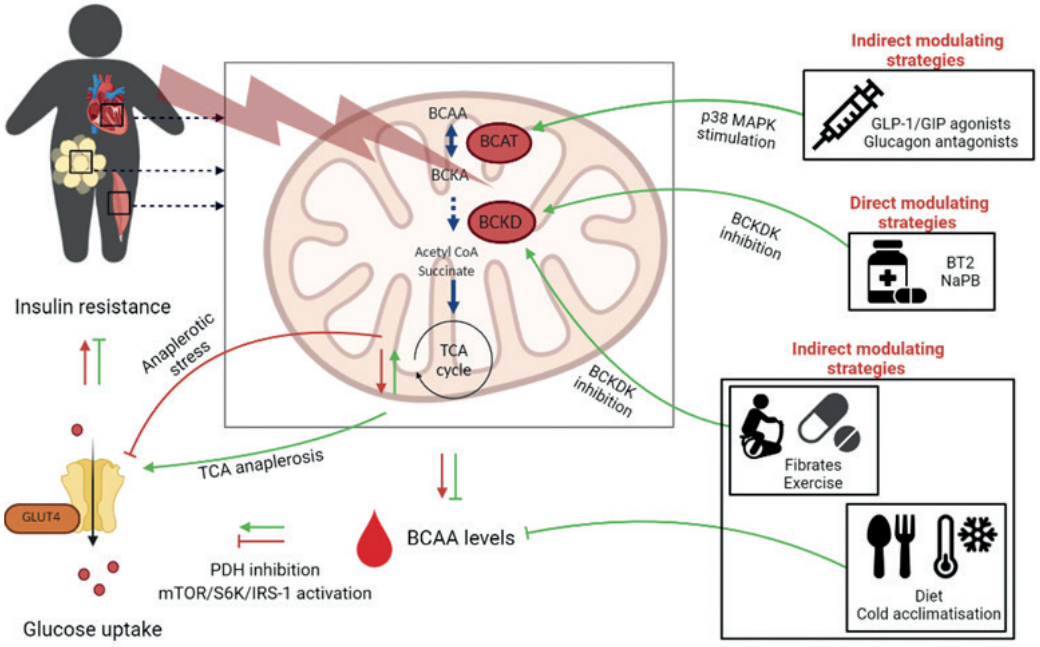


Figure 3. Schematic overview of pharmaceutical and alternative strategies and their hypothesized way of action to boost BCAA oxidation and lower BCAA levels.

BCAA: branched-chain amino acids; mTOR, mammalian target of rapamycin complex; S6K: ribosomal S6 kinase; IRS-1: insulin receptor substrate-1; PDH: pyruvate dehydrogenase complex; GLUT4: glucose transporter type 4; BT2: 3,6-dichlorobenzo(b)thiopene-2-carboxylic acid; NaPB: sodium phenylbutyrate; GLP-1: GCR-like peptide-1; GIP: glucose-dependent insulinotropic polypeptide.

NaPB

Sodium phenylbutyrate (NaPB) is a commonly used medication for the treatment of patients with urea cycle disorders (207). NaPB is an aromatic fatty acid that is converted *in vivo* by β-oxidation into phenylacetate followed by conjugation with glutamine to form phenylacetylglutamine, which is excreted in the urine (208). Via this mechanism NaPB act as an ammonia scavenger in patients with urea cycle disorders (209). Interestingly, it has been demonstrated in mice (210) and human cells (208) that NaPB, as BT2, also directly enhance BCAA catabolism through stimulation of the BCKD complex by preventing the phosphorylation of BCKDK (Figure 3). Holecek et al. (211) showed that *in vitro* and *in vivo* administration of NaPB resulted in augmented BCAA catabolism resulting in reduced BCAA levels in plasma and muscle (211). In another *in vitro* study in mice, NaPB treatment resulted in lower BCAA concentrations paralleled by improved insulin-stimulated glucose uptake (189, 212) via an improved insulin signaling in skeletal muscle cells (189). This result was confirmed in a diabetic mouse model showing substantial improved glucose

metabolism upon NaPB treatment (213). These data postulate that NaPB-induced lowering of BCAA levels alleviate the inhibition of insulin signaling leading to an improved glucose uptake, in which skeletal muscle plays an important role.

Although limited research has been performed in humans, some studies show that NaPB lowers BCAA levels in patients with urea cycle disorders, patients with MSUD and healthy subjects (207, 208, 214-217). In a study with male people with overweight or obesity, NaPB administration was effective in partially improving lipid-induced insulin resistance, although circulating plasma BCAA levels were not measured (218). As previously done in mouse skeletal muscle cells (189), it would be of interest to study effects of NaPB administration on insulin signaling and glucose uptake in primary human muscle cells, to acquire missing physiological insights on the metabolic consequences of modulating BCAA catabolism in humans.

Fibrates

Fibrate is a class of drugs widely used to treat dyslipidaemia by reducing cholesterol and triglyceride levels, decreasing the risk for the development of cardiovascular diseases (219, 220). Fibrate mechanism of action includes activation of peroxisome proliferator-activated receptor alpha (PPARα), a transcriptional factor of genes involved in fatty acid oxidation (219, 220). The major adverse effect of the clinical use of fibrates is the development of myopathy (221-223), however, the pathogenesis of fibrate-induced myopathy is still unclear.

In rodents, several studies showed that fibrate treatment decreased BCAA and BCKA plasma levels (224-226) as well in skeletal muscle and liver tissue (227). Fibrates inhibit gene expression of the BCKDK in the liver (Figure 3) (225, 226, 228-231), an effect which was not found in skeletal muscle (228). This could imply that fibrates enhance BCAA catabolism specifically in the liver.

Interestingly, it has been shown that fibrate treatment improved insulin sensitivity in patients with T2D, although the underlying mechanisms were not investigated (232-234). Fibrate treatment decreased the activation of the mTOR/p70S6K pathway in rats (226), as well lowered BCAA plasma levels in humans (235). Whether the fibrate-induced improvement in insulin sensitivity is attributable to improved BCAA catabolism, lower BCAA levels and/or decreased activation of the mTOR-pathway, cannot be deduced from these studies.

Novel T2D therapies targeting incretin and glucagon receptors

In recent years, new therapies targeting receptors including GCR-like peptide-1 (GLP-1), glucose-dependent insulinotropic polypeptide (GIP) and glucagon have

been developed. Tirzepatide, a dual GIP and GLP-1 agonist and potential new glucose-lowering medication for patients with T2D, has been shown to improve hyperglycaemia (236). Obese insulin resistant mouse models feature improved glycaemic control in the presence of reduced BCAA and BCKA plasma levels upon Trizepatide treatment (237). The observed effects were accompanied by an increased expression of BCAT via the p38-MAPK pathway particularly in BAT (Figure 3) (237). Interestingly, in humans, Tirzepatide treatment reduced BCAA, BCKA and other BCAA-derived metabolites in plasma, including 3-HIB and 2-HB, previously shown to associate with insulin resistance and T2D (238). Together, tirzepatide may alter expression of genes regulating BCAA catabolism explaining these results (238). Also, antagonising the glucagon receptors has shown to be effective in improving insulin sensitivity in models of diabetes and obesity (239). In failing heart, inhibition of the glucagon receptor improved insulin-stimulated glucose oxidation and enhanced cardiac function, which were attributable to an improved BCAA catabolism via the p38-MAPK pathway (240). Although these findings suggests that T2D treatment targeting receptors as GLP-1, GIP and glucagon may activate BCAA catabolism, future studies will be required to investigate if and how activated BCAA catabolism helps to improve glycaemic control upon this treatment in individuals with insulin resistance and T2D.

Alternative strategies

Physical activity and exercise

Generally, it has been assumed that amino acids do not contribute substantially to energy supply during endurance exercise training (241). In contrast, others suggest that this assumption may underestimate the role of proteins and that endurance exercise may result in promotion of amino acid catabolism in general, and especially the oxidation of BCAA (242). To provide energy, endurance training promotes the transamination of BCAA to BCKA (75), which are further metabolized into acyl-coenzymes which can enter the TCA cycle (84). Indeed, it is well established that endurance exercise training in rodents (243) and combined endurance and resistance training in humans with overweight (244) decreased plasma BCAA levels and toxic intermediates of BCAA catabolism, such as acylcarnitines. Consistent with this finding, a recognized effect of endurance exercise training is an accelerated BCAA catabolism represented by an increased BCKD activity (245). More specifically, it has been found that BCKD is activated due to decreased phosphorylation by BCKD kinase (Figure 3) (246-250). Several exercise intervention studies in rats found that BCKD complex was activated in skeletal muscle (78, 251), as well as in liver (248, 249). The mechanisms responsible for activating these enzymes are not fully understood. One report demonstrated that inactivity potently downregulated expression of BCAA metabolic genes in

mice and vice versa that expression of BCAA metabolic enzymes were upregulated in response to endurance exercise training (25). Contrarily, others suggest that the relative short exercise training sessions, as performed in the beforementioned studies, could not underlie altered gene expression or phosphorylation status of the kinase and that other mechanisms are possibly involved (248, 252).

Recently, we found that levels of BCAA were lower in more active individuals compared to less active individuals (56), which is in line with another observational study showing an association between high physical activity level and low plasma BCAA levels (253). Nevertheless, 12-week combined endurance and resistance-exercise training in people with obesity did not result in decreased plasma BCAA levels (56). Although prolonged intense exercise has been shown to increase the activity of the BCKD complex in skeletal muscle of trained, healthy individuals (254), this effect might be blunted in people with insulin resistance. Controversy does exist on the effect of exercise on BCAA catabolism. Howarth et al. (255) showed that a single bout of endurance exercise increased BCKD kinase content in human skeletal muscle, which was associated with a training-induced decrease in BCKD activity, although Poortmans et al. did not find a change in plasma BCAA levels (256). The inconsistent responses of the different studies could be explained by different work load, duration of physical activity and exercise training, and individuals' training status. In addition, changes in plasma BCAA levels upon exercise are not a good reflection of BCAA catabolism since exercise influence protein turnover, and therefore also BCAA levels. Exercise training studies combined with stable isotope would elucidate the impact of exercise on BCAA catabolism. The question, however, remains if improved BCAA catabolism is involved in the improvement in metabolic health after physical activity and exercise.

Dietary restriction of BCAA

As mentioned before, diet may contribute to the elevation of BCAA as observed in humans, and therefore diet intervention could potentially help to improve BCAA metabolism. Indeed, it has been shown that restricting dietary BCAA restores metabolic health, including lower adiposity and improved insulin sensitivity in obese rodents (257-259). The positive metabolic effects were independent of alterations in BCKD activity (260) suggesting that low protein diets restrict plasma BCAA levels thereby alleviating its inhibitory effect on glucose uptake.

In humans, BCAA dietary restriction studies are limited since feasibility is a challenge: interpretation can be limited in case nitrogen and caloric content is different between intervention arms, and therefore any reported effects cannot be asserted as solely due to BCAA restriction. It has been shown, that BCAA levels

decreased after a weight loss program, but was not related to changes in BCAA intake (10).

One study reported only modest changes in fasting BCAA levels, associated with an increase in insulin sensitivity upon short-term dietary restriction in healthy individuals (261). Patients with T2D are characterized by higher plasma BCAA levels compared to healthy controls and therefore probably may benefit more from a BCAA restricted diet. Indeed, short-term dietary reduction of BCAA was effective in decreasing BCAA levels coinciding with improved postprandial insulin sensitivity and gut microbiome composition in patients with T2D (262). Although, reports showed *in vivo* and *in vitro* that lowering BCAA levels alleviates the inhibition of the insulin signaling pathway by decreasing mTOR/S6K1 signaling resulting in increased insulin sensitivity (191, 262), when and how BCAA restriction influences metabolic health, particularly glucose homeostasis, remains unclear. Long-term studies in humans are needed to evaluate the safety and the metabolic efficiency in individuals with obesity and insulin resistance.

Cold acclimatization

Several rodent reports noted that cold exposure significantly decreases plasma BCAA levels, possibly by an increased BCAA uptake and oxidation merely located in BAT (263-266). Consistent with their findings, it was recently reported that BCAA are actively utilized in BAT mitochondria for UCP1-mediated thermogenesis upon cold exposure in mice (266). In turn, impaired capacity to take up BCAA and defective BCAA catabolism in BAT results in impaired BCAA clearance and thermogenesis leading to impairments in lipid and glucose metabolism (266, 267). Thus, besides glucose and fatty acids, BCAA are likely to be important energy substrates in BAT during cold exposure, however, the relationship of BCAA metabolism to thermogenesis is still unclear.

Also in humans, Yoneshiro et al. (266) observed that cold exposure for 2h preferentially decreased BCAA plasma levels in participants with high BAT activity, suggesting a potential link between BAT and BCAA metabolism. Surprisingly, muscle mass showed no correlation with cold-induced changes in BCAA levels although skeletal muscle is a major organ that utilizes BCAA (266). Feasibility

To summarize, catabolism and levels of BCAA can be modulated by several pharmaceutical and alternative strategies, although their mechanisms are not completely known in humans. Further research would be needed to study feasibility and optimization for alternative strategies. As a side note, BT2 and NaPB are the only interventions able to directly target the BCAA-catabolic defect to improve

glucose homeostasis. Other pharmaceutical and alternative interventions, known to improve metabolic health, have also shown to influence BCAA catabolism and levels, however, it has not yet been investigated whether this improved metabolic health is attributable to change in BCAA catabolism and levels.

CONCLUSION

Dysregulation of BCAA catabolism is closely related to obesity- and T2D related metabolic disturbances since BCAA levels plays a key role in interorgan metabolic crosstalk. Findings from animal and human studies provided evidence that dysfunctional BCAA catabolism in several tissues could be a plausible explanation for the elevated plasma BCAA levels seen in obesity and T2D, however, huge knowledge gaps exist in tissue-specific BCAA catabolism in humans. Insulin resistance can occur via dysfunctional BCAA catabolism or BCAA levels acting as signaling molecules hampering the insulin signaling pathways. Therefore, exploring intervention strategies to increase BCAA oxidation and/or lower BCAA levels is important to investigate whether this could be a new potential strategy in the treatment of metabolic diseases, including obesity and T2D.

ACKNOWLEDGEMENTS

E.P. was granted with a senior fellowship from the Dutch Diabetes Foundation (Grant No. 2017.82.010), a "VENI" Research Grant for innovative research from the Netherlands Organization for Scientific Research (91613132), and an EFSD/Lilly grant from the European Foundation for the Study of Diabetes (EFSD).

AUTHOR CONTRIBUTIONS

F.V. and E.P. were responsible for writing the manuscript. E.P and P.S. were responsible for designing the review protocol, and provided feedback on the manuscript. All authors reviewed and approved the final version of the manuscript.

DECLARATION OF INTEREST

The authors declare no conflicts of interest

REFERENCES

1. Organization WH. Global Report on Diabetes. France; 2016.
2. Abdul-Ghani MA, DeFronzo RA. Pathogenesis of insulin resistance in skeletal muscle. J Biomed Biotechnol. 2010;2010:476279.
3. Newgard CB, An J, Bain JR, Muehlbauer MJ, Stevens RD, Lien LF, et al. A branched-chain amino acid-related metabolic signature that differentiates obese and lean humans and contributes to insulin resistance. Cell Metab. 2009;9(4):311-26.
4. Tai ES, Tan ML, Stevens RD, Low YL, Muehlbauer MJ, Goh DL, et al. Insulin resistance is associated with a metabolic profile of altered protein metabolism in Chinese and Asian-Indian men. Diabetologia. 2010;53(4):757-67.
5. Krebs M, Krssak M, Bernroider E, Anderwald C, Brehm A, Meyerspeer M, et al. Mechanism of amino acid-induced skeletal muscle insulin resistance in humans. Diabetes. 2002;51(3):599-605.
6. Huffman KM, Shah SH, Stevens RD, Bain JR, Muehlbauer M, Slentz CA, et al. Relationships Between Circulating Metabolic Intermediates and Insulin Action in Overweight to Obese, Inactive Men and Women. Diabetes Care. 2009;32(9):1678-83.
7. Perng W, Gillman MW, Fleisch AF, Michalek RD, Watkins SM, Isganaitis E, et al. Metabolomic profiles and childhood obesity. Obesity (Silver Spring). 2014;22(12):2570-8.
8. Palmer ND, Stevens RD, Antinozzi PA, Anderson A, Bergman RN, Wagenknecht LE, et al. Metabolomic profile associated with insulin resistance and conversion to diabetes in the Insulin Resistance Atherosclerosis Study. J Clin Endocrinol Metab. 2015;100(3):E463-8.
9. Xu F, Tavintharan S, Sum CF, Woon K, Lim SC, Ong CN. Metabolic signature shift in type 2 diabetes mellitus revealed by mass spectrometry-based metabolomics. J Clin Endocrinol Metab. 2013;98(6):E1060-5.
10. Shah SH, Crosslin DR, Haynes CS, Nelson S, Turer CB, Stevens RD, et al. Branched-chain amino acid levels are associated with improvement in insulin resistance with weight loss. Diabetologia. 2012;55(2):321-30.
11. Walford GA, Ma Y, Clish C, Florez JC, Wang TJ, Gerszten RE, et al. Metabolite Profiles of Diabetes Incidence and Intervention Response in the Diabetes Prevention Program. Diabetes. 2016;65(5):1424-33.
12. Wurtz P, Soininen P, Kangas AJ, Ronnemaa T, Lehtimaki T, Kahonen M, et al. Branched-chain and aromatic amino acids are predictors of insulin resistance in young adults. Diabetes Care. 2013;36(3):648-55.
13. Vanweert F, de Ligt M, Hoeks J, Hesselink MKC, Schrauwen P, Phielix E. Elevated Plasma Branched-Chain Amino Acid Levels Correlate With Type 2 Diabetes-Related Metabolic Disturbances. J Clin Endocrinol Metab. 2021;106(4):e1827-e36.
14. Nishimura J, Masaki T, Arakawa M, Seike M, Yoshimatsu H. Isoleucine prevents the accumulation of tissue triglycerides and upregulates the expression of PPARalpha and uncoupling protein in diet-induced obese mice. J Nutr. 2010;140(3):496-500.
15. Nie C, He T, Zhang W, Zhang G, Ma X. Branched Chain Amino Acids: Beyond Nutrition Metabolism. Int J Mol Sci. 2018;19(4).
16. Gancheva S, Jelenik T, Alvarez-Hernandez E, Roden M. Interorgan Metabolic Crosstalk in Human Insulin Resistance. Physiol Rev. 2018;98(3):1371-415.
17. Wang TJ, Larson MG, Vasan RS, Cheng S, Rhee EP, McCabe E, et al. Metabolite profiles and the risk of developing diabetes. Nat Med. 2011;17(4):448-53.
18. Floegel A, Stefan N, Yu Z, Muhlenbruch K, Drogan D, Joost HG, et al. Identification of serum metabolites associated with risk of type 2 diabetes using a targeted metabolomic approach. Diabetes. 2013;62(2):639-48.

19. McCormack SE, Shaham O, McCarthy MA, Deik AA, Wang TJ, Gerszten RE, et al. Circulating branched-chain amino acid concentrations are associated with obesity and future insulin resistance in children and adolescents. Pediatr Obes. 2013;8(1):52-61.
20. Hamley S, Kloosterman D, Duthie T, Dalla Man C, Visentin R, Mason SA, et al. Mechanisms of hyperinsulinaemia in apparently healthy non-obese young adults: role of insulin secretion, clearance and action and associations with plasma amino acids. Diabetologia. 2019;62(12):2310-24.
21. Merino J, Leong A, Liu CT, Porneala B, Walford GA, von Grotthuss M, et al. Metabolomics insights into early type 2 diabetes pathogenesis and detection in individuals with normal fasting glucose. Diabetologia. 2018;61(6):1315-24.
22. Tremblay F, Krebs M, Dombrowski L, Brehm A, Bernroider E, Roth E, et al. Overactivation of S6 kinase 1 as a cause of human insulin resistance during increased amino acid availability. Diabetes. 2005;54(9):2674-84.
23. Lynch CJ, Adams SH. Branched-chain amino acids in metabolic signalling and insulin resistance. Nat Rev Endocrinol. 2014;10(12):723-36.
24. Eagle H, Piez KA, Fleischman R, Oyama VI. Protein turnover in mammaliar cell cultures. J Biol Chem. 1959;234(3):592-7.
25. Lerin C, Goldfine AB, Boes T, Liu M, Kasif S, Dreyfuss JM, et al. Defects in muscle branched-chain amino acid oxidation contribute to impaired lipid metabolism. Mol Metab. 2016;5(10):926-36.
26. Cheng S, Wiklund P, Autio R, Borra R, Ojanen X, Xu L, et al. Adipose Tissue Dysfunction and Altered Systemic Amino Acid Metabolism Are Associated with Non-Alcoholic Fatty Liver Disease. PLoS One. 2015;10(10):e0138889.
27. Lake AD, Novak P, Shipkova P, Aranibar N, Robertson DG, Reily MD, et al. Branched chain amino acid metabolism profiles in progressive human nonalcoholic fatty liver disease. Amino Acids. 2015;47(3):603-15.
28. Meek SE, Persson M, Ford GC, Nair KS. Differential regulation of amino acid exchange and protein dynamics across splanchnic and skeletal muscle beds by insulin in healthy human subjects. Diabetes. 1998;47(12):1824-35.
29. Flakoll PJ, Kulaylat M, Frexes-Steed M, Hourani H, Brown LL, Hill JO, et al. Amino acids augment insulin's suppression of whole body proteolysis. Am J Physiol. 1989;257(6 Pt 1):E839-47.
30. Fukagawa NK, Minaker KL, Rowe JW, Goodman MN, Matthews DE, Bier DM, et al. Insulin-mediated reduction of whole body protein breakdown. Dose-response effects on leucine metabolism in postabsorptive men. J Clin Invest. 1985;76(6):2306-11.
31. Glass DJ. PI3 kinase regulation of skeletal muscle hypertrophy and atrophy. Curr Top Microbiol Immunol. 2010;346:267-78.
32. Schiaffino S, Mammucari C. Regulation of skeletal muscle growth by the IGF1-Akt/PKB pathway: insights from genetic models. Skelet Muscle. 2011;1(1):4.
33. Milan G, Romanello V, Pescatore F, Armani A, Paik JH, Frasson L, et al. Regulation of autophagy and the ubiquitin-proteasome system by the FoxO transcriptional network during muscle atrophy. Nat Commun. 2015;6:6670.
34. Sanchez AM, Candau RB, Bernardi H. FoxO transcription factors: their roles in the maintenance of skeletal muscle homeostasis. Cell Mol Life Sci. 2014;71(9):1657-71.
35. O'Neill BT, Bhardwaj G, Penniman CM, Krumpoch MT, Suarez Beltran PA, Klaus K, et al. FoxO Transcription Factors Are Critical Regulators of Diabetes-Related Muscle Atrophy. Diabetes. 2019;68(3):556-70.
36. Abdulla H, Smith K, Atherton PJ, Idris I. Role of insulin in the regulation of human skeletal muscle protein synthesis and breakdown: a systematic review and meta-analysis. Diabetologia. 2016;59(1):44-55.

37. Wang X, Hu Z, Hu J, Du J, Mitch WE. Insulin resistance accelerates muscle protein degradation: Activation of the ubiquitin-proteasome pathway by defects in muscle cell signaling. Endocrinology. 2006;147(9):4160-8.
38. Stitt TN, Drujan D, Clarke BA, Panaro F, Timofeyva Y, Kline WO, et al. The IGF-1/PI3K/Akt pathway prevents expression of muscle atrophy-induced ubiquitin ligases by inhibiting FOXO transcription factors. Mol Cell. 2004;14(3):395-403.
39. Sandri M, Sandri C, Gilbert A, Skurk C, Calabria E, Picard A, et al. Foxo transcription factors induce the atrophy-related ubiquitin ligase atrogin-1 and cause skeletal muscle atrophy. Cell. 2004;117(3):399-412.
40. Lee SW, Dai G, Hu Z, Wang X, Du J, Mitch WE. Regulation of muscle protein degradation: coordinated control of apoptotic and ubiquitin-proteasome systems by phosphatidylinositol 3 kinase. J Am Soc Nephrol. 2004;15(6):1537-45.
41. Greenhaff PL, Karagounis LG, Peirce N, Simpson EJ, Hazell M, Layfield R, et al. Disassociation between the effects of amino acids and insulin on signaling, ubiquitin ligases, and protein turnover in human muscle. Am J Physiol Endocrinol Metab. 2008;295(3):E595-604.
42. Everman S, Meyer C, Tran L, Hoffman N, Carroll CC, Dedmon WL, et al. Insulin does not stimulate muscle protein synthesis during increased plasma branched-chain amino acids alone but still decreases whole body proteolysis in humans. Am J Physiol Endocrinol Metab. 2016;311(4):E671-E7.
43. Hoffer LJ, Taveroff A, Robitaille L, Hamadeh MJ, Mamer OA. Effects of leucine on whole body leucine, valine, and threonine metabolism in humans. Am J Physiol. 1997;272(6 Pt 1):E1037-42.
44. Nair KS, Schwartz RG, Welle S. Leucine as a regulator of whole body and skeletal muscle protein metabolism in humans. Am J Physiol. 1992;263(5 Pt 1):E928-34.
45. Pozefsky T, Felig P, Tobin JD, Soeldner JS, Cahill GF, Jr. Amino acid balance across tissues of the forearm in postabsorptive man. Effects of insulin at two dose levels. J Clin Invest. 1969;48(12):2273-82.
46. Louard RJ, Fryburg DA, Gelfand RA, Barrett EJ. Insulin sensitivity of protein and glucose metabolism in human forearm skeletal muscle. J Clin Invest. 1992;90(6):2348-54.
47. Adeva MM, Calvino J, Souto G, Donapetry C. Insulin resistance and the metabolism of branched-chain amino acids in humans. Amino Acids. 2012;43(1):171-81.
48. Castellino P, Luzi L, Simonson DC, Haymond M, DeFronzo RA. Effect of insulin and plasma amino acid concentrations on leucine metabolism in man. Role of substrate availability on estimates of whole body protein synthesis. J Clin Invest. 1987;80(6):1784-93.
49. Zanetti M, Barazzoni R, Kiwanuka E, Tessari P. Effects of branched-chain-enriched amino acids and insulin on forearm leucine kinetics. Clin Sci (Lond). 1999;97(4):437-48.
50. Felig P. Amino acid metabolism in man. Annu Rev Biochem. 1975;44:933-55.
51. Aoki TT, Brennan MF, Muller WA, Soeldner JS, Alpert JS, Saltz SB, et al. Amino acid levels across normal forearm muscle and splanchnic bed after a protein meal. Am J Clin Nutr. 1976;29(4):340-50.
52. Elia M, Livesey G. Effects of ingested steak and infused leucine on forelimb metabolism in man and the fate of the carbon skeletons and amino groups of branched-chain amino acids. Clin Sci (Lond). 1983;64(5):517-26.
53. Hagenfeldt L, Eriksson LS, Wahren J. Amino acids in liver disease. Proc Nutr Soc. 1983;42(3):497-506.
54. Nair KS, Garrow JS, Ford C, Mahler RF, Halliday D. Effect of poor diabetic control and obesity on whole body protein metabolism in man. Diabetologia. 1983;25(5):400-3.

55. Nair KS, Ford GC, Ekberg K, Fernqvist-Forbes E, Wahren J. Protein dynamics in whole body and in splanchnic and leg tissues in type I diabetic patients. J Clin Invest. 1995;95(6):2926-37.
56. Vanweert F, Boone SC, Brouwers B, Mook-Kanamori DO, de Mutsert R, Rosendaal FR, et al. The effect of physical activity level and exercise training on the association between plasma branched-chain amino acids and intrahepatic lipid content in participants with obesity. Int J Obes (Lond). 2021.
57. Forlani G, Vannini P, Marchesini G, Zoli M, Ciavarella A, Pisi E. Insulin-dependent metabolism of branched-chain amino acids in obesity. Metabolism. 1984;33(2):147-50.
58. Chevalier S, Burgess SC, Malloy CR, Gougeon R, Marliss EB, Morais JA. The greater contribution of gluconeogenesis to glucose production in obesity is related to increased whole-body protein catabolism. Diabetes. 2006;55(3):675-81.
59. Felig P, Marliss E, Cahill GF, Jr. Plasma amino acid levels and insulin secretion in obesity. N Engl J Med. 1969;281(15):811-6.
60. Mahendran Y, Jonsson A, Have CT, Allin KH, Witte DR, Jorgensen ME, et al. Genetic evidence of a causal effect of insulin resistance on branched-chain amino acid levels. Diabetologia. 2017;60(5):873-8.
61. Wang Q, Holmes MV, Davey Smith G, Ala-Korpela M. Genetic Support for a Causal Role of Insulin Resistance on Circulating Branched-Chain Amino Acids and Inflammation. Diabetes care. 2017;40(12):1779-86.
62. Lotta LA, Scott RA, Sharp SJ, Burgess S, Luan J, Tillin T, et al. Genetic Predisposition to an Impaired Metabolism of the Branched-Chain Amino Acids and Risk of Type 2 Diabetes: A Mendelian Randomisation Analysis. PLoS Med. 2016;13(11):e1002179.
63. Harper AE, Miller RH, Block KP. Branched-chain amino acid metabolism. Annu Rev Nutr. 1984;4:409-54.
64. Ridaura VK, Faith JJ, Rey FE, Cheng J, Duncan AE, Kau AL, et al. Gut microbiota from twins discordant for obesity modulate metabolism in mice. Science. 2013;341(6150):1241214.
65. Jennings A, MacGregor A, Pallister T, Spector T, Cassidy A. Associations between branched chain amino acid intake and biomarkers of adiposity and cardiometabolic health independent of genetic factors: A twin study. Int J Cardiol. 2016;223:992-8.
66. Zheng Y, Li Y, Qi Q, Hruby A, Manson JE, Willett WC, et al. Cumulative consumption of branched-chain amino acids and incidence of type 2 diabetes. Int J Epidemiol. 2016;45(5):1482-92.
67. Lopez AM, Noriega LG, Diaz M, Torres N, Tovar AR. Plasma branched-chain and aromatic amino acid concentration after ingestion of an urban or rural diet in rural Mexican women. BMC Obes. 2015;2:8.
68. Watson E, MacNeil LT, Ritter AD, Yilmaz LS, Rosebrock AP, Caudy AA, et al. Interspecies Systems Biology Uncovers Metabolites Affecting C. elegans Gene Expression and Life History Traits. Cell. 2014;156(6):1336-7.
69. Saad MJ, Santos A, Prada PO. Linking Gut Microbiota and Inflammation to Obesity and Insulin Resistance. Physiology (Bethesda). 2016;31(4):283-93.
70. Pedersen HK, Gudmundsdottir V, Nielsen HB, Hyotylainen T, Nielsen T, Jensen BA, et al. Human gut microbes impact host serum metabolome and insulin sensitivity. Nature. 2016;535(7612):376-81.
71. Kappel BA, Federici M. Gut microbiome and cardiometabolic risk. Rev Endocr Metab Disord. 2019;20(4):399-406.
72. Holecek M. Branched-chain amino acids in health and disease: metabolism, alterations in blood plasma, and as supplements. Nutr Metab (Lond). 2018;15:33.
73. Neinast M, Murashige D, Arany Z. Branched Chain Amino Acids. Annu Rev Physiol. 2019;81:139-64.

74. Holecek M. Branched-Chain Amino Acids and Branched-Chain Keto Acids in Hyperammonemic States: Metabolism and as Supplements. Metabolites. 2020;10(8).
75. She P, Van Horn C, Reid T, Hutson SM, Cooney RN, Lynch CJ. Obesity-related elevations in plasma leucine are associated with alterations in enzymes involved in branched-chain amino acid metabolism. Am J Physiol Endocrinol Metab. 2007;293(6):E1552-63.
76. Suryawan A, Hawes JW, Harris RA, Shimomura Y, Jenkins AE, Hutson SM. A molecular model of human branched-chain amino acid metabolism. Am J Clin Nutr. 1998;68(1):72-81.
77. Wynn RM, Kato M, Machius M, Chuang JL, Li J, Tomchick DR, et al. Molecular mechanism for regulation of the human mitochondrial branched-chain alpha-ketoacid dehydrogenase complex by phosphorylation. Structure. 2004;12(12):2185-96.
78. Shimomura Y, Obayashi M, Murakami T, Harris RA. Regulation of branched-chain amino acid catabolism: nutritional and hormonal regulation of activity and expression of the branched-chain alpha-keto acid dehydrogenase kinase. Curr Opin Clin Nutr Metab Care. 2001;4(5):419-23.
79. Joshi M, Jeoung NH, Popov KM, Harris RA. Identification of a novel PP2C-type mitochondrial phosphatase. Biochem Biophys Res Commun. 2007;356(1):38-44.
80. Zhou M, Lu G, Gao C, Wang Y, Sun H. Tissue-specific and nutrient regulation of the branched-chain alpha-keto acid dehydrogenase phosphatase, protein phosphatase 2Cm (PP2Cm). J Biol Chem. 2012;287(28):23397-406.
81. Adams SH, Hoppel CL, Lok KH, Zhao L, Wong SW, Minkler PE, et al. Plasma acylcarnitine profiles suggest incomplete long-chain fatty acid beta-oxidation and altered tricarboxylic acid cycle activity in type 2 diabetic African-American women. J Nutr. 2009;139(6):1073-81.
82. Chuang JL, Wynn RM, Chuang DT. The C-terminal hinge region of lipoic acid-bearing domain of E2b is essential for domain interaction with branched-chain alpha-keto acid dehydrogenase kinase. J Biol Chem. 2002;277(40):36905-8.
83. Islam MM, Wallin R, Wynn RM, Conway M, Fujii H, Mobley JA, et al. A novel branched-chain amino acid metabolon. Protein-protein interactions in a supramolecular complex. J Biol Chem. 2007;282(16):11893-903.
84. Neinast MD, Jang C, Hui S, Murashige DS, Chu Q, Morscher RJ, et al. Quantitative Analysis of the Whole-Body Metabolic Fate of Branched-Chain Amino Acids. Cell Metab. 2019;29(2):417-29 e4.
85. Wahren J, Felig P, Hagenfeldt L. Effect of protein ingestion on splanchnic and leg metabolism in normal man and in patients with diabetes mellitus. J Clin Invest. 1976;57(4):987-99.
86. O'Connell TM. The complex role of branched chain amino acids in diabetes and cancer. Metabolites. 2013;3(4):931-45.
87. Felig P. The glucose-alanine cycle. Metabolism. 1973;22(2):179-207.
88. Sugden MC, Holness MJ. Recent advances in mechanisms regulating glucose oxidation at the level of the pyruvate dehydrogenase complex by PDKs. Am J Physiol Endocrinol Metab. 2003;284(5):E855-62.
89. Nurjhan N, Bucci A, Perriello G, Stumvoll M, Dailey G, Bier DM, et al. Glutamine: a major gluconeogenic precursor and vehicle for interorgan carbon transport in man. J Clin Invest. 1995;95(1):272-7.
90. Hutson SM, Wallin R, Hall TR. Identification of mitochondrial branched chain aminotransferase and its isoforms in rat tissues. J Biol Chem. 1992;267(22):15681-6.
91. Hutson SM, Sweatt AJ, Lanoue KF. Branched-chain (corrected] amino acid metabolism: implications for establishing safe intakes. J Nutr. 2005;135(6 Suppl):1557S-64S.
92. Ding C, Li Y, Guo F, Jiang Y, Ying W, Li D, et al. A Cell-type-resolved Liver Proteome. Mol Cell Proteomics. 2016;15(10):3190-202.

93. Shin AC, Fasshauer M, Filatova N, Grundell LA, Zielinski E, Zhou JY, et al. Brain insulin lowers circulating BCAA levels by inducing hepatic BCAA catabolism. Cell Metab. 2014;20(5):898-909.
94. Li T, Zhang Z, Kolwicz SC, Jr., Abell L, Roe ND, Kim M, et al. Defective Branched-Chain Amino Acid Catabolism Disrupts Glucose Metabolism and Sensitizes the Heart to Ischemia-Reperfusion Injury. Cell Metab. 2017;25(2):374-85.
95. Uddin GM, Zhang L, Shah S, Fukushima A, Wagg CS, Gopal K, et al. Impaired branched chain amino acid oxidation contributes to cardiac insulin resistance in heart failure. Cardiovasc Diabetol. 2019;18(1):86.
96. Buse MG, Biggers JF, Friderici KH, Buse JF. Oxidation of branched chain amino acids by isolated hearts and diaphragms of the rat. The effect of fatty acids, glucose, and pyruvate respiration. J Biol Chem. 1972;247(24):8085-96.
97. Lackey DE, Lynch CJ, Olson KC, Mostaedi R, Ali M, Smith WH, et al. Regulation of adipose branched-chain amino acid catabolism enzyme expression and cross-adipose amino acid flux in human obesity. Am J Physiol Endocrinol Metab. 2013;304(11):E1175-87.
98. Herman MA, She P, Peroni OD, Lynch CJ, Kahn BB. Adipose tissue branched chain amino acid (BCAA) metabolism modulates circulating BCAA levels. J Biol Chem. 2010;285(15):11348-56.
99. Pietilainen KH, Naukkarinen J, Rissanen A, Saharinen J, Ellonen P, Keranen H, et al. Global transcript profiles of fat in monozygotic twins discordant for BMI: pathways behind acquired obesity. PLoS Med. 2008;5(3):e51.
100. Leskinen T, Rinnankoski-Tuikka R, Rintala M, Seppanen-Laakso T, Pollanen E, Alen M, et al. Differences in muscle and adipose tissue gene expression and cardio-metabolic risk factors in the members of physical activity discordant twin pairs. PLoS One. 2010;5(9).
101. Klimcakova E, Roussel B, Marquez-Quinones A, Kovacova Z, Kovacikova M, Combes M, et al. Worsening of obesity and metabolic status yields similar molecular adaptations in human subcutaneous and visceral adipose tissue: decreased metabolism and increased immune response. J Clin Endocrinol Metab. 2011;96(1):E73-82.
102. Stancakova A, Civelek M, Saleem NK, Soininen P, Kangas AJ, Cederberg H, et al. Hyperglycemia and a common variant of GCKR are associated with the levels of eight amino acids in 9,369 Finnish men. Diabetes. 2012;61(7):1895-902.
103. Doisaki M, Katano Y, Nakano I, Hirooka Y, Itoh A, Ishigami M, et al. Regulation of hepatic branched-chain alpha-keto acid dehydrogenase kinase in a rat model for type 2 diabetes mellitus at different stages of the disease. Biochem Biophys Res Commun. 2010;393(2):303-7.
104. Adams SH. Emerging Perspectives on Essential Amino Acid Metabolism in Obesity and the Insulin-Resistant State. Adv Nutr. 2011;2(6):445-56.
105. Zhou M, Shao J, Wu CY, Shu L, Dong W, Liu Y, et al. Targeting BCAA Catabolism to Treat Obesity-Associated Insulin Resistance. Diabetes. 2019;68(9):1730-46.
106. She P, Reid TM, Bronson SK, Vary TC, Hajnal A, Lynch CJ, et al. Disruption of BCATm in mice leads to increased energy expenditure associated with the activation of a futile protein turnover cycle. Cell Metab. 2007;6(3):181-94.
107. White PJ, McGarrah RW, Grimsrud PA, Tso SC, Yang WH, Haldeman JM, et al. The BCKDH Kinase and Phosphatase Integrate BCAA and Lipid Metabolism via Regulation of ATP-Citrate Lyase. Cell Metabolism. 2018;27(6):1281-+.
108. Lian K, Du C, Liu Y, Zhu D, Yan W, Zhang H, et al. Impaired adiponectin signaling contributes to disturbed catabolism of branched-chain amino acids in diabetic mice. Diabetes. 2015;64(1):49-59.
109. Lu G, Sun H, She P, Youn JY, Warburton S, Ping P, et al. Protein phosphatase 2Cm is a critical regulator of branched-chain amino acid catabolism in mice and cultured cells. J Clin Invest. 2009;119(6):1678-87.

110. Sun H, Olson KC, Gao C, Prosdocimo DA, Zhou M, Wang Z, et al. Catabolic Defect of Branched-Chain Amino Acids Promotes Heart Failure. Circulation. 2016;133(21):2038-49.

111. Kuzuya T, Katano Y, Nakano I, Hirooka Y, Itoh A, Ishigami M, et al. Regulation of branched-chain amino acid catabolism in rat models for spontaneous type 2 diabetes mellitus. Biochem Biophys Res Commun. 2008;373(1):94-8.

112. Bajotto G, Murakami T, Nagasaki M, Sato Y, Shimomura Y. Decreased enzyme activity and contents of hepatic branched-chain alpha-keto acid dehydrogenase complex subunits in a rat model for type 2 diabetes mellitus. Metabolism. 2009;58(10):1489-95.

113. Biswas D, Duffley L, Pulinilkunnil T. Role of branched-chain amino acid-catabolizing enzymes in intertissue signaling, metabolic remodeling, and energy homeostasis. Faseb J. 2019;33(8):8711-31.

114. Hernandez-Alvarez MI, Thabit H, Burns N, Shah S, Brema I, Hatunic M, et al. Subjects with early-onset type 2 diabetes show defective activation of the skeletal muscle PGC-1{alpha}/Mitofusin-2 regulatory pathway in response to physical activity. Diabetes care. 2010;33(3):645-51.

115. Takeuchi Y, Yahagi N, Aita Y, Murayama Y, Sawada Y, Piao X, et al. KLF15 Enables Rapid Switching between Lipogenesis and Gluconeogenesis during Fasting. Cell Rep. 2016;16(9):2373-86.

116. Salinas-Rubio D, Tovar AR, Torre-Villalvazo I, Granados-Portillo O, Torres N, Pedraza-Chaverri J, et al. Interaction between leucine and palmitate catabolism in 3T3-L1 adipocytes and primary adipocytes from control and obese rats. J Nutr Biochem. 2018;59:29-36.

117. Hernandez-Alvarez MI, Diaz-Ramos A, Berdasco M, Cobb J, Planet E, Cooper D, et al. Early-onset and classical forms of type 2 diabetes show impaired expression of genes involved in muscle branched-chain amino acids metabolism. Sci Rep. 2017;7(1):13850.

118. Sperringer JE, Addington A, Hutson SM. Branched-Chain Amino Acids and Brain Metabolism. Neurochem Res. 2017;42(6):1697-709.

119. Greco D, Kotronen A, Westerbacka J, Puig O, Arkkila P, Kiviluoto T, et al. Gene expression in human NAFLD. Am J Physiol-Gastr L. 2008;294(5):G1281-G7.

120. Hirata Y, Nomura K, Senga Y, Okada Y, Kobayashi K, Okamoto S, et al. Hyperglycemia induces skeletal muscle atrophy via a WWP1/KLF15 axis. JCI Insight. 2019;4(4).

121. Fisch S, Gray S, Heymans S, Haldar SM, Wang B, Pfister O, et al. Kruppel-like factor 15 is a regulator of cardiomyocyte hypertrophy. Proc Natl Acad Sci U S A. 2007;104(17):7074-9.

122. Hsiao G, Chapman J, Ofrecio JM, Wilkes J, Resnik JL, Thapar D, et al. Multi-tissue, selective PPARgamma modulation of insulin sensitivity and metabolic pathways in obese rats. Am J Physiol Endocrinol Metab. 2011;300(1):E164-74.

123. Zhang B, Zhao Y, Harris RA, Crabb DW. Molecular defects in the E1 alpha subunit of the branched-chain alpha-ketoacid dehydrogenase complex that cause maple syrup urine disease. Mol Biol Med. 1991;8(1):39-47.

124. Strauss KA, Puffenberger EG, Carson VJ. Maple Syrup Urine Disease. In: Adam MP, Ardinger HH, Pagon RA, Wallace SE, Bean LJH, Mirzaa G, et al., editors. GeneReviews((R)). Seattle (WA)1993.

125. Strauss KA, Wardley B, Robinson D, Hendrickson C, Rider NL, Puffenberger EG, et al. Classical maple syrup urine disease and brain development: principles of management and formula design. Mol Genet Metab. 2010;99(4):333-45.

126. Homanics GE, Skvorak K, Ferguson C, Watkins S, Paul HS. Production and characterization of murine models of classic and intermediate maple syrup urine disease. BMC Med Genet. 2006;7:33.

127. Oyarzabal A, Martinez-Pardo M, Merinero B, Navarrete R, Desviat LR, Ugarte M, et al. A novel regulatory defect in the branched-chain alpha-keto acid dehydrogenase complex due to a mutation in the PPM1K gene causes a mild variant phenotype of maple syrup urine disease. Hum Mutat. 2013;34(2):355-62.

128. Lu G, Ren S, Korge P, Choi J, Dong Y, Weiss J, et al. A novel mitochondrial matrix serine/threonine protein phosphatase regulates the mitochondria permeability transition pore and is essential for cellular survival and development. Genes Dev. 2007;21(7):784-96.

129. Lefort N, Glancy B, Bowen B, Willis WT, Bailowitz Z, De Filippis EA, et al. Increased reactive oxygen species production and lower abundance of complex I subunits and carnitine palmitoyltransferase 1B protein despite normal mitochondrial respiration in insulin-resistant human skeletal muscle. Diabetes. 2010;59(10):2444-52.

130. Laferrere B, Reilly D, Arias S, Swerdlow N, Gorroochurn P, Bawa B, et al. Differential metabolic impact of gastric bypass surgery versus dietary intervention in obese diabetic subjects despite identical weight loss. Sci Transl Med. 2011;3(80):80re2.

131. Arany Z, Neinast M. Branched Chain Amino Acids in Metabolic Disease. Curr Diab Rep. 2018;18(10):76.

132. Cheng Y, Meng Q, Wang C, Li H, Huang Z, Chen S, et al. Leucine deprivation decreases fat mass by stimulation of lipolysis in white adipose tissue and upregulation of uncoupling protein 1 (UCP1) in brown adipose tissue. Diabetes. 2010;59(1):17-25.

133. Newgard CB. Interplay between Lipids and Branched-Chain Amino Acids in Development of Insulin Resistance. Cell Metabolism. 2012;15(5):606-14.

134. Steele RD, Weber H, Patterson JI. Characterization of alpha-ketobutyrate metabolism in rat tissues: effects of dietary protein and fasting. J Nutr. 1984;114(4):701-10.

135. Gillim SE, Paxton R, Cook GA, Harris RA. Activity state of the branched chain alpha-ketoacid dehydrogenase complex in heart, liver, and kidney of normal, fasted, diabetic, and protein-starved rats. Biochem Biophys Res Commun. 1983;111(1):74-81.

136. Randle PJ. alpha-Ketoacid dehydrogenase complexes and respiratory fuel utilisation in diabetes. Diabetologia. 1985;28(8):479-84.

137. She P, Olson KC, Kadota Y, Inukai A, Shimomura Y, Hoppel CL, et al. Leucine and protein metabolism in obese Zucker rats. PLoS One. 2013;8(3):e59443.

138. Olson KC, Chen G, Xu Y, Hajnal A, Lynch CJ. Alloisoleucine differentiates the branched-chain aminoacidemia of Zucker and dietary obese rats. Obesity (Silver Spring). 2014;22(5):1212-5.

139. Burgos SA, Chandurkar V, Tsoukas MA, Chevalier S, Morais JA, Lamarche M, et al. Insulin resistance of protein anabolism accompanies that of glucose metabolism in lean, glucose-tolerant offspring of persons with type 2 diabetes. BMJ Open Diabetes Res Care. 2016;4(1):e000312.

140. Luzi L, Castellino P, DeFronzo RA. Insulin and hyperaminoacidemia regulate by a different mechanism leucine turnover and oxidation in obesity. Am J Physiol. 1996;270(2 Pt 1):E273-81.

141. Glynn EL, Piner LW, Huffman KM, Slentz CA, Elliot-Penry L, AbouAssi H, et al. Impact of combined resistance and aerobic exercise training on branched-chain amino acid turnover, glycine metabolism and insulin sensitivity in overweight humans. Diabetologia. 2015;58(10):2324-35.

142. Matthews DE. Observations of branched-chain amino acid administration in humans. J Nutr. 2005;135(6 Suppl):1580S-4S.

143. Tan HC, Hsu JW, Khoo CM, Tai ES, Yu S, Chacko S, et al. Alterations in branched-chain amino acid kinetics in nonobese but insulin-resistant Asian men. Am J Clin Nutr. 2018;108(6):1220-8.

144. Harris LLS, Smith GI, Patterson BW, Ramaswamy RS, Okunade AL, Kelly SC, et al. Alterations in 3-Hydroxyisobutyrate and FGF21 Metabolism Are Associated With Protein Ingestion-Induced Insulin Resistance. Diabetes. 2017;66(7):1871-8.

145. Krebs M, Brehm A, Krssak M, Anderwald C, Bernroider E, Nowotny P, et al. Direct and indirect effects of amino acids on hepatic glucose metabolism in humans. Diabetologia. 2003;46(7):917-25.

146. Jang C, Oh SF, Wada S, Rowe GC, Liu L, Chan MC, et al. A branched-chain amino acid metabolite drives vascular fatty acid transport and causes insulin resistance. Nature medicine. 2016;22(4):421-6.

147. Zhang Y, Cao J, Zhang S, Lee AJ, Sun G, Larsen CN, et al. Genetic changes found in a distinct clade of Enterovirus D68 associated with paralysis during the 2014 outbreak. Virus Evol. 2016;2(1):vew015.

148. Phielix E, Meex R, Moonen-Kornips E, Hesselink MK, Schrauwen P. Exercise training increases mitochondrial content and ex vivo mitochondrial function similarly in patients with type 2 diabetes and in control individuals. Diabetologia. 2010;53(8):1714-21.

149. Phielix E, Jelenik T, Nowotny P, Szendroedi J, Roden M. Reduction of non-esterified fatty acids improves insulin sensitivity and lowers oxidative stress, but fails to restore oxidative capacity in type 2 diabetes: a randomised clinical trial. Diabetologia. 2014;57(3):572-81.

150. Bridi R, Braun CA, Zorzi GK, Wannmacher CM, Wajner M, Lissi EG, et al. alpha-keto acids accumulating in maple syrup urine disease stimulate lipid peroxidation and reduce antioxidant defences in cerebral cortex from young rats. Metab Brain Dis. 2005;20(2):155-67.

151. Funchal C, Latini A, Jacques-Silva MC, Dos Santos AQ, Buzin L, Gottfried C, et al. Morphological alterations and induction of oxidative stress in glial cells caused by the branched-chain alpha-keto acids accumulating in maple syrup urine disease. Neurochem Int. 2006;49(7):640-50.

152. Jackson RH, Singer TP. Inactivation of the 2-ketoglutarate and pyruvate dehydrogenase complexes of beef heart by branched chain keto acids. J Biol Chem. 1983;258(3):1857-65.

153. Walajtys-Rode E, Williamson JR. Effects of branched chain alpha-ketoacids on the metabolism of isolated rat liver cells. III. Interactions with pyruvate dehydrogenase. J Biol Chem. 1980;255(2):413-8.

154. Petersen KF, Dufour S, Befroy D, Garcia R, Shulman GI. Impaired mitochondrial activity in the insulin-resistant offspring of patients with type 2 diabetes. N Engl J Med. 2004;350(7):664-71.

155. Mogensen M, Sahlin K, Fernstrom M, Glintborg D, Vind BF, Beck-Nielsen H, et al. Mitochondrial respiration is decreased in skeletal muscle of patients with type 2 diabetes. Diabetes. 2007;56(6):1592-9.

156. Ritov VB, Menshikova EV, He J, Ferrell RE, Goodpaster BH, Kelley DE. Deficiency of subsarcolemmal mitochondria in obesity and type 2 diabetes. Diabetes. 2005;54(1):8-14.

157. Morino K, Petersen KF, Dufour S, Befroy D, Frattini J, Shatzkes N, et al. Reduced mitochondrial density and increased IRS-1 serine phosphorylation in muscle of insulin-resistant offspring of type 2 diabetic parents. J Clin Invest. 2005;115(12):3587-93.

158. Befroy DE, Petersen KF, Dufour S, Mason GF, de Graaf RA, Rothman DL, et al. Impaired mitochondrial substrate oxidation in muscle of insulin-resistant offspring of type 2 diabetic patients. Diabetes. 2007;56(5):1376-81.

159. Magkos F, Bradley D, Schweitzer GG, Finck BN, Eagon JC, Ilkayeva O, et al. Effect of Roux-en-Y gastric bypass and laparoscopic adjustable gastric banding on branched-chain amino acid metabolism. Diabetes. 2013;62(8):2757-61.

160. Fiehn O, Garvey WT, Newman JW, Lok KH, Hoppel CL, Adams SH. Plasma metabolomic profiles reflective of glucose homeostasis in non-diabetic and type 2 diabetic obese African-American women. PLoS One. 2010;5(12):e15234.

161. Aguer C, McCoin CS, Knotts TA, Thrush AB, Ono-Moore K, McPherson R, et al. Acylcarnitines: potential implications for skeletal muscle insulin resistance. Faseb J. 2015;29(1):336-45.

162. Goossens GH. The role of adipose tissue dysfunction in the pathogenesis of obesity-related insulin resistance. Physiol Behav. 2008;94(2):206-18.

163. Koves TR, Ussher JR, Noland RC, Slentz D, Mosedale M, Ilkayeva O, et al. Mitochondrial overload and incomplete fatty acid oxidation contribute to skeletal muscle insulin resistance. Cell Metab. 2008;7(1):45-56.

164. Arner P. Human fat cell lipolysis: biochemistry, regulation and clinical role. Best Pract Res Clin Endocrinol Metab. 2005;19(4):471-82.

165. Lu G, Sun H, Korge P, Koehler CM, Weiss JN, Wang Y. Functional characterization of a mitochondrial Ser/Thr protein phosphatase in cell death regulation. Methods Enzymol. 2009;457:255-73.

166. Kettunen J, Tukiainen T, Sarin AP, Ortega-Alonso A, Tikkanen E, Lyytikainen LP, et al. Genome-wide association study identifies multiple loci influencing human serum metabolite levels. Nat Genet. 2012;44(3):269-76.

167. Nilsen MS, Jersin RA, Ulvik A, Madsen A, McCann A, Svensson PA, et al. 3-Hydroxyisobutyrate, A Strong Marker of Insulin Resistance in Type 2 Diabetes and Obesity That Modulates White and Brown Adipocyte Metabolism. Diabetes. 2020;69(9):1903-16.

168. Trico D, Prinsen H, Giannini C, de Graaf R, Juchem C, Li F, et al. Elevated alpha-Hydroxybutyrate and Branched-Chain Amino Acid Levels Predict Deterioration of Glycemic Control in Adolescents. J Clin Endocrinol Metab. 2017;102(7):2473-81.

169. Cobb J, Eckhart A, Motsinger-Reif A, Carr B, Groop L, Ferrannini E. alpha-Hydroxybutyric Acid Is a Selective Metabolite Biomarker of Impaired Glucose Tolerance. Diabetes Care. 2016;39(6):988-95.

170. Gall WE, Beebe K, Lawton KA, Adam KP, Mitchell MW, Nakhle PJ, et al. alpha-hydroxybutyrate is an early biomarker of insulin resistance and glucose intolerance in a nondiabetic population. PLoS One. 2010;5(5):e10883.

171. Ferrannini E, Natali A, Camastra S, Nannipieri M, Mari A, Adam KP, et al. Early metabolic markers of the development of dysglycemia and type 2 diabetes and their physiological significance. Diabetes. 2013;62(5):1730-7.

172. Plummer DT, Elliott BA, Cooke KB, Wilkinson JH. Organ specificity and lactate-dehydrogenase activity. 1. The relative activities with pyruvate and 2-oxobutyrate of electrophoretically separated fractions. Biochem J. 1963;87:416-22.

173. Nepstad I, Hatfield KJ, Gronningsaeter IS, Aasebo E, Hernandez-Valladares M, Hagen KM, et al. Effects of insulin and pathway inhibitors on the PI3K-Akt-mTOR phosphorylation profile in acute myeloid leukemia cells. Signal Transduct Target Ther. 2019;4:20.

174. Yoon MS. The Emerging Role of Branched-Chain Amino Acids in Insulin Resistance and Metabolism. Nutrients. 2016;8(7).

175. Jewell JL, Russell RC, Guan KL. Amino acid signalling upstream of mTOR. Nat Rev Mol Cell Biol. 2013;14(3):133-9.

176. Thirone AC, Huang C, Klip A. Tissue-specific roles of IRS proteins in insulin signaling and glucose transport. Trends Endocrinol Metab. 2006;17(2):72-8.

177. Sakamoto K, Holman GD. Emerging role for AS160/TBC1D4 and TBC1D1 in the regulation of GLUT4 traffic. Am J Physiol Endocrinol Metab. 2008;295(1):E29-37.

178. Hay N, Sonenberg N. Upstream and downstream of mTOR. Genes Dev. 2004;18(16):1926-45.

179. Sancak Y, Peterson TR, Shaul YD, Lindquist RA, Thoreen CC, Bar-Peled L, et al. The Rag GTPases bind raptor and mediate amino acid signaling to mTORC1. Science. 2008;320(5882):1496-501.

180. Gleason CE, Lu D, Witters LA, Newgard CB, Birnbaum MJ. The role of AMPK and mTOR in nutrient sensing in pancreatic beta-cells. J Biol Chem. 2007;282(14):10341-51.

181. Um SH, D'Alessio D, Thomas G. Nutrient overload, insulin resistance, and ribosomal protein S6 kinase 1, S6K1. Cell Metab. 2006;3(6):393-402.

182. Um SH, Frigerio F, Watanabe M, Picard F, Joaquin M, Sticker M, et al. Absence of S6K1 protects against age- and diet-induced obesity while enhancing insulin sensitivity. Nature. 2004;431(7005):200-5.

183. Krebs M, Brunmair B, Brehm A, Artwohl M, Szendroedi J, Nowotny P, et al. The Mammalian target of rapamycin pathway regulates nutrient-sensitive glucose uptake in man. Diabetes. 2007;56(6):1600-7.

184. Tremblay F, Brule S, Hee Um S, Li Y, Masuda K, Roden M, et al. Identification of IRS-1 Ser-1101 as a target of S6K1 in nutrient- and obesity-induced insulin resistance. Proc Natl Acad Sci U S A. 2007;104(35):14056-61.

185. Macotela Y, Emanuelli B, Bang AM, Espinoza DO, Boucher J, Beebe K, et al. Dietary leucine--an environmental modifier of insulin resistance acting on multiple levels of metabolism. PLoS One. 2011;6(6):e21187.

186. Jeganathan S, Abdullahi A, Zargar S, Maeda N, Riddell MC, Adegoke OA. Amino acid-induced impairment of insulin sensitivity in healthy and obese rats is reversible. Physiol Rep. 2014;2(7).

187. Patti ME, Brambilla E, Luzi L, Landaker EJ, Kahn CR. Bidirectional modulation of insulin action by amino acids. J Clin Invest. 1998;101(7):1519-29.

188. Zhenyukh O, Civantos E, Ruiz-Ortega M, Sanchez MS, Vazquez C, Peiro C, et al. High concentration of branched-chain amino acids promotes oxidative stress, inflammation and migration of human peripheral blood mononuclear cells via mTORC1 activation. Free Radic Biol Med. 2017;104:165-77.

189. Crossland H, Smith K, Idris I, Phillips BE, Atherton PJ, Wilkinson DJ. Exploring mechanistic links between extracellular branched-chain amino acids and muscle insulin resistance: an in vitro approach. Am J Physiol Cell Physiol. 2020;319(6):C1151-C7.

190. Xiao F, Yu J, Guo Y, Deng J, Li K, Du Y, et al. Effects of individual branched-chain amino acids deprivation on insulin sensitivity and glucose metabolism in mice. Metabolism. 2014;63(6):841-50.

191. Xiao F, Huang Z, Li H, Yu J, Wang C, Chen S, et al. Leucine deprivation increases hepatic insulin sensitivity via GCN2/mTOR/S6K1 and AMPK pathways. Diabetes. 2011;60(3):746-56.

192. Rivera ME, Rivera CN, Vaughan RA. Branched-chain amino acids at supraphysiological but not physiological levels reduce myotube insulin sensitivity. Diabetes Metab Res Rev. 2021:e3490.

193. Weickert MO, Roden M, Isken F, Hoffmann D, Nowotny P, Osterhoff M, et al. Effects of supplemented isoenergetic diets differing in cereal fiber and protein content on insulin sensitivity in overweight humans. Am J Clin Nutr. 2011;94(2):459-71.

194. Lee IK. The role of pyruvate dehydrogenase kinase in diabetes and obesity. Diabetes Metab J. 2014;38(3):181-6.

195. Kelley DE, Mandarino LJ. Fuel selection in human skeletal muscle in insulin resistance: a reexamination. Diabetes. 2000;49(5):677-83.

196. Randle PJ, Garland PB, Hales CN, Newsholme EA. The glucose fatty-acid cycle. Its role in insulin sensitivity and the metabolic disturbances of diabetes mellitus. Lancet. 1963;1(7285):785-9.

197. Muoio DM, Noland RC, Kovalik JP, Seiler SE, Davies MN, DeBalsi KL, et al. Muscle-specific deletion of carnitine acetyltransferase compromises glucose tolerance and metabolic flexibility. Cell Metab. 2012;15(5):764-77.

198. Williamson JR, Walajtys-Rode E, Coll KE. Effects of branched chain alpha-ketoacids on the metabolism of isolated rat liver cells. I. Regulation of branched chain alpha-ketoacid metabolism. J Biol Chem. 1979;254(22):11511-20.

199. Randle PJ, Newsholme EA, Garland PB. Regulation of glucose uptake by muscle. 8. Effects of fatty acids, ketone bodies and pyruvate, and of alloxan-diabetes and starvation, on the uptake and metabolic fate of glucose in rat heart and diaphragm muscles. Biochem J. 1964;93(3):652-65.

200. Lian K, Guo X, Wang Q, Liu Y, Wang RT, Gao C, et al. PP2Cm overexpression alleviates MI/R injury mediated by a BCAA catabolism defect and oxidative stress in diabetic mice. Eur J Pharmacol. 2020;866:172796.

201. Lewandowski ED, White LT. Pyruvate dehydrogenase influences postischemic heart function. Circulation. 1995;91(7):2071-9.

202. Stanley WC, Lopaschuk GD, Hall JL, McCormack JG. Regulation of myocardial carbohydrate metabolism under normal and ischaemic conditions. Potential for pharmacological interventions. Cardiovasc Res. 1997;33(2):243-57.

203. Blomstrand E, Saltin B. BCAA intake affects protein metabolism in muscle after but not during exercise in humans. Am J Physiol Endocrinol Metab. 2001;281(2):E365-74.

204. Tso SC, Gui WJ, Wu CY, Chuang JL, Qi X, Skvora KJ, et al. Benzothiophene carboxylate derivatives as novel allosteric inhibitors of branched-chain alpha-ketoacid dehydrogenase kinase. J Biol Chem. 2014;289(30):20583-93.

205. Wang W, Zhang F, Xia Y, Zhao S, Yan W, Wang H, et al. Defective branched chain amino acid catabolism contributes to cardiac dysfunction and remodeling following myocardial infarction. Am J Physiol Heart Circ Physiol. 2016;311(5):H1160-H9.

206. Chen M, Gao C, Yu J, Ren S, Wang M, Wynn RM, et al. Therapeutic Effect of Targeting Branched-Chain Amino Acid Catabolic Flux in Pressure-Overload Induced Heart Failure. J Am Heart Assoc. 2019;8(11):e011625.

207. Burrage LC, Jain M, Gandolfo L, Lee BH, Members of the Urea Cycle Disorders C, Nagamani SC. Sodium phenylbutyrate decreases plasma branched-chain amino acids in patients with urea cycle disorders. Mol Genet Metab. 2014;113(1-2):131-5.

208. Brunetti-Pierri N, Lanpher B, Erez A, Ananieva EA, Islam M, Marini JC, et al. Phenylbutyrate therapy for maple syrup urine disease. Hum Mol Genet. 2011;20(4):631-40.

209. Brusilow SW. Phenylacetylglutamine may replace urea as a vehicle for waste nitrogen excretion. Pediatr Res. 1991;29(2):147-50.

210. Tso SC, Qi X, Gui WJ, Chuang JL, Morlock LK, Wallace AL, et al. Structure-based design and mechanisms of allosteric inhibitors for mitochondrial branched-chain alpha-ketoacid dehydrogenase kinase. Proc Natl Acad Sci U S A. 2013;110(24):9728-33.

211. Holecek M, Vodenicarovova M, Siman P. Acute effects of phenylbutyrate on glutamine, branched-chain amino acid and protein metabolism in skeletal muscles of rats. Int J Exp Pathol. 2017;98(3):127-33.

212. Hu H, Li L, Wang C, He H, Mao K, Ma X, et al. 4-Phenylbutyric acid increases GLUT4 gene expression through suppression of HDAC5 but not endoplasmic reticulum stress. Cell Physiol Biochem. 2014;33(6):1899-910.

213. Ozcan U, Yilmaz E, Ozcan L, Furuhashi M, Vaillancourt E, Smith RO, et al. Chemical chaperones reduce ER stress and restore glucose homeostasis in a mouse model of type 2 diabetes. Science. 2006;313(5790):1137-40.

214. Darmaun D, Welch S, Rini A, Sager BK, Altomare A, Haymond MW. Phenylbutyrate-induced glutamine depletion in humans: effect on leucine metabolism. Am J Physiol. 1998;274(5 Pt 1):E801-7.

215. Scaglia F, Carter S, O'Brien WE, Lee B. Effect of alternative pathway therapy on branched chain amino acid metabolism in urea cycle disorder patients. Mol Genet Metab. 2004;81 Suppl 1:S79-85.

216. Seminara J, Tuchman M, Krivitzky L, Krischer J, Lee HS, Lemons C, et al. Establishing a consortium for the study of rare diseases: The Urea Cycle Disorders Consortium. Mol Genet Metab. 2010;100 Suppl 1:S97-105.

217. Tuchman M, Lee B, Lichter-Konecki U, Summar ML, Yudkoff M, Cederbaum SD, et al. Cross-sectional multicenter study of patients with urea cycle disorders in the United States. Mol Genet Metab. 2008;94(4):397-402.

218. Xiao C, Giacca A, Lewis GF. Sodium phenylbutyrate, a drug with known capacity to reduce endoplasmic reticulum stress, partially alleviates lipid-induced insulin resistance and beta-cell dysfunction in humans. Diabetes. 2011;60(3):918-24.

219. Goya K, Sumitani S, Xu X, Kitamura T, Yamamoto H, Kurebayashi S, et al. Peroxisome proliferator-activated receptor alpha agonists increase nitric oxide synthase expression in vascular endothelial cells. Arterioscler Thromb Vasc Biol. 2004;24(4):658-63.

220. Walker AE, Kaplon RE, Lucking SM, Russell-Nowlan MJ, Eckel RH, Seals DR. Fenofibrate improves vascular endothelial function by reducing oxidative stress while increasing endothelial nitric oxide synthase in healthy normolipidemic older adults. Hypertension. 2012;60(6):1517-23.

221. Hodel C. Myopathy and rhabdomyolysis with lipid-lowering drugs. Toxicol Lett. 2002;128(1-3):159-68.

222. Tahmaz M, Kumbasar B, Ergen K, Ure U, Karatemiz G, Kazancioglu R. Acute renal failure secondary to fenofibrate monotherapy-induced rhabdomyolysis. Ren Fail. 2007;29(7):927-30.

223. Wang D, Wang Y. Fenofibrate monotherapy-induced rhabdomyolysis in a patient with hypothyroidism: A rare case report and literature review. Medicine (Baltimore). 2018;97(14):e0318.

224. Paul HS, Adibi SA. Paradoxical effects of clofibrate on liver and muscle metabolism in rats. Induction of myotonia and alteration of fatty acid and glucose oxidation. J Clin Invest. 1979;64(2):405-12.

225. Kadota Y, Kazama S, Bajotto G, Kitaura Y, Shimomura Y. Clofibrate-induced reduction of plasma branched-chain amino acid concentrations impairs glucose tolerance in rats. JPEN J Parenter Enteral Nutr. 2012;36(3):337-43.

226. Ishiguro H, Katano Y, Nakano I, Ishigami M, Hayashi K, Honda T, et al. Clofibrate treatment promotes branched-chain amino acid catabolism and decreases the phosphorylation state of mTOR, eIF4E-BP1, and S6K1 in rat liver. Life Sci. 2006;79(8):737-43.

227. Holecek M, Vodenicarovova M. Effects of low and high doses of fenofibrate on protein, amino acid, and energy metabolism in rat. Int J Exp Pathol. 2020;101(5):171-82.

228. Paul HS, Liu WQ, Adibi SA. Alteration in gene expression of branched-chain keto acid dehydrogenase kinase but not in gene expression of its substrate in the liver of clofibrate-treated rats. Biochem J. 1996;317 (Pt 2):411-7.

229. Kobayashi R, Murakami T, Obayashi M, Nakai N, Jaskiewicz J, Fujiwara Y, et al. Clofibric acid stimulates branched-chain amino acid catabolism by three mechanisms. Arch Biochem Biophys. 2002;407(2):231-40.

230. Knapik-Czajka M, Gozdzialska A, Jaskiewicz J. Adverse effect of fenofibrate on branched-chain alpha-ketoacid dehydrogenase complex in rat's liver. Toxicology. 2009;266(1-3):1-5.

231. Zhao Y, Jaskiewicz J, Harris RA. Effects of clofibric acid on the activity and activity state of the hepatic branched-chain 2-oxo acid dehydrogenase complex. Biochem J. 1992;285 (Pt 1):167-72.

232. Kobayashi M, Shigeta Y, Hirata Y, Omori Y, Sakamoto N, Nambu S, et al. Improvement of glucose tolerance in NIDDM by clofibrate. Randomized double-blind study. Diabetes Care. 1988;11(6):495-9.

233. Murakami K, Nambu S, Koh H, Kobayashi M, Shigeta Y. Clofibrate enhances the affinity of insulin receptors in non-insulin dependent diabetes mellitus. Br J Clin Pharmacol. 1984;17(1):89-91.

234. Ferrari C, Frezzati S, Romussi M, Bertazzoni A, Testori GP, Antonini S, et al. Effects of short-term clofibrate administration on glucose tolerance and insulin secretion in patients with chemical diabetes or hypertriglyceridemia. Metabolism. 1977;26(2):129-39.

235. Wolfe BM, Kane JP, Havel RJ, Brewster HP. Mechanism of the hypolipemic effect of clofibrate in postabsorptive man. J Clin Invest. 1973;52(9):2146-59.

236. Frias JP, Nauck MA, Van J, Kutner ME, Cui X, Benson C, et al. Efficacy and safety of LY3298176, a novel dual GIP and GLP-1 receptor agonist, in patients with type 2 diabetes: a randomised, placebo-controlled and active comparator-controlled phase 2 trial. Lancet. 2018;392(10160):2180-93.

237. Samms RJ, Christe ME, Collins KA, Pirro V, Droz BA, Holland AK, et al. GIPR agonism mediates weight-independent insulin sensitization by tirzepatide in obese mice. J Clin Invest. 2021;131(12).

238. Pirro V, Roth KD, Lin Y, Willency JA, Milligan PL, Wilson JM, et al. Effects of Tirzepatide, a Dual GIP and GLP-1 RA, on Lipid and Metabolite Profiles in Subjects with Type 2 Diabetes. J Clin Endocrinol Metab. 2021.

239. Unger RH, Cherrington AD. Glucagonocentric restructuring of diabetes: a pathophysiologic and therapeutic makeover. J Clin Invest. 2012;122(1):4-12.

240. Karwi QG, Zhang L, Wagg CS, Wang W, Ghandi M, Thai D, et al. Targeting the glucagon receptor improves cardiac function and enhances insulin sensitivity following a myocardial infarction. Cardiovasc Diabetol. 2019;18(1):1.

241. Consolazio CF, Johnson HL, Nelson RA, Dramise JG, Skala JH. Protein metabolism during intensive physical training in the young adult. Am J Clin Nutr. 1975;28(1):29-35.

242. Rennie MJ. Influence of exercise on protein and amino acid metabolism. Compr Physiol. 2011;25(1):995-1035.

243. Xiao W, Chen P, Liu X, Zhao L. The Impaired Function of Macrophages Induced by Strenuous Exercise Could Not Be Ameliorated by BCAA Supplementation. Nutrients. 2015;7(10):8645-56.

244. Glynn EL, Piner LW, Huffman KM, Slentz CA, Elliot-Penry L, AbouAssi H, et al. Impact of combined resistance and aerobic exercise training on branched-chain amino acid turnover, glycine metabolism and insulin sensitivity in overweight humans. Diabetologia. 2015;58(10):2324-35.

245. Fujii H, Shimomura Y, Murakami T, Nakai N, Sato T, Suzuki M, et al. Branched-chain alpha-keto acid dehydrogenase kinase content in rat skeletal muscle is decreased by endurance training. Biochem Mol Biol Int. 1998;44(6):1211-6.

246. Shimomura Y, Murakami T, Nakai N, Nagasaki M, Harris RA. Exercise promotes BCAA catabolism: effects of BCAA supplementation on skeletal muscle during exercise. J Nutr. 2004;134(6 Suppl):1583S-7S.

247. Shimomura Y, Fujii H, Suzuki M, Murakami T, Fujitsuka N, Nakai N. Branched-chain alpha-keto acid dehydrogenase complex in rat skeletal muscle: regulation of the activity and gene expression by nutrition and physical exercise. J Nutr. 1995;125(6 Suppl):1762S-5S.

248. Xu M, Nagasaki M, Obayashi M, Sato Y, Tamura T, Shimomura Y. Mechanism of activation of branched-chain alpha-keto acid dehydrogenase complex by exercise. Biochem Bioph Res Co. 2001;287(3):752-6.

249. Kobayashi R, Shimomura Y, Murakami T, Nakai N, Otsuka M, Arakawa N, et al. Hepatic branched-chain alpha-keto acid dehydrogenase complex in female rats: activation by exercise and starvation. J Nutr Sci Vitaminol (Tokyo). 1999;45(3):303-9.

250. Murakami T, Matsuo M, Shimizu A, Shimomura Y. Dissociation of branched-chain alpha-keto acid dehydrogenase kinase (BDK) from branched-chain alpha-keto acid dehydrogenase complex (BCKDC) by BDK inhibitors. J Nutr Sci Vitaminol (Tokyo). 2005;51(1):48-50.

251. Kasperek GJ, Dohm GL, Snider RD. Activation of branched-chain keto acid dehydrogenase by exercise. Am J Physiol. 1985;248(2 Pt 2):R166-71.

252. Shimomura Y, Fujii H, Suzuki M, Fujitsuka N, Naoi M, Sugiyama S, et al. Branched-chain 2-oxo acid dehydrogenase complex activation by tetanic contractions in rat skeletal muscle. Biochim Biophys Acta. 1993;1157(3):290-6.

253. Kujala UM, Peltonen M, Laine MK, Kaprio J, Heinonen OJ, Sundvall J, et al. Branched-Chain Amino Acid Levels Are Related with Surrogates of Disturbed Lipid Metabolism among Older Men. Front Med (Lausanne). 2016;3:57.

254. Wagenmakers AJ, Brookes JH, Coakley JH, Reilly T, Edwards RH. Exercise-induced activation of the branched-chain 2-oxo acid dehydrogenase in human muscle. Eur J Appl Physiol Occup Physiol. 1989;59(3):159-67.

255. Howarth KR, Burgomaster KA, Phillips SM, Gibala MJ. Exercise training increases branched-chain oxoacid dehydrogenase kinase content in human skeletal muscle. Am J Physiol Regul Integr Comp Physiol. 2007;293(3):R1335-41.

256. Poortmans JR, Siest G, Galteau MM, Houot O. Distribution of plasma amino acids in humans during submaximal prolonged exercise. Eur J Appl Physiol Occup Physiol. 1974;32(2):143-7.

257. Cummings NE, Williams EM, Kasza I, Konon EN, Schaid MD, Schmidt BA, et al. Restoration of metabolic health by decreased consumption of branched-chain amino acids. J Physiol. 2018;596(4):623-45.

258. White PJ, Lapworth AL, An J, Wang L, McGarrah RW, Stevens RD, et al. Branched-chain amino acid restriction in Zucker-fatty rats improves muscle insulin sensitivity by enhancing efficiency of fatty acid oxidation and acyl-glycine export. Mol Metab. 2016;5(7):538-51.

259. Fontana L, Cummings NE, Arriola Apelo SI, Neuman JC, Kasza I, Schmidt BA, et al. Decreased Consumption of Branched-Chain Amino Acids Improves Metabolic Health. Cell Rep. 2016;16(2):520-30.

260. McGarrah RW, Zhang GF, Christopher BA, Deleye Y, Walejko JM, Page S, et al. Dietary branched-chain amino acid restriction alters fuel selection and reduces triglyceride stores in hearts of Zucker fatty rats. Am J Physiol Endocrinol Metab. 2020;318(2):E216-E23.

261. Cavallaro NL, Garry J, Shi X, Gerszten RE, Anderson EJ, Walford GA. A pilot, short-term dietary manipulation of branched chain amino acids has modest influence on fasting levels of branched chain amino acids. Food Nutr Res. 2016;60:28592.

262. Karusheva Y, Koessler T, Strassburger K, Markgraf D, Mastrototaro L, Jelenik T, et al. Short-term dietary reduction of branched-chain amino acids reduces meal-induced insulin secretion and modifies microbiome composition in type 2 diabetes: a randomized controlled crossover trial. Am J Clin Nutr. 2019;110(5):1098-107.

263. Cannon B, Nedergaard J. Nonshivering thermogenesis and its adequate measurement in metabolic studies. J Exp Biol. 2011;214(Pt 2):242-53.

264. Chondronikola M, Volpi E, Borsheim E, Chao T, Porter C, Annamalai P, et al. Brown Adipose Tissue Is Linked to a Distinct Thermoregulatory Response to Mild Cold in People. Front Physiol. 2016;7:129.

265. Lopez-Soriano FJ, Fernandez-Lopez JA, Mampel T, Villarroya F, Iglesias R, Alemany M. Amino acid and glucose uptake by rat brown adipose tissue. Effect of cold-exposure and acclimation. Biochem J. 1988;252(3):843-9.

266. Yoneshiro T, Wang Q, Tajima K, Matsushita M, Maki H, Igarashi K, et al. BCAA catabolism in brown fat controls energy homeostasis through SLC25A44. Nature. 2019;572(7771):614-9.

267. Cannavino J, Shao M, An YA, Bezprozvannaya S, Chen S, Kim J, et al. Regulation of cold-induced thermogenesis by the RNA binding protein FAM195A. Proc Natl Acad Sci U S A. 2021;118(23).

3

Elevated plasma branched-chain amino acid levels correlate with type 2 diabetes-related metabolic disturbances

Froukje Vanweert[1], Marlies de Ligt[1], Joris Hoeks[1], Matthijs K. C. Hesselink[1], Patrick Schrauwen[1], and Esther Phielix[1]

[1] Department of Nutrition and Movement Sciences, NUTRIM, School of Nutrition and Translational Research in Metabolism, Maastricht University Medical Center, Maastricht, the Netherlands

This chapter has been published in: J Clin Endocrinol Metab. 2021;106(4):e1827-e36.

ABSTRACT

Context: Patients with type 2 diabetes (T2D) have elevated plasma branched-chain amino acid levels (BCAA). The underlying cause is, however, not known. Low mitochondrial oxidation of BCAA could contribute to higher plasma BCAA levels.
Objective: We aimed to investigate *ex vivo* muscle mitochondrial oxidative capacity and *in vivo* BCAA oxidation measured by whole-body leucine oxidation rates in patients with T2D, first-degree relatives (FDR) and control participants (CON) with overweight or obesity.
Design and Setting: An observational, community-based study was conducted.
Participants: Fifteen patients with T2D, thirteen FDR and seventeen CON were included (age between 40-70 years and body mass index ranging from 27-35 kg/m^2).
Main Outcome Measures: High-resolution respirometry was used to examine *ex vivo* mitochondrial oxidative capacity in permeabilized muscle fibers. A subgroup of five patients with T2D and five CON underwent hyperinsulinemic-euglycemic clamps combined with 1-^{13}C leucine-infusion to determine whole-body leucine oxidation.
Results: Total BCAA levels were higher in patients with T2D compared to CON, but not in FDR, and correlated negatively with muscle mitochondrial oxidative capacity ($r = -0.44$, $p<0.001$). Consistently, whole-body leucine oxidation rate was lower in patients with T2D vs. CON under basal conditions (0.202 ± 0.049 vs. 0.275 ± 0.043 mmol kg^{-1} min^{-1}, $p<0.05$) and tended to be lower during high insulin infusion (0.326 ± 0.024 vs. 0.382 ± 0.013 mmol kg^{-1} min^{-1}, $p=0.075$).
Conclusions: In patients with T2D, a compromised whole-body leucine oxidation rate supports our hypothesis that higher plasma BCAA levels may at least originate partly from a low mitochondrial oxidative capacity.

INTRODUCTION

Patients with type 2 diabetes (T2D) are characterized by the presence of higher levels of branched-chain amino acids (BCAA) in plasma, coinciding with reduced whole-body insulin sensitivity (1-8). Some (9, 10), but not all studies (11), also found elevated plasma BCAA levels in people prior to the actual onset of T2D.

Overt T2D, however, comprises a broad scale of metabolic disturbances closely related to insulin resistance. We, as well as others, have repeatedly reported that patients with T2D have low muscle mitochondrial oxidative capacity compared to age- and BMI- matched control groups (12-14). In addition, patients with T2D are less metabolic flexible, which is the inability to readily adjust substrate oxidation to changes in substrate availability (15-17). Previous reports showed that first-degree relatives of patients with T2D (FDR) feature both low mitochondrial oxidative capacity and reduced insulin sensitivity when compared to control groups (18-20). Evidence, however, seems to be inconclusive as to whether plasma BCAA may function as an effective biomarker to identify people at risk of developing T2D. To date, it has not been investigated whether plasma BCAA levels correlate with key metabolic parameters, like mitochondrial oxidative capacity and metabolic flexibility, which are linked to insulin sensitivity. The mechanism underlying elevated plasma levels in patients with T2D remains largely unknown, although it has been reported that low mitochondrial oxidation of BCAA (21) and/or a blunted inhibitory effect on proteolysis (22-24) may contribute.

Therefore, in the present study we aimed to investigate whether high plasma BCAA levels correlate with low *ex vivo* muscle mitochondrial oxidative capacity in patients with T2D, FDR and age and BMI matched control participants (CON). To specifically characterize BCAA oxidation *in vivo*, subsequently, whole-body leucine oxidation rates were measured in a subgroup of patients with T2D and CON. We hypothesized that low mitochondrial oxidation of BCAA partly contributes to the high plasma BCAA levels.

MATERIAL AND METHODS

Participant characteristics

Fifteen patients with T2D, thirteen FDR and seventeen CON were included in the study. Groups were male and had comparable BMI ranging between 27 and 35 kg/m^2. Age ranged between 40 and 70 years and on average CON were significant younger compared to patients with T2D. All characteristics are shown in Table

1. Participants previously participated in metabolic research performed by our research group, with similar methodology (25-28). All participants underwent physical examination and routine medical laboratory tests. Patients with T2D were using oral glucose-lowering medication only. FDR was defined as previously reported (28) and CON had no family history of type 2 diabetes. Participants underwent an incremental cycling test to determine maximal oxygen uptake (VO_2 peak) for characterization of participants. A subset of five patients with T2D and five CON (Table 2) were enrolled for leucine oxidation measurements. All participants gave their written informed consent and the local medical ethical committee of Maastricht University approved the studies.

Table 1. Participant characteristics (males) presented as mean ± SE with $^{*}p \leq 0.05$ compared to CON and $^{\#}p \leq 0.05$ compared to FDR.

	T2D (n=15)	FDR (n=13)	CON (n=17)	Anova p-value	Post-hoc p-value
Age (y)	62.3 ± 2.2*	60.2 ± 2.0	53.6 ± 1.7	0.006	0.006
BMI (kg/m^2)	30.9 ± 0.6	29.8 ± 0.6	31.7 ± 0.1	0.085	
Body weight (kg)	95.1 ± 2.4	87.7 ± 2.3*	99.6 ± 3.1	0.015	0.011
Fat (%)	27.1 ± 1.7	33.7 ± 2.5	30.0 ± 1.6	0.073	
VO_2 peak (ml kg^{-1} min^{-1})	24.5 ± 1.2	28.5 ± 0.7	25.7 ± 1.2	0.07	
Fasting glucose (mmol/l)	7.2 ± 0.4*#	5.8 ± 0.1	5.6 ± 0.2	<0.001	<0.001 T2D>CON <0.001 T2D>FDR
Fasting FFA (mmol/l)	0.76 ± 0.06*#	0.45 ± 0.04	0.59 ± 0.09		<0.001 T2D>CON 0.002 T2D>FDR

Differences between groups tested by one-way ANOVA with Tukey post hoc correction. *BMI, body mass index; FFA, free fatty acids; T2D, type 2 diabetes mellitus; VO_2 peak, maximal oxygen uptake.*

Table 2. Subgroup characteristics presented as mean ± SE with *p≤0.05 compared to CON.

	T2D (n=5)	CON (n=5)	p-value
Age (y)	58.2 ± 2.7	55.6 ± 3.5	0.572
BMI (kg/m^2)	32.9 ± 1.2	32.1 ± 1.3	0.668
Body weight (kg)	98.1 ± 4.4	100.4 ± 7.4	0.793
VO_2 peak (ml kg^{-1} min $^{-1}$)	26.1 ±2.0	28.0 ± 1.6	0.479
Fasting glucose (mmol/l)	7.6 ± 0.8*	5.7 ± 0.2	0.046
FFA fasting (mmol/l)	0.83 ± 0.14	0.66 ± 0.06	0.548
FFA low insulin clamp (mmol/l)	0.33 ± 0.04*	0.24 ± 0.01	0.048
FFA high insulin clamp (mmol/l)	0.17 ± 0.02	0.12 ± 0.02	0.206
insulin fasting(pmol/l)	104 ± 23	95 ± 17	0.308
insulin low insulin clamp (pmol/l)	221 ± 24	211 ± 25	0.778
insulin high insulin clamp (pmol/l)	940 ± 90	681 ± 73	0.111

Differences between groups tested by Mann Whitney-test. *BMI, body mass index; FFA, free fatty acids; T2D, type 2 diabetes mellitus; VO_2 peak, maximal oxygen uptake.*

Study design

Participants reported to the metabolic research center in the morning after an overnight fast. All participants provided a fasting blood sample and a muscle biopsy was taken from the vastus lateralis after which the fresh tissue was immediately processed for high-resolution respirometry measurements (described as follows) and subsequently a 2-step hyperinsulinemic-euglycemic clamp was performed with target glucose value of 5 to 5.5 mmol/L as previously described (25, 26, 28, 29). Peripheral insulin sensitivity was defined as glucose infusion rate (GIR) during the 40mU m^{-2} min^{-1} insulin infusion step to maintain euglycemia. Before and during the clamp, blood sampling and ventilated hood measurements (Omnical, Maastricht Instruments, Maastricht, the Netherlands) were performed to compute the respiratory exchange ratio (RER). Metabolic flexibility was expressed as the change in RER (ΔRER) from fasted to the insulin-stimulated state (16).

Leucine oxidation measurements

In vivo leucine oxidation rate was measured in a subgroup of patients with T2D and CON. All received a single dose prime (7.6 µmol/kg) –continuous (7.6 µmol/kg/min) 1-^{13}C leucine isotopic tracer during the hyperinsulinemic euglycemic clamp. Infusion rates for 1-^{13}C leucine were based on previous studies (30, 31). Breath samples were taken using a nonrebreathing, 2-way valve (ADinstruments, Oxford, UK) every 10 min during the steady-state phases. Simultaneously, venous blood was sampled and ventilated hood measurements were performed to acquire synchronized rates of CO_2 production (ml/min), enriched concentrations of α-ketoisocaproate (KIC) in

plasma (mole percent excess (MPE)) (32) and $^{13}C/^{12}C$ of CO_2 in expired breath (atom percent excess (APE)) (33). A pre-infusion of 3 hours for the 1-^{13}C leucine infusion was maintained to reach isotopic equilibrium during the basal period (i.e. before the start of the low insulin infusion). Leucine oxidation (mmol kg^{-1} min^{-1}) was calculated by dividing the enrichment of expired CO_2 (APE $^{13}C/^{12}C$) through the enrichment of KIC (MPE) and multiplied by the total CO_2 production rate (mmol kg^{-1} min^{-1}). The rates of appearance (Ra) of leucine were calculated from the equation: Ra = [(Ei/Ep)-1] x I, where Ei is the isotopic enrichment of the tracer infused (99%), Ep is the isotopic enrichment of the tracer in plasma, and I is the infusion rate of the tracer, as previously described (34). The rate of expired $^{13}CO_2$ (mmol kg^{-1} min^{-1}) was calculated by multiplying the CO_2 production rate by the APE $^{13}C/^{12}C$ (35). As C-labels must pass through the bicarbonate pool, a correction factor of 0.81 was used to correct $^{13}CO_2$ for bicarbonate retention (36). Recovery factors of ^{13}C from the bicarbonate pool for the calculation of postabsorptive and clamp leucine oxidation, were 0.67 and 0.80, respectively (37). Of note, in the clamps performed here, amino acids were not replaced with a co-infusion of an amino acid mixture.

High-resolution respirometry

High-resolution respirometry was performed to assess mitochondrial oxidative capacity. Part of the muscle biopsy was immediately used for *ex vivo* analysis of mitochondrial oxidative capacity in permeabilized muscle fibers using a two-chamber oxygraph (OROBOROS Instruments, Innsbruck, Austria) as previously described (26). In short, mitochondrial ADP-driven state 3 respiration was measured with and without the presence of octanoyl-carnitine on parallel electron input to both complex I and II (malate + glutamate + succinate). Also, carbonyl cyanide p-(trifluoromethoxyl]-phenyl hydrozone (FCCP)-driven uncoupled respiration (state u) was evaluated. Oxygen consumption was corrected for muscle wet mass (2-4 mg) and given as oxygen flux expressed as pmol mg^{-1} s^{-1} (38).

Plasma assays

Plasma free fatty acids (FFA) and glucose concentrations were measured with enzymatic assays as previously reported (39). Plasma amino acid levels were determined as previously described (40). In short, acetonitrile (200 ul) was added to 100 ul plasma for deproteinization, vortex-mixed and analyzed by liquid chromatography–mass spectrometry (LCMS) for the measurement of concentrated amino acid peaks.

Statistical analyses

Statistical analyses were performed using the statistics program SPSS 24 for Mac OS. The Kolmogorov-Smirnov/Shapiro-Wilk normality test was performed to evaluate normal distribution. Differences between the three groups were tested by one-way analysis of variance (ANOVA) with Tukey post hoc correction for multiple testing. P-values were adjusted for age by General Linear Model Univariate analysis. Differences for 1-^{13}C leucine isotopic tracer kinetics between T2D and CON throughout the clamp were tested using a 2-way ANOVA for repeated measures. Correlations were computed with the Pearson correlation coefficient. Data are expressed as mean ± SE and differences were considered statistically significant when p-value was ≤ 0.05.

RESULTS

Amino acid profiles

Fasting amino acid profiles are shown in Figure 1. Total plasma BCAA levels (Figure 1a) were significantly higher in T2D compared to both CON (p=0.001) and FDR (p=0.016), but did not differ between FDR and CON. Of the BCAA, levels of valine (Figure 1b) and isoleucine (Figure 1c) were higher in T2D compared to CON (p=0.001 for valine; p=0.047 for isoleucine), but did not between FDR and CON. Levels of valine were higher in T2D compared to FDR (p=0.043), but levels of isoleucine were not significantly different between T2D and FDR. Plasma levels of leucine (Figure 1d) were higher in T2D compared to both CON (p=0.002) and FDR (p=0.009). The significantly higher BCAA levels in T2D are not a reflection of higher essential amino acids as is displayed by lower methionine levels (Figure 1e) in T2D compared to both CON (p=0.001) and FDR (p<0.001). Of the aromatic amino acids, tryptophan was lower in T2D vs. FDR (Figure 1e, p<0.001), but both groups did not differ compared to CON. Other aromatic amino acids, like phenylalanine, tyrosine and histidine were not significantly different between groups (Figure 1e).

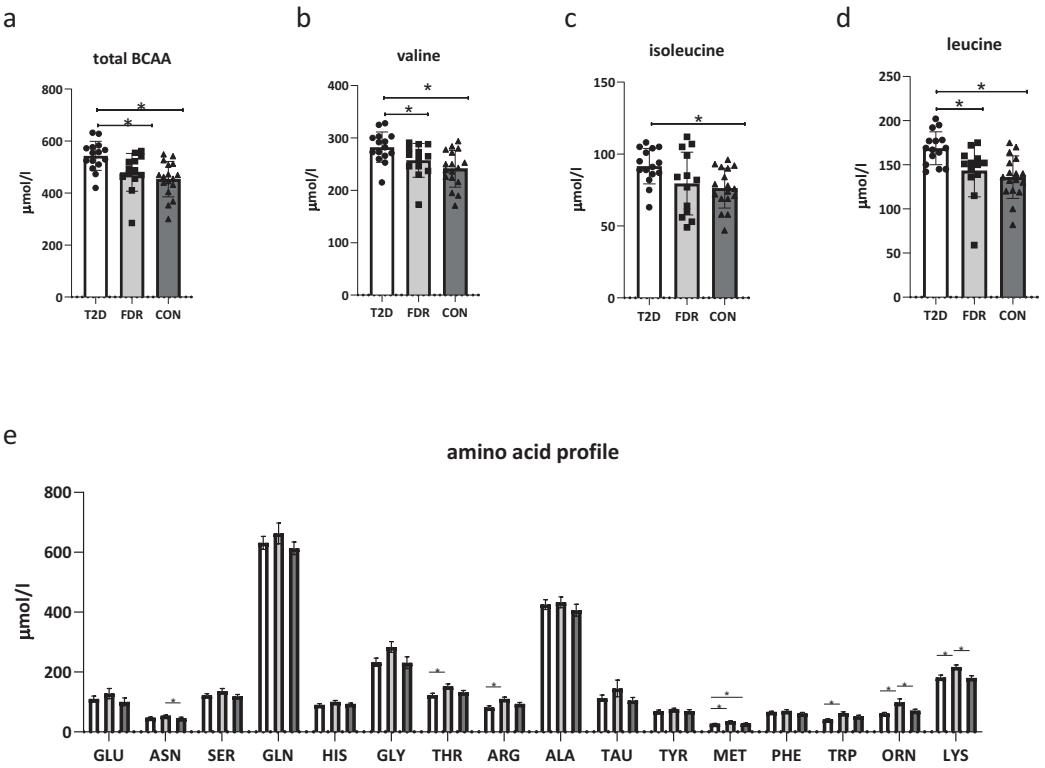


Figure 1. Fasting plasma levels (µmol/l) of total BCAA (a), valine (b), isoleucine (c), leucine (d) and other amino acids (e) in patients with T2D (white bars, n=15), FDR (light grey, n=13) and CON (dark grey, n=17).

Differences between groups tested by one-way ANOVA with Tukey post hoc correction. Data are expressed as mean ± SE; * p≤0.05. Differences between groups tested by one-way ANOVA with Tukey post hoc correction. ALA, alanine; ARG, arginine; ASN, asparagine; BCAA, branched-chain amino acids; CON, control participants; FDR, first-degree relatives with T2D; *GLN, glutamine; GLU, glutamic acid; GLY, glycine; HIS, histidine; LYS, lysine. MET, Methionine; ORN, ornithine; PHE, phenylalanine; SER, serine; T2D, type 2 diabetes mellitus; THR, threonine; TRP, tryptophan; TYR, tyrosine;*

Peripheral insulin sensitivity

GIR (Figure 2a) on high insulin infusion was significantly lower in people with T2D compared to CON (p=0.009) and to FDR (p=0.011). GIR was not significant different between FDR and CON (Figure 2a).

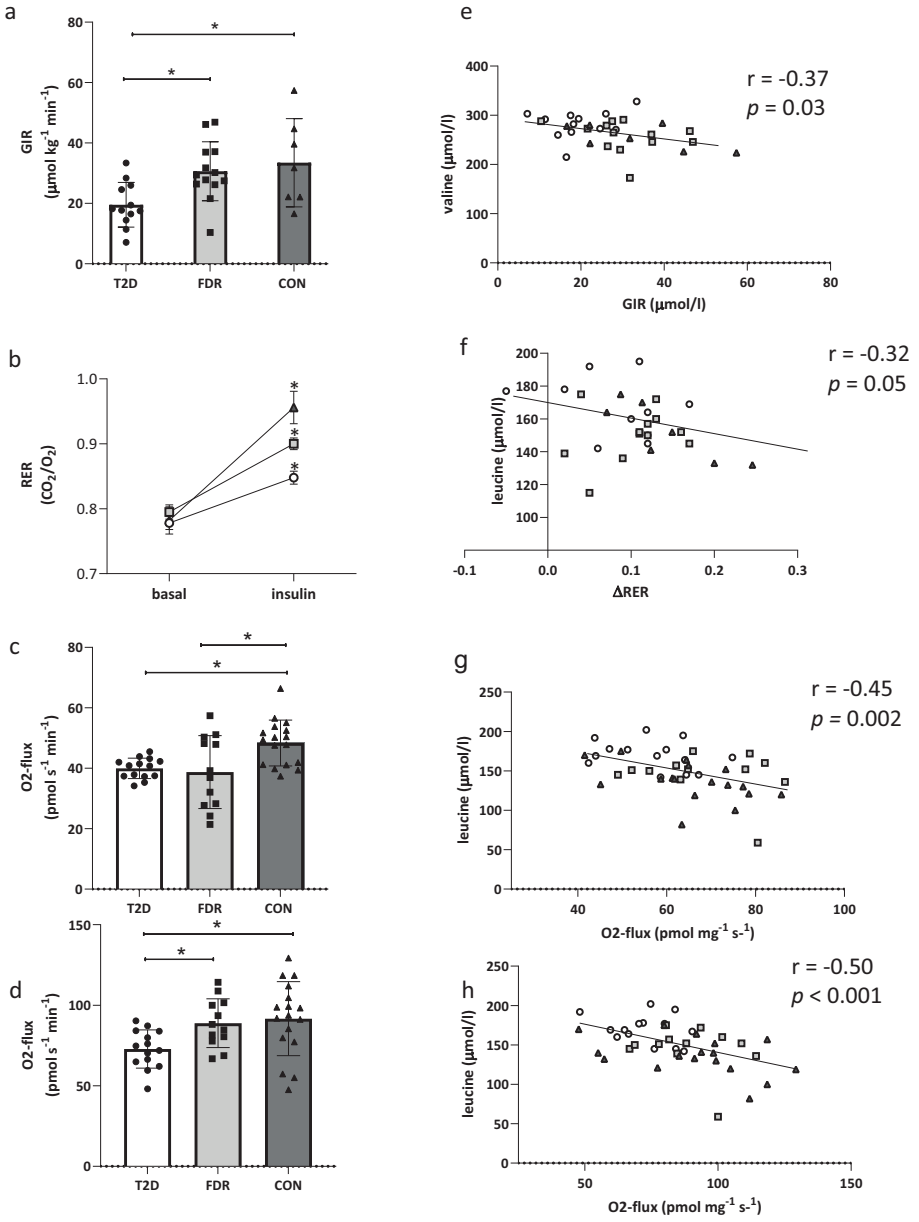


Figure 2. Peripheral insulin sensitivity expressed as the glucose infusion rate (GIR, µmol kg^{-1} min^{-1}) (a), metabolic flexibility expressed as the respiratory exchange ratio (ΔRER, VCO_2/VO_2) under basal condition and upon insulin stimulation (b), mitochondrial oxidative capacity expressed as state 3 respiration on malate and glutamate (O_2 flux, pmol s^{-1} min^{-1}) (c) and as uncoupled (state u) respiration upon FCCP (O_2 flux, pmol s^{-1} min^{-1}) (d).

Differences between groups tested by one-way ANOVA with Tukey post hoc correction. Pearson correlation between plasma BCAA levels and peripheral insulin sensitivity (e), metabolic flexibility (f), mitochondrial oxidative capacity (state 3 respiration) (g), and mitochondrial oxidative capacity (state u respiration) (g). Analyses were done in patients with T2D (white (dots), n=15), FDR (light grey (squares), n=13) and CON (dark grey (triangles), n=17). Data are expressed as mean ± SE; * p≤0.05. *CON, control participants; FDR, first-degree relatives; GIR, glucose infusion rate; T2D, type 2 diabetes mellitus; RER, respiratory exchange ratio;*

Metabolic flexibility

RER (Figure 2b) under basal conditions (before the start of the insulin infusion) was similar between the three groups and significantly increased on insulin stimulation in all groups ($p<0.05$ vs. basal RER for all groups). Metabolic flexibility, expressed as the ΔRER from the fasted state to the insulin-stimulated state, was significantly lower in T2D (0.06 ± 0.02) compared to CON (0.14 ± 0.02, p=0.044). No differences were found between FDR (0.11 ± 0.01) and CON or patients with T2D.

Ex vivo mitochondrial oxidative capacity

ADP-driven state 3 respiration upon malate and glutamate (Figure 2c) was significantly lower in T2D and in FDR both compared to CON (p=0.015 and p=0.005, respectively). State 3 respiration on malate and glutamate did not differ between T2D and FDR (Figure 2c). With the addition of octanoyl-carnitine, no differences were found between T2D (41.3 ± 1.8 mmol mg^{-1} s^{-1}), FDR (41.9 ± 2.9 mmol mg^{-1} s^{-1}) or CON (45.3 ± 2.0 mmol mg^{-1} s^{-1}). ADP-driven state 3 respiration upon malate, glutamate and succinate was lower in T2D (61.2 ± 1.8 mmol mg^{-1} s^{-1}) compared to CON (71.9 ± 2.9 mmol mg^{-1} s^{-1}, p=0.002). No differences were found between FDR (66.4 ± 3.7 mmol mg^{-1} s^{-1}) and CON, neither between FDR and T2D. With the addition of octanoyl-carnitine, T2D (56.7 ± 2.5 mmol mg^{-1} s^{-1}) had lower levels compared to FDR (68.2 ± 3.4 mmol mg^{-1} s^{-1}, p=0.018), and tended to be lower compared to CON (65.4 ± 2.9 mmol mg^{-1} s^{-1}, p=0.083). Maximal FCCP-driven state u respiration (Figure 2d) was significantly lower in T2D compared to FDR (p=0.03) and CON (p=0.013). No differences were observed in FCCP-driven state u respiration between FDR and CON (Figure 2d).

Correlations between plasma BCAA levels and metabolic parameters

Total plasma BCAA, leucine and isoleucine levels positively correlated with fasting FFA levels (total BCAA: r =0.32, p=0.036, leucine: r=0.41, p=0.007, isoleucine: r=0.40, p=0.007). Leucine plasma levels correlated positively as well with fasting glucose levels (r=0.30, p=0.044).

Total plasma BCAA levels (r = -0.35, p=0.049), plasma valine (Figure 2e, p=0.03,) and leucine (r = -0.35, p=0.050) negatively correlated with peripheral insulin sensitivity, expressed as the GIR in the whole group of participants. In addition, plasma leucine levels also negatively correlated with metabolic flexibility (Figure 2f, p=0.02), illustrating that the most metabolically inflexible and insulin-resistant people had the highest plasma BCAA levels. These correlations were not significant between groups. Total plasma BCAA levels (r = -0.44, p=0.004), plasma leucine (Figure 2g, p=0.002) and valine (r = -0.46, p=0.002) showed a strong negative correlation with ADP-driven state 3 respiration upon octanoyl-carnitine, glutamate and succinate

in all participants. The correlation between state 3 respiration with BCAA (r = -0.64, p<0.01), leucine (r = -0.65, p<0.01), and valine (r = -0.63, p<0.02) were also found in the T2D group. Similarly, total plasma BCAA levels (r = -0.49, p<0.001), plasma leucine (Figure 2h, p<0.001), valine (r = -0.47, p=0.001) and isoleucine (r = -0.37, p=0.02) showed a negative correlation with maximal FCCP-driven state u respiration in the whole group of participants.

Whole-body leucine oxidation rate

As shown in Figure 3, the subgroup who underwent the 1-^{13}C leucine infusion protocol showed a tendency towards an enhanced BCAA signature between patients with T2D vs. CON (p=0.09 for total BCAA, p=0.08 for valine and p=0.03 for isoleucine; however, with an absent difference found for leucine p=0.40).

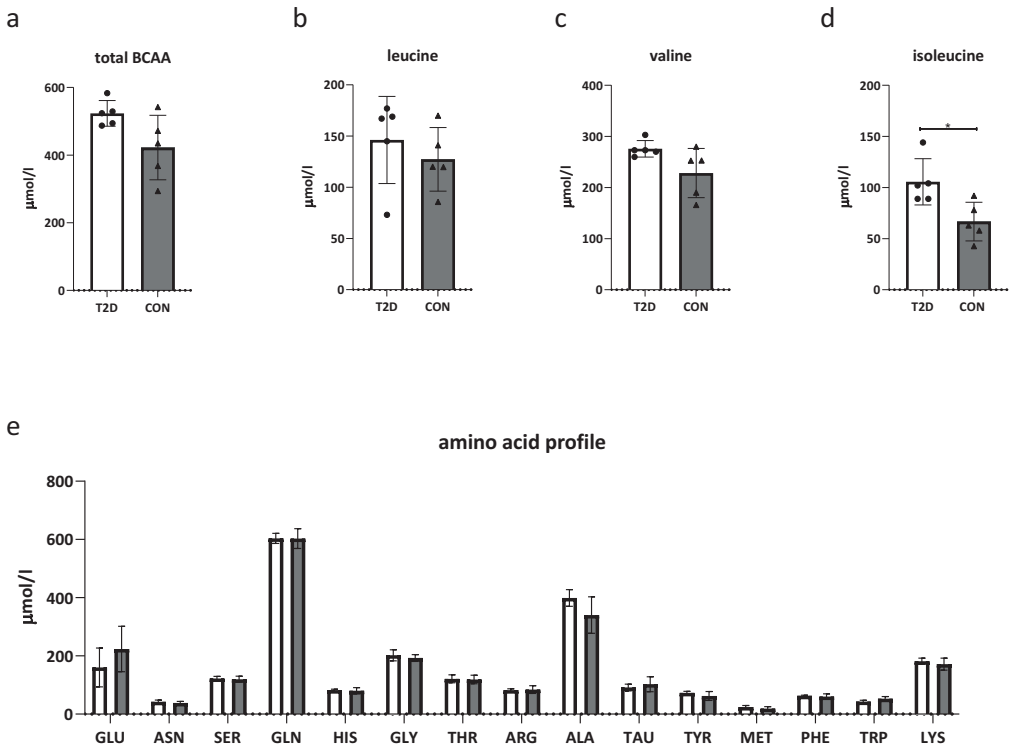


Figure 3. Fasting plasma levels (µmol/l) of total BCAA (a), leucine (b), valine (c), isoleucine (d) and other amino acids (e) in patients with T2D (white bars, n=5) and CON (grey bars, n=17).

Differences between groups tested by Mann Whitney-test. Data are expressed as mean ± SE; * p≤0.05. *ALA, alanine; ARG, arginine; ASN, asparagine; BCAA, branched-chain amino acids; CON, control participants; GLN, glutamine; GLU, glutamic acid; GLY, glycine; HIS, histidine; LYS, lysine. MET, Methionine; ORN, ornithine; PHE, phenylalanine; SER, serine; T2D, type 2 diabetes mellitus; THR, threonine; TRP, tryptophan; TYR, tyrosine.*

The enrichment of plasma KIC (MPE) and of CO_2 in the expired air (APE) are shown in Figure 4a and c, respectively. No differences were seen between the individuals with T2D and CON for the average MPE values for KIC during the basal phase (T2D: 7.28 ± 0.91% vs. CON: 7.22 ± 0.30%), nor for the low-insulin (T2D: 7.72 ± 0.83% vs. CON: 8.08 ± 0.56%) or high-insulin phase (T2D: 8.39 ± 1.02% vs. CON: 8.69 ± 0.46%). Average enrichment of CO_2 (APE) under basal conditions did not differ between T2D patients and CON (T2D: 0.014 ± 0.004% and CON: 0.0167 ± 0.003%). However, CO_2 enrichment tended to be lower in T2D vs. CON during the low-insulin (T2D: 0.019 ± 0.003% vs. CON: 0.023 ± 0.03%, p=0.084) and high-insulin phases (T2D: 0.022 ± 0.002% vs. CON: 0.026 ± 0.03%, p=0.077). A stepwise increase in the rates of leucine oxidation was seen throughout the clamp (Figure 4c). Leucine oxidation rate was lower in patients with T2D (0.202 ± 0.049 mmol kg^{-1} min^{-1}) vs. CON (0.275 ± 0.043 mmol kg^{-1} min^{-1}) under basal conditions (Figure 4c, p=0.038) and tended to be lower

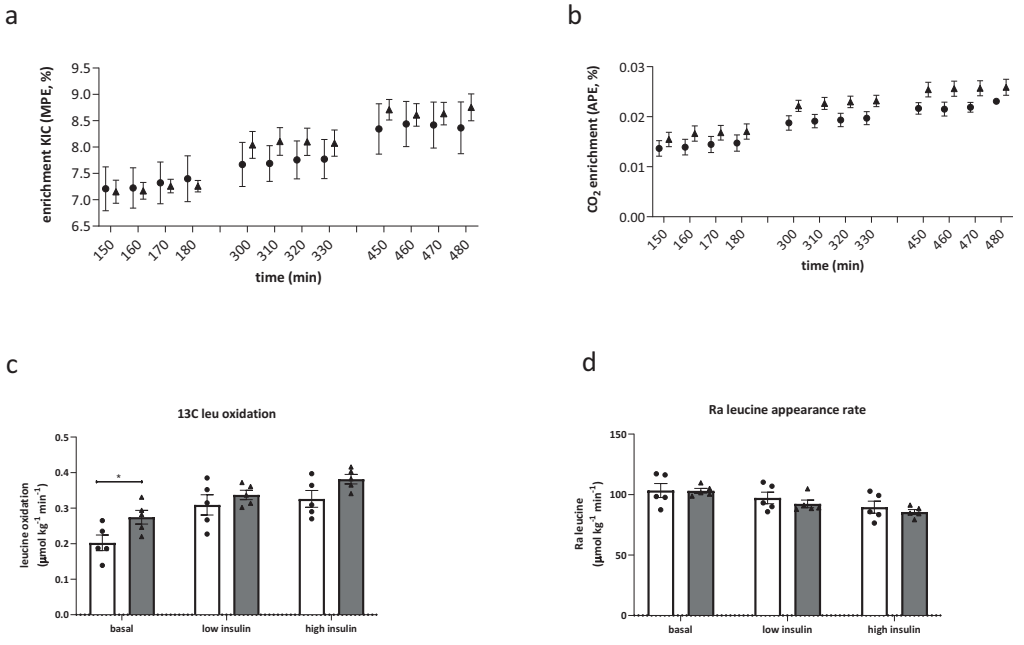


Figure 4. The enrichment of plasma KIC (mole percent excess, %) (a) and of CO_2 in the expired air (atom percent excess, %) (b), rate of leucine oxidation (µmol kg^{-1} min^{-1}) (c) and leucine appearance (Ra) rate (d) during the basal (150 – 180 min), low insulin (300 – 330 min), and high insulin phase (450 – 480 min) measured in patients with T2D (white bars, n=5) and CON (grey bars, n=5).

Differences between groups tested using the Mann-Whitney test. Leucine oxidation and Ra rates between basal, low and high insulin phases were tested using a 2-way analysis of variance for repeated measures Data are expressed as mean ± SE; * p≤0.05. *APE, atom percent excess; CON, control participants; KIC, ketoisocaproic acid; MPE, mole percent excess; Ra, rate of appearance. T2D, type 2 diabetes mellitus.*

in T2D vs. CON during high insulin infusion (0.326 ± 0.053 vs. 0.382 ± 0.0.030 mmol kg^{-1} min^{-1}, Figure 4c, p=0.075). No differences were observed during the low-insulin phase (Figure 4c). No differences in leucine Ra were observed between individuals with T2D and CON throughout the clamp test (Figure 4d). In both T2D and CON, leucine oxidation rates increased, and Ra decreased significantly from basal to insulin-stimulated conditions.

DISCUSSION

In the present study, we report higher plasma BCAA levels in patients with T2D and show that plasma BCAA levels in patients with T2D, FDR and CON correlate significantly with diabetes-related metabolic disturbances, like *ex vivo* mitochondrial oxidative capacity and metabolic flexibility. Subsequently, whole-body leucine oxidation was measured *in vivo* as a reflection of BCAA oxidation and was significantly lower in patients with T2D compared to CON. Together, these results suggest that a low mitochondrial oxidation of BCAA may contribute to higher plasma BCAA levels and affect metabolic health in T2D.

The higher plasma BCAA cluster (isoleucine, leucine and valine) observed in patients with T2D substantiates and extends observations by others (1-3). In contrast, we did not observe higher plasma BCAA levels, or higher aromatic amino acid levels in FDR. Because insulin sensitivity was not different between FDR and CON, which implies that FDR were rather metabolically healthy, it is conceivable that no higher plasma BCAA levels were observed in FDR in line with a previous report (11).

The present study is the first to investigate the correlation between plasma BCAA levels and peripheral insulin sensitivity, measured with the gold-standard technique. In accordance with previous findings (3, 5, 8, 10, 41), we also report negative correlations between peripheral insulin sensitivity and plasma BCAA levels. Patients with T2D were more insulin resistant and metabolically inflexible in comparison with CON, with intermediate values seen in FDR. Furthermore, we sought to investigate whether plasma BCAA levels correlate with muscle mitochondrial oxidative capacity, in the presence of a fatty acid-like substrate as well as other complex I and -II-linked substrates. ADP- and maximal FCCP-driven mitochondrial respiration showed lowest values in T2D and FDR compared to CON participants, in line with previous reports (12-14, 18). Interestingly, we show that plasma BCAA levels negatively correlates with metabolic flexibility and mitochondrial oxidative capacity, both linked to insulin sensitivity. These data

match with previously reported correlations between elevated plasma BCAA levels and insulin resistance in obese humans (2, 42). The correlations presented were significant, albeit not very strong, which could be attributed to the rather small population size together with the variations accompanying the measurement of these biological parameters.

The difference in mitochondrial oxidative capacity observed between groups is not expected to be caused by differences in mitochondrial content. In several previous reports, we did not observe differences in markers for mitochondrial content between patients with T2D, FDR and CON (19, 43, 44). Differences in physical activity is a well-known factor explaining differences in mitochondrial content. In previous studies as in the present study, by design, all participants had, however, comparable levels of physical fitness reflected by a similar VO_2 peak.

Because low mitochondrial oxidation of BCAA could underlie the higher plasma BCAA levels seen in T2D, we subsequently measured *in vivo* whole-body leucine oxidation rates -using the KIC tracer enrichment as reciprocal pool model as proxy measures of intracellular leucine enrichment- in a small group of patients with T2D and CON. We found that patients with T2D featured lower whole-body leucine oxidation rates. The low *in vivo* leucine rates were accompanied by a tendency toward lower total BCAA and significantly lower isoleucine levels, but absent a difference in leucine concentrations. Without the co-infusion of amino acids during the clamp, we observed insulin-stimulated BCAA oxidation in both T2D and CON. Insulin is well-known to increase BCAA clearance out of the circulation mainly via protein synthesis, however, as recently published by Neinast et al. (45), insulin more than doubled the preference for BCAA oxidation specifically in muscle and the heart. In line with this observation, also others reported enhanced leucine oxidation rates during insulin stimulation in humans (46, 47), which needs further investigation.

A limitation of our study is the small number of participants who underwent the 1-^{13}C leucine infusion protocol. Also, we did not measure valine and isoleucine oxidation rates, meaning that we cannot conclude whether patients with T2D have lower total BCAA oxidation. The first two steps of the BCAA oxidative pathway, however, are common for all three BCAA, which are the only amino acids to share common metabolic steps. Therefore, the measurement of leucine oxidation with the 1-^{13}C leucine infusion protocol is a good index of total BCAA oxidation

(48-50). These exploratory results may form a lead to further investigate whether defective BCAA oxidation underlies elevated plasma BCAA levels in T2D. In this observational study, owing to the small sample size, we could not investigate sex-specific effects, which is a limitation of the study. Future research is warranted to investigate whether the results can be translated to the female population.

To conclude, our data shows that plasma BCAA levels in overweight/obese humans correlate with key metabolic parameters, like insulin sensitivity, metabolic flexibility and mitochondrial oxidative capacity. Reduced mitochondrial BCAA oxidation could underlie higher plasma BCAA levels and may affect metabolic health in T2D. Future research is required to investigate whether boosting BCAA oxidation would improve metabolic health in patients with T2D.

ACKNOWLEDGMENTS

E.P., M.d.L and F.V. performed the experiments and analyzed data. F.V. and E.P. wrote the manuscript. E.P., J.H., P.S. and M.H. assisted during the acquisition, analysis and interpretation of data and reviewed the manuscript. E.P., P.S., and M.H. designed the study. All authors reviewed and approved the final version of the manuscript. E.P. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

ADDITIONAL INFORMATION

Disclosure summary: The authors have nothing to disclosure

DATA PROCESSING AND AVAILABILITY

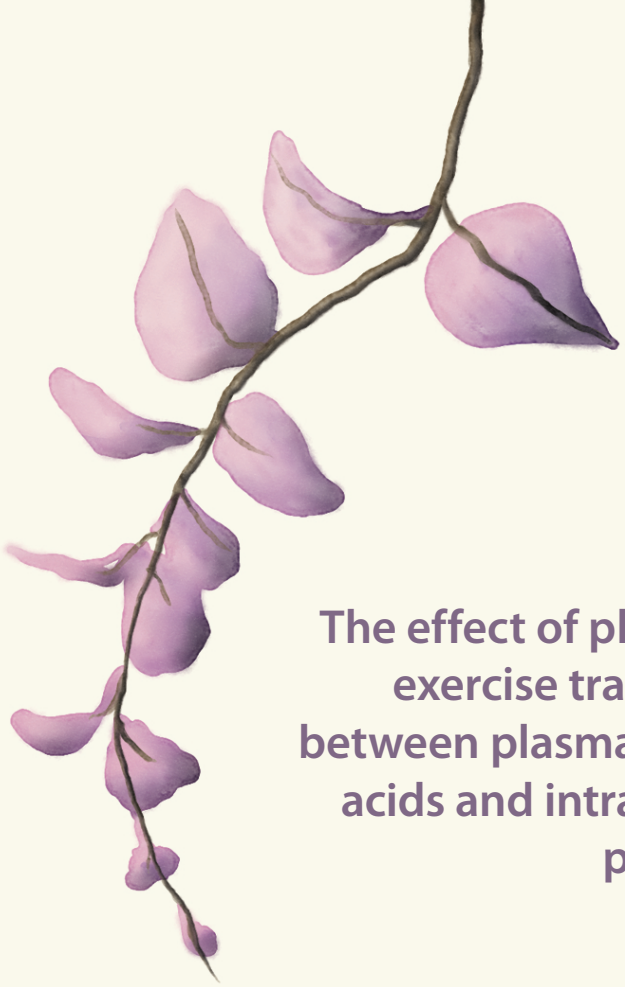
All data generated or analyzed during this study are included in this published article or in the data repositories listed in "References." All data were analyzed in a blinded fashion. The data sets generated during and/or analyzed during the study are available from the corresponding author on reasonable request.

REFERENCES

1. Huffman KM, Shah SH, Stevens RD, Bain JR, Muehlbauer M, Slentz CA, et al. Relationships Between Circulating Metabolic Intermediates and Insulin Action in Overweight to Obese, Inactive Men and Women. Diabetes Care. 2009;32(9):1678-83.
2. Newgard CB, An J, Bain JR, Muehlbauer MJ, Stevens RD, Lien LF, et al. A branched-chain amino acid-related metabolic signature that differentiates obese and lean humans and contributes to insulin resistance. Cell Metab. 2009;9(4):311-26.
3. Palmer ND, Stevens RD, Antinozzi PA, Anderson A, Bergman RN, Wagenknecht LE, et al. Metabolomic profile associated with insulin resistance and conversion to diabetes in the Insulin Resistance Atherosclerosis Study. J Clin Endocrinol Metab. 2015;100(3):E463-8.
4. Perng W, Gillman MW, Fleisch AF, Michalek RD, Watkins SM, Isganaitis E, et al. Metabolomic profiles and childhood obesity. Obesity (Silver Spring). 2014;22(12):2570-8.
5. Shah SH, Crosslin DR, Haynes CS, Nelson S, Turer CB, Stevens RD, et al. Branched-chain amino acid levels are associated with improvement in insulin resistance with weight loss. Diabetologia. 2012;55(2):321-30.
6. Tai ES, Tan ML, Stevens RD, Low YL, Muehlbauer MJ, Goh DL, et al. Insulin resistance is associated with a metabolic profile of altered protein metabolism in Chinese and Asian-Indian men. Diabetologia. 2010;53(4):757-67.
7. Walford GA, Ma Y, Clish C, Florez JC, Wang TJ, Gerszten RE, et al. Metabolite Profiles of Diabetes Incidence and Intervention Response in the Diabetes Prevention Program. Diabetes. 2016;65(5):1424-33.
8. Wurtz P, Soininen P, Kangas AJ, Ronnemaa T, Lehtimaki T, Kahonen M, et al. Branched-chain and aromatic amino acids are predictors of insulin resistance in young adults. Diabetes Care. 2013;36(3):648-55.
9. Wang TJ, Larson MG, Vasan RS, Cheng S, Rhee EP, McCabe E, et al. Metabolite profiles and the risk of developing diabetes. Nat Med. 2011;17(4):448-53.
10. Floegel A, Stefan N, Yu Z, Muhlenbruch K, Drogan D, Joost HG, et al. Identification of serum metabolites associated with risk of type 2 diabetes using a targeted metabolomic approach. Diabetes. 2013;62(2):639-48.
11. Hamley S, Kloosterman D, Duthie T, Dalla Man C, Visentin R, Mason SA, et al. Mechanisms of hyperinsulinaemia in apparently healthy non-obese young adults: role of insulin secretion, clearance and action and associations with plasma amino acids. Diabetologia. 2019;62(12):2310-24.
12. Phielix E, Jelenik T, Nowotny P, Szendroedi J, Roden M. Reduction of non-esterified fatty acids improves insulin sensitivity and lowers oxidative stress, but fails to restore oxidative capacity in type 2 diabetes: a randomised clinical trial. Diabetologia. 2014;57(3):572-81.
13. Phielix E, Meex R, Moonen-Kornips E, Hesselink M, Schrauwen P. Exercise training increases mitochondrial content and ex vivo mitochondrial function similarly in patients with type 2 diabetes and in control individuals. Diabetologia. 2010;53(8):1714-21.
14. Phielix E, Schrauwen-Hinderling VB, Mensink M, Lenaers E, Meex R, Hoeks J, et al. Lower intrinsic ADP-stimulated mitochondrial respiration underlies in vivo mitochondrial dysfunction in muscle of male type 2 diabetic patients. Diabetes. 2008;57(11):2943-9.
15. Galgani JE, Moro C, Ravussin E. Metabolic flexibility and insulin resistance. Am J Physiol Endocrinol Metab. 2008;295(5):E1009-17.
16. van de Weijer T, Sparks LM, Phielix E, Meex RC, van Herpen NA, Hesselink MKC, et al. Relationships between mitochondrial function and metabolic flexibility in type 2 diabetes mellitus. PLoS One. 2013;8(2):e51648.
17. Phielix E, Mensink M. Type 2 diabetes mellitus and skeletal muscle metabolic function. Physiology & behavior. 2008;94(2):252-8.

18. Petersen KF, Dufour S, Befroy D, Garcia R, Shulman GI. Impaired mitochondrial activity in the insulin-resistant offspring of patients with type 2 diabetes. N Engl J Med. 2004;350(7):664-71.
19. Phielix E, Schrauwen-Hinderling VB, Mensink M, Lenaers E, Meex R, Hoeks J, et al. Lower intrinsic ADP-stimulated mitochondrial respiration underlies in vivo mitochondrial dysfunction in muscle of male type 2 diabetic patients. Diabetes. 2008;57(11):2943-9.
20. Morino K, Petersen KF, Dufour S, Befroy D, Frattini J, Shatzkes N, et al. Reduced mitochondrial density and increased IRS-1 serine phosphorylation in muscle of insulin-resistant offspring of type 2 diabetic parents. J Clin Invest. 2005;115(12):3587-93.
21. Lotta LA, Scott RA, Sharp SJ, Burgess S, Luan J, Tillin T, et al. Genetic Predisposition to an Impaired Metabolism of the Branched-Chain Amino Acids and Risk of Type 2 Diabetes: A Mendelian Randomisation Analysis. PLoS Med. 2016;13(11):e1002179.
22. Mahendran Y, Jonsson A, Have CT, Allin KH, Witte DR, Jorgensen ME, et al. Genetic evidence of a causal effect of insulin resistance on branched-chain amino acid levels. Diabetologia. 2017;60(5):873-8.
23. Welle S, Nair KS. Failure of glyburide and insulin treatment to decrease leucine flux in obese type II diabetic patients. Int J Obes. 1990;14(8):701-10.
24. Halvatsiotis P, Short KR, Bigelow M, Nair KS. Synthesis rate of muscle proteins, muscle functions, and amino acid kinetics in type 2 diabetes. Diabetes. 2002;51(8):2395-404.
25. Van De Weijer T, Phielix E, Bilet L, Williams EG, Ropelle ER, Bierwagen A, et al. Evidence for a direct effect of the NAD+ precursor acipimox on muscle mitochondrial function in humans. Diabetes. 2015;64(4):1193-201.
26. Timmers S, de Ligt M, Phielix E, van de Weijer T, Hansen J, Moonen-Kornips E, et al. Resveratrol as Add-on Therapy in Subjects With Well-Controlled Type 2 Diabetes: A Randomized Controlled Trial. Diabetes Care. 2016;39(12):2211-7.
27. Timmers S, Konings E, Bilet L, Houtkooper RH, van de Weijer T, Goossens GH, et al. Calorie restriction-like effects of 30 days of resveratrol supplementation on energy metabolism and metabolic profile in obese humans. Cell Metab. 2011;14(5):612-22.
28. de Ligt M, Bruls YMH, Hansen J, Habets MF, Havekes B, Nascimento EBM, et al. Resveratrol improves ex vivo mitochondrial function but does not affect insulin sensitivity or brown adipose tissue in first degree relatives of patients with type 2 diabetes. Mol Metab. 2018;12:39-47.
29. DeFronzo RA, Tobin JD, Andres R. Glucose clamp technique: a method for quantifying insulin secretion and resistance. Am J Physiol. 1979;237(3):E214-23.
30. Koopman R, Pannemans DL, Jeukendrup AE, Gijsen AP, Senden JM, Halliday D, et al. Combined ingestion of protein and carbohydrate improves protein balance during ultra-endurance exercise. Am J Physiol Endocrinol Metab. 2004;287(4):E712-20.
31. Balagopal P, Ford GC, Ebenstein DB, Nadeau DA, Nair KS. Mass spectrometric methods for determination of (13C]Leucine enrichment in human muscle protein. Anal Biochem. 1996;239(1):77-85.
32. van den Akker CH, Schierbeek H, Minderman G, Vermes A, Schoonderwaldt EM, Duvekot JJ, et al. Amino acid metabolism in the human fetus at term: leucine, valine, and methionine kinetics. Pediatr Res. 2011;70(6):566-71.
33. van den Akker CH, te Braake FW, Wattimena DJ, Voortman G, Schierbeek H, Vermes A, et al. Effects of early amino acid administration on leucine and glucose kinetics in premature infants. Pediatr Res. 2006;59(5):732-5.
34. Wolfe RR, Goodenough RD, Wolfe MH, Royle GT, Nadel ER. Isotopic analysis of leucine and urea metabolism in exercising humans. J Appl Physiol Respir Environ Exerc Physiol. 1982;52(2):458-66.
35. Toth MJ, MacCoss MJ, Poehlman ET, Matthews DE. Recovery of (13)CO(2) from infused (1-(13)C]leucine and (1,2-(13)C(2)]leucine in healthy humans. Am J Physiol Endocrinol Metab. 2001;281(2):E233-41.

36. Allsop JR, Wolfe RR, Burke JF. Tracer priming the bicarbonate pool. J Appl Physiol Respir Environ Exerc Physiol. 1978;45(1):137-9.
37. Chevalier S, Gougeon R, Kreisman SH, Cassis C, Morais JA. The hyperinsulinemic amino acid clamp increases whole-body protein synthesis in young subjects. Metabolism. 2004;53(3):388-96.
38. Phielix E, Jelenik T, Nowotny P, Szendroedi J, Roden M. Reduction of non-esterified fatty acids improves insulin sensitivity and lowers oxidative stress, but fails to restore oxidative capacity in type 2 diabetes: a randomised clinical trial. Diabetologia. 2014;57(3):572-81.
39. van de Weijer T, Phielix E, Bilet L, Williams EG, Ropelle ER, Bierwagen A, et al. Evidence for a direct effect of the NAD+ precursor acipimox on muscle mitochondrial function in humans. Diabetes. 2015;64(4):1193-201.
40. van Eijk HM, Wijnands KA, Bessems BA, Olde Damink SW, Dejong CH, Poeze M. High sensitivity measurement of amino acid isotope enrichment using liquid chromatography-mass spectrometry. J Chromatogr B Analyt Technol Biomed Life Sci. 2012;905:31-6.
41. Wang Q, Holmes MV, Davey Smith G, Ala-Korpela M. Genetic Support for a Causal Role of Insulin Resistance on Circulating Branched-Chain Amino Acids and Inflammation. Diabetes Care. 2017;40(12):1779-86.
42. Lynch CJ, Adams SH. Branched-chain amino acids in metabolic signalling and insulin resistance. Nat Rev Endocrinol. 2014;10(12):723-36.
43. Mogensen M, Sahlin K, Fernstrom M, Glintborg D, Vind BF, Beck-Nielsen H, et al. Mitochondrial respiration is decreased in skeletal muscle of patients with type 2 diabetes. Diabetes. 2007;56(6):1592-9.
44. Kelley DE, He J, Menshikova EV, Ritov VB. Dysfunction of mitochondria in human skeletal muscle in type 2 diabetes. Diabetes. 2002;51(10):2944-50.
45. Neinast MD, Jang C, Hui S, Murashige DS, Chu Q, Morscher RJ, et al. Quantitative Analysis of the Whole-Body Metabolic Fate of Branched-Chain Amino Acids. Cell Metab. 2019;29(2):417-29 e4.
46. Fukagawa NK, Minaker KL, Rowe JW, Goodman MN, Matthews DE, Bier DM, et al. Insulin-mediated reduction of whole body protein breakdown. Dose-response effects on leucine metabolism in postabsorptive men. J Clin Invest. 1985;76(6):2306-11.
47. Burgos SA, Chandurkar V, Tsoukas MA, Chevalier S, Morais JA, Lamarche M, et al. Insulin resistance of protein anabolism accompanies that of glucose metabolism in lean, glucose-tolerant offspring of persons with type 2 diabetes. BMJ Open Diabetes Res Care. 2016;4(1):e000312.
48. Glynn EL, Piner LW, Huffman KM, Slentz CA, Elliot-Penry L, AbouAssi H, et al. Impact of combined resistance and aerobic exercise training on branched-chain amino acid turnover, glycine metabolism and insulin sensitivity in overweight humans. Diabetologia. 2015;58(10):2324-35.
49. Matthews DE. Observations of branched-chain amino acid administration in humans. J Nutr. 2005;135(6 Suppl):1580S-4S.
50. Tan HC, Hsu JW, Khoo CM, Tai ES, Yu S, Chacko S, et al. Alterations in branched-chain amino acid kinetics in nonobese but insulin-resistant Asian men. Am J Clin Nutr. 2018;108(6):1220-8.



4

The effect of physical activity level and exercise training on the association between plasma branched-chain amino acids and intrahepatic lipid content in participants with obesity

Froukje Vanweert[1], Sebastiaan C. Boone[2], Bram Brouwers[1], Dennis O. Mook-Kanamori[2], Renée de Mutsert[2], Frits R. Rosendaal[2], Hildo J. Lamb[2], Vera B. Schrauwen-Hinderling[1,3], Patrick Schrauwen[1], Matthijs K.C. Hesselink[1] and Esther Phielix[1]

1 Department of Nutrition and Movement Sciences, NUTRIM School of Nutrition and Translational Research in Metabolism, Maastricht University, Maastricht, The Netherlands

2 Department of Clinical Epidemiology, Leiden University Medical Center, Leiden, The Netherlands

3 Department of Radiology and Nuclear Medicine, Maastricht University Medical Center, Maastricht, The Netherlands

This chapter has been published in: Int J Obes (Lond). 2021. May

ABSTRACT

Aims: To evaluate whether the association between plasma branched-chain amino acids (BCAA) and intrahepatic lipid (IHL) was affected by physical activity level. Furthermore, to investigate if a conventional exercise training program, a subcategory of physical activity, could lower plasma BCAA along with alterations in IHL content in patients with type 2 diabetes (T2D) and people with non-alcoholic fatty liver (NAFL).

Methods: To investigate the effect of physical activity on the association between plasma BCAA and IHL content, linear regression analyses were performed in 1 983 individuals from the Netherlands Epidemiology of Obesity (NEO) stratified by physical activity frequency. Furthermore, the effect of a 12-week supervised combined aerobic resistance-exercise program on plasma BCAA, insulin sensitivity (hyperinsulinemic-euglycemic clamp) and IHL (proton-magnetic resonance spectroscopy (^{1}H-MRS)) was investigated in seven patients with T2D, seven individuals with NAFL and seven BMI-matched control participants (CON).

Results: We observed positive associations between plasma valine, isoleucine and leucine level and IHL content (1.29 (95%CI:1.21, 1.38), 1.52 (95%CI:1.43, 1.61), and 1.54 (95%CI:1.44, 1.64) times IHL, respectively, per standard deviation of plasma amino acid level). Similar associations were observed in less active versus more active individuals. Exercise training did not change plasma BCAA levels among groups, but reduced IHL content in NAFL (from 11.6% ± 3.0% pre-exercise to 8.1% ± 2.0% post-exercise, $p<0.05$) and CON (from 2.4% ± 0.6% pre-exercise to 1.6% ± 1.4% post-exercise, $p<0.05$), and improved peripheral insulin sensitivity in NAFL as well by ~23% ($p<0.05$).

Conclusions: The association between plasma BCAA levels and IHL is not affected by physical activity level. Exercise training reduced IHL without affecting plasma BCAA levels in individuals with NAFL and CON. We conclude that exercise training-induced reduction in IHL content is not related to changes in plasma BCAA levels.

INTRODUCTION

Type 2 diabetes patients are characterized by the presence of high levels of branched-chain amino acids (BCAA) in plasma (1-6). In addition, elevated systemic BCAA levels have been reported prior to the actual onset of type 2 diabetes (5, 7). Furthermore, the accumulation of plasma BCAA levels is strongly associated with insulin resistance (1-4, 8-11) and mitochondrial dysfunction (12, 13). It is not known why plasma BCAA are elevated. Some studies relate a higher protein intake that characterize the Western diet, to the increase of BCAA in plasma (2), however, others found no relationship (14, 15). In addition, it has been suggested that elevated plasma BCAA in individuals with T2D originates from a blunted inhibitory effect of insulin on proteolysis (16-18) and/or a compromised mitochondrial BCAA metabolism (19-22).

Recently, a cross-sectional study in the Young Finns Study cohort including 338 middle-aged, individuals with overweight/obesity, reported a positive association between plasma BCAA and intrahepatic lipid (IHL) content (23). Iwasa et al. also described a positive association between plasma BCAA levels and IHL content (24). Additionally, they observed that plasma BCAA levels and fasting glucose levels decreased upon initiation of glucose-lowering therapy (24), but it was not investigated whether the lowering of plasma BCAA levels was associated with a lowering of IHL content. In addition, Kaikkonen et al. showed in a 10-year prospective study that changes in plasma BCAA levels may predict development of non-alcoholic fatty liver (NAFL) in the Young Finns cohort (25). The mechanism of elevated BCAA levels leading to hepatic fat accumulation is still unknown. However, animal research point towards BCAA-stimulated hepatic lipogenesis via activation of lipogenic genes, such as SREBP-1c, FAS, and ACC(26, 27). Taken together, we hypothesize that elevated plasma BCAA levels could be a contributing factor to insulin resistance, possibly via modulation of IHL content.

We, as well as others, have previously reported that physical activity and/or exercise training is an effective strategy to improve insulin sensitivity and to reduce IHL content. It is, however, never investigated whether the reduction in IHL relates to a reduction in plasma BCAA levels. Physical activity is defined as movement that require energy expenditure, including walking, cycling sports and recreation. Exercise training, however, is a subcategory of physical activity that is planned, structured and repetitive aiming to improve or maintain physical fitness (28). Whether physical activity and/or exercise affect plasma BCAA levels is still unknown. An observational study showed an association between high physical activity level and low plasma BCAA levels (29). In contrast, a 12-week endurance

and resistance exercise training study did not result in reduced BCAA levels, while insulin sensitivity did improve (30). In these studies, IHL content has not been measured.

Based on the strong association between plasma BCAA and IHL content and considering that physical activity level or exercise training may affect both plasma BCAA and IHL, we aimed to investigate whether the association between plasma BCAA and IHL was affected by physical activity level. Furthermore, we investigated if a conventional exercise training program could lower plasma BCAA levels along with alterations in IHL in patients with T2D and in people with NAFL. We hypothesized that moderate to high physical activity and exercise training will result in lower plasma BCAA levels paralleled by a decreased IHL content.

MATERIALS AND METHODS

Cross-sectional study

Analyses were performed in 1 983 individuals from the Netherlands Epidemiology of Obesity (NEO) study. The NEO study includes middle-aged (45-65 year) individuals, with an oversampling of individuals with overweight and obesity. At baseline, information on demography, lifestyle and medical history were collected. Physical activity was self-reported using the Short QUestionnaire to ASsess Health enhancing physical activity (SQUASH). Additionally, fasting blood samples were collected, and IHL content was quantified by proton-magnetic resonance spectroscopy (^{1}H-MRS) on a 1.5 T whole-body scanner (Philips Medical Systems, Best, the Netherlands) as previously described (31). The data was collected from September 2008 until September 2012 (31). In 2015, plasma amino acid levels were measured in stored samples (-80°C) by the Nightingale nuclear magnetic resonance (NMR) platform and levels were standardized (i.e. mean of zero and standard deviation of one for each metabolite). The Medical Ethical Committee of the LUMC approved the study and all participants gave their written informed consent.

Intervention study

Participants

We analyzed BCAA levels in plasma samples derived from the participants previously enrolled in the exercise intervention study as published by Brouwers et al. (32). In our analysis, we selected 7 participants with NAFL, 7 patients with T2D and 7 BMI-matched control participants (CON), all males, based on exercise-induced changes in IHL. Participants had no signs of active cardiac disease, impaired renal or hepatic function (32). NAFL was defined as having an IHL content of ≥5.0%

(33), measured with ^{1}H-MRS, and a fasting plasma glucose (FPG) concentration of <7.0mmol/l. Patients with T2D were treated with oral glucose lowering agents solely and for at least 6 months prior to the onset of the study. Participants were sedentary and maintained their regular dietary behavior throughout the study. Other inclusion criteria were as previously described (32). All data was collected at the Maastricht University Medical Center, Maastricht, The Netherlands. The study was approved by the local ethics committee and carried out in compliance with the Declaration of Helsinki.

Exercise training protocol

The 12-week combined progressive aerobic and resistance exercise training program, previously performed within our research group (32), included three exercise session per week: two times a week aerobic cycling for 30 min at 70% of maximal work load (W_{max}) and once a week resistance exercise including three series of 10 repetitions at 60% of the maximal voluntary contraction (MVC) with focus at large muscle groups (chest press, leg extension, lat pull down, leg press, triceps curls, biceps curls, abdominal crunched, and horizontal row). MVC was predicted from five multiple repeated maximum testing, as previously described by Reynolds et al. (34). Total muscle strength was calculated as the sum of the predicted MVC for all eight muscle groups. Warming-up and cooling-down cycling sessions (5 min) were performed at 45% of W_{max} before and after every exercise session. The progressive exercise training program was re-assessed for W_{max} and MVC after 6 and 4 weeks, respectively, as previously described (35). Training sessions were performed with three to four individuals at a time.

Test day including 1H-MRS and 2-step clamp

The test days were performed 4 days before the start of the exercise training protocol and between 48-72h after the last exercise bout, as previously reported (32). In the morning of the test day all participants underwent ^{1}H-MRS on a 3 T whole body scanner (Achieva 3Tx; Philips Healthcare, Best, The Netherland) to measure IHL content. Then, a primed continuous infusion of (6,6-$^{2}H_{2}$]glucose (0.04 $mg \cdot kg^{-2} \cdot min^{-1}$) was started after which at t=180 min the 2-step hyperinsulinemic-euglycemic clamp (10 and 40 $mU \cdot m^{-2} \cdot min^{-1}$) was started with target glucose value of 5 – 5.5 mmol/L (Supplemental Figure 1).

The 10 $mU \cdot m^{-2} \cdot min^{-1}$ insulin infusion step was performed for 4h to assess hepatic insulin sensitivity and the 40 $mU \cdot m^{-2} \cdot min^{-1}$ step for 2h to measure peripheral insulin sensitivity. Steele's single-pool non-steady state equations were used to calculate glucose rate of appearance (Ra) and glucose rate of disappearance (Rd) (36). Endogenous glucose production (EGP) was calculated as R_a minus

exogenous glucose infusion rate (GIR). Hepatic insulin sensitivity was computed as percent insulin-suppressed EGP during the 10 mU m^{-2} min^{-1} low-dose insulin phase. Peripheral insulin sensitivity was measured by insulin-stimulated glucose disposal (R_d) under the 40 mU m^{-2} min^{-1} high-dose insulin phase. Due to technical difficulties, ΔRd and insulin-suppressed EGP were determined in six patients with T2D and six individuals with NAFL. Total plasma aromatic amino acid (AAA) levels were computed as the sum of phenylalanine, tryptophan and tyrosine level. Total plasma BCAA levels consist of the sum of valine, isoleucine and leucine level. The insulin-suppressive effect on total plasma BCAA levels (in %) was calculated as the difference in total plasma BCAA levels during the high- and low-dose insulin phase. Further details of the clamp test were previously reported (32).

Laboratory analysis

Arterialized blood samples were collected and immediately centrifuged at high speed. Plasma was frozen in liquid nitrogen and stored at -80°C until assayed. Amino acid levels were determined as previously described (37). In short, acetonitrile (200 ul) was added to 100 ul plasma for deproteinization, vortex-mixed and analysed by liquid chromatography–mass spectrometry (LCMS) for the measurement of concentrated amino acid peaks. This approach, as well as analysis sensitivity, was well validated as previously reported (37). Calibration curves were constructed to determine the linear ranges of levels.

Statistics

Cross-sectional study

To correct for the oversampling of individuals with a high BMI, all analyses in the NEO study were weighted towards the BMI distribution of the general Dutch population. First, we estimated the difference in amino acid levels between men and women and between a low vs. high physical active group (i.e. less or more than two times per week at least 30 minutes of moderate intensity) using linear regression analysis. Second, we performed linear regression analyses with fasting plasma amino acid levels as exposure and ln-transformed IHL content as outcome, adjusting for age, sex, total body fat, alcohol intake, energy intake and leisure time physical activity. In addition, we tested for interaction with sex and physical activity by adding product terms of plasma amino acid levels with sex and physical activity in the regression models. All reported regression coefficients were back transformed and represent relative changes in IHL content per standard deviation of plasma amino acid levels. Participant characteristics are expressed as mean ± standard deviation (SD) for normally distributed data or median with interquartile ranges (IQR) for non-normally distributed data, and differences were assumed to be significant when $p \leq 0.05$.

Exercise intervention study

Baseline comparisons between the three groups were computed with a one-way ANOVA. Linear mixed model analyses were used to analyze differences among groups before and upon exercise. Bonferroni post-hoc testing was performed to correct for multiple testing. Pearson correlations were used to evaluate associations between parameters. Sample size calculation was calculated with an α of 0.05 and a power of 80% using the formula of correlation sample size ($Z_{0.05}$ = 1.9600 and $Z_{0.8}$ = 0.8416). Based on the study of Sliz et al. (23), the correlation coefficient is expected to be r = 0.57. With the formula, we calculated a sample size of 22 participants. Participant characteristics are presented as mean ± SD. The (insulin-suppressed) plasma amino acids, IHL, Rd and EGP are presented as mean ± SEM and differences were assumed to be significant when p≤0.05. All analyses were computed with SPSS software for MacOS or Windows.

RESULTS

Cross-sectional study

In the cross-sectional analyses, we examined 1983 participants whose characteristics are reported in Supplemental Table 1. The mean plasma levels of isoleucine, leucine and valine were 50.3 ± 14.7 mmol/l, 66.0 ± 13.7 mmol/l, and 153.9 ± 27.6 mmol/l respectively in the 1983 individuals of the NEO study. The correlation coefficients between dietary intake of protein, derived from food frequency questionnaires, and amino acids were close to zero, indicating no relation with plasma levels of isoleucine (r=-0.09, p=0.004), leucine (r=-0.06, p=0.061), valine (r=0.03, p=0.391), phenylalanine (r=0.03, p=0.414) , tyrosine (r=-0.05, p=0.120) and histidine (r=-0.01, p=0.786). The median IHL content in men was higher than in women (Supplemental Table 1). Median IHL content in individuals who performed 30 minutes of moderate intensity activity less than two times per week was 3.3% (IQR: 1.6-7.7%) compared with 2.0% (IQR: 1.2-5.6%) in those who performed such physical activity two or more times per week.

On average, individual BCAA concentrations were significantly higher in men than in women (p<0.05 for all three amino acids). Plasma BCAA levels were positively associated with IHL content after adjusting for age, sex, total body fat, alcohol intake, energy intake and leisure time physical activity. In women, the association between BCAA levels and IHL content was somewhat stronger (p-value for interaction <0.05 for all three amino acids), than in men (Supplemental Table 2).

Upon stratification by physical activity frequency, levels of isoleucine, leucine and valine were 51.9 ± 16.2 mmol/l, 67.1 ± 14.8 mmol/l, and 155.0 ± 29.9 mmol/l, respectively, in less active individuals and 48.1 ± 12.3 mmol/l, 64.6 ± 12.1 mmol/l, and 152.3 ± 24.3 mmol/l, respectively, in more active individuals. Levels of isoleucine and leucine were significantly lower ($p<0.001$ and $p<0.005$, respectively) in more active individuals compared to less active individuals. The associations between BCAAs and IHL content were similar in individuals with less or more than two times per week at least 30 minutes of moderate intensity activity (Table 1).

Table 1. Relative change in IHL content and 95% confidence intervals per SD of plasma amino acid level in participants of the NEO study (45 to 65 years), stratified by frequency of physical activity.

	Physical activity frequency		*P* value for interaction
	< 2 times per week (n=1 307)	≥ 2 times per week (n=676)	
Isoleucine	1.52 (1.43, 1.62)	1.49 (1.33, 1.67)	0.698
Leucine	1.54 (1.43, 1.66)	1.53 (1.39, 1.69)	0.945
Valine	1.28 (1.19, 1.37)	1.32 (1.20, 1.46)	0.529
Phenylalanine	1.13 (1.05, 1.22)	1.15 (1.04, 1.27)	0.745
Tyrosine	1.29 (1.21, 1.38)	1.34 (1.21, 1.48)	0.512
Histidine	1.03 (0.97, 1.10)	1.03 (0.93, 1.14)	0.927

Linear regression analysis including standardized fasting plasma BCAA and aromatic amino acids levels as exposure and log-transformed IHL content as outcome, and were weighted towards the BMI distribution of the general population. Model adjusted for age, sex, body fat %, alcohol and energy intake as well as for leisure time physical activity and include an interaction term between the frequency of physical activity and amino acid concentration. The regression coefficients represent relative changes in IHL content per SD of plasma amino acid level. Such ratio, for example 1.2, can be interpreted as 1.2 times IHL content for each extra SD in amino acid concentration, which would reflect an increase in IHL content from, for example, 5% to 6%. Asterisks (*) indicate a significant interaction with frequency of physical activity.

Intervention study

Baseline characteristics

Baseline characteristics of participants are reported in Supplemental Table 3. The groups did not differ significantly in age, BMI, fasting plasma FFA, aspartate aminotransferase, alanine aminotransferase, glutamyl transferase, high density lipoprotein, C-reactive protein, $VO_{2\ max,}$ and $W_{max.}$ Data on compliance and exercise training results were previously reported (32).

IHL content was significantly different among groups (Anova $p=0.012$). By design, people with NAFL had high IHL content (≥ 5%). Patients with T2D had higher IHL content compared to CON (Figure 1a, $p<0.05$). IHL content was not significantly different between NAFL and patients with T2D (Figure 1a). Baseline peripheral

insulin sensitivity (Figure 1b) and hepatic insulin sensitivity (Figure 1c) were also significantly different between groups (Anova p<0.0001 for both). Specifically, they were ~60% and ~38%, respectively, higher in CON compared to NAFL and patients with T2D (p<0.05 for all). Glucose values throughout the clamp are shown in Supplemental Figure 1.

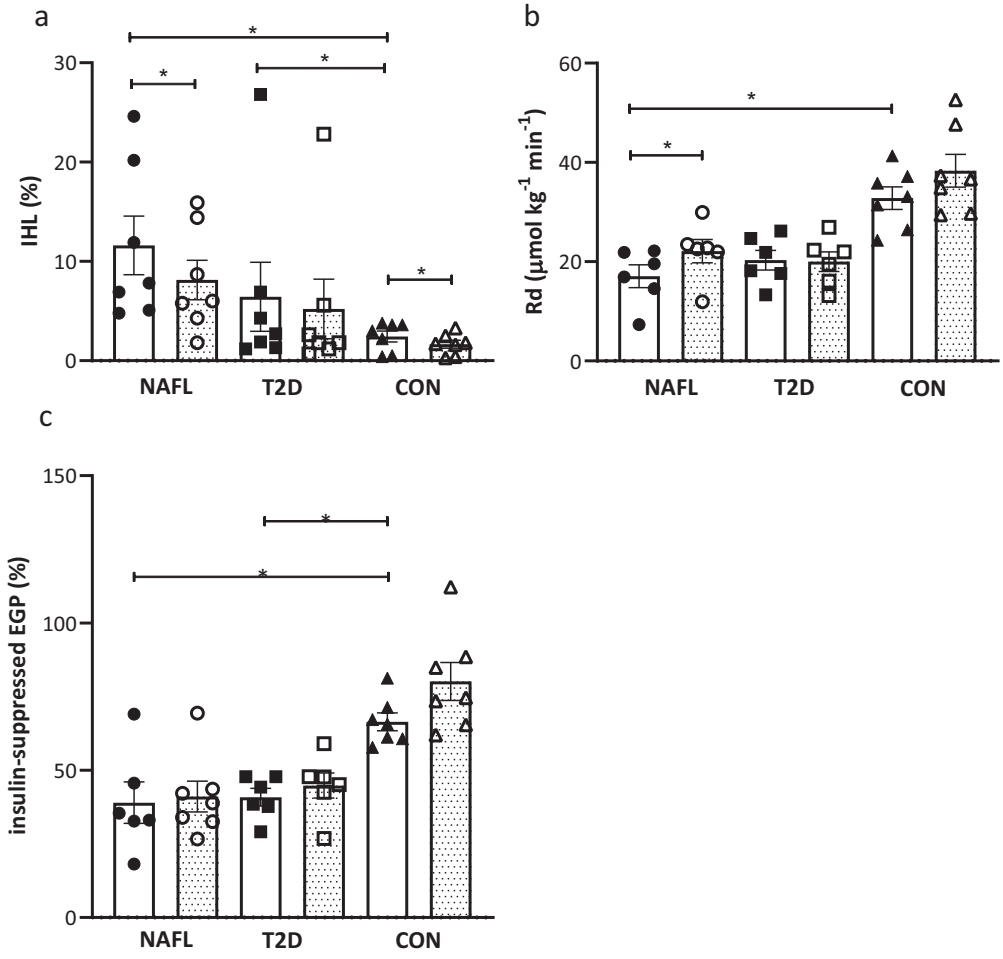


Figure 1. Changes in IHL content, peripheral and hepatic insulin sensitivity after exercise training: IHL content (%) (a), peripheral insulin sensitivity expressed as the insulin-stimulated change in glucose uptake (ΔRd mmol kg^{-1} min^{-1}) (b) and hepatic insulin sensitivity expressed as the insulin-suppressed EGP (%) (c) before (open bars) vs after (dotted bars) exercise training in people with NAFL, T2D, and CON.

IHL was determined in n=7 T2D, n=7 NAFL and n=7 CON, however due to technical difficulties, ΔRd were determined in n=6 T2D and n=6 NAFL. Data are expressed as mean ± SEM and tested using Anova for repeated measurements; * p< 0.05. *CON, control participants; EGP, endogenous glucose production; IHL, intrahepatic lipid content; NAFL, non-alcoholic fatty liver; Rd, rate of disappearance; T2D, type 2 diabetes mellitus.*

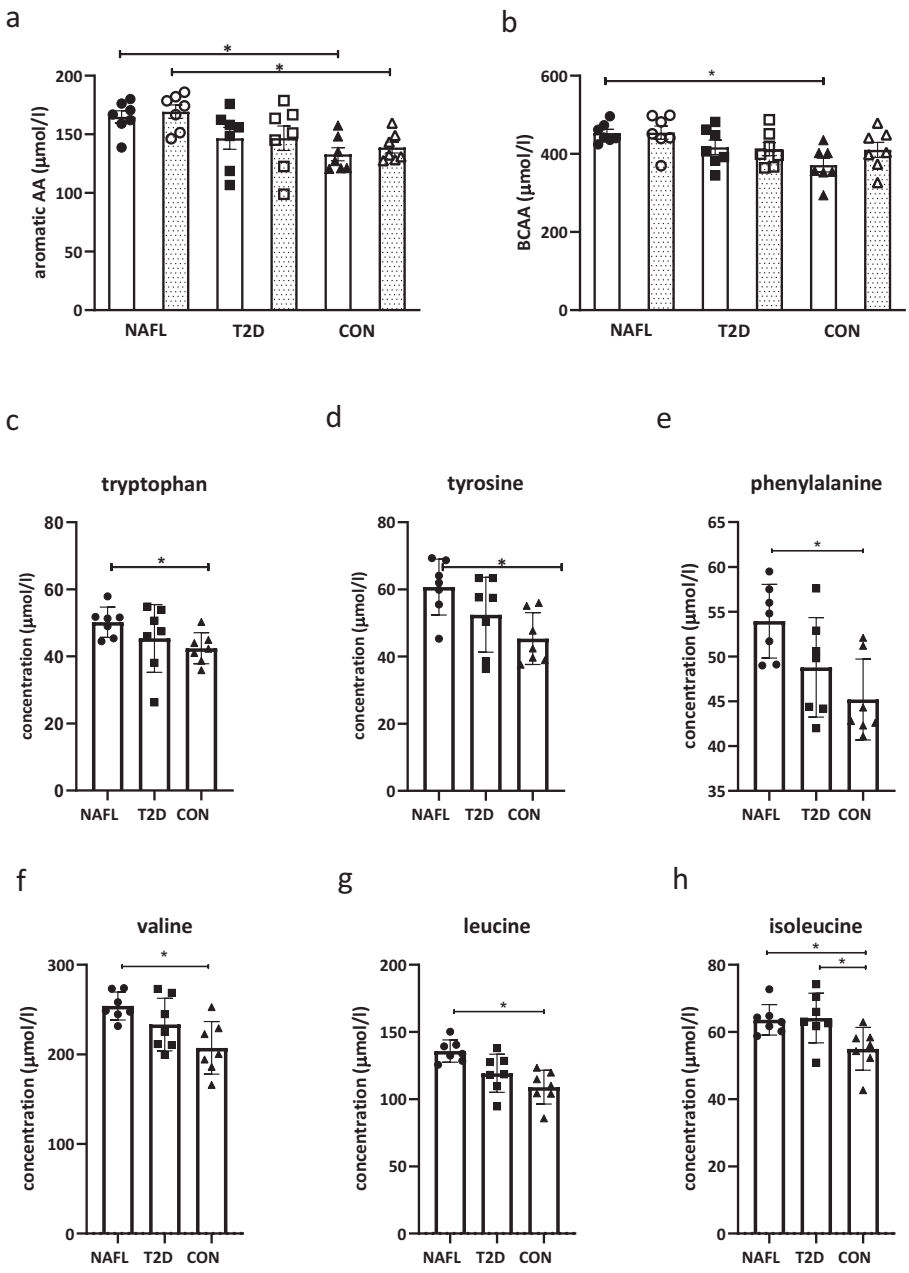


Figure 2. Changes in fasting total AAA and total BCAA levels after exercise training.

Fasting total AAA (including phenylalanine, tryptophan and tyrosine) (a) and total BCAA (including leucine, isoleucine and valine) (b) measured in plasma before (open bars) and after (dotted bars) the exercise program. Pre-exercise fasting plasma levels of tryptophan (c), tyrosine (d) and phenylalanine (e), valine (f), leucine (g) and isoleucine (h). People with NAFL (dots, n=7), patients with T2D (squares, n=7) and in CON (triangles, n=7). Data are expressed as mean ± SEM and tested using Anova for repeated measurements; * $p < 0.05$. *AA, amino acids; BCAA, branched-chain amino acids; CON, control participants; EGP, endogenous glucose production; IHL, intrahepatic lipid content; NAFL, non-alcoholic fatty liver; Rd, rate of disappearance; T2D, type 2 diabetes mellitus.*

Figure 2 shows fasting plasma levels for total plasma AAA (a) and total BCAA (b) levels in people with NAFL, patients with T2D and the CON. Data on essential amino acids are shown in Supplemental Figure 2. Total plasma AAA levels were significantly different among groups (Anova $p=0.015$), with higher levels in NAFL compared to CON (Figure 2a, $p<0.05$). Also, total BCAA levels were significantly different among groups (Anova $p=0.006$), with significant elevated levels in NAFL compared to CON (Figure 2b, $p<0.05$). Of the AAA, plasma tryptophan (Figure 2c, Anova $p=0.05$), tyrosine (Figure 2d, Anova $p=0.02$) and phenylalanine (Figure 2e, Anova $p=0.01$) were all different between groups with higher levels in NAFL compared to CON ($p<0.05$ for all). Of the BCAA, plasma valine (Figure 2f, Anova $p=0.011$), leucine (Figure 2g, Anova $p=0.002$) and isoleucine (Figure 2h, Anova $p=0.02$) also differed among groups with higher levels in NAFL compared to CON ($p<0.05$ for all). Plasma isoleucine was also higher in patients with T2D compared to the CON (Figure 2h, $p<0.05$).

Associations between fasting plasma BCAA levels, IHL content and insulin sensitivity

A positive correlation was found between fasting plasma BCAA levels and IHL content (Figure 3a, $p<0.05$). Peripheral insulin sensitivity expressed as insulin-stimulated glucose disposal correlated negatively with BCAA levels (Figure 3b, $p<0.05$). Furthermore, hepatic insulin sensitivity, expressed as insulin-suppressed EGP, strongly correlated with total BCAA levels (Figure 3c, $p<0.01$), as well with all separate BCAA (isoleucine $r=0.62$, $p<0.01$, valine $r=0.54$, $p<0.01$ and leucine $r=0.56$, $p<0.05$). These results indicate that people with high IHL content and those with low peripheral and hepatic insulin sensitivity feature highest plasma BCAA levels.

Differences in the insulin-suppressive effect on plasma BCAA levels among groups

During the clamp, the insulin-suppressive effect on plasma BCAA levels was determined as the change of plasma BCAA levels during the high insulin phase vs. levels measured at basal, expressed as percentage suppression. Insulin suppression for isoleucine (Figure 4a) and leucine (Figure 4b) differed between groups (Anova $p=0.004$ and $p=0.001$, respectively) and was larger in CON compared to both NAFL and patients with T2D ($p<0.05$ for both). Insulin suppression of valine (Figure 4c) differed as well between groups (Anova $p=0.01$), with lower suppression values seen in NAFL vs. CON ($p<0.05$).

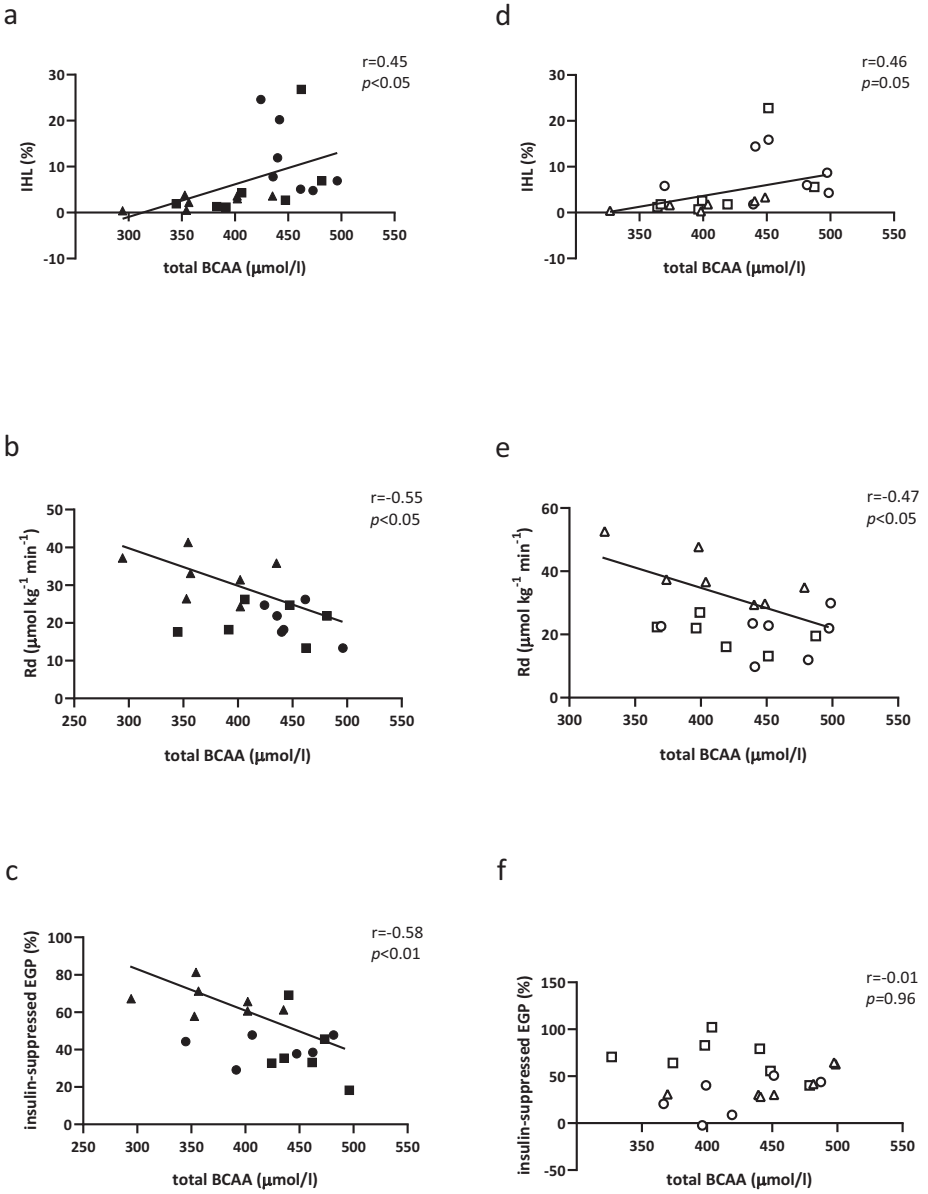


Figure 3. Pearson correlations between fasting total BCAA levels and IHL content, peripheral and hepatic insulin sensitivity.

Pre-exercise training (closed symbols): Pearson correlations between plasma BCAA (mmol/l) levels and (a) IHL content (%), (b) peripheral insulin sensitivity expressed as the change in insulin-stimulated glucose disposal (ΔRd mmol kg^{-1} min^{-1}) and (c) with the insulin-suppressed EGP (%). Post-exercise (open symbols): correlations between plasma BCAA levels and (d) IHL content, (e) peripheral insulin sensitivity and (f) with insulin-suppressed EGP (%). Correlations based on the whole group including people with NAFL (dots, n=7), T2D (squares, n=7) and CON (triangles, n=7), however, due to technical differences only n=6 T2D and n=6 NAFL were included for the association with ΔRd and insulin-suppressed EGP. *BCAA, branched-chain amino acids; EGP, endogenous glucose production; IHL, intrahepatic lipid content; NAFL, non-alcoholic fatty liver; Rd, rate of disappearance.*

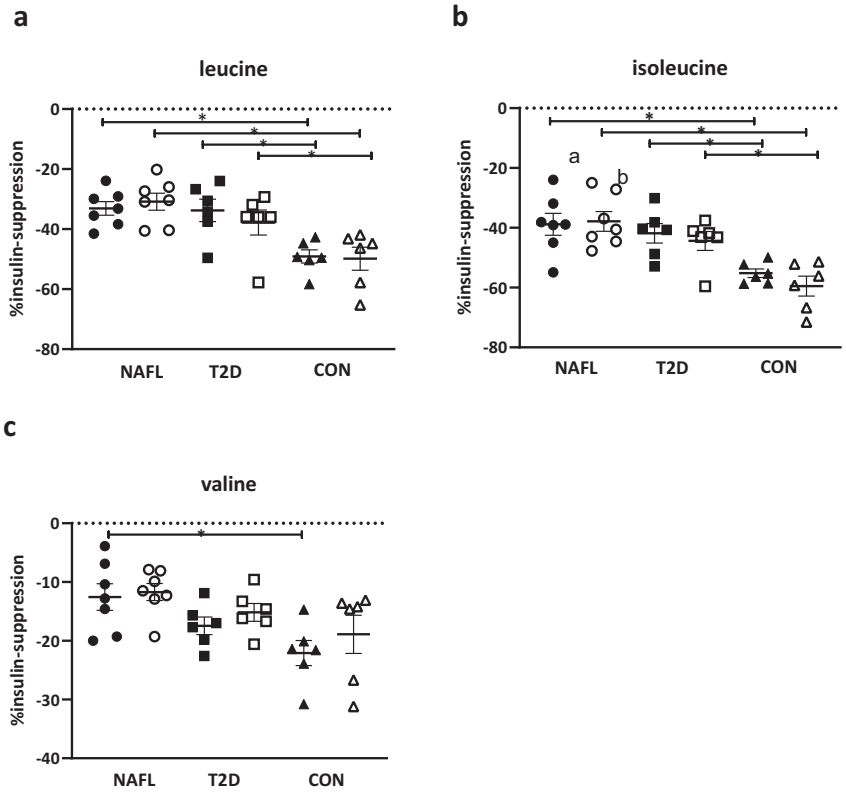


Figure 4. Changes in the insulin-suppressive effect on fasting BCAA levels after exercise training.

The percentage of insulin-suppressed leucine (a), isoleucine (b) and valine (c) measured in fasting plasma before (solid symbols) and after exercise training (open symbols) in people with NAFL (NAFL, dots), people with patients with type 2 diabetes (T2D, squares) and control participants (CON, triangles). Due to technical difficulties, the percentage of insulin-suppressed leucine was determined in n=6 T2D, n=6 NAFL and n=7 CON. Data are expressed as mean ± SEM and tested using Anova for repeated measurements; * p< 0.05. *CON, control participants; NAFL, non-alcoholic fatty liver; T2D, type 2 diabetes mellitus.*

Exercise training does not affect AAA and BCAA levels

Exercise training had no effect on total plasma AAA (Figure 2a) or total BCAA (Figure 2b) levels in NAFL, patients with T2D, or in CON. Also when groups were pooled, we did not observe exercise-induced changes in total AAA (148 ± 5 mmol/l pre-exercise vs. 152 ± 5 mol/l post-exercise) neither in total BCAA (414 ± 11 mmol/l pre-exercise vs. 425 ± 11 mmol/l post-exercise). However, upon exercise, BCAA levels between the groups lost significance (Supplemental Figure 3a). These absent differences in BCAA concentration between groups upon exercise training were not caused by altered levels in T2D or NAFL, but due to a slight, non-significant increase in the CON (Supplemental Figure 3b, c, d and e).

Exercise-mediated effects on IHL content and insulin sensitivity in people with NAFL

Exercise training mediated a reduction in IHL content in NAFL ($p<0.05$) and in CON ($p<0.05$), but not in patients with T2D (Figure 1a, p-value Anova repeated measures <0.0001). Exercise training improved peripheral insulin sensitivity in NAFL ($p<0.05$), but not in people with T2D nor in CON (Figure 1b, p-value Anova repeated measures =0.019). Hepatic insulin sensitivity did not significantly change with exercise training (Figure 1c, Anova p-value for repeated measures = 0.086). After exercise training, the associations between plasma BCAA levels with IHL content (Figure 3d, $p=0.05$) and peripheral insulin sensitivity (Figure 3e, $p<0.05$) persisted, however, no correlation was seen between plasma BCAA levels and peripheral insulin sensitivity (Figure 3f, $p=0.96$).

Exercise training did not improve the suppressive effect of insulin on plasma BCAA levels

Exercise training had no effect on the insulin-suppressive effect on plasma leucine (Figure 4a), isoleucine (Figure 4b) and valine (Figure 4c) in patients with T2D, individuals with NAFL, nor in CON. After exercise training, the insulin-suppressive effect on plasma BCAA levels remained highest in CON compared to both NAFL and patients with T2D ($p<0.05$ for both).

DISCUSSION

We here aimed to explore the relationship between plasma BCAA levels and IHL content based on data from a Dutch population-based study and a 12-week exercise intervention program performed in patients with T2D, NAFL and CON. We hypothesized that a conventional exercise training program lowers plasma BCAA levels along with alterations in IHL content in patients with T2D and in people with NAFL. Our results indicate that the association between plasma BCAA levels and IHL content is not affected by physical activity level and/or exercise training. Exercise training reduced IHL content without affecting plasma BCAA levels in individuals with NAFL and CON. We conclude that exercise training does not change plasma BCAA levels, despite reductions in IHL content.

In the present study, we aimed to investigate the relationship between plasma BCAA levels and IHL content and therefore compared amino acid levels between people with NAFL, patients with T2D and a BMI-matched CON. We found that both AAA and BCAA levels were higher in people with NAFL, who had the highest IHL content, compared to both T2D and CON. In line with this data, a recent study

demonstrated elevated plasma BCAA levels in people with NAFL and concluded that elevated plasma BCAA levels play a role in the progression from NAFL towards the development of type 2 diabetes (38). Therefore, it seems plausible that both elevated plasma BCAA levels and IHL content may impact insulin sensitivity.

Currently, it is not known why plasma BCAA levels are elevated in insulin resistant people, in patients with T2D or in people with NAFL. In theory, a higher protein intake that characterize the Western diet, combined with a mismatch in BCAA oxidation could result in an accumulation of BCAA in plasma. There are reports with of a self-administered food frequency questionnaire, protein consumption seemed to be slightly higher in people with obesity when compared to lean participants (2). Others reported that the overall dietary pattern, rather than the dietary intake of BCAA per se, contributes to high BCAA plasma concentrations thereby modulating the risk for chronic diseases (14, 39). Importantly, using protein intake derived from food frequency questionnaires in the Dutch NEO cohort showed no relation between dietary intake of protein and BCAA levels. Several reports point however towards diminished or altered function of the key enzymes involved in BCAA catabolism, indicating that disturbances in BCAA metabolism could underlie the elevated plasma levels observed (19-22). Since insulin inhibits amino acid release by muscle, insulin resistance towards this inhibition may underlie higher plasma BCAA levels in patients with T2D and humans with NAFL (40). In line, results from the present study showed a ~15% reduced peripheral suppression of plasma BCAA levels under hyperinsulinemic conditions in both patients with T2D and in people with NAFL when compared to CON. Whether the resistance towards insulin-suppression of plasma BCAA indeed contributes to elevated levels seen upon an overnight fast, cannot be deduced from the current study.

In the present study, we observed a positive association between plasma BCAA levels and IHL content in 1 983 participants of a Dutch population-based study. This is in in line with the results of a cross-sectional analysis in the Young Finns Study cohort including 338 middle-aged individuals with overweight and obesity (23). The association between plasma BCAA levels and IHL content was also supported in a smaller group of people who participated in the exercise intervention study. Whether elevated plasma BCAA contribute to the development of NAFL cannot be concluded from this study. A large prospective study of Kaikkonen et al. (25) points out that higher plasma BCAA levels precede the onset of NAFL in healthy young individuals (25). Therefore, it is plausible that elevated plasma BCAA levels could contribute to a high IHL content. In line, a recent observational study evaluated relations of plasma BCAA levels and NAFL with incident type 2 diabetes (38). It was found that elevated BCAA levels in part mediated the associations between

NAFL and type 2 diabetes. This suggests that both elevated BCAA levels and IHL content play a role in type 2 diabetes development (38), however, causality cannot be concluded from the present association analysis and needs to be further investigated.

We found that physically active people (≥ 2 times per week moderate intensity exercise for at least 30 min) had slightly, but significant lower plasma BCAA levels. The level of physical activity did not alter the positive association between plasma BCAA levels and IHL content. Also, we found that a conventional exercise program including both resistance and endurance training, was effective in lowering IHL content in NAFL and CON, but did not coincide with lower plasma BCAA levels. Moreover, the association between IHL content and plasma BCAA levels persisted upon exercise training, albeit less strong. The exercise intervention program did also not induce changes in plasma AAA levels. In the NEO cohort, we investigated the long-term effects of physical activity on plasma BCAA levels, while the 12-week exercise training represents the short-term exercise-induced changes. Based on our results, we can conclude that long-term physical activity leads to a slightly reduction in plasma BCAA levels, but the exercise training showed no short-term changes. In line with our results, an observational study showed an association between high physical activity level and low plasma BCAA levels (29), although an exercise intervention study reported that plasma BCAA levels in insulin-resistant individuals did not reduce after a 12-week endurance resistance training program (30). These results indicate that exercise training or physical activity differently affect plasma BCAA levels, but not the relation between plasma BCAA and IHL content and suggest that BCAA do not play a role in mediating the beneficial metabolic effects of exercise training on, among others, IHL content. The mechanism of long-term physical activity leading to lower plasma BCAA levels is however unknown and needs further research.

A limitation of the present study is that physical activity in the observational study was self-reported with use of the SQUASH. The main limitation of these questionnaire is that individuals may overestimate their true rate of activity, although, SQUASH is a previously validated tool to assess physical activity in the Dutch population (41, 42). Nevertheless, in our manuscript we stratified participants into high and low physical activity and therefore we do not expect potential overreporting would have led to misclassification in these groups. In the exercise intervention study, we were not able to investigate gender-specific effects, which however is important. In these kinds of invasive intervention studies, unfortunately only a small number of participants can be included, however, this

study had enough power to pick up changes in insulin sensitivity and changes in IHL, which were the main objectives of the study.

To summarize, we showed an association between plasma BCAA levels and IHL content in a large Dutch populations-based study, which remained similar in strata of physical activity frequency. Levels of isoleucine and leucine were lower in more active individuals compared to less active individuals. The present study furthermore extends the finding of elevated plasma BCAA levels in people with NAFL as well as the positive association with IHL content which was not affected after a conventional exercise training program. The exercise training program did not decrease BCAA levels, neither was able to overcome diminished insulin suppression of BCAA levels in people with or without NAFL and/or T2D. Moreover, our results showed that lower IHL content and improved hepatic insulin sensitivity in people with NAFL upon the exercise training did not coincide with reduced BCAA levels. We conclude that BCAA levels and IHL are associated, but differently affected by physical activity and/or exercise training.

ACKNOWLEDGMENTS

Dr. E. Phielix was supported by a 'VENI' Research Grant for innovative research from the Netherlands Organization for Scientific Research (91613132), an EFSD/Lilly grant from the European Foundation for the Study of Diabetes (EFSD) and obtained a senior fellowship from the Dutch Diabetes Foundation (DFN) (2017.82.010). V.B. Schrauwen-Hinderling is a recipient of an ERC starting grant (grant no. 759161 'MRS in diabetes'). This work is partly financed by the Netherlands Cardiovascular Research Initiative: an initiative with support of the Dutch Heart Foundation Grant CVON2014-02 ENERGISE.

DECLARATION OF COMPETING INTEREST

The authors declare no competing interest.

CONTRIBUTION STATEMENT

S.B., F.V. and B.B. performed the experiments and analyzed data. F.V., S.B. and E.P. wrote the manuscript. E.P., D.M., R.M., F.R., V.S., P.S. and M.H. assisted during the

acquisition, analysis and interpretation of data and reviewed the manuscript. P.S., M.H. and E.P. designed the study. All authors reviewed and approved the final version of the manuscript. E.P. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

DATA PROCESSING AND AVAILABILITY

All data were analyzed in a blinded fashion. The datasets generated during and/or analyzed during the study are available from the corresponding author on reasonable request.

SUPPLEMENTARY MATERIAL FOR CHAPTER 4

Supplementary table 1. Characteristics of the NEO study population

	Total (n=1 983)	Men (n=1 042)	Women (n=941)
Age (years)	56 (50-61)	56 (50-61)	55 (51-60)
Sex (% men)	47.3	-	-
BMI (kg/m^2)	25.9 ± 3.8	26.6 ± 3.4	25.3 ± 4.0
FM (%)	30.4 (23.8-37.0)	23.9 (21.2-27.9)	36.1 (31.8-40.6)
IHL content (%)	2.73 (1.36-6.34)	3.81 (2.03-8.66)	1.82 (1.09-4.55)
Education (% high)	46.2	50.8	41.9
Ethnicity (%white)	96.2	95.9	95.9
Smoking			
Current	14.2	15.5	13.3
Former	45.8	46.7	44.9
Never	40.0	37.8	41.9
Physical activity (MET-h per week leisure)	30.4 (16.0-51.2)	31.4 (15.8-52.5)	29.8 (16.5-50)
Energy intake (kJ/day)	9475 ± 2904	10592 ± 3234	8474 ± 2238
Alcohol intake (g/day)	10.4 (2.8 – 21.5)	16.9 (5.2-27.9)	7.3 (1.4-14.5)
Glucose (mmol/L)	5.3 (5.0-5.7)	5.4 (5.1-5.9)	5.1 (4.8-5.6)
Isoleucine (µmol/L)	50.3 ± 14.7	58.1 ± 15.6	43.4 ± 10.4
Leucine (µmol/L)	66.0 ± 13.7	74.2 ±13.4	58.7 ± 9.8
Valine (µmol/L)	153.9 ± 27.6	169.4 ± 26.0	140.1 ± 21.7
Phenylalanine (µmol/L)	52.6 ± 5.2	54.0 ± 5.5	51.3 ± 4.6
Tyrosine (µmol/L)	53.0 ± 8.8	55.0 ± 9.2	51.2 ± 8.1
Histidine (µmol/L)	54.8 ± 6.3	55.8 ± 6.9	54.0 ± 5.7

Participant characteristics in 1983 participants. Results are weighted towards the BMI distribution of the general population, and are expressed as mean ± standard deviation (SD) for normally distributed data or median with interquartile ranges (IQR) for non-normally distributed data. *BMI, body mass index; FM, fat mass; IHL, intrahepatic lipid content; MET, metabolic equivalent of tasks.*

Supplementary table 2. Relative change in IHL content and 95% confidence intervals per SD of plasma amino acid level in participants of the NEO study (45 to 65 years), stratified by sex.

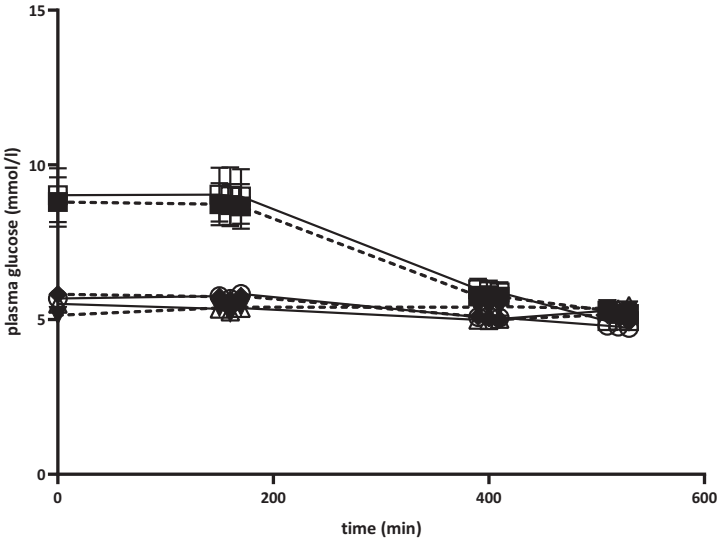
	Total (n=1 983)	Male (n=1 042)	Female (n=941)	*P* value for interaction
Isoleucine	1.52 (1.43, 1.61)*	1.34 (1.25, 1.43)	1.89 (1.72, 2.08)	< 0.001
Leucine	1.54 (1.44, 1.64)*	1.35 (1.25, 1.46)	1.85 (1.68, 2.03)	< 0.001
Valine	1.29 (1.21, 1.38)*	1.18 (1.09, 1.28)	1.42 (1.29, 1.56)	0.003
Phenylalanine	1.14 (1.07, 1.21)*	1.07 (0.99, 1.15)	1.22 (1.10, 1.34)	0.032
Tyrosine	1.31 (1.24, 1.39)	1.27 (1.19, 1.37)	1.35 (1.24, 1.47)	0.310
Histidine	1.03 (0.97, 1.09)	1.01 (0.93, 1.10)	1.05 (0.97, 1.14)	0.515

Linear regression analysis including fasting plasma BCAA and AAA levels as exposure and log-transformed IHL content as outcome, and were weighted towards the BMI distribution of the general population. Model adjusted for age, sex, total body fat, alcohol and energy intake as well as for leisure time physical activity and include an interaction term between sex and amino acid concentration. The regression coefficients with 95% CI represent relative changes in IHL content per SD of plasma amino acid level. Such ratio, for example 1.2, can be interpreted as 1.2 times IHL content for each extra SD in amino acid concentration, which would reflect an increase in IHL content from, for example, 5% to 6%. Asterisks (*) indicate a significant interaction with sex ($p < 0.05$).

Supplementary table 3. Participant characteristics of the exercise intervention study

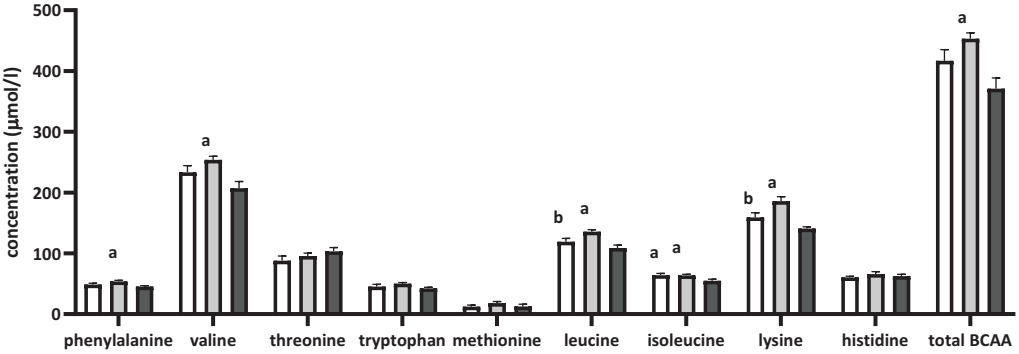
	NAFL (n=7)	T2D (n=7)	CON (n=7)
Age (years)	55.6 ± 5.4	63.1 ± 5.8	59.0 ± 7.9
BW (kg)	104.7 ± 8.1	91.1 ± 5.0 *	89.4 ± 5.3 *
BMI (kg/m^2)	31.3 ± 2.7	29.2 ± 2.0	29.0 ± 1.7
FM (kg)	30.9 ± 4.5	25.6 ± 3.3 *	25.4 ± 2.0 *
FFM (kg)	71.0 ± 4.7	63.6 ± 3.4 *	61.9 ± 3.9 *
FM (%)	29.4 ± 0.03	27.8 ± 0.03	28.2 ± 0.02
FFA fasting (mmol/l)	786 ± 177	666 ± 108	671 ± 188
ASAT (U/l)	25.4 ± 4.4	25.7 ± 6.7	21.7 ± 4.3
ALAT (U/l)	36.3 ± 5.0	41.1 ± 16.7	28.4 ± 13.0
GGT (U/l)	39.9 ± 12.2	38.2 ± 16.4	33.0 ± 16.2
CRP (mg/l)	1.51 ± 0.85	1.94 ± 1.38	1.31 ± 0.77
VO_2 max (ml/kg/min)	24.7 ± 4.2	25.3 ± 3.8	28.1 ± 4.6
Wmax (watt/kg)	1.9 ± 0.3	1.9 ± 0.3	2.2 ± 0.4

Participant characteristics (all males) measured before the start of the exercise training program for people with NAFL (n=7), T2D (n=7) and CON (n=7). Data are expressed as mean ± SD; * $p<0.05$ vs. NAFL. *BW, body mass; BMI, body mass index; FFM, fat free mass; FM, fat mass; FFA, free fatty acids; ASAT, aspartate-amino-transferase; ALAT, alanine-aminotransferase; GGT, γ-glutamyltransferase; HDL, high density lipoprotein; CRP, C-reactive protein; VO2 max, maximal oxygen uptake; Wmax, maximal Watt output.*



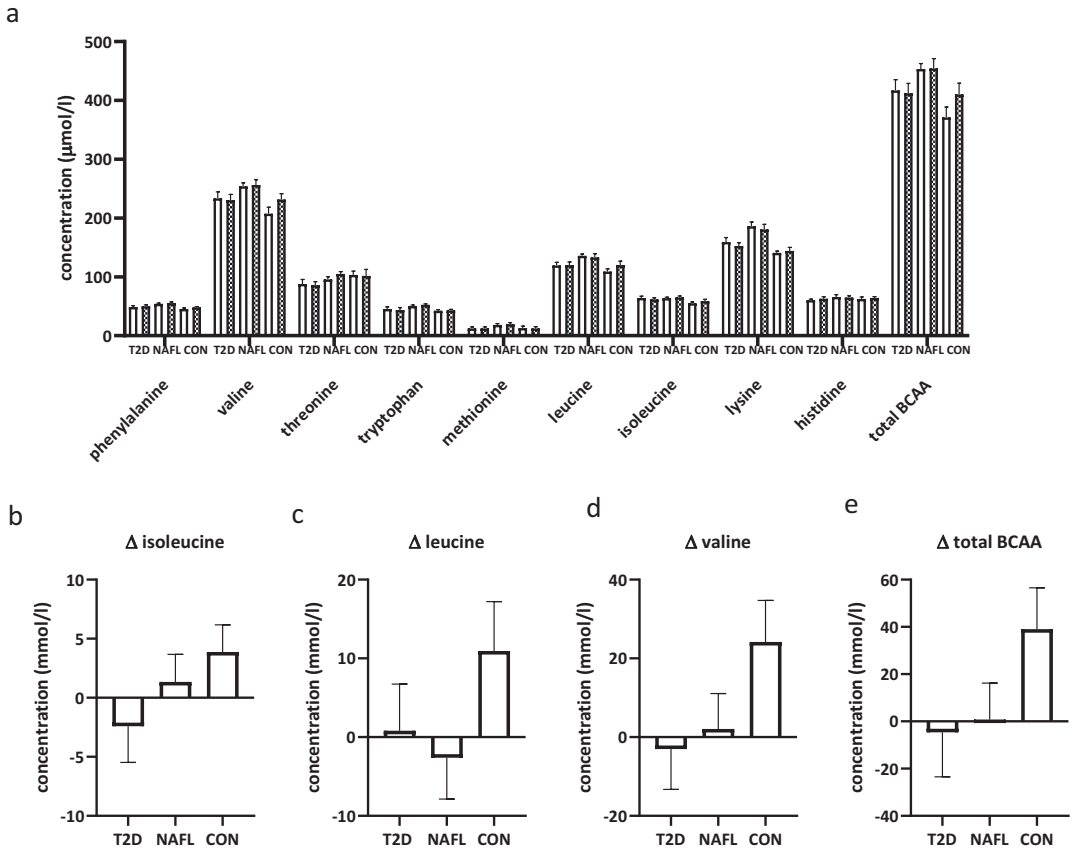
Supplementary Figure 1. Plasma glucose values during the steady state phases of the 2-step hyperinsulinemic euglycemic clamp test.

Plasma glucose values at t=0 min, the pre-infusion phase t=150-170 min, low insulin phase t=390-410 min and high insulin phase t=510-530 min. Solid lines represent pre-exercise training and dotted lines indicate post-exercise training program; triangles represent CON, squares represent T2D and dots represent NAFL. Data are expressed as mean ± SE.



Supplementary Figure 2. Baseline fasting essential amino acids levels.

Fasting essential amino acids (mmol/l) measured in plasma before the exercise program in people with T2D (white bars, n=7), NAFL (light grey bars, n=7) and in CON (dark grey bars, n=7). Data are expressed as mean ± SE and tested using Anova for repeated measurements; 'a' indicates $p<0.05$ vs. CON and 'b' indicates $p<0.05$ vs. NAFL.



Supplementary Figure 3. Changes in fasting essential amino acid levels after exercise training.

Fasting essential amino acids (mmol/l) measured in plasma before (open bars) vs. after (dashed bars) the exercise program (a) in people with T2D (n=7), NAFL (n=7) and in CON (n=7). The delta changes (absolute values) between post minus pre-exercise were calculated for isoleucine (b), leucine (c), valine (d) and total BCAA (e) and compared between people with T2D (n=7), NAFL (n=7), CON (n=7). Data are expressed as mean ± SE and tested using Anova for repeated measurements. *CON, control participants; NAFL, non-alcoholic fatty liver; T2D, type 2 diabetes mellitus.*

REFERENCES

1. Huffman KM, Shah SH, Stevens RD, Bain JR, Muehlbauer M, Slentz CA, et al. Relationships Between Circulating Metabolic Intermediates and Insulin Action in Overweight to Obese, Inactive Men and Women. Diabetes Care. 2009;32(9):1678-83.
2. Newgard CB, An J, Bain JR, Muehlbauer MJ, Stevens RD, Lien LF, et al. A branched-chain amino acid-related metabolic signature that differentiates obese and lean humans and contributes to insulin resistance. Cell Metab. 2009;9(4):311-26.
3. Palmer ND, Stevens RD, Antinozzi PA, Anderson A, Bergman RN, Wagenknecht LE, et al. Metabolomic profile associated with insulin resistance and conversion to diabetes in the Insulin Resistance Atherosclerosis Study. J Clin Endocrinol Metab. 2015;100(3):E463-8.
4. Tai ES, Tan ML, Stevens RD, Low YL, Muehlbauer MJ, Goh DL, et al. Insulin resistance is associated with a metabolic profile of altered protein metabolism in Chinese and Asian-Indian men. Diabetologia. 2010;53(4):757-67.
5. Wang TJ, Larson MG, Vasan RS, Cheng S, Rhee EP, McCabe E, et al. Metabolite profiles and the risk of developing diabetes. Nat Med. 2011;17(4):448-53.
6. Xu F, Tavintharan S, Sum CF, Woon K, Lim SC, Ong CN. Metabolic signature shift in type 2 diabetes mellitus revealed by mass spectrometry-based metabolomics. J Clin Endocrinol Metab. 2013;98(6):E1060-5.
7. Floegel A, Stefan N, Yu Z, Muhlenbruch K, Drogan D, Joost HG, et al. Identification of serum metabolites associated with risk of type 2 diabetes using a targeted metabolomic approach. Diabetes. 2013;62(2):639-48.
8. Perng W, Gillman MW, Fleisch AF, Michalek RD, Watkins SM, Isganaitis E, et al. Metabolomic profiles and childhood obesity. Obesity (Silver Spring). 2014;22(12):2570-8.
9. Shah SH, Crosslin DR, Haynes CS, Nelson S, Turer CB, Stevens RD, et al. Branched-chain amino acid levels are associated with improvement in insulin resistance with weight loss. Diabetologia. 2012;55(2):321-30.
10. Walford GA, Ma Y, Clish C, Florez JC, Wang TJ, Gerszten RE, et al. Metabolite Profiles of Diabetes Incidence and Intervention Response in the Diabetes Prevention Program. Diabetes. 2016;65(5):1424-33.
11. Wurtz P, Soininen P, Kangas AJ, Ronnemaa T, Lehtimaki T, Kahonen M, et al. Branched-chain and aromatic amino acids are predictors of insulin resistance in young adults. Diabetes Care. 2013;36(3):648-55.
12. Koves TR, Ussher JR, Noland RC, Slentz D, Mosedale M, Ilkayeva O, et al. Mitochondrial overload and incomplete fatty acid oxidation contribute to skeletal muscle insulin resistance. Cell Metab. 2008;7(1):45-56.
13. Muoio DM, Newgard CB. Molecular and metabolic mechanisms of insulin resistance and beta-cell failure in type 2 diabetes. Nat Rev Mol Cell Bio. 2008;9(3):193-205.
14. Merz B, Frommherz L, Rist MJ, Kulling SE, Bub A, Watzl B. Dietary Pattern and Plasma BCAA-Variations in Healthy Men and Women-Results from the KarMeN Study. Nutrients. 2018;10(5).
15. Lopez AM, Noriega LG, Diaz M, Torres N, Tovar AR. Plasma branched-chain and aromatic amino acid concentration after ingestion of an urban or rural diet in rural Mexican women. BMC Obes. 2015;2:8.
16. Mahendran Y, Jonsson A, Have CT, Allin KH, Witte DR, Jorgensen ME, et al. Genetic evidence of a causal effect of insulin resistance on branched-chain amino acid levels. Diabetologia. 2017;60(5):873-8.
17. Welle S, Nair KS. Failure of glyburide and insulin treatment to decrease leucine flux in obese type II diabetic patients. Int J Obes. 1990;14(8):701-10.

18. Halvatsiotis P, Short KR, Bigelow M, Nair KS. Synthesis rate of muscle proteins, muscle functions, and amino acid kinetics in type 2 diabetes. Diabetes. 2002;51(8):2395-404.
19. Doisaki M, Katano Y, Nakano I, Hirooka Y, Itoh A, Ishigami M, et al. Regulation of hepatic branched-chain alpha-keto acid dehydrogenase kinase in a rat model for type 2 diabetes mellitus at different stages of the disease. Biochem Biophys Res Commun. 2010;393(2):303-7.
20. She P, Van Horn C, Reid T, Hutson SM, Cooney RN, Lynch CJ. Obesity-related elevations in plasma leucine are associated with alterations in enzymes involved in branched-chain amino acid metabolism. Am J Physiol Endocrinol Metab. 2007;293(6):E1552-63.
21. Adams SH. Emerging Perspectives on Essential Amino Acid Metabolism in Obesity and the Insulin-Resistant State. Adv Nutr. 2011;2(6):445-56.
22. Zhou M, Shao J, Wu CY, Shu L, Dong W, Liu Y, et al. Targeting BCAA Catabolism to Treat Obesity-Associated Insulin Resistance. Diabetes. 2019;68(9):1730-46.
23. Sliz E, Sebert S, Wurtz P, Kangas AJ, Soininen P, Lehtimaki T, et al. NAFLD risk alleles in PNPLA3, TM6SF2, GCKR and LYPLAL1 show divergent metabolic effects. Hum Mol Genet. 2018;27(12):2214-23.
24. Iwasa M, Ishihara T, Mifuji-Moroka R, Fujita N, Kobayashi Y, Hasegawa H, et al. Elevation of branched-chain amino acid levels in diabetes and NAFL and changes with antidiabetic drug treatment. Obes Res Clin Pract. 2015;9(3):293-7.
25. Kaikkonen JE, Wurtz P, Suomela E, Lehtovirta M, Kangas AJ, Jula A, et al. Metabolic profiling of fatty liver in young and middle-aged adults: Cross-sectional and prospective analyses of the Young Finns Study. Hepatology. 2017;65(2):491-500.
26. Du Y, Meng Q, Zhang Q, Guo F. Isoleucine or valine deprivation stimulates fat loss via increasing energy expenditure and regulating lipid metabolism in WAT. Amino Acids. 2012;43(2):725-34.
27. Cheng Y, Meng Q, Wang C, Li H, Huang Z, Chen S, et al. Leucine deprivation decreases fat mass by stimulation of lipolysis in white adipose tissue and upregulation of uncoupling protein 1 (UCP1) in brown adipose tissue. Diabetes. 2010;59(1):17-25.
28. Caspersen CJ, Powell KE, Christenson GM. Physical activity, exercise, and physical fitness: definitions and distinctions for health-related research. Public Health Rep. 1985;100(2):126-31.
29. Kujala UM, Peltonen M, Laine MK, Kaprio J, Heinonen OJ, Sundvall J, et al. Branched-Chain Amino Acid Levels Are Related with Surrogates of Disturbed Lipid Metabolism among Older Men. Front Med (Lausanne). 2016;3:57.
30. Glynn EL, Piner LW, Huffman KM, Slentz CA, Elliot-Penry L, AbouAssi H, et al. Impact of combined resistance and aerobic exercise training on branched-chain amino acid turnover, glycine metabolism and insulin sensitivity in overweight humans. Diabetologia. 2015;58(10):2324-35.
31. de Mutsert R, den Heijer M, Rabelink TJ, Smit JW, Romijn JA, Jukema JW, et al. The Netherlands Epidemiology of Obesity (NEO) study: study design and data collection. Eur J Epidemiol. 2013;28(6):513-23.
32. Brouwers B, Schrauwen-Hinderling VB, Jelenik T, Gemmink A, Sparks LM, Havekes B, et al. Exercise training reduces intrahepatic lipid content in people with and people without nonalcoholic fatty liver. Am J Physiol Endocrinol Metab. 2018;314(2):E165-E73.
33. Neuschwander-Tetri BA. Nonalcoholic steatohepatitis and the metabolic syndrome. Am J Med Sci. 2005;330(6):326-35.
34. Reynolds JM, Gordon TJ, Robergs RA. Prediction of one repetition maximum strength from multiple repetition maximum testing and anthropometry. J Strength Cond Res. 2006;20(3):584-92.

35. Meex RC, Schrauwen-Hinderling VB, Moonen-Kornips E, Schaart G, Mensink M, Phielix E, et al. Restoration of muscle mitochondrial function and metabolic flexibility in type 2 diabetes by exercise training is paralleled by increased myocellular fat storage and improved insulin sensitivity. Diabetes. 2010;59(3):572-9.
36. Steele R. Influences of glucose loading and of injected insulin on hepatic glucose output. Ann N Y Acad Sci. 1959;82:420-30.
37. van Eijk HM, Wijnands KA, Bessems BA, Olde Damink SW, Dejong CH, Poeze M. High sensitivity measurement of amino acid isotope enrichment using liquid chromatography-mass spectrometry. J Chromatogr B Analyt Technol Biomed Life Sci. 2012;905:31-6.
38. van den Berg EH, Flores-Guerrero JL, Gruppen EG, de Borst MH, Wolak-Dinsmore J, Connelly MA, et al. Non-Alcoholic Fatty Liver Disease and Risk of Incident Type 2 Diabetes: Role of Circulating Branched-Chain Amino Acids. Nutrients. 2019;11(3).
39. Lynch CJ, Adams SH. Branched-chain amino acids in metabolic signalling and insulin resistance. Nat Rev Endocrinol. 2014;10(12):723-36.
40. Adeva MM, Calvino J, Souto G, Donapetry C. Insulin resistance and the metabolism of branched-chain amino acids in humans. Amino Acids. 2012;43(1):171-81.
41. de Hollander EL, Zwart L, de Vries SI, Wendel-Vos W. The SQUASH was a more valid tool than the OBiN for categorizing adults according to the Dutch physical activity and the combined guideline. J Clin Epidemiol. 2012;65(1):73-81.
42. Wendel-Vos GC, Schuit AJ, Saris WH, Kromhout D. Reproducibility and relative validity of the short questionnaire to assess health-enhancing physical activity. J Clin Epidemiol. 2003;56(12):1163-9.

5

A randomized placebo-controlled clinical trial for pharmacological activation of BCAA catabolism in patients with type 2 diabetes

Froukje Vanweert[1], Michael Neinast[2], Edmundo Erazo Tapia[1], Tineke van de Weijer[1,3], Joris Hoeks[1], Vera B. Schrauwen-Hinderling[1,3], Megan C. Blair[2], Marc R. Bornstein[2], Matthijs K.C. Hesselink[1], Patrick Schrauwen[1], Zoltan Arany[2] and Esther Phielix[1]

[1] Department of Nutrition and Movement Sciences, NUTRIM School of Nutrition and Translational Research in Metabolism, Maastricht University, Maastricht, 6229 ER, The Netherlands

[2] Cardiovascular Institute, Perelman School of Medicine, University of Pennsylvania, Philadelphia, Pennsylvania, PA 19104, USA

[3] Department of Radiology and Nuclear Medicine, Maastricht University Medical Center, Maastricht, 6229 ER, The Netherlands

This chapter has been published in: Nat Commun. 2022;13(1):3508

ABSTRACT

Elevations in plasma branched-chain amino acid (BCAA) levels associate with insulin resistance and type 2 diabetes (T2D). Pre-clinical models suggest that lowering BCAA levels improve glucose tolerance, but data in humans are lacking. Here, we used sodium phenylbutyrate (NaPB), an accelerator of BCAA catabolism, as tool to lower plasma BCAA levels in patients with T2D, and evaluate its effect on metabolic health. This trial (NetherlandsTrialRegister: NTR7426) had a randomized, placebo-controlled, double-blind cross-over design and was performed in the Maastricht University Medical Center (MUMC+), the Netherlands, between February 2019 and February 2020. Patients were eligible for the trial if they were 40-75years, BMI of 25-38 kg/m², relatively well-controlled T2D (HbA1C<8.5%) and treated with oral glucose-lowering medication. Eighteen participants were randomly assigned to receive either NaPB 4.8g/m²/day and placebo for 2 weeks via controlled randomization and sixteen participants completed the study. The primary outcome was peripheral insulin sensitivity. Secondary outcomes were *ex vivo* muscle mitochondrial oxidative capacity, substrate oxidation and ectopic fat accumulation. Fasting blood samples were collected to determine levels of BCAA, their catabolic intermediates, insulin, triglycerides, free fatty acids (FFA) and glucose. NaPB led to a robust 27% improvement in peripheral insulin sensitivity compared to placebo (ΔRd:13.2±1.8 vs. 9.6±1.8 μmol/kg/min, p=0.02). This was paralleled by an improvement in pyruvate-driven muscle mitochondrial oxidative capacity and whole-body insulin-stimulated carbohydrate oxidation, and a reduction in plasma BCAA and glucose levels. No effects were observed on levels of insulin, triglycerides and FFA, or ectopic fat accumulation. No adverse events were reported. These data establish the proof-of-concept in humans that modulating the BCAA oxidative pathway may represent a potential treatment strategy for patients with T2D.

INTRODUCTION

In the past three decades, the prevalence of type 2 diabetes (T2D) has risen dramatically and developed into a major global health problem (1). Extensive research has shown that T2D is multifactorial disease characterized by insulin resistance accompanied with a broad scale of tissue-specific and whole-body metabolic disturbances, such as low mitochondrial function, metabolic inflexibility and ectopic lipid accumulation (2-4). In recent years, several observational studies, including work of our own, identified elevated plasma branched-chain amino acids (BCAA) levels in obese people and patients with T2D, associating with insulin resistance (5-8). A rise of BCAA plasma levels may even predict the onset of T2D (9, 10). Why BCAA levels accumulate in plasma is currently unknown, but recent data -predominantly obtained from animal models- hypothesize that levels accumulate through suppression of the BCAA-catabolic pathway (11-14).

BCAA catabolism involves initial transamination of BCAA to branched-chain a-keto acids (BCKAs) by the BCAA aminotransferase (BCAT), followed by decarboxylation of BCKAs by the BCKA dehydrogenase complex (BCKD), the rate limiting enzyme of BCAA catabolism (11). The latter complex is activated via dephosphorylation by the PPM1K phosphatase, and inactivated via phosphorylation by the BCKD kinase. There is evidence that BCKD kinase activity increases with the progression of insulin resistance and T2D, resulting in reduced BCAA oxidation and a subsequent rise of BCAA levels in plasma (11, 13, 15, 16). In line with this hypothesis, we recently reported lower whole-body leucine oxidation rates in patients with T2D compared to healthy control participants (8).

Several rodent studies have compellingly demonstrated that promoting BCAA oxidation benefits glucose metabolism and alleviates insulin resistance. Administration of the compound BT2 (3,6-dichlorobenzothiophene-2-carboxylic acid), a potent and specific inhibitor of the BCKD kinase (17), in mice accelerated BCAA oxidation and reduced plasma BCAA levels (18-23). As a result, hepatic steatosis decreased and glucose disposal in peripheral tissues improved. Administration of this BCKD kinase inhibitor furthermore attenuated insulin resistance in high-fat diet-induced obese mice (24). These studies provide proof-of-concept evidence for the therapeutic potential of manipulating BCAA metabolism (24), and raise the question if this strategy may form a treatment strategy in patients with T2D. BT2 binds BCKD kinase in an allosteric pocket, leading to inhibition of kinase activity. BT2 is not suitable for human use, but sodium phenylbutyrate (NaPB), an FDA approved drug regularly prescribed in patients suffering from urea cycle disorders, binds the same allosteric pocket inhibiting inhibits BCKD kinase (17), and lowers plasma BCAA levels in humans (25).

In the present study, NaPB was administered 'off-label' as add-on medication to patients with T2D as a tool to lower BCAA plasma levels. We evaluated a broad range of metabolic parameters after a 2-week intervention period, and compare results to a placebo arm. We hypothesize that NaPB treatment effectively decreases BCAA plasma levels and improves patients' metabolic health, including peripheral insulin sensitivity, muscle mitochondrial oxidative capacity, whole-body substrate oxidation and ectopic fat accumulation in muscle and liver.

METHODS

Clinical study design

Participants were enrolled between February 2019 and September 2019 at the Maastricht University Medical Center (MUMC+), the Netherlands, and the last subject completed in February 2020. Two dropouts were reported during the study (Figure 1). The protocol was reviewed and approved by the Medical Ethical Review Committee of the MUMC+ (Netherlands Trial Register ID: NTR7426) and conducted in accordance with the declaration of Helsinki. All participants were informed about the nature and risk of the experimental procedures before their written informed consent was obtained.

Participants

Sixteen male and postmenopausal females diagnosed with T2D for at least 1.5 years, participated in the study. Participants underwent a medical screening to check eligibility. Inclusion criteria were 40 - 75 y of age, BMI of 25 – 38 kg/m^2, relatively well-controlled T2D (HbA1C < 8.5%) treated with oral glucose lowering medication (metformin only, or in combination with sulphonylurea agents and/or DPPIV inhibitors) or drug naive for at least 3 months prior to the onset of the study. Patients had no signs of active cardiovascular diseases, liver or renal insufficiency. Exclusion criteria were unstable body weight (i.e. weight gain or loss > 5 kg in the last three months), participation in physical activity ≥ 3 times a week, insulin treatment, and MRI contra-indications.

Experimental design

The study had a randomized, double-blind, placebo-controlled, crossover design (Figure 2). Each participant underwent 2 intervention arms, which involved daily administration of 4.8 g/m^2/d NaPB or placebo. The participants were randomly assigned to receive either the NaPB or the placebo treatment, separated by a washout

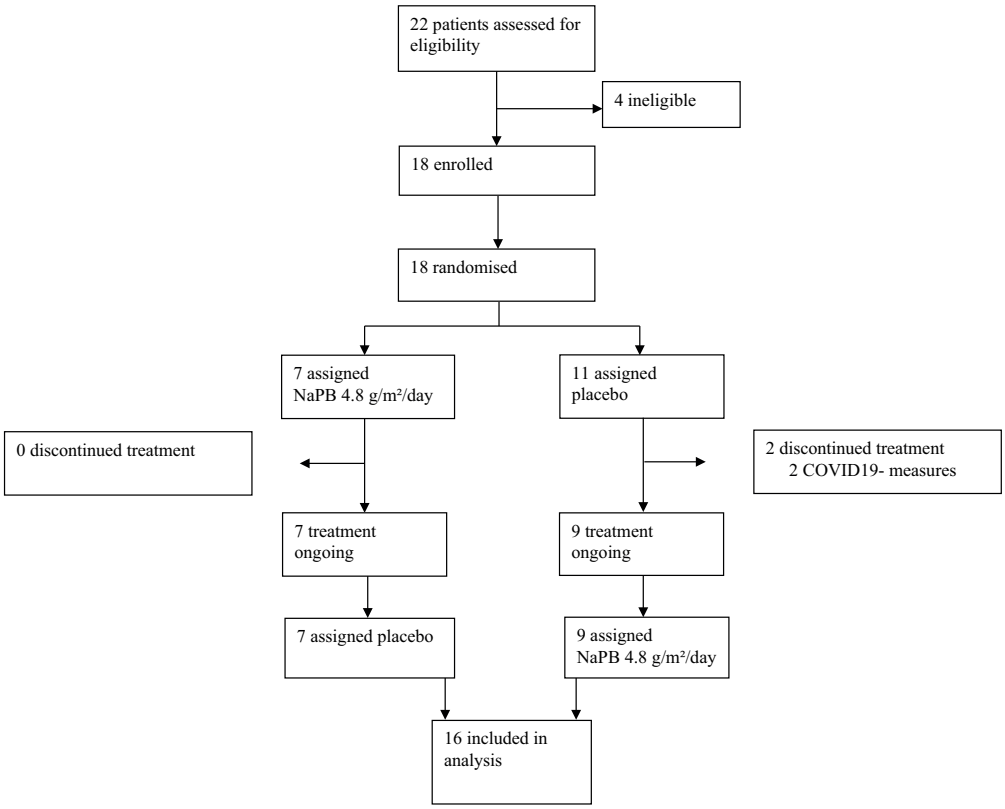


Figure 1. Trial profile.

Sixteen participants completed the treatment and are included in analysis. Two patients discontinued treatment prematurely due to COVID19-measures. *T2D, patients with type 2 diabetes mellitus; NaPB, sodium phenylbutyrate.*

period of 6 to 8 weeks via controlled randomization. After 2 weeks, all participants underwent several measurements to evaluate patients' metabolic health. Three days before the start of these measurements, participants were instructed to refrain from strenuous physical activities and to continue their antidiabetic medication with the last dose taken on the evening before the hyperinsulinemic-euglycemic clamp test. Throughout the study, patients were asked to maintain their habitual diet and regular physical activity pattern.

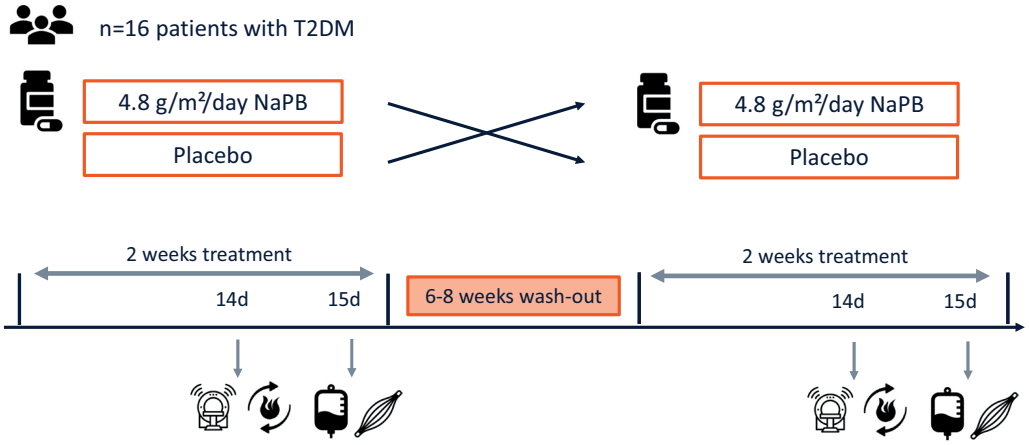


Figure 2. Experimental design.

In this crossover study, participants were randomly assigned to start with 2-week NaPB supplementation or placebo treatment. After a washout period of 6-8 weeks, participants switched from intervention arm such that all participants served as their own control. In each treatment arm, measurements were performed after 2 weeks treatment, including magnetic resonance spectroscopy (day 14), whole-body 24h energy metabolism and substrate oxidation (day 14), 2-step euglycemic hyperinsulinemic clamp (day 15) and muscle biopsies (day 15) were taken. *T2D, patients with type 2 diabetes mellitus; NaPB, sodium phenylbutyrate.*

Study medication

The study medication Pheburane (Lucane Pharma, Paris, France provided by Eurocept International, Ankeveen, The Netherlands) contained 483 mg/g NaPB and inactive ingredients (sucrose, maize starch, sodium, Hypromellose, ethylcellulose N7, macrogel 1500 and povidone K25). The placebo (produced by Tiofarma, Oud-Beijerland, the Netherlands) only contained the inactive ingredients of Pheburane. The daily dose of 4.8 g/m^2/day (NaPB and placebo) was far below the minimal, clinically prescribed (9.9-13.0 g/m^2/day NaPB), to prevent the development of unwanted side effects. The study medication was administered in the form of granules, taken orally 3 times a day, divided into 3 equal amounts and given with breakfast, lunch and dinner. The granules could be directly swallowed with a drink (e.g. water, fruit juices) or sprinkled on solid foods (e.g. mashed potatoes, yoghurt). Administration of 4.8 g/m^2/day NaPB was well tolerated and no adverse events or side effects were reported throughout the study.

Overview of specified outcomes

The primary outcome was peripheral insulin sensitivity, measured by the hyperinsulinemic-euglycemic clamps, expressed as the change in insulin-stimulated glucose disposal rate minus baseline (ΔRd).

Secondary outcomes were *ex vivo* mitochondrial oxidative capacity in skeletal muscle, measured with high-resolution respirometry expressed as O_2-flux, substrate oxidation, assessed with indirect calorimetry and ectopic fat accumulation in muscle and liver measured with proton magnetic resonance spectroscopy (^{1}H-MRS). Fasting blood samples were collected to determine levels of BCAA and their intermediates, insulin, triglycerides, FFA and glucose. In addition, phenylbutyrate levels were determined by LCMS to check compliance to the intervention.

2-step hyperinsulinemic-euglycemic clamp

A 2-step hyperinsulinemic-euglycemic clamp with co-infusion of D-(6.6-$^2H_2$] glucose tracer (0.04 mg $\cdot$ kg^{-1} $\cdot$ min^{-1}) started in the morning of day 15 at 06:30 to assess hepatic and whole-body insulin sensitivity. After a pre-infusion of D-(6.6-$^2H_2$] glucose tracer (0.04 mg/kg/min) for 3h (basal phase), low dose insulin was infused at 10 mU $\cdot$ m^{-2} $\cdot$ min^{-1} for 3h to assess hepatic insulin sensitivity (low insulin phase), with a subsequently raise in insulin concentration to 40 mU $\cdot$ m^{-2} $\cdot$ min^{-1} for 2.5h (high insulin phase) to determine peripheral insulin sensitivity. Blood was frequently sampled to measure glucose concentration in arterialized blood. In addition, 20% glucose (enriched with D-(6.6-$^2H_2$] glucose tracer) was co-infused at a variable rate to maintain euglycemia (~ 6.0 mmol/L). During the last 30 min of each phase, blood samples were collected at 10 minutes interval to determine glucose tracer kinetics and indirect calorimetry was performed to measure substrate oxidation. Steele's single pool non-steady state equations were used to calculate the rate of glucose appearance (Ra) and disappearance (Rd) (46). Volume of distribution was assumed to be 0.160 l/kg for glucose. The change in insulin-stimulated glucose disposal (ΔRd) was calculated by the difference between Rd measured under insulin-stimulated condition and basal conditions. Endogenous glucose production (EGP) was calculated as Ra minus exogenous glucose infusion rate. Hepatic insulin sensitivity was calculated as the percentage of EGP suppression during the low and high insulin phase. Nonoxidative glucose disposal (NOGD) was calculated as Rd minus carbohydrate oxidation, determined with indirect calorimetry. Isotopic enrichment of plasma glucose was determined by electron ionization gas chromatography-mass spectrometry as described previously (47).

Indirect calorimetry

Before and during the clamp test, indirect calorimetry was performed to measure energy expenditure and substrate utilization. Gas exchange was measured by open-circuit respirometry with an automated ventilated hood system (Omnical, Maastricht, the Netherlands) for 30 min. The Weir equation (48) was used to calculate whole-body resting energy expenditure from measurements of oxygen consumption and carbon dioxide production. Carbohydrate, fat and protein

oxidation rates were calculated according to Frayn (49) and nitrogen was measured in 24h collected urine samples.

Skeletal muscle biopsies

In the morning at day 15, before the start of the clamp test, a muscle biopsy was obtained from the m.vastus lateralis under local anesthesia (1% lidocaine without epinephrine), according to the technique of Bergström et al. (50). A portion of muscle tissue was directly frozen in isopentane and stored at -80°C until further analysis. Another portion was immediately placed in ice-cold preservation medium and processed for high resolution respirometry.

High-resolution respirometry in permeabilized muscle fibers

A small portion of the muscle biopsy sample was immediately placed in ice-cold biopsy preservation medium (BIOPS; OROBOROS Instruments, Innsbruck, Austria). Muscle fibers were permeabilized with saponin according to the technique of Veksler et al (51). After permeabilization, muscle fibers were transferred into ice-cold mitochondrial respiration buffer (MiRO5; OROBOROS Instruments). Subsequently, permeabilized muscle fibers (~ 2.5 g wet weight) were used for *ex vivo* high-resolution respirometry (Oxygraph, OROBOROS Instruments) by measuring oxygen consumption rate upon addition of several substrates. In every protocol applied, first, 4.0 mM malate was added to obtain state 2 respiration followed by addition of 1.0 mM octanoyl-carnitine or in presence of 5 mM pyruvate. In addition, 2 mM ADP with 10 mM glutamate was added to obtain ADP-driven state 3 respiration of complex I. Then 10 mM succinate was added to obtain state 3 respiration by activating both complex I and II. Finally, 1.0 mM carbonylcyanide p-trifluoromethoxyphenylhydrozone (FCCP) was added (in stepwise titration) to evaluate maximal respiratory capacity.

Magnetic resonance spectroscopy: IHL and IMCL content

On day 14, directly after the BodPod measurement, participants also underwent proton magnetic ^{1}H-MRS to quantify intrahepatic lipid (IHL) and intramyocellular lipid (IMCL) content on a 3T whole body scanner (Achieva 3T-X, Philips Healthcare, Best, the Netherlands). IHL and hepatic fatty acid composition was quantified as previously described (52). Values were corrected for T2 relaxation (T2 water: 26.3 ms and T2 CH_2: 57.8 ms) and given as ratios of CH_2 peak relative to the sum of CH_2 resonance and the unsuppressed water peak (in %). IMCL was measured in the m. tibialis anterior of the left leg, as previously described (53). Values are given as T1- and T2-corrected ratios of the CH_2 peak (54) relative to the unsuppressed water peak (in %). Due to analytical problems only 13 participants could be included in the analyses of IMCL.

Respiration chamber

After the MRS measurements, in the late afternoon of day 14 of each intervention arm, participants consumed a standardized dinner before they went into the respiration chamber: a small room with a bed, toilet, TV and computer. During the overnight stay (for 12 hours) in this chamber, oxygen consumption and carbohydrate production were measured continuously in sampled room air. Sleep metabolic rate (SMR), substrate oxidation and sleep respiration quotient (RQ) were measured using direct calorimetry equipment (Omnical, Maastricht, the Netherlands). SMR was calculated as the lowest average 3-h energy expenditure during the sleep. At 6 AM the next morning, participants were woken up and left the respiration chamber.

Blood parameters

Venous blood samples were taken throughout the study in which routine medical laboratory analysis were performed (Table 1 and Table 2). The metabolites phenylbutyrate, BCAA, BCKA and 3-HIB were analyzed in plasma by LC-MS, as previously described (23).

To extract metabolites from serum samples, 100 µl −20° 40:40:20 methanol:acetonitrile:water (extraction solvent) was added to 5 µl of serum sample and incubated in −20°C for 1 hour, followed by vortexing and centrifugation at 16,000 x g for 10 min at 4°C. The supernatant (first extract) was transferred to a new tube. Then, 50 µl extraction solution was added to resuspend the pellet, followed by vortexing and centrifugation at 16,000 x g for 10 min at 4°C. The supernatant (second extract) was combined with the first extract. Then, 3 µl among the 150 µl extract was loaded to LC-MS. A quadrupole-orbitrap mass spectrometer (Q Exactive, Thermo Fisher Scientific, San Jose, CA) operating in negative or positive ion mode was coupled to hydrophilic interaction chromatography via electrospray ionization and used to scan from m/z 70 to 1000 at 1 Hz and 75,000 resolution. LC separation was on a XBridge BEH Amide column (2.1 mm × 150 mm, 2.5 µm particle size, 130 Å pore size; Waters, Milford, MA) using a gradient of solvent A (20 mM ammonium acetate, 20 mM ammonium hydroxide in 95:5 water: acetonitrile, pH 9.45) and solvent B (acetonitrile). Data were analyzed using the MAVEN software (55). Isotope labeling was corrected for natural ^{13}C abundance (56). Flow rate was 150 µl/min. The LC gradient was: 0 min, 85% B; 2 min, 85% B; 3 min, 80% B; 5 min, 80% B; 6 min, 75% B; 7 min, 75% B; 8 min, 70% B; 9 min, 70% B; 10 min, 50% B; 12 min, 50% B; 13 min, 25% B; 16 min, 25% B; 18 min, 0% B; 23 min, 0% B; 24 min, 85% B; 30 min, 85% B. Autosampler temperature is 5 °C, and injection volume is 3 µL.

Body composition

On day 14 of each intervention period, participants were advised to have a lunch at 12:00 and to remain fasted until they arrived the research unit. In the afternoon, participants underwent a body composition measurement with the BodPod ® (Cosmed, California, USA). Body mass and body volume were assessed as previously described (57).

Statistical analysis

All results were normally distributed and presented as mean ± SE. The intervention effect was analyzed using the paired student t-test and correlations by using Pearson's correlation coefficient. Statistics were performed using SPSS 26.0 for Mac and a two-sided $p<0.05$ was considered statistically significant.

RESULTS

Experimental design

The study had a randomized, double-blinded, placebo-controlled, crossover design (Figure 2). Sixteen participants underwent 2 intervention arms, which involved daily administration of 4.8 g/m^2/d NaPB or placebo, separated by a washout period of 6 to 8 weeks. After 2 weeks of each treatment, participants underwent comprehensive metabolic evaluation. The primary outcome was insulin sensitivity, measured by a the 2-step euglycemic/hyperinsulinemic. Secondary outcomes were *ex vivo* mitochondrial oxidative capacity in skeletal muscle, measured with high-resolution respirometry, substrate oxidation, assessed with indirect calorimetry, and ectopic fat accumulation in muscle and liver measured with proton magnetic resonance spectroscopy (^{1}H-MRS).

Baseline characteristics and treatment compliance

Baseline characteristics are reported in Table 1. Compliance was determined by weighing the medication granules and by analysis of concentrations of phenylbutyrate and phenylacetylglutamine in plasma at the end of treatment period. Compliance rate (ratio taken dose/prescribed dose) in the NaPB arm was 95.8 ± 13.8% and 95.4 ± 11.0 % for placebo. Concentrations of phenylbutyrate and phenylacetylglutamine in plasma were significantly higher in the NaPB arm compared to placebo ($p<0.0001$, Suppl. Figure 1), which together confirms compliance to the treatment intervention.

Table 2 Baseline characteristics [a]

	Mean ± SD
Gender, *n* (F/M)	3/13
Age, years	66 ± 6
Body weight, kg	90.8 ± 15.4
Height, cm	174.7 ± 8.1
BMI, kg/m^2	29.6 ± 3.3
Fasting glucose, mmol/L	8.4 ± 1.5
HbA1c, %	6.5 ± 0.6
ASAT, U/L	23.8 ± 3.9
ALAT, U/L	30.3 ± 7.7
GGT, U/L	32.6 ± 14.6
Potassium (mmol/L)	4.5 ± 0.1
Creatinine (µmol/L)	84.8 ±4.8
Bilirubin	12.1 ± 2.8
Hemoglobin (mmol/L)	8.8 ± 0.2
Oral glucose lowering medication, *n*	16
Metformin only	8
Sulphonylurea derivates only	2
Metformin + sulphonylurea derivates	6

[a] *n*=16. Screening values are means ± SD. *BMI, body mass index; ASAT, aspartate aminotransferase; ALAT, alanine aminotransferase; GGT, gamma glutamyltransferase.*

NaPB treatment improved peripheral insulin sensitivity and whole-body carbohydrate oxidation

NaPB treatment improved whole-body insulin sensitivity, as assessed by a 2-step hyperinsulinemic-euglycemic clamp. The change in hyperinsulinemic-stimulated glucose disposal rate (ΔRd), robustly improved by 27% (p=0.02) after NaPB treatment compared to placebo (Figure 3a, Table 2). During the low-insulin phase of the clamp, insulin-suppressed EGP did not change (p=0.84, Table 2), but EGP became 6% more suppressed during the high-insulin phase with NaPB compared to placebo (p=0.02, Figure 3b, Table 2). Plasma FFA levels were suppressed to a similar extent between NaPB and placebo during both the low and high-insulin

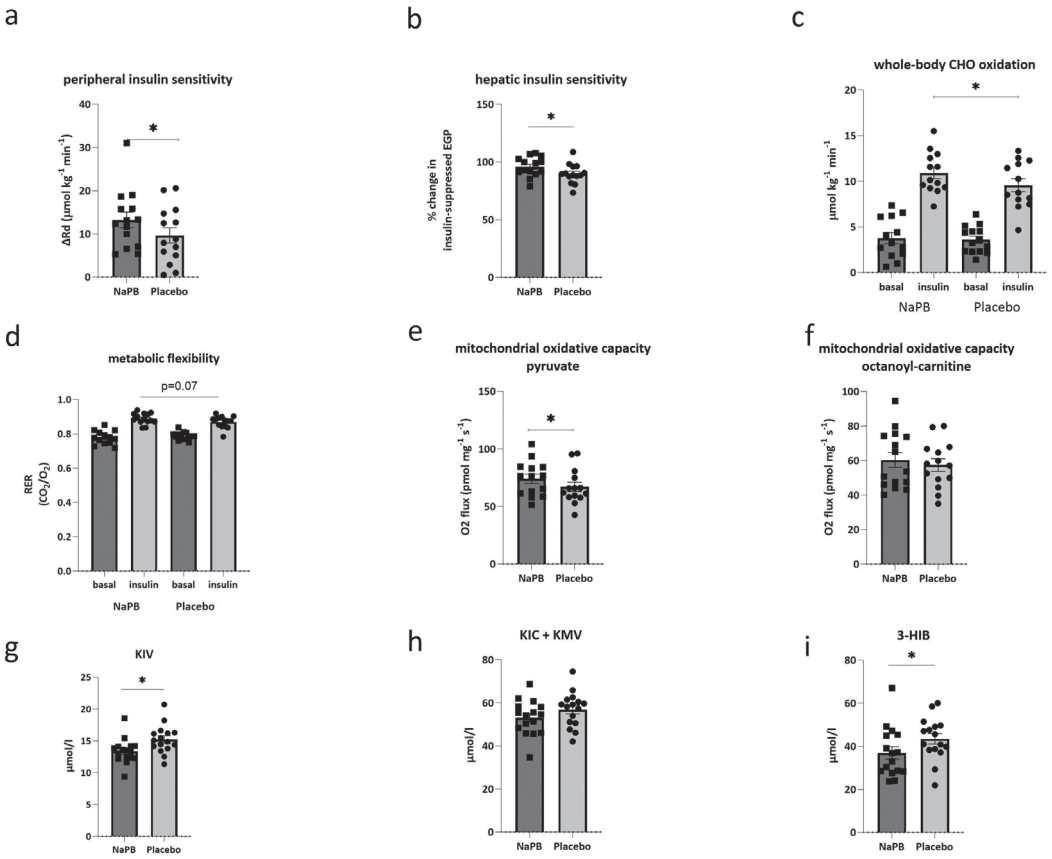


Figure 3. NaPB treatment effects on metabolic read-out parameters and plasma metabolites.

Metabolic parameters measured in patients with T2D after 2-week treatment with NaPB (grey bars, n=16) and placebo (white bars, n=16). (a) peripheral insulin sensitivity expressed as the change in insulin-stimulated Rd (µmol kg^{-1} min^{-1}, p=0.02), calculated as the difference between Rd under high-insulin infusion and Rd during basal conditions, (b) hepatic insulin sensitivity expressed as the change in insulin-suppressed EGP (%, p=0.02) under basal conditions versus high insulin infusion, (c) insulin-stimulated carbohydrate oxidation (µmol kg^{-1} min^{-1}, p=0.03), and (d) metabolic flexibility, expressed as change in RER from the basal to the high-insulin infusion (p=0.07), skeletal muscle ex vivo mitochondrial respiratory capacity (e) upon carbohydrate-derived substrate pyruvate (pmol mg^{-1} s^{-1}, p=0.03), and (f) upon lipid-derived substrate octanoyl carnitine (pmol mg^{-1} s^{-1}, p=0.25) with parallel electron input to complex II (malate + glutamate + succinate), Fasting plasma metabolites measured after 2-week treatment with NaPB: (g) fasting total KIV levels (µmol/l, p=0.02), (h) fasting KIC + KMV levels (µmol/l, p=0.12) and (i) fasting concentration of 3-HIB (µmol/l, p=0.02). Data are expressed as mean ± SE. The intervention effect was analyzed using the paired student t-test. *P < 0.05. *BCAA, branched-chain amino acids; NaPB, sodium-phenylbutyrate; T2D, type 2 diabetes; Rd, glucose disposal; EGP, endogenous glucose production; RER, respiratory exchange ratio, 3-HIB, 3-hydroxyisobutyrate; KIC, a-ketoisocaproate; KIV, a-ketoisovalerate; KMV, a-keto-ß-methylvalerate. Source data are provided as a Source Data file.*

phases of the clamp (p=0.57 and p=0.84, respectively, Table 2). These data suggest that NaPB treatment specifically improves muscle insulin sensitivity, with a significant, albeit modest, improvement of hepatic insulin sensitivity upon high insulin concentration.

Whole body carbohydrate, fat and protein oxidation measured during the basal and low-insulin phase, remained similar between NaPB and placebo groups (Table 2). During high-insulin phase, carbohydrate oxidation was 10% higher after NaPB treatment (p=0.03, Figure 3c, Table 2), while fat and protein oxidation did not change (p=0.16 and p=0.45, Table 2). The change in insulin-stimulated non-oxidative glucose disposal (ΔNOGD) was similar between conditions (p=0.46, Table 2). In addition, metabolic flexibility, expressed as the change from basal respiratory exchange ratio (RER) to insulin-stimulated RER, tended to improve under high insulin conditions with NaPB vs. placebo (p=0.07, Figure 3d, Table 2).

NaPB treatment elevates muscle mitochondrial oxidative capacity

Mitochondrial oxidative capacity, measured in permeabilized muscle fibers was higher after NaPB treatment compared to placebo. In the presence of pyruvate, a carbohydrate-derived substrate, ADP-driven state 3 respiration with parallel electron input to complex II (malate + glutamate + succinate), significantly improved by 10% (Figure 3e, p=0.04). NaPB treatment tended to improve mitochondrial oxygen consumption upon the stimulation of complex I (malate + glutamate) (Suppl. Table 1, p=0.07), without differences found in the maximal respiratory capacity upon the chemical uncoupler FCCP (Suppl. Table 1, p=0.12). In contrast, ADP-driven state 3 respiration fueled by the lipid-derived substrate octanoyl-carnitine, did not change (p=0.25, Figure 3f). Also, no differences were observed for other respiratory states in the presence of octanoyl-carnitine, as shown in Suppl. Table 1. Together, these respiratory data in permeabilized skeletal muscle fibers indicate an improvement in the capacity for the oxidation of carbohydrate-derived substrates. The complete data set for the different respiratory states are reported in Suppl. Table 1.

NaPB treatment did not alter ectopic lipid storage

^{1}H-MRS was applied in the m.tibialis anterior and liver to measure *in vivo* ectopic lipid content. NaPB treatment had no effect on IMCL content (NaPB: 0.61 ± 0.08 % vs. placebo: 0.50 ± 0.06 %; p=0.14), or IHL content (NaPB: 13.5 ± 3.1 % vs. placebo: 11.7 ± 2.4 %; p=0.20, Suppl. Table 2). Further analysis of hepatic lipid composition revealed no effect of NaPB compared to placebo (%PUFA: 13.15 ± 1.49 vs 14.09 ± 1.39, p = 0.50; %MUFA: 42.93 ± 1.79 vs. 41.70 ± 1.62, p = 0.51; %SFA: 43.91 ± 1.29 vs. 44.21 ± 1.67, p =0.88, Suppl. Table 2).

Table 3 NaPB treatment improved peripheral insulin sensitivity and whole-body carbohydrate oxidation [a]

	NaPB	Placebo	*P* value
Ra (μmol · kg^{-1} · min^{-1}) [b]			
Baseline	10.6 ± 0.7	12.1 ± 1.1	0.19
Low insulin	10.5 ± 0.7	10.6 ± 0.5	0.81
High insulin	23.4 ± 2.4	21.1 ± 2.2	0.01*
$\Delta_{\text{baseline - low}}$	-0.1 ± 0.5	-1.5 ± 0.9	0.32
$\Delta_{\text{baseline – high}}$	11.2 ± 2.0	7.9 ± 1.9	0.01*
Rd (μmol · kg^{-1} · min^{-1}) [b]			
Baseline	10.9 ± 0.9	12.2 ± 1.1	0.11
Low insulin	10.6 ± 0.8	10.6 ± 0.6	0.92
High insulin	24.1 ± 2.3	21.9 ± 2.1	0.01*
$\Delta_{\text{low ins - baseline}}$	-0.3 ± 0.7	-1.4 ± 0.7	0.23
$\Delta_{\text{high ins - baseline}}$	13.2 ± 1.8	9.6 ± 1.8	0.02*
EGP (μmol · kg^{-1} · min^{-1}) [c]			
Baseline	10.6 ± 0.9	12.1 ± 1.1	0.19
Low insulin	5.9 ± 0.4	6.4 ± 0.6	0.17
High insulin	1.0 ± 0.2	1.8 ± 0.3	0.02*
% suppression $_{\text{low vs baseline}}$	49.2 ± 2.3	49.6 ± 2.6	0.84
% suppression $_{\text{high vs baseline}}$	95.9 ± 2.3	89.9 ± 2.3	0.02*
NOGD (μmol · kg^{-1} · min^{-1}) [c]			
Baseline	7.3 ± 0.8	8.1 ± 1.2	0.54
Low insulin	3.6 ± 0.6	4.0 ± 0.7	0.70
High insulin	11.8 ± 1.6	11.8 ± 1.5	0.99
$\Delta_{\text{high ins - baseline}}$	3.3 ± 1.4	3.2 ± 1.4	0.46
Carbohydrate oxidation (μmol · kg^{-1} · min^{-1}) [b]			
Baseline	3.8 ± 0.6	3.6 ± 0.4	0.57
Low insulin	6.6 ± 0.7	6.6 ± 0.6	0.97
High insulin	10.9 ± 0.6	9.6 ± 0.7	0.03*
Fat oxidation (μmol · kg^{-1} · min^{-1}) [b]			
Baseline	2.6 ± 0.1	2.6 ± 0.2	0.57
Low insulin	2.2 ± 0.2	2.3 ± 0.2	0.75
High insulin	1.5 ± 0.1	1.7 ± 0.2	0.16
Protein oxidation (μmol · kg^{-1} · min^{-1}) [b]			
Baseline	6.8 ± 0.5	6.9 ± 0.6	0.77
Low insulin	4.4 ± 0.4	4.4 ± 0.5	0.84
High insulin	4.0 ± 0.2	4.5 ± 0.6	0.45
Plasma FFA's (μmol/L) [b]			
Baseline	612 ± 37	628 ± 31	0.52
Low insulin	212 ± 20	226 ± 20	0.31
High insulin	89 ± 15	92 ± 12	0.67
% suppression $_{\text{low insulin}}$	65 ± 3	64 ± 3	0.57
% suppression $_{\text{high insulin}}$	85 ± 3	85 ± 2	0.84
Respiratory exchange ratio [c]			
Baseline	0.78 ± 0.01	0.78 ± 0.01	0.66
Low insulin	0.83 ± 0.01	0.82 ± 0.01	0.94
High insulin	0.89 ± 0.01	0.87 ± 0.01	0.07
$\Delta_{\text{high ins - baseline}}$	0.11 ± 0.01	0.10 ± 0.01	0.37

[a] Data expressed as mean ± SE. The intervention effect was analyzed using the paired student t-test. *P values <0.05 NaPB vs. Placebo. *EGP, endogenous glucose production; FFA free fatty acids; NOGD, nonoxidative glucose disposal; NaPB, sodium phenylbutyrate; Ra, rate of glucose appearance; Rd, rate of glucose disappearance*
[b]n = 14, [c]n=15,

NaPB treatment reduces plasma BCAA levels

As hypothesized, 2 weeks NaPB treatment resulted in 8% lower total BCAA levels (p=0.03, Figure 4a) compared to placebo, with a significant decline of all three individual BCAA (-10% for valine, p=0.009; -7% for leucine, p=0.03; -6% for isoleucine, p=0.05; Figure 4b, c and d, respectively). The complete amino acids profile after both treatment arms are presented in Suppl. Table 3. In addition, fasting glucose levels tended to be lower after NaPB treatment compared to placebo (7.7 ± 0.4 mmol/L vs. 8.2 ± 0.5 mmol/L, p=0.06, Table 3), without any effects observed for insulin, triglycerides and FFA (Table 3).

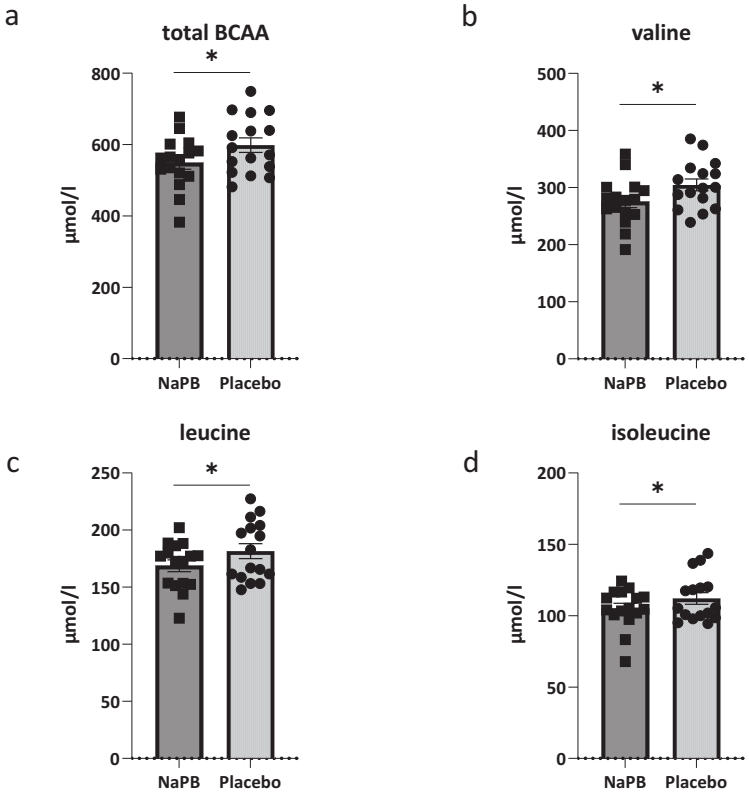


Figure 4. NaPB treatment reduces plasma BCAA levels.

Fasting plasma BCAA's were measured in patients with T2D after 2-week treatment with NaPB (grey bars, n=16) and placebo (white bars, n=16). (a) total BCAA levels (µmol/l, p=0.03), (b) valine levels (µmol/l, p=0.009) and (c) leucine values (µmol/l, p=0.03), and (d) isoleucine (µmol/l, p=0.05). Data are expressed as mean ± SE. The intervention effect was analyzed using the paired student t-test. *P < 0.05. *NaPB, sodium-phenylbutyrate; PLC, placebo; T2D, type 2 diabetes.* Source data are provided as a Source Data file.

Table 4 NaPB treatment reduces fasting glucose levels [a]

Parameter	NaPB	Placebo	*P* value
Glucose, mmol/L [b]			
day 0	8.7 ± 0.5	8.5 ± 0.5	0.53
day 15	7.7 ± 0.4	8.2 ± 0.5	0.06
$\Delta_{\text{day 0-day 15}}$	-1.0 ± 0.2	-0.3 ± 0.3	0.09
Insulin, mU/L	10.9 ± 1.2	11.1 ± 1.4	0.78
Triglycerides, mmol/L	1.9 ± 0.2	1.8 ± 0.2	0.50
Free fatty acids, µmol//L	614 ± 42	632 ± 50	0.51

[a] *n*=16. Data expressed as mean ± SE. The intervention effect was analyzed using the paired student t-test. **P* values <0.05 NaPB vs. Placebo. Fasting blood samples were taken after 2 weeks (15 days) of NaPB treatment or placebo after an overnight fast. *NaPB, sodium phenylbutyrate; BCAA, branched-chain amino acids*
[b] *n*=15.

Effect of NaPB on BCAA catabolism-related metabolites in plasma

Metabolomic analysis in plasma showed a significant decrease of α-ketoisovalerate (KIV), the valine-derived BCKA (p=0.02, Figure 3g) with NaPB treatment compared to placebo. Leucine- and isoleucine-derived BCKA's, (α-ketoisocaproate (KIC) and α-keto b-methylvalerate (KMV); not distinguishable by mass spectroscopy) did not change (p=0.12, Figure 3h), however trended in the same direction as KIV. The reduction of plasma BCAA levels and KIV reflects reduced accumulation of plasma substrates upstream of the BCKD-complex. 3-hydroxyisobutyrate (3-HIB) is an intermediate of valine catabolism downstream of the BCKD complex, and is unique in its ability to escape the mitochondria and appear in the plasma. Interestingly, 3-HIB significantly decreased in the NaPB treatment arm compared to placebo (p=0.02, Figure 3i). The change in 3-HIB concentrations between placebo and NaPB arms negatively correlated with the change in whole-body carbohydrate oxidation (r=-0.55, p=0.05), i.e. the subjects with highest NaPB-improved whole body carbohydrate oxidation showed the largest decrease in plasma 3-HIB, suggesting that the decrease of 3-HIB in plasma after NaPB treatment reflects improved mitochondrial TCA flux. Consistent with our findings, elevated levels of 3-HIB were previously shown to be higher under insulin resistant conditions (21, 26, 27). In contrast, we did not found associations between 3-HIB concentrations and other measures for insulin sensitivity, like Rd (r= 0.08, p=0.76), EGP (r=0.13, p=0.64) and fasting glucose values (r=0.27, p=0.31).

No change in sleeping metabolic rate and nocturnal substrate oxidation with NaPB treatment

Sleeping metabolic rate, measured during an overnight stay in the respiration chamber, was not affected by NaPB treatment compared to placebo (7.0 ± 0.3 MJ/d vs. 7.1 ± 0.2 MJ/d, respectively, p=0.92, Suppl. Table 4). In addition, RER and substrate oxidation during the night were similar between the two conditions (Suppl. Table 4).

Unchanged body composition upon treatment arms

Two weeks of NaPB treatment did not affect body composition. Percentage fat free mass (NaPB: 63.6 ± 2.1 % vs. placebo: 63.4 ± 2.0 %, p=0.79) and fat mass (NaPB: 36.1 ± 2.2 % vs. placebo: 36.6 ± 2.0 %, p=0.50) remained similar. In line, no effect of NaPB was observed for the change in total body weight (NaPB: -0.01 ± 0.5 kg vs. placebo: -0.19 ± 0.49 kg, p=0.56).

DISCUSSION

Recent metabolomics and comprehensive metabolic profiling studies, including work of our own, consistently show elevated BCAA plasma levels in obese/T2D rodent models as well as in patients with T2D (5, 9, 10, 28-30). Here, we prescribed NaPB 'off-label' to patients with T2D to stimulate the oxidation of BCAA aiming to lower its systemic concentrations. We show that 2 weeks of NaPB treatment effectively reduced plasma BCAA levels. This reduction was accompanied by a 27% improved peripheral glucose disposal, mainly exerted by enhanced insulin-stimulated carbohydrate oxidation. In addition, NaPB treatment increased *ex vivo* mitochondrial oxidative capacity upon pyruvate in muscle by 10%. These data provide evidence in humans that pharmacologically boosting BCAA oxidation, lowers BCAA plasma levels in patients with T2D and results in beneficial outcomes on patients' glucose metabolism.

NaPB-enhanced BCAA catabolism resulted in improved peripheral insulin sensitivity, which mainly involved glucose uptake by muscle, accompanied by a tendency towards lower fasting plasma glucose levels. The improved insulin-stimulated glucose uptake was attributed to enhanced glucose oxidation, without changes in NOGD, the latter reflecting glycogen synthesis. These findings align with cell and rodent studies, in which the BCKD kinase inhibitor BT2, like NaPB, effectively improved glucose tolerance of peripheral tissues, attenuated insulin resistance and enhanced glucose oxidation via stimulated insulin signaling in high-fat diet-induced obese mice. We found that NaPB treatment did not show major effects on hepatic insulin sensitivity, which suggest that insulin resistance

in the liver is less responsive to NaPB treatment. Together, our results show that the effects of NaPB treatment in patients with T2D for a great part take place in peripheral tissues, mainly muscle, which matches with the observation that skeletal muscle in humans has the highest capacity for BCAA catabolism (31, 32).

We found a decrease in 3-HIB plasma concentrations with NaPB treatment, paralleled by improved insulin sensitivity and furthermore associated with improved whole-body carbohydrate oxidation. This finding aligns with observational human studies, which showed that elevated 3-HIB plasma levels associate with insulin resistance and risk of incident T2D (21, 27). 3-HIB is formed from valine breakdown and becomes hydrolyzed by 3-HIB-CoA hydrolase, whereafter it can leave the mitochondria and cell to the extracellular fluid or plasma (33). As NaPB boosts BCAA catabolism, the decrease in 3-HIB plasma levels could be a resultant of better mitochondrial TCA cycling. Alternatively, oxidation of BCAA may have been repartitioned to tissues less amenable to 3-HIB secretion.

The obtained metabolic results are consistent in showing improved glucose handling at different levels: enhanced insulin-stimulated glucose disposal rate, improved insulin-stimulated whole-body carbohydrate oxidation and tendency towards a higher RER, as well indirectly, by increased mitochondrial oxidative capacity for pyruvate oxidation in muscle fibers. The mechanistic link between elevated plasma BCAA levels and insulin resistance remains poorly understood, although several hypotheses have been proposed (14, 34). Our data support that the lowering of BCAA plasma levels, and the concomitant reduction in plasma BCKA levels, may alleviate the inhibition on insulin signaling and glucose oxidation, leading to improved insulin sensitivity. A hypothesis is that the NaPB-induced improvement in insulin sensitivity could be explained by the reduced activation of the mammalian target of rapamycin complex (mTOR) (14), as elevated plasma BCAA levels are thought to impair insulin signaling via activation of S6 kinase (p70S6K) and mTOR (35-38). In addition, previous publications performed in animal heart tissue collectively showed that the accumulation of BCAA levels and its derived metabolites inhibit PDH activity, thereby hampering glucose oxidation and insulin sensitivity (39, 40). Therefore, NaPB-induced improved glucose oxidation could also be explained by higher PDH activity.

Previously we reported 60% improved insulin sensitivity and 33% improved mitochondrial oxidative capacity after a 3 months progressive exercise training program in patients with T2D (41, 42). Exercising is by far the most effective strategy to reduce diabetes-related metabolic disturbances, as well prevent or delay the onset. The 27% improvement in insulin sensitivity we observed in the current study

is therefore quite significant, amounting to about half of that achieved by exercise. The results emphasize the relevance of BCAA catabolism in insulin resistance in humans, as well the potential impact of this treatment strategy on metabolic health in metabolically compromised people. With this short duration time we observed these significant improvements, as well as tendencies for decreasing fasting glucose values and improved metabolic flexibility, which may form the lead for future studies with longer treatment duration.

Our study has limitations. We recognize unequal gender distribution. Although there were no restrictions for females participating in the study, a majority of male patients subscribed to the study. Therefore, future studies are needed to investigate whether similar effects occur in both sexes. Participants' wide range of BMI (25 – 38 kg/m^2) could influence metabolic responses. However, after adjustment for BMI, p-values remained significant meaning that it is unlikely BMI affected the metabolic responses seen. Due to the invasive character of the study, we prioritized evaluating effects on skeletal muscle and liver. Reports, however, highlight the role of BCAA catabolism in adipose tissue (43, 44). Therefore, it would be of interest to study NaPB treatment effects in human adipocytes and investigate its contributing effect on defining plasma BCAA levels. NaPB was given as add-on treatment, combined with oral antidiabetic agents of various mechanisms of action. For example, 14 patients were on metformin treatment throughout the study, and 8 patients received metformin only/or a combined therapy with sulphonylurea derivates. Recently, it has been shown that metformin does not alter BCAA plasma levels (45), while the effect of sulphonylureas on BCAA metabolism or BCAA plasma levels have not yet been investigated. It would be of interest to perform sub-group analysis comparing two groups of patients' medication in order to investigate drug-drug interactions, however, our sample size was too small to de reliable sub-group analysis. Therefore, we cannot conclude to what extent the effects of NaPB depend on the co-medication given, which should be investigated in future trials.

In summary, the present randomized double-blind placebo-controlled trial shows that NaPB treatment decreases BCAA levels together with an improvement in peripheral insulin sensitivity and muscle mitochondrial oxidative capacity on pyruvate in patients with T2D. These findings demonstrate in humans that pharmacologically boosting BCAA catabolism exerts substantial beneficial effects on glucose homeostasis in patients with T2D, as has previously been shown in numerous rodent models. Our work strongly justifies future efforts to investigate this potential treatment strategy for this prevalent and debilitating disease.

ACKNOWLEDGEMENTS

E.P. was granted with a senior fellowship from the Dutch Diabetes Foundation (Grant No. 2017.82.010), which supported the present study. E.P. furthermore received a "VENI" Research Grant for innovative research from the Netherlands Organization for Scientific Research (91613132), an EFSD/Lilly grant from the European Foundation for the Study of Diabetes (EFSD). Z.A. is supported by NIH (DK114103). T.W. received a junior fellowship from the Dutch Diabetes Foundation (Grant No. 2015.81.1833). V.S. was supported by an ERC starting grant (Grant no. 759161) 'MRS in diabetes'. Metabolomics were performed with support from the DRC Regional Metabolomics and Fluxomics Core (NIH 5P30DK019525-45 7239). The funding agencies had no role in study design, data collection and analysis or manuscript writing.

AUTHOR CONTRIBUTIONS

F.V, E.E.T, M.C.B. and M.N. performed the experiments and analyzed data. F.V. and E.P. wrote the manuscript. E.P., Z.A. J.H., P.S., T.W., M.C.M., V.S. and M.H. assisted during the acquisition, analysis and interpretation of data and reviewed the manuscript. E.P., P.S. and M.H. designed the study. All authors reviewed and approved the final version of the manuscript. E.P. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

DECLARATION OF INTERESTS

The authors declare no competing interests

SUPPLEMENTARY MATERIAL FOR CHAPTER 5

Supplementary table 1. NaPB treatment elevates muscle mitochondrial oxidative capacity [a]

	NaPB	Placebo	*P* value
	2 weeks intervention		
State 3 MOG [b]	39.58 ± 3.39	38.30 ± 2.72	0.41
State 3 MOGS [b]	61.25 ± 4.52	57.49 ± 3.62	0.25
State u MOGS [b]	73.33 ± 6.13	71.84 ± 5.91	0.78
State 3 MPG [c]	52.27 ± 3.15	47.90 ± 3.44	0.09
State 3 MPGS [c]	74.01 ± 4.05	67.05 ± 4.05	0.04*
State u MPGS [c]	109.38 ± 7.14	99.46 ± 7.56	0.12

[a] Data expressed as mean ± SE and are pmol $\cdot mg^{-1} \cdot s^{-1}$. The intervention effect was analyzed using the paired student t-test. **P* values <0.05 NaPB vs. Placebo. *NaPB, sodium phenylbutyrate; MOG, malate + octanoyl carnitine + glutamate; MOGS, malate + octanoyl carnitine + glutamate + succinate; MPG, malate + pyruvate + glutamate; MPGS, malate + pyruvate + glutamate + succinate;*
[b]*n* = 14, [c]*n*=13

Supplementary table 2. NaPB treatment did not alter ectopic lipid storage [a]

	NaPB	Placebo	*P* value
IMCL [b] (%)	0.61 ± 0.09	0.50 ± 0.07	0.15
IHL (CH_2/ ($CH_{2+}H_2O$)) [c] (%)	11.11 ± 2.26	11.70 ± 2.36	0.20
PUFA [b] (%)	13.15 ± 1.48	14.70 ± 1.39	0.50
MUFA [b] (%)	42.93 ± 1.79	41.70 ± 1.62	0.51
SFA [b] (%)	43.91 ± 1.27	44.21 ± 1.67	0.88

[a] Data expressed as mean ± SE. The intervention effect was analyzed using the paired student t-test. **P* values <0.05 NaPB vs. Placebo. *IMCL, intramyocellular lipid; IHL, intrahepatic lipid; PUFA, poly-unsaturated fatty acids; MUFA, mono-unsaturated fatty acids; SFA, saturated fatty acids; NaPB, sodium phenylbutyrate;*
[b]*n* = 13, [c]*n*=14

Supplementary table 3. NaPB treatment reduces plasma BCAA levels [a]

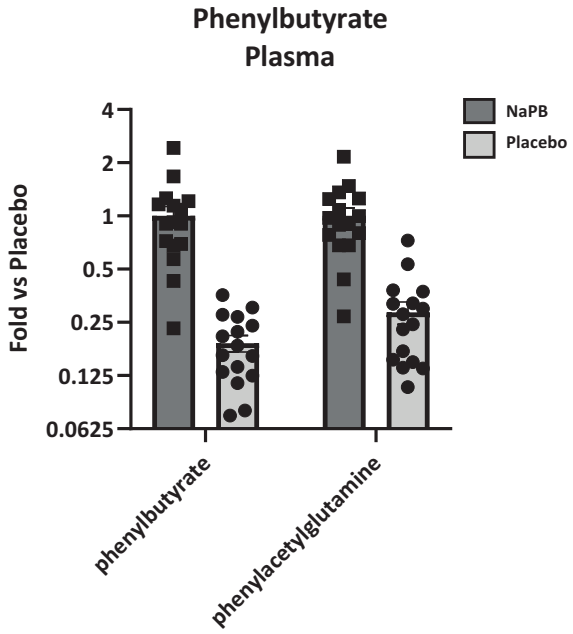
Amino acid (μmol/L)	**NaPB**	**Placebo**	***P* value**
Alanine	426 ± 16	440 ± 15	0.76
Arginine	71 ± 4	71 ± 4	0.83
Asparagine	64 ± 4	65 ± 5	0.78
Asparagine	2.2 ± 0.3	2.8 ± 0.9	0.51
Cysteine	68 ± 2	66 ± 2	0.16
Glutamic acid	122 ± 6	112 ± 7	0.17
Glycine	206 ± 12	194 ± 9	0.15
Histidine	70 ± 3	75 ± 2	0.08
Lysine	175 ± 5	183 ± 5	0.16
Methionine	23 ± 1	24 ± 1	0.07
Phenylalanine	61 ± 2	62 ± 2	0.11
Serine	89 ± 5	91 ± 4	0.63
Threonine	107 ± 5	111 ± 4	0.49
Tryptophan	49 ± 2	53 ± 2	0.01**
Tyrosine	64 ± 3	66 ± 3	0.09
Total AAA, μmol/L	174 ± 6	181 ± 6	0.004**
Total EAA, μmol/L	964 ± 18	1008 ± 24	0.004**

[a] n=16. Data expressed as mean ± SE. The intervention effect was analyzed using the paired student t-test. ***P* values < 0.01 NaPB vs. Placebo. Blood samples were taken after 2 weeks of NaPB treatment and placebo after an overnight fast. Total AAA includes phenylalanine, tryptophan, tyrosine; total EAA includes histidine, lysine, methionine, phenylalanine, tryptophan, tyrosine, isoleucine, leucine, valine. *NaPB, sodium phenylbutyrate; AAA, aromatic amino acids; EAA, essential amino acid*

Supplementary table 4. No change in sleeping metabolic rate and nocturnal substrate oxidation with NaPB treatment [a]

	NaPB	**Placebo**	***P* value**
SMR (MJ/d) [b]	7.0 ± 0.3	7.1 ± 0.3	0.92
Sleep RER [b]	0.81 ± 0.01	0.82 ± 0.01	0.58
Carbohydrate oxidation (μmol · kg^{-1} · min^{-1}) [c]	5.3 ± 0.5	5.4 ± 0.4	0.76
Fat oxidation (μmol · kg^{-1} · min^{-1}) c	2.2 ± 0.1	2.1 ± 0.1	0.61
Protein oxidation (μmol · kg^{-1} · min^{-1}) [c]	6.7 ± 0.5	7.0 ± 0.58	0.55

[a] Data expressed as mean ± SE. The intervention effect was analyzed using the paired student t-test. **P* values <0.05 NaPB vs. Placebo. *NaPB, sodium phenylbutyrate; SMR, sleep metabolic rate*
[b]n = 16, [c]n=15



Supplementary Figure. 1 Treatment compliance.

Study compliance was measured in patients with T2D after 2-week treatment with NaPB (grey bars, n=16) and placebo (white bars, n=16) in plasma. Data are expressed as mean ± SE. The intervention effect was analyzed using the paired student t-test. *P <0.05. *T2D, type 2 diabetes; NaPB, sodium phenylbutyrate*

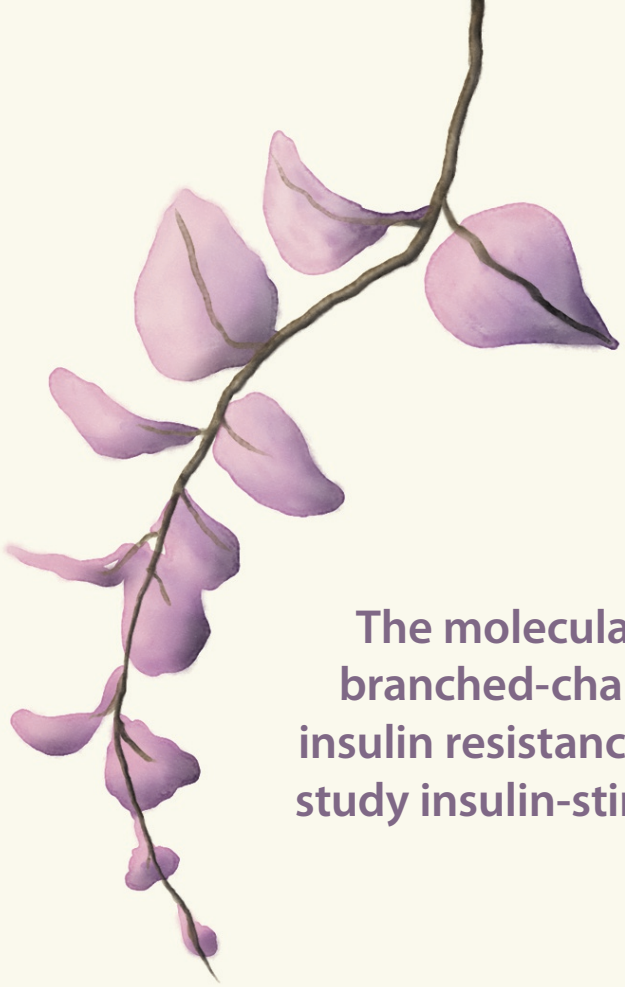
REFERENCES

1. Organization WH. Global Report on Diabetes. France; 2016.
2. Kahn SE. The relative contributions of insulin resistance and beta-cell dysfunction to the pathophysiology of Type 2 diabetes. Diabetologia. 2003;46(1):3-19.
3. Szendroedi J, Phielix E, Roden M. The role of mitochondria in insulin resistance and type 2 diabetes mellitus. Nat Rev Endocrinol. 2011;8(2):92-103.
4. van de Weijer T, Sparks LM, Phielix E, Meex RC, van Herpen NA, Hesselink MK, et al. Relationships between mitochondrial function and metabolic flexibility in type 2 diabetes mellitus. PLoS One. 2013;8(2):e51648.
5. Newgard CB, An J, Bain JR, Muehlbauer MJ, Stevens RD, Lien LF, et al. A branched-chain amino acid-related metabolic signature that differentiates obese and lean humans and contributes to insulin resistance. Cell Metab. 2009;9(4):311-26.
6. Wurtz P, Soininen P, Kangas AJ, Ronnemaa T, Lehtimaki T, Kahonen M, et al. Branched-chain and aromatic amino acids are predictors of insulin resistance in young adults. Diabetes Care. 2013;36(3):648-55.
7. Vanweert F, Boone SC, Brouwers B, Mook-Kanamori DO, de Mutsert R, Rosendaal FR, et al. The effect of physical activity level and exercise training on the association between plasma branched-chain amino acids and intrahepatic lipid content in participants with obesity. Int J Obes (Lond). 2021.
8. Vanweert F, de Ligt M, Hoeks J, Hesselink MKC, Schrauwen P, Phielix E. Elevated Plasma Branched-Chain Amino Acid Levels Correlate With Type 2 Diabetes-Related Metabolic Disturbances. J Clin Endocrinol Metab. 2021;106(4):e1827-e36.
9. Wang TJ, Larson MG, Vasan RS, Cheng S, Rhee EP, McCabe E, et al. Metabolite profiles and the risk of developing diabetes. Nat Med. 2011;17(4):448-53.
10. Floegel A, Stefan N, Yu Z, Muhlenbruch K, Drogan D, Joost HG, et al. Identification of serum metabolites associated with risk of type 2 diabetes using a targeted metabolomic approach. Diabetes. 2013;62(2):639-48.
11. She P, Van Horn C, Reid T, Hutson SM, Cooney RN, Lynch CJ. Obesity-related elevations in plasma leucine are associated with alterations in enzymes involved in branched-chain amino acid metabolism. Am J Physiol Endocrinol Metab. 2007;293(6):E1552-63.
12. Doisaki M, Katano Y, Nakano I, Hirooka Y, Itoh A, Ishigami M, et al. Regulation of hepatic branched-chain alpha-keto acid dehydrogenase kinase in a rat model for type 2 diabetes mellitus at different stages of the disease. Biochem Biophys Res Commun. 2010;393(2):303-7.
13. Adams SH. Emerging Perspectives on Essential Amino Acid Metabolism in Obesity and the Insulin-Resistant State. Adv Nutr. 2011;2(6):445-56.
14. Lynch CJ, Adams SH. Branched-chain amino acids in metabolic signalling and insulin resistance. Nat Rev Endocrinol. 2014;10(12):723-36.
15. Harris RA, Kobayashi R, Murakami T, Shimomura Y. Regulation of branched-chain alpha-keto acid dehydrogenase kinase expression in rat liver. J Nutr. 2001;131(3):841S-5S.
16. Shimomura Y, Obayashi M, Murakami T, Harris RA. Regulation of branched-chain amino acid catabolism: nutritional and hormonal regulation of activity and expression of the branched-chain alpha-keto acid dehydrogenase kinase. Curr Opin Clin Nutr Metab Care. 2001;4(5):419-23.
17. Tso SC, Gui WJ, Wu CY, Chuang JL, Qi X, Skvora KJ, et al. Benzothiophene carboxylate derivatives as novel allosteric inhibitors of branched-chain alpha-ketoacid dehydrogenase kinase. J Biol Chem. 2014;289(30):20583-93.

18. Uddin GM, Zhang L, Shah S, Fukushima A, Wagg CS, Gopal K, et al. Impaired branched chain amino acid oxidation contributes to cardiac insulin resistance in heart failure. Cardiovasc Diabetol. 2019;18(1):86.
19. Lian K, Guo X, Wang Q, Liu Y, Wang RT, Gao C, et al. PP2Cm overexpression alleviates MI/R injury mediated by a BCAA catabolism defect and oxidative stress in diabetic mice. Eur J Pharmacol. 2020;866:172796.
20. White PJ, McGarrah RW, Grimsrud PA, Tso SC, Yang WH, Haldeman JM, et al. The BCKDH Kinase and Phosphatase Integrate BCAA and Lipid Metabolism via Regulation of ATP-Citrate Lyase. Cell Metab. 2018;27(6):1281-93 e7.
21. Jang C, Oh SF, Wada S, Rowe GC, Liu L, Chan MC, et al. A branched-chain amino acid metabolite drives vascular fatty acid transport and causes insulin resistance. Nat Med. 2016;22(4):421-6.
22. Chen M, Gao C, Yu J, Ren S, Wang M, Wynn RM, et al. Therapeutic Effect of Targeting Branched-Chain Amino Acid Catabolic Flux in Pressure-Overload Induced Heart Failure. J Am Heart Assoc. 2019;8(11):e011625.
23. Neinast MD, Jang C, Hui S, Murashige DS, Chu Q, Morscher RJ, et al. Quantitative Analysis of the Whole-Body Metabolic Fate of Branched-Chain Amino Acids. Cell Metab. 2019;29(2):417-29 e4.
24. Zhou M, Shao J, Wu CY, Shu L, Dong W, Liu Y, et al. Targeting BCAA Catabolism to Treat Obesity-Associated Insulin Resistance. Diabetes. 2019;68(9):1730-46.
25. Burrage LC, Jain M, Gandolfo L, Lee BH, Members of the Urea Cycle Disorders C, Nagamani SC. Sodium phenylbutyrate decreases plasma branched-chain amino acids in patients with urea cycle disorders. Mol Genet Metab. 2014;113(1-2):131-5.
26. Harris LLS, Smith GI, Patterson BW, Ramaswamy RS, Okunade AL, Kelly SC, et al. Alterations in 3-Hydroxyisobutyrate and FGF21 Metabolism Are Associated With Protein Ingestion-Induced Insulin Resistance. Diabetes. 2017;66(7):1871-8.
27. Mardinoglu A, Gogg S, Lotta LA, Stancakova A, Nerstedt A, Boren J, et al. Elevated Plasma Levels of 3-Hydroxyisobutyric Acid Are Associated With Incident Type 2 Diabetes. EBioMedicine. 2018;27:151-5.
28. Yoon MS. The Emerging Role of Branched-Chain Amino Acids in Insulin Resistance and Metabolism. Nutrients. 2016;8(7).
29. Vanweert F, Boone SC, Brouwers B, Mook-Kanamori DO, de Mutsert R, Rosendaal FR, et al. The effect of physical activity level and exercise training on the association between plasma branched-chain amino acids and intrahepatic lipid content in participants with obesity. Int J Obes (Lond). 2021;45(7):1510-20.
30. Vanweert F, de Ligt M, Hoeks J, Hesselink MKC, Schrauwen P, Phielix E. Elevated plasma branched-chain amino acid levels correlate with type 2 diabetes-related metabolic disturbances. J Clin Endocrinol Metab. 2020.
31. Adeva MM, Calvino J, Souto G, Donapetry C. Insulin resistance and the metabolism of branched-chain amino acids in humans. Amino Acids. 2012;43(1):171-81.
32. Suryawan A, Hawes JW, Harris RA, Shimomura Y, Jenkins AE, Hutson SM. A molecular model of human branched-chain amino acid metabolism. Am J Clin Nutr. 1998;68(1):72-81.
33. Letto J, Brosnan ME, Brosnan JT. Valine metabolism. Gluconeogenesis from 3-hydroxyisobutyrate. Biochem J. 1986;240(3):909-12.
34. Arany Z, Neinast M. Branched Chain Amino Acids in Metabolic Disease. Curr Diab Rep. 2018;18(10):76.
35. Krebs M, Brunmair B, Brehm A, Artwohl M, Szendroedi J, Nowotny P, et al. The Mammalian target of rapamycin pathway regulates nutrient-sensitive glucose uptake in man. Diabetes. 2007;56(6):1600-7.

36. Um SH, D'Alessio D, Thomas G. Nutrient overload, insulin resistance, and ribosomal protein S6 kinase 1, S6K1. Cell Metab. 2006;3(6):393-402.
37. Tremblay F, Brule S, Hee Um S, Li Y, Masuda K, Roden M, et al. Identification of IRS-1 Ser-1101 as a target of S6K1 in nutrient- and obesity-induced insulin resistance. Proc Natl Acad Sci U S A. 2007;104(35):14056-61.
38. Thirone AC, Huang C, Klip A. Tissue-specific roles of IRS proteins in insulin signaling and glucose transport. Trends Endocrinol Metab. 2006;17(2):72-8.
39. Jackson RH, Singer TP. Inactivation of the 2-ketoglutarate and pyruvate dehydrogenase complexes of beef heart by branched chain keto acids. J Biol Chem. 1983;258(3):1857-65.
40. Li T, Zhang Z, Kolwicz SC, Jr., Abell L, Roe ND, Kim M, et al. Defective Branched-Chain Amino Acid Catabolism Disrupts Glucose Metabolism and Sensitizes the Heart to Ischemia-Reperfusion Injury. Cell Metab. 2017;25(2):374-85.
41. Phielix E, Meex R, Moonen-Kornips E, Hesselink M, Schrauwen P. Exercise training increases mitochondrial content and ex vivo mitochondrial function similarly in patients with type 2 diabetes and in control individuals. Diabetologia. 2010;53(8):1714-21.
42. Meex RC, Schrauwen-Hinderling VB, Moonen-Kornips E, Schaart G, Mensink M, Phielix E, et al. Restoration of muscle mitochondrial function and metabolic flexibility in type 2 diabetes by exercise training is paralleled by increased myocellular fat storage and improved insulin sensitivity. Diabetes. 2010;59(3):572-9.
43. Lackey DE, Lynch CJ, Olson KC, Mostaedi R, Ali M, Smith WH, et al. Regulation of adipose branched-chain amino acid catabolism enzyme expression and cross-adipose amino acid flux in human obesity. Am J Physiol Endocrinol Metab. 2013;304(11):E1175-87.
44. Zimmerman HA, Olson KC, Chen G, Lynch CJ. Adipose transplant for inborn errors of branched chain amino acid metabolism in mice. Mol Genet Metab. 2013;109(4):345-53.
45. Irving BA, Carter RE, Soop M, Weymiller A, Syed H, Karakelides H, et al. Effect of insulin sensitizer therapy on amino acids and their metabolites. Metabolism. 2015;64(6):720-8.
46. Steele R. Influences of glucose loading and of injected insulin on hepatic glucose output. Ann N Y Acad Sci. 1959;82:420-30.
47. Ackermans MT, Pereira Arias AM, Bisschop PH, Endert E, Sauerwein HP, Romijn JA. The quantification of gluconeogenesis in healthy men by (2)H2O and (2-(13)C]glycerol yields different results: rates of gluconeogenesis in healthy men measured with (2)H2O are higher than those measured with (2-(13)C]glycerol. J Clin Endocrinol Metab. 2001;86(5):2220-6.
48. Weir JB. New methods for calculating metabolic rate with special reference to protein metabolism. 1949. Nutrition. 1990;6(3):213-21.
49. Frayn KN. Calculation of substrate oxidation rates in vivo from gaseous exchange. J Appl Physiol Respir Environ Exerc Physiol. 1983;55(2):628-34.
50. Bergstrom J, Hermansen L, Hultman E, Saltin B. Diet, muscle glycogen and physical performance. Acta Physiol Scand. 1967;71(2):140-50.
51. Veksler VI, Kuznetsov AV, Sharov VG, Kapelko VI, Saks VA. Mitochondrial respiratory parameters in cardiac tissue: a novel method of assessment by using saponin-skinned fibers. Biochim Biophys Acta. 1987;892(2):191-6.
52. Roumans KHM, Lindeboom L, Veeraiah P, Remie CME, Phielix E, Havekes B, et al. Hepatic saturated fatty acid fraction is associated with de novo lipogenesis and hepatic insulin resistance. Nat Commun. 2020;11(1):1891.
53. Lindeboom L, Nabuurs CI, Hesselink MK, Wildberger JE, Schrauwen P, Schrauwen-Hinderling VB. Proton magnetic resonance spectroscopy reveals increased hepatic lipid content after a single high-fat meal with no additional modulation by added protein. Am J Clin Nutr. 2015;101(1):65-71.
54. Krssak M, Mlynarik V, Meyerspeer M, Moser E, Roden M. 1H NMR relaxation times of skeletal muscle metabolites at 3 T. MAGMA. 2004;16(4):155-9.

55. Melamud E, Vastag L, Rabinowitz JD. Metabolomic analysis and visualization engine for LC-MS data. Anal Chem. 2010;82(23):9818-26.
56. Su X, Lu W, Rabinowitz JD. Metabolite Spectral Accuracy on Orbitraps. Anal Chem. 2017;89(11):5940-8.
57. Plasqui G, Soenen S, Westerterp-Plantenga MS, Westerterp KR. Measurement of longitudinal changes in body composition during weight loss and maintenance in overweight and obese subjects using air-displacement plethysmography in comparison with the deuterium dilution technique. Int J Obes (Lond). 2011;35(8):1124-30.



6

The molecular link between elevated branched-chain amino acid levels and insulin resistance: a novel *in vitro* tool to study insulin-stimulated glucose uptake in C2C12 cells

Froukje Vanweert[1], Nima Gharahdaghi[2], Hannah Crossland[2], Esther Phielix[1], Matthijs K.C. Hesselink, Kenneth Smith[2], Daniel J. Wilkinson[2] and Philip J. Atherton[2]

[1] *Department of Nutrition and Movement Sciences, NUTRIM School of Nutrition and Translational Research in Metabolism, Maastricht University, Maastricht, The Netherlands*

[2] *Medical Research Council-Versus Arthritis Centre for Musculoskeletal Ageing Research, National Institute for Health Research Nottingham Biomedical Research Centre, Clinical, Metabolic and Molecular Physiology, University of Nottingham, Royal Derby Hospital, Derby, United Kingdom*

ABSTRACT

Branched-chain amino acids (BCAA) are known to be essential substrates for protein synthesis in skeletal muscle. Recent research has highlighted the role BCAA may have in regulating glucose metabolism. Moreover, people with insulin resistance and type 2 diabetes (T2D) feature elevated BCAA levels in plasma, which associates with insulin sensitivity. Here, we focused on the impact of BCAA supplementation on insulin signaling and glucose uptake in an *in vitro* skeletal muscle cell model (C2C12). Under insulin-stimulation, exposing cells to high BCAA medium (1.6 mM) increased S6K phosphorylation (0.4 mM: 0.55 ± 0.18 vs 1.6mM: 1.93 ± 0.50 RAU, $p<0.05$) and blunted Akt activation (0.4 mM: 1.00 ± 0.05 vs 1.6mM: 0.43 ± 0.06 RAU, $p<0.01$) compared to control medium (0.4 mM BCAA). The anticipated decreased intracellular glucose uptake was however not observed, as measured by 2-DG6P uptake under insulin-stimulated conditions. In addition to S6K, the high BCAA concentrations induced anabolic signaling as indicated by activation of 4EBP1 (0.4 mM: 0.49 ± 0.06 vs 1.6mM: 2.27 ± 0.15 RAU, $p<0.01$). To conclude, exposure of C2C12 myotubes to high levels of BCAA levels introduced impairments in intracellular insulin signaling exerted via the mTOR/S6K pathway.

INTRODUCTION

Branched-chain amino acids (BCAA) include the amino acids leucine, isoleucine and valine and are grouped as they share a structural feature with a branched-side chain, and common initiation steps of catabolism (1). BCAA impact on several metabolic routes and recent research highlights their regulatory role in glucose metabolism (2). We (3), as well as others (4-8), have repeatedly reported that people with insulin resistance and patients with type 2 diabetes (T2D) feature elevated BCAA plasma levels, which associate with peripheral insulin resistance. In the present study, we studied the effect of high (1.6mM) BCAA levels in medium on glucose uptake under basal and insulin-stimulated conditions. Moreover, we aimed to identify the impact of high BCAA on intracellular insulin signaling in an *in vitro* skeletal muscle murine cell model.

Over the last decade, BCAA metabolism has increasingly been considered to play a role in the development of insulin resistance in people with obesity and patients with T2D (9-11). Moreover, it has been demonstrated that elevated BCAA levels in people with obesity were related to a higher risk of the development of T2D (12). Although, it is still unknown why BCAA levels are elevated in obese and T2D prone individuals and why they associate with insulin resistance, a dysfunctional BCAA catabolism could hypothetically be an underlying factor (6, 11, 13).

One hypothesized route is that elevated BCAA in plasma of metabolically compromised people may act as signaling molecules hampering the insulin signaling. Data, predominantly derived from animal studies, indicates the involvement of the mammalian target of rapamycin (mTOR)/ribosomal protein S6 kinase (S6K) pathway (14-16). mTOR is a nutrient sensor and functions as a central signaling molecule of crosstalk between insulin and amino acids (17, 18). Under physiological conditions, insulin activates the insulin receptor (IRS-1) via phosphorylation, which in turn stimulates the phosphatidylinositol 3-kinase (PI3K)/Protein kinase B (Akt) pathway (19). Subsequently, Akt, phosphorylates Akt substrate 160 kDA (AS160) to trigger glucose transporter type 4 (GLUT4) translocation from intracellular sites to the surface of the cell. When more GLUT4 is on the cell membrane, more glucose can be taken up by the cell (13, 20-22). Next to activating GLUT4 translocation, Akt can also phosphorylate and inhibit tuberous sclerosis complex 1/2 (TSC 1/2), which activates mTOR resulting in degradation of IRS-1 (19). As a negative feedback system, insulin-mediated activation of IRS-1 indirectly activates mTOR. Alternatively, BCAA can activate mTOR, albeit independently of TSC 1/2. The exact molecular pathways involved in this process, however, are not completely understood (18, 23). It has been proposed that elevated

levels of BCAA in plasma, just like in insulin resistant conditions, may result in to hyperactivation of mTOR followed by activation of S6K. Thus, chronic accumulation of plasma BCAA levels and subsequent mTOR activation could impair insulin signaling for glucose uptake and consequently lead to insulin resistance (24-27). Examples from cell culture and rodent models, however, reveal inconsistent results about the effect of BCAA on insulin resistance via mTOR-activation (4, 9). Also in human obesity, it has not yet been investigated whether the elevated BCAA concentrations suffice to promote mTOR-activation (11, 28).

Besides the role of BCAA on glucose metabolism, BCAA are also essential for muscle protein synthesis and thus function as anabolic signaling molecules (29). It has been suggested that the anabolic effect of the BCAA is mediated in part through the activation of the mTOR (30). Activation of mTOR and subsequent phosphorylation of downstream targets, eukaryotic translation initiation factor 4E-binding protein 1 (4EBP1) and S6K stimulates protein synthesis with concomitant increases in skeletal muscle fibre size and muscle mass (31).

Skeletal muscle not only is the dominant tissue for BCAA catabolism (32), it also accounts for ~80% of the insulin-stimulated glucose uptake (33). Therefore, studying muscle cells in culture, is a valuable tool to investigate molecular links between BCAA and glucose metabolism. In the present study, insulin-stimulated glucose uptake will be assessed with the glucose-analogue tracer, 2-deoxy-D-glucose (2-DG) in an *in vitro* model using murine C2C12 cells. A recently developed method for gas chromatography combined with a triple quadrupole mass spectrometry system (GC-MS/MS) will be used to measure the intracellular glucose uptake. This method has the potential to be a sensitive and reliable method to detect low changes of labelled glucose-analogue (34). Here, with use of this methodology, we aim to investigate whether elevated BCAA levels exposed to skeletal muscle cells inhibit insulin-stimulated glucose uptake, via activation of the mTOR-S6K pathway. In addition, surrogate markers of muscle protein synthesis and anabolic signaling will be taken into account.

METHODS

C2C12 cell culture experiments

Murine C2C12 myoblasts (passage 8-10; ECACC, Salisbury, UK) were cultured at 37°C and 5% CO_2 in Dulbecco's Modified Eagle Medium (DMEM; Thermo Fisher Scientific) supplemented with 10% (vol/vol) fetal bovine serum (FBS) and 1% (vol/vol) antibiotic-antimycotic solution (all from Sigma-Aldrich, UK). Cells were seeded

onto six-well multidishes (Nunclon Delta; Thermo Fisher Scientific), and once cells reached ~90% confluency, differentiation was induced by changing the medium to minimum essential medium alpha (MEMα; Thermo Fisher Scientific) containing 2% (vol/vol) horse serum. In the MEMα used, control medium BCAA concentrations were all ~0.4 mM, according to the manufacturer's manual. A medium change was carried out every 48h.

Day 3 post-induction of differentiation, myotubes were exposed for 48h with control (0.4mM) or high (1.6mM) BCAA concentrations Simultaneously, deuterium water (D_2O) was added to the control (0.4 mM) and high BCAA (1.6mM) medium. After 48h, 5 days after induction of differentiation, medium was replaced with fresh medium supplemented without (basal) or with insulin (100nM). 2-DG (200µM) was added to both conditions (basal and insulin). Cells were collected after a further 1h, 4h and 6h of insulin stimulation (n=3 replicates for each treatment group). When medium replaced, control (0.4mM) or high (1.6mM) BCAA concentrations were maintained in the respective treatment groups. Media were collected and cells were harvested in homogenization buffer (50mM Tris-HCl, pH7.5, 1 mM EDTA, 1mM EGTA, 10 mM β-glycerophosphate, 50mM NaF, and complete protease inhibitor cocktail tablet; Roche, UK) for immunoblotting and D_2O analysis and in 75% methanol for measurement of 2-DG6P.

Measurement of 2-deoxy-D-glucose-6-phosphate (2-DG6P)

Non-stimulated time controls (1h, 4h and 6h) were run alongside insulin-stimulated cells. Cells were harvested in Methanol:Water (75:25 v/v) and homogenised via repeat pipetting to extract out intracellular metabolites. The supernatant containing the 2DGP was dried under nitrogen and derivatised to its methyloxime-TMS ester via addition of methoxylamine hydrochloride in pyridine and BSTFA. 2DGP concentration was analysed using a TRACE 1310 Gas Chromatograph connected to TSQ 8000 triple quadrupole gas chromatography (GC)-MS/MS (Thermo Fisher Scientific, Hemel Hempstead, UK) with reference to a standard curve of know concentrations ranging from 25 to 0.78µM 2-DG6P. Thereafter, standard curve was converted to mass per volume of sample and normalised for ug of cellular protein content.

Measurement of D_2O tracer

The deuterium enrichment was measured in medium collected from cell culture plates by incubating 100µl of each sample with 2µl of 10M NaOH and 1µl of acetone for 24h at room temperature. Following incubation, the acetone was extracted into 200µl of n-heptane, and 0.5µl of the heptane phase was injected into the GC-MS/MS for analysis. A standard curve of known D_2O enrichment was run alongside the samples for calculation of enrichment.

Protein-bound alanine intracellular fraction enrichment and calculation of fractional synthesis rate.

Samples were centrifuged at 13.000*xg* for 5 min at 4 °C, and the supernatant was collected. The pellet was solubilized in 0.3M NaOH and separated from the insoluble collagen by centrifugation, and the intracellular protein was precipitated using 1M perchloric acid. Protein-bound amino acids were released using acid hydrolysis by incubating in 0.1M HCl in Dowex H^+ resin slurry overnight before being eluted from the resin with 2M NH_4OH and evaporated to dryness; amino acids were then derivatized as their N-methoxycarbonyl methyl ester. Dried samples were suspended in 60µl of distilled water and 8µl of methyl chloroformate were added. Samples were vortexed for 30s and left to react at room temperature for 5 min. The newly formed N-methoxycarbonyl methyl ester amino acids were then extracted into 100µl chloroform. A molecular sieve was added to each sample for ~20 S before being transferred to a clean glass GC insert, removing any remaining water by size exclusion absorption. The intracellular protein-bound alanine enrichment was determined by gas chromatography-tandem mass spectrometry 4 °C alongside a standard curve of known $_{DL}$ -alanine-2,3,3,3-d_4 enrichment to validate measurement accuracy of the machine. Intracellular MPS, in terms of fractional synthetic rate (FSR), was calculated from the incorporation of deuterium-labelled alanine into protein using the enrichment of body water [corrected for the mean number of deuterium moieties incorporated per alanine (3.7) and the dilution from the total number of hydrogens in the derivative (i.e., 11)] as the surrogate precursor labelling between time points. The equation used was:

$$FSR = -\ln\left[\frac{\frac{APE(ala)}{APE(pre)}}{t}\right]$$

where APE_{ala} equals deuterium enrichment of protein-bound alanine, APE_p indicates mean precursor enrichment over the time period, and t is the time cell collection.

Western blot analysis

Samples were lysed by repeatedly passing through gel-loading pipette tips, and lysates were centrifuged at 13.000*xg* for 10 min at 4°C. Ten micrograms of protein were loaded onto Criterion XT 12% Bis-Tris gels (Bio-Rad, UK), and samples were electrophoresed at 200 V for 45 min. Sample transfer to PDVF membranes were performed at 100V for 45 min; and membranes were blocked in 5% (wt/vol) non-fat dry milk for 1h at room temperature. Primary antibody incubation was carried out overnight at 4°C with the following antibodies (Cell Signaling Technology, UK): Akt Ser473 (no. 4060), S6K Thr389 (no. 92345), mTOR Ser2448 (no. 9271S) and

4EBP1 Thr37/46 (no. 2855). After antibody incubations, membranes were washed with 1x TBS-Tween and incubated with anti-rabbit horseradisch peroxidase (HRP)-conjugated secondary antibody (no. 7074; Cell Signaling Technology; 1:2000 dilution) for 1h at room temperature. After further washing with TBS-Tween, bands were detected using Chemiluminescent HRP substrate (Millipore EMD) with a Chemidoc XRS imaging system (Bio-Rad, UK). Staining of the membrane with Coomassie Brilliant Blue was used for total protein normalization.

Statistical analyses

Shapiro-Wilk normality test was performed to evaluate normal distribution. A two-way ANOVA (using a Bonferroni's correction for multiple testing) was performed to evaluate the differences between treatments (control vs. high BCAA concentrations) at different time-points (1h, 4h and 6h). A two-tailed unpaired Student's *t*-test was used to evaluate the effect of high BCAA concentrations on FSR. $P<0.05$ was considered as statistically significant. All data are presented as means ±SEM.

RESULTS

Effects of elevated BCAA on insulin signaling in C2C12 myotubes

First, we aimed to explore whether supplementation of BCAA in medium for 48h would affect insulin signaling pathways. The change in phosphorylation status upon the transition from basal to insulin stimulated conditions (1h, 4h and 6h) of proteins involved in insulin signaling was determined, after a control (0.4mM) vs. high (1.6mM) concentration exposure of BCAA concentrations. The delta of the phosphorylation status of the insulin-signaling mediated proteins in the insulin condition vs. the basal condition of the different timepoints (1h, 4h and 6h) was computed to represent the stimulatory effect of insulin (Figure 1).

The inhibitory effect of BCAA on insulin signaling after 1h of insulin stimulation is shown by a higher phosphorylation status of mTOR, albeit non-significantly (0.4mM: 0.01 ± 0.17 vs 1.6mM: 1.6 ± 1.0 RAU, p=0.07, Figure 1a). This inhibitory effect was alleviated after 4h and 6h of insulin incubation. In addition, a significant BCAA-effect was found for S6K (p=0.04) and 4EPB1 (p=0.03). The results showed that after 1h insulin stimulation high BCAA levels in medium significantly increased activity of S6K (0.4 mM: 0.55 ± 0.18 vs 1.6mM: 1.93 ± 0.50 RAU, p=0.04, Figure 1b) and 4EBP1 (0.4mM: 0.49 ± 0.06 vs 1.6mM: 2.27 ± 0.15 RAU, p=0.04, Figure 1c), matching with increased protein synthesis. This significant increase was attenuated at later time points. Akt (Figure 1d), a more downstream target of the

mTOR/S6K signaling, was also affected by BCAA supplementation (p=0.01). After 4h (0.4mM: 0.95 ± 0.14 vs. 1.6mM: 0.48 ± 0.15 RAU, p=0.02) and 6h (0.4 mM: 1.00 ± 0.05 vs. 1.6mM: 0.43 ± 0.06 RAU, p=0.007) of insulin stimulation, the phosphorylation of Akt was blunted in the high BCAA condition.

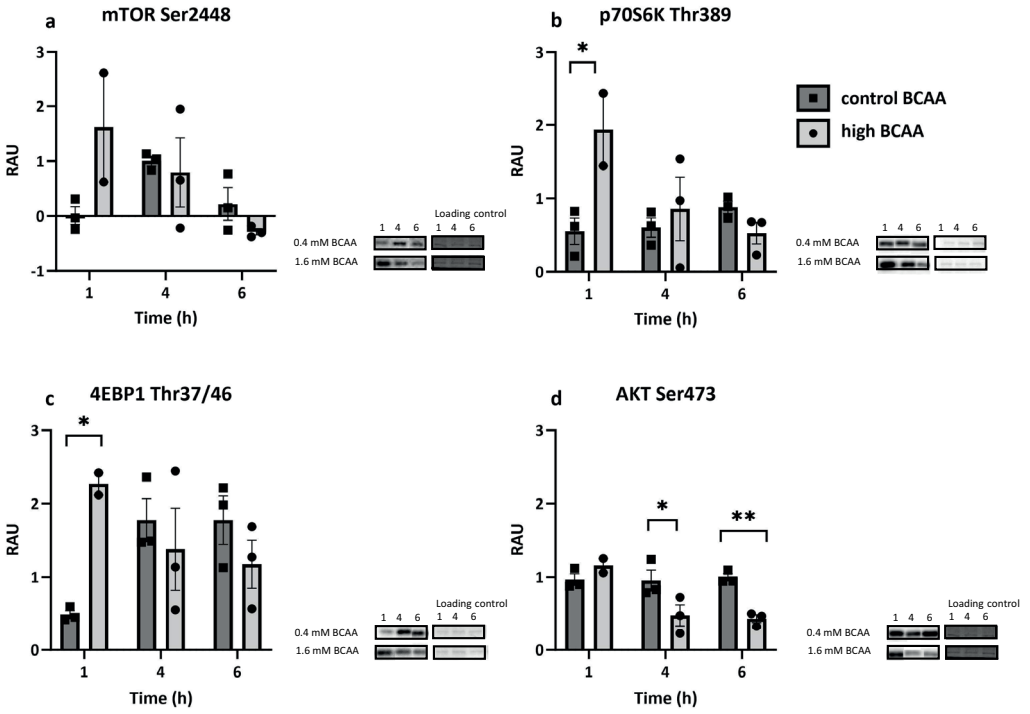


Figure 1. Phosphorylation status of proteins involved in insulin signaling under control (0.4mM; squares) and high BCAA (1.6mM; dots) concentrations in C2C12 cells after 1h, 4h and 6h with insulin stimulation vs. basal condition.

Values are means ± standard error of the mean. * *P*<0.05, ** *P*<0.01. P-value reflect treatment effect by ANOVA. *4EBP1, eukaryotic translation initiation factor 4E-binding protein 1; AKT, protein kinase B; BCAA, branched-chain amino acids; mTOR, mammalian target of rapamycin; p70S6K, ribosomal S6 kinase.*

Effects of elevated BCAA on glucose uptake in C2C12 myotubes.

Intracellular 2-DG6P concentration was used as marker of glucose uptake and measured in vitro after a control (0.4mM) vs. high (1.6mM) concentration exposure of BCAA for 48h, subsequently without (termed as basal condition) and with the presence of insulin, and at different incubation times (1h, 4h and 6h).

In the absence of insulin stimulation, the basal condition (Figure 2a), no significant change was found on the uptake of 2-DG6P after high exposure of BCAA

concentrations vs. control concentrations at different incubation times. However, a significant time-effect was found (p=0.0003), 2-DG6P uptake increased significantly from 1h to 4h incubation time in both control and high BCAA condition. (0.4mM: p = 0.006, 1.6mM: p = 0.003), and from 1h to 6h in the high BCAA condition (p=0.003).

The results showed a similar pattern under insulin stimulation (Figure 4b, time-effect: p=0.001). The uptake of 2-DG6P increased from 1h to 4h (0.4mM: p = 0.0007, 1.6mM: p = 0.004) and 6h of insulin stimulation (0.4mM: p = 0.009, 1.6mM: p = 0.02), both in control as well as under high BCAA concentrations.

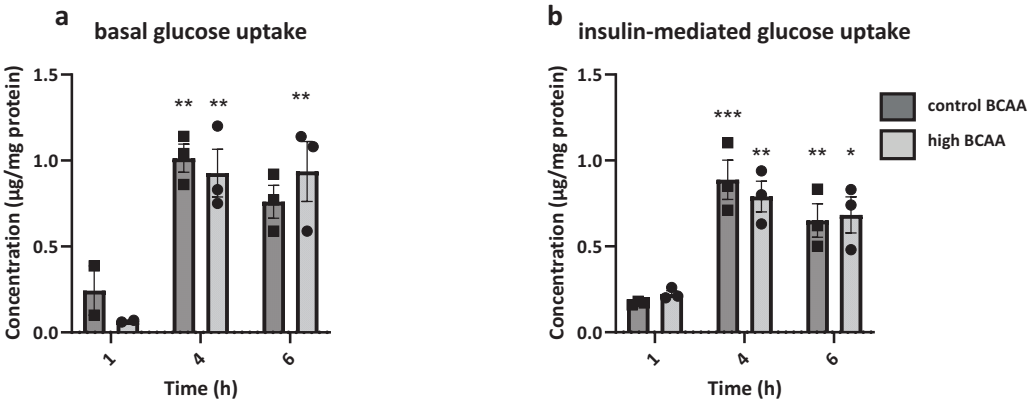


Figure 2. Intracellular 2DG6P concentration as marker of glucose uptake under control (0.4mM; squares) and high BCAA (1.6mM; dots) concentrations in C2C12 cells after 1h, 4h, and 6h without (A) and with (B) insulin stimulation.

Values are means ± standard error of the mean. * *P*<0.05 vs. 1, ** *P* <0.01 vs. 1, *** *P* <0.001. P-value reflect time-effect by ANOVA. *BCAA, branched-chain amino acids. 2-DG6P, 2-deoxy-D-glucose-6-phosphate.*

Effects of elevated BCAA on FSR in C2C12 myotubes.

FSR was measured without the presence of insulin. The addition of 10% D_2O to cell medium have led to a medium enrichment of 9.83 ± 0.11% in the control condition (0.4mM) BCAA and 10.14 ± 0.11% for the media with high BCAA concentrations (1.6mM) after 48h.

In the myotubes, FSR decreased significantly after exposure with high BCAA concentration (1.6mM: 0.76 ± 0.01%/h) compared to the control condition (0.4mM: 0.81 ± 0.02%/h (Figure 3).

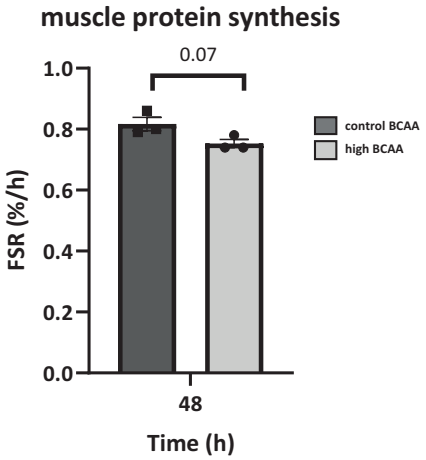


Figure 3. FSR under control (0.4mM; squares) and high BCAA (1.6mM; dots) concentrations in C2C12 cells after 48h.

Values are means ± standard error of the mean. P-value reflect treatment effect by student t-test. *BCAA, branched-chain amino acids; FSR, fractional synthetic rate.*

DISCUSSION

BCAA has been considered to have an emerging role in the pathogenesis of T2D. Others (4-8) and we (6, 7) have shown elevated plasma BCAA levels in insulin resistant states. Although the underlying mechanisms are unknown, several pathways have been hypothesized on how BCAA levels may impede peripheral insulin signaling and hence insulin-stimulated glucose uptake. BCAA are recognized substrates for protein synthesis and may perform the role of anabolic signals (29, 35). Our aim was, therefore, to investigate the effects of BCAA supplementation on regulators of insulin signaling with inclusion of markers of anabolic protein signaling. We hypothesized that exposing C2C12 myotubes to BCAA would impede the insulin signaling pathway with blunted glucose uptake but enhanced protein synthesis as a consequence. The results of the study showed that supplementation of BCAA 1) impedes insulin signaling via phosphorylation of S6K, 2) failed to impede downstream glucose uptake, 3) stimulated anabolic signaling via 4EBP1 phosphorylation.

Effect of elevated BCAA levels on insulin signaling

One of the proposed mechanisms is that plasma BCAA interfere with insulin signaling via stimulation of mTOR and S6K and, therefore, contribute to the

development of insulin resistance in humans (4, 5). In this study, we focused on the effect of BCAA on insulin signaling in an *in vitro* skeletal muscle cell model in basal and insulin-stimulated conditions.

Previous work showed that *in vitro* supplementation of BCAA resulted in activation of S6K in rat pancreatic ß-cells (36) and depressed IRS-1/Akt signaling in mice skeletal muscle cells (37, 38). These findings were confirmed *in vitro* and *in vivo* studies performed by Tremblay et al. (39, 40), although in these studies, a mixture of amino acids that were not exclusively BCAA, was used and therefore the isolated effect of BCAA cannot be deduced from these data. As expected, our data showed that exposing C2C12 muscle cells to high BCAA levels did augment the phosphorylation status of S6K, a downstream target of mTOR, indicating an activation of S6K, in an insulin-stimulated setting. Moreover, we demonstrated that BCAA supplementation resulted in a diminished activation of Akt (by phosphorylation), an important enzyme regulating glucose uptake in tissues. Therefore, compromised insulin-stimulated glucose uptake was anticipated. For reasons, currently unknown, glucose uptake was not diminished under the high BCAA blunted insulin signaling condition.

In contrast to our hypothesis, we did not observe an BCAA-induced effect on mTOR activation after insulin stimulation. The regulation of mTOR signaling remains complex reflected by the inconsistent data reporting stimulatory as well as inhibitory functions for mTOR in insulin signal transduction (41). In this study, we measured Ser2448 phosphorylation site of mTOR, which is only one of many of the phosphorylation sites of mTOR (41). Thus, effects on other phosphorylation sites cannot be excluded. Additionally, it is very challenging to detect and quantify mTOR signaling (42). Here we used the downstream mTOR target, S6K phosphorylation (43), as a proxy for mTOR activity (44). Follow-up studies should focus on other phosphorylation sites of mTOR, such as Thr2446, Ser2481, Ser1261 (45) to investigate which phosphorylation site is involved in the BCAA-induced activation of mTOR/S6k pathway.

Another interesting finding is that we observed an order-of-event effect of BCAA supplementation on the phosphorylation status of important insulin signaling proteins after different insulin incubation times. The results point out that under high BCAA conditions, the downstream target of mTOR, S6K, was phosphorylated (activated) first after 1h of insulin stimulation. The preceding role of S6K in the inhibition of the insulin signaling was underlined further by an increased Akt phosphorylation (inactivation) after 4h and 6h of insulin treatment. One could

speculate that this timing of action is due to the duration of insulin stimulation since it is noted that insulin in elevated BCAA conditions activates phosphorylation sites of different proteins time-dependent, which differentially regulate signal transductions. This might confirm our hypothesis that BCAA-induced activation of mTOR/S6K will eventually result in a decreased Akt-activation.

Despite several lines of experimental evidence supporting the view that the effect of BCAA on insulin resistance occurs through mTOR/S6K activation, some observations are controversial. In contrast to observations by others who reported no effect of leucine or valine on mTOR/Akt signaling upon insulin stimulation in rodent myotubes (46-48), we observed that high BCAA concentrations blunted the insulin stimulatory effect on Akt via mTOR/S6K. Interestingly, Newgard et al. (49) suggested a dissociation between BCAA-induced mTOR activity and insulin resistance, which is in contrast with our data but has been confirmed by others (50-52). Other routes than in impaired insulin signaling can be involved in the link between BCAA levels and insulin sensitivity, such as a dysfunctional mitochondrial BCAA catabolism resulting in disturbed substrate oxidation (53).

Effect of elevated BCAA levels on intracellular glucose uptake

The model used in the present study allowed us to directly assess the impact of increased extracellular BCAA on the glucose uptake in skeletal muscle cells in basal and insulin-stimulated conditions. To assess insulin-stimulated glucose uptake, labelling with 2-DG has been widely used for the measurement of intracellular glucose uptake (54). After uptake in the cell, 2-DG will be immediately phosphorylated via hexokinase forming 2DG-6P. Because the hydroxyl group is missing, formed 2DG-6P is not able to entry glycolysis causing it to be trapped in the cell (55). In that way, levels of 2-DG-6P can be used as a proxy of glucose uptake. Some tissues, such as liver, kidney and intestine feature high levels of glucose-6-phosphatase (G6Pase) activity, and therefore, a small fraction of 2DG-6P can enter glycogenesis or can be dephosphorylated back to 2-DG, however, is negligible in muscle tissue (55-57). A sensitive and reliable method is needed to quantify small amounts of 2-DG-6P, and various techniques, such as nuclear magnetic resonance (NMR) (58), enzymatic assays (59-61), and scintillation of radiolabelled metabolites (56, 62-65) has been developed. Although these techniques all have their own advantages, important difficulties need to be kept in mind, including lack of sensitivity, time-consuming and requirement for expertise. Using GC-MS/MS could overcome these complications due to its superior selectivity and sensitivity (34).

Although an increase in intracellular glucose uptake was observed over time, no change was observed between insulin stimulation and basal conditions. In insulin-stimulated conditions, glucose transport is almost entirely accounted for by the GLUT4-isoform, which is also the most abundant glucose transporter in human skeletal muscle (66). However, in established skeletal muscle cell lines, including C2C12, limited GLUT4 is present, while GLUT1 is expressed to a greater extend (67). Also others suggests that GLUT1 plays a major role in glucose transport in C2C12 myoblast (68). Since the GLUT-1 isoform is responsible for non-insulin mediated glucose transport, it is conceivable that the observed increased glucose uptake over time was GLUT-1 mediated, established by a concentration gradient, and not by GLUT-4. Therefore, this study should be repeated in differentiated human myotubes, who shown to have a lower GLUT1:GLUT4 (69) action making it a suitable experimental tool to study the effect of BCAA supplementation on insulin action.

While the impact of BCAA supplementation, especially leucine, on glucose uptake in muscle has been broadly investigated, no consensus on its main effect has been reached. Previous observations showed that BCAA supplementation blunted insulin-mediated glucose uptake, measured by Akt (38). In contrast, others found that BCAA supplementation did increase glucose uptake in skeletal muscle tissue (50, 70) and myotubes (48, 71) of rats. Similarly, in medium containing supraphysiological leucine concentrations (5-10 mM) glucose uptake in C2C12 skeletal muscle cells was substantially elevated, even without insulin-stimulation (72). In our hands, we did not found effects of BCAA supplementation on insulin-stimulated glucose uptake, measured by 2DG-6P, which is in contrast to the BCAA-induced inactivation of Akt observed under insulin-stimulation.

Effect of elevated BCAA levels on muscle protein synthesis

BCAA are key components for muscle protein synthesis (73) through activation of anabolic signaling pathways (29), possibly mediated in part through the activation of the mTOR (30) and subsequent phosphorylation of downstream targets, 4EBP1 and S6K (31). Previous reports (44, 74, 75) showed that leucine had the unique capacity to stimulate mTOR via S6K and 4EBP1 phosphorylation, even in the absence of insulin stimulation in skeletal muscle cells and tissue of rodents. This indicates that leucine functions independently as a nutritional signaling molecule stimulating protein synthesis (75). In agreement, in the present study, we report that BCAA stimulates mTOR signaling using 4EBP1 and S6K phosphorylation as surrogate measures, which is indicative of its unique role in stimulating protein synthesis, in presence of insulin.

Surprisingly, we found that despite a stimulated anabolic protein signaling, muscle protein synthesis, measured with D_2O, was decreased after exposure to BCAA. In contrast, previous studies in rats revealed that BCAAs primarily stimulate muscle protein synthesis in rats, independently of insulin (75-77), which was also confirmed in humans (78). The reason why the observed decreased muscle protein synthesis did not match with the stimulated anabolic protein signaling is unclear, however, duration of exposure to D_2O (>48h) must be considered by interpreting the analysis (79) since C2C12 skeletal muscle cells feature relative high protein turnover (80). A further exploration is needed about the exposure time of the D_2O tracer.

BCAA paradox

Current evidence suggests that the metabolic effects of BCAA can be either beneficial or detrimental (73). In line, our results point out that two perspectives on BCAA exists. Conflicting data with regards to the role of BCAA in metabolic health might be coined the BCAA paradox (81). The mechanisms of this paradox remain unclear, but a plausible explanation is that BCAA can play different roles in glucose metabolism in individuals with different levels of insulin sensitivity. For example, BCAA supplementation can be beneficial for healthy individuals, but detrimental in insulin-resistant states who might suffer from a defective BCAA catabolism, and therefore, are unable to handle the supplemented BCAA resulting in metabolic disturbances. These findings highlight the complexity about the role of BCAA in the regulation of insulin resistance and metabolic dysfunction. Future studies are warranted to delineate these and other possible signaling mechanisms linking BCAA to skeletal muscle insulin resistance.

Strengths and limitations

The present work is the first successful GS-MS/MS method able to quantify trace amounts of the phosphorylated metabolite (2-DG6P) of the isotopic glucose tracer (2-DG), and thus has the sensitivity to determine glucose uptake in biological samples. Previously, an liquid chromatography (LC)-MS method has been developed to quantify 2-DG6P (34, 82), but is challenging as a consequence of its polarity and poor ionization (34). These difficulties can be overcome with GC-MS/MS. An advantage of MS/MS is that the detection sensitivity can be enhanced in comparison with other detection methods and, therefore, very low concentrations of glucose tracer can be measured in the samples. In this study, no insulin-mediated effects on 2-DG6P uptake were observed, and therefore, insulin stimulation protocol needs to be optimized and adapted, and eventually, validated.

A limitation of this study is that C2C12 skeletal muscle cells were exposed to sustained supraphysiological levels of BCAA, hence, it is not established whether small physiological changes in BCAA levels are sufficient to cause insulin resistance. Furthermore, it is also important to highlight that an *in vitro* skeletal muscle model with continuous exposure of BCAA makes it difficult to extrapolate findings *in vivo*. Another limitation is that intra- and extracellular levels of BCAA and BCKA were not measured in these experiments, which would have given further insight into downstream impact of elevated BCAA in muscle cells. As a compromised BCAA catabolism has also been suggested to be linked with insulin resistance (83), it would be interesting to investigate the effect of branched-chain keto acids (BCKA), as marker for defective BCAA catabolism, on insulin signaling pathway. Previous studies have been shown that exposure of muscle cells to higher BCKA resulted in decreased glucose uptake, measured by phosphorylation of Akt (84). As our pilot study data points towards that elevated BCAA levels might hamper insulin signaling pathway, more targets of the mTOR/S6K pathway affecting insulin signaling should be explored in later research.

Conclusion

In the present study, we demonstrated that exposing C2C12 skeletal muscle cells to increases in extracellular BCAA caused impairments in intracellular signaling via the mTOR/S6K pathway *in vitro*. In parallel, this increase in BCAA modulated anabolic signaling via activation of 4EBP1, in addition to S6K. Our results contribute substantially to further knowledge about the link between BCAA and glucose metabolism in skeletal muscle cells. Our results could indicate that the elevation of BCAA levels, which reflects elevation in plasma seen in patient with T2D, can be responsible for insulin resistance. Further investigation in human cells to further increase the insight about the underlying mechanisms of elevated BCAA levels in insulin resistant states, such as T2D, should be warranted.

GRANTS

This work was financially supported by the Foundation “De Drie Lichten” in The Netherlands and by the EMBO Scientific Exchange Grant (number 9142).

REFERENCES

1. Nishimura J, Masaki T, Arakawa M, Seike M, Yoshimatsu H. Isoleucine prevents the accumulation of tissue triglycerides and upregulates the expression of PPARalpha and uncoupling protein in diet-induced obese mice. J Nutr. 2010;140(3):496-500.
2. Nie C, He T, Zhang W, Zhang G, Ma X. Branched Chain Amino Acids: Beyond Nutrition Metabolism. Int J Mol Sci. 2018;19(4).
3. Wang TJ, Larson MG, Vasan RS, Cheng S, Rhee EP, McCabe E, et al. Metabolite profiles and the risk of developing diabetes. Nat Med. 2011;17(4):448-53.
4. Newgard CB, An J, Bain JR, Muehlbauer MJ, Stevens RD, Lien LF, et al. A branched-chain amino acid-related metabolic signature that differentiates obese and lean humans and contributes to insulin resistance. Cell Metab. 2009;9(4):311-26.
5. Krebs M, Krssak M, Bernroider E, Anderwald C, Brehm A, Meyerspeer M, et al. Mechanism of amino acid-induced skeletal muscle insulin resistance in humans. Diabetes. 2002;51(3):599-605.
6. Vanweert F, de Ligt M, Hoeks J, Hesselink MKC, Schrauwen P, Phielix E. Elevated Plasma Branched-Chain Amino Acid Levels Correlate With Type 2 Diabetes-Related Metabolic Disturbances. J Clin Endocrinol Metab. 2021;106(4):e1827-e36.
7. Vanweert F, Boone SC, Brouwers B, Mook-Kanamori DO, de Mutsert R, Rosendaal FR, et al. The effect of physical activity level and exercise training on the association between plasma branched-chain amino acids and intrahepatic lipid content in participants with obesity. Int J Obes (Lond). 2021.
8. Palmer ND, Stevens RD, Antinozzi PA, Anderson A, Bergman RN, Wagenknecht LE, et al. Metabolomic profile associated with insulin resistance and conversion to diabetes in the Insulin Resistance Atherosclerosis Study. J Clin Endocrinol Metab. 2015;100(3):E463-8.
9. Adams SH. Emerging Perspectives on Essential Amino Acid Metabolism in Obesity and the Insulin-Resistant State. Adv Nutr. 2011;2(6):445-56.
10. Xu F, Tavintharan S, Sum CF, Woon K, Lim SC, Ong CN. Metabolic signature shift in type 2 diabetes mellitus revealed by mass spectrometry-based metabolomics. J Clin Endocrinol Metab. 2013;98(6):E1060-5.
11. She P, Van Horn C, Reid T, Hutson SM, Cooney RN, Lynch CJ. Obesity-related elevations in plasma leucine are associated with alterations in enzymes involved in branched-chain amino acid metabolism. Am J Physiol Endocrinol Metab. 2007;293(6):E1552-63.
12. Ramzan I, Ardavani A, Vanweert F, Mellett A, Atherton PJ, Idris I. The Association between Circulating Branched Chain Amino Acids and the Temporal Risk of Developing Type 2 Diabetes Mellitus: A Systematic Review & Meta-Analysis. Nutrients. 2022;14(20).
13. Lynch CJ, Adams SH. Branched-chain amino acids in metabolic signalling and insulin resistance. Nat Rev Endocrinol. 2014;10(12):723-36.
14. Hutson SM, Sweatt AJ, Lanoue KF. Branched-chain [corrected] amino acid metabolism: implications for establishing safe intakes. J Nutr. 2005;135(6 Suppl):1557S-64S.
15. Harper AE, Miller RH, Block KP. Branched-chain amino acid metabolism. Annu Rev Nutr. 1984;4:409-54.
16. Ding C, Li Y, Guo F, Jiang Y, Ying W, Li D, et al. A Cell-type-resolved Liver Proteome. Mol Cell Proteomics. 2016;15(10):3190-202.
17. Ma XM, Blenis J. Molecular mechanisms of mTOR-mediated translational control. Nat Rev Mol Cell Biol. 2009;10(5):307-18.
18. Gleason CE, Lu D, Witters LA, Newgard CB, Birnbaum MJ. The role of AMPK and mTOR in nutrient sensing in pancreatic beta-cells. J Biol Chem. 2007;282(14):10341-51.
19. Yoon MS. The Emerging Role of Branched-Chain Amino Acids in Insulin Resistance and Metabolism. Nutrients. 2016;8(7).

20. Jewell JL, Russell RC, Guan KL. Amino acid signalling upstream of mTOR. Nat Rev Mol Cell Biol. 2013;14(3):133-9.
21. Thirone AC, Huang C, Klip A. Tissue-specific roles of IRS proteins in insulin signaling and glucose transport. Trends Endocrinol Metab. 2006;17(2):72-8.
22. Sakamoto K, Holman GD. Emerging role for AS160/TBC1D4 and TBC1D1 in the regulation of GLUT4 traffic. Am J Physiol Endocrinol Metab. 2008;295(1):E29-37.
23. Sancak Y, Peterson TR, Shaul YD, Lindquist RA, Thoreen CC, Bar-Peled L, et al. The Rag GTPases bind raptor and mediate amino acid signaling to mTORC1. Science. 2008;320(5882):1496-501.
24. Krebs M, Brunmair B, Brehm A, Artwohl M, Szendroedi J, Nowotny P, et al. The Mammalian target of rapamycin pathway regulates nutrient-sensitive glucose uptake in man. Diabetes. 2007;56(6):1600-7.
25. Um SH, D'Alessio D, Thomas G. Nutrient overload, insulin resistance, and ribosomal protein S6 kinase 1, S6K1. Cell Metab. 2006;3(6):393-402.
26. Tremblay F, Brule S, Hee Um S, Li Y, Masuda K, Roden M, et al. Identification of IRS-1 Ser-1101 as a target of S6K1 in nutrient- and obesity-induced insulin resistance. Proc Natl Acad Sci U S A. 2007;104(35):14056-61.
27. Um SH, Frigerio F, Watanabe M, Picard F, Joaquin M, Sticker M, et al. Absence of S6K1 protects against age- and diet-induced obesity while enhancing insulin sensitivity. Nature. 2004;431(7005):200-5.
28. Laferrere B, Reilly D, Arias S, Swerdlow N, Gorroochurn P, Bawa B, et al. Differential metabolic impact of gastric bypass surgery versus dietary intervention in obese diabetic subjects despite identical weight loss. Sci Transl Med. 2011;3(80):80re2.
29. Laplante M, Sabatini DM. Regulation of mTORC1 and its impact on gene expression at a glance. J Cell Sci. 2013;126(Pt 8):1713-9.
30. Gran P, Cameron-Smith D. The actions of exogenous leucine on mTOR signalling and amino acid transporters in human myotubes. BMC Physiol. 2011;11:10.
31. Bodine SC, Stitt TN, Gonzalez M, Kline WO, Stover GL, Bauerlein R, et al. Akt/mTOR pathway is a crucial regulator of skeletal muscle hypertrophy and can prevent muscle atrophy in vivo. Nat Cell Biol. 2001;3(11):1014-9.
32. Neinast MD, Jang C, Hui S, Murashige DS, Chu Q, Morscher RJ, et al. Quantitative Analysis of the Whole-Body Metabolic Fate of Branched-Chain Amino Acids. Cell Metab. 2019;29(2):417-29 e4.
33. Petersen KF, Dufour S, Savage DB, Bilz S, Solomon G, Yonemitsu S, et al. The role of skeletal muscle insulin resistance in the pathogenesis of the metabolic syndrome. Proc Natl Acad Sci U S A. 2007;104(31):12587-94.
34. Williams A MD, Zhang G. A Fast and Sensitive Method Combining Reversed-Phase Chromatography with High Resolution Mass Spectrometry to Quantify 2-Fluoro-2-Deoxyglucose and Its Phosphorylated Metabolite for Determining Glucose Uptake. ChemRxiv Cambridge: Cambridge Open Engage;. 2019.
35. Holecek M. Branched-chain amino acids in health and disease: metabolism, alterations in blood plasma, and as supplements. Nutr Metab (Lond). 2018;15:33.
36. Xu G, Kwon G, Marshall CA, Lin TA, Lawrence JC, Jr., McDaniel ML. Branched-chain amino acids are essential in the regulation of PHAS-I and p70 S6 kinase by pancreatic beta-cells. A possible role in protein translation and mitogenic signaling. J Biol Chem. 1998;273(43):28178-84.
37. Crossland H, Smith K, Idris I, Phillips BE, Atherton PJ, Wilkinson DJ. Exploring mechanistic links between extracellular branched-chain amino acids and muscle insulin resistance: an in vitro approach. Am J Physiol Cell Physiol. 2020;319(6):C1151-C7.

38. Rivera ME, Rivera CN, Vaughan RA. Branched-chain amino acids at supraphysiological but not physiological levels reduce myotube insulin sensitivity. Diabetes Metab Res Rev. 2021:e3490.
39. Tremblay F, Krebs M, Dombrowski L, Brehm A, Bernroider E, Roth E, et al. Overactivation of S6 kinase 1 as a cause of human insulin resistance during increased amino acid availability. Diabetes. 2005;54(9):2674-84.
40. Tremblay F, Marette A. Amino acid and insulin signaling via the mTOR/p70 S6 kinase pathway. A negative feedback mechanism leading to insulin resistance in skeletal muscle cells. J Biol Chem. 2001;276(41):38052-60.
41. Weigert C, Kron M, Kalbacher H, Pohl AK, Runge H, Haring HU, et al. Interplay and effects of temporal changes in the phosphorylation state of serine-302, -307, and -318 of insulin receptor substrate-1 on insulin action in skeletal muscle cells. Mol Endocrinol. 2008;22(12):2729-40.
42. Truillet C, Cunningham JT, Parker MFL, Huynh LT, Conn CS, Ruggero D, et al. Noninvasive Measurement of mTORC1 Signaling with (89)Zr-Transferrin. Clin Cancer Res. 2017;23(12):3045-52.
43. Magnuson B, Ekim B, Fingar DC. Regulation and function of ribosomal protein S6 kinase (S6K) within mTOR signalling networks. Biochem J. 2012;441(1):1-21.
44. Atherton PJ, Smith K, Etheridge T, Rankin D, Rennie MJ. Distinct anabolic signalling responses to amino acids in C2C12 skeletal muscle cells. Amino Acids. 2010;38(5):1533-9.
45. Acosta-Jaquez HA, Keller JA, Foster KG, Ekim B, Soliman GA, Feener EP, et al. Site-specific mTOR phosphorylation promotes mTORC1-mediated signaling and cell growth. Mol Cell Biol. 2009;29(15):4308-24.
46. Rivera ME, Lyon ES, Johnson MA, Vaughan RA. Leucine increases mitochondrial metabolism and lipid content without altering insulin signaling in myotubes. Biochimie. 2020;168:124-33.
47. Rivera ME, Lyon ES, Johnson MA, Sunderland KL, Vaughan RA. Effect of valine on myotube insulin sensitivity and metabolism with and without insulin resistance. Mol Cell Biochem. 2020;468(1-2):169-83.
48. Yin Q, Brameld JM, Parr T, Murton AJ. Leucine and mTORc1 act independently to regulate 2-deoxyglucose uptake in L6 myotubes. Amino Acids. 2020;52(3):477-86.
49. D'Antona G, Ragni M, Cardile A, Tedesco L, Dossena M, Bruttini F, et al. Branched-chain amino acid supplementation promotes survival and supports cardiac and skeletal muscle mitochondrial biogenesis in middle-aged mice. Cell Metab. 2010;12(4):362-72.
50. Zeanandin G, Balage M, Schneider SM, Dupont J, Hebuterne X, Mothe-Satney I, et al. Differential effect of long-term leucine supplementation on skeletal muscle and adipose tissue in old rats: an insulin signaling pathway approach. Age (Dordr). 2012;34(2):371-87.
51. She P, Reid TM, Bronson SK, Vary TC, Hajnal A, Lynch CJ, et al. Disruption of BCATm in mice leads to increased energy expenditure associated with the activation of a futile protein turnover cycle. Cell Metab. 2007;6(3):181-94.
52. Magkos F, Bradley D, Schweitzer GG, Finck BN, Eagon JC, Ilkayeva O, et al. Effect of Roux-en-Y gastric bypass and laparoscopic adjustable gastric banding on branched-chain amino acid metabolism. Diabetes. 2013;62(8):2757-61.
53. Vanweert F, Schrauwen P, Phielix E. Role of branched-chain amino acid metabolism in the pathogenesis of obesity and type 2 diabetes-related metabolic disturbances BCAA metabolism in type 2 diabetes. Nutr Diabetes. 2022;12(1):35.
54. Yamamoto N, Ueda M, Sato T, Kawasaki K, Sawada K, Kawabata K, et al. Measurement of glucose uptake in cultured cells. Curr Protoc Pharmacol. 2011;Chapter 12:Unit 12 4 1-22.

55. Virkamaki A, Rissanen E, Hamalainen S, Utriainen T, Yki-Jarvinen H. Incorporation of [3-3H]glucose and 2-[1-14C]deoxyglucose into glycogen in heart and skeletal muscle in vivo: implications for the quantitation of tissue glucose uptake. Diabetes. 1997;46(7):1106-10.
56. Doenst T, Taegtmeyer H. Complexities underlying the quantitative determination of myocardial glucose uptake with 2-deoxyglucose. J Mol Cell Cardiol. 1998;30(8):1595-604.
57. Sweet IR, Najafi H, Li G, Grodberg J, Matschinsky FM. Measurement and modeling of glucose-6-phosphatase in pancreatic islets. Am J Physiol. 1997;272(4 Pt 1):E696-711.
58. Hopkins JC, Radda GK, Veech RL, Clarke K. Accumulation of 2-deoxy-D-glucose-6-phosphate as a measure of glucose uptake in the isolated perfused heart: a 31P NMR study. Metab Eng. 2004;6(1):36-43.
59. Sasson S, Oron R, Cerasi E. Enzymatic assay of 2-deoxyglucose 6-phosphate for assessing hexose uptake rates in cultured cells. Anal Biochem. 1993;215(2):309-11.
60. Yamamoto N, Kawasaki K, Sato T, Hirose Y, Muroyama K. A nonradioisotope, enzymatic microplate assay for in vivo evaluation of 2-deoxyglucose uptake in muscle tissue. Anal Biochem. 2008;375(2):397-9.
61. Tong H, Chen W, London RE, Murphy E, Steenbergen C. Preconditioning enhanced glucose uptake is mediated by p38 MAP kinase not by phosphatidylinositol 3-kinase. J Biol Chem. 2000;275(16):11981-6.
62. Thompson AL, Lim-Fraser MY, Kraegen EW, Cooney GJ. Effects of individual fatty acids on glucose uptake and glycogen synthesis in soleus muscle in vitro. Am J Physiol Endocrinol Metab. 2000;279(3):E577-84.
63. Halseth AE, Bracy DP, Wasserman DH. Overexpression of hexokinase II increases insulinand exercise-stimulated muscle glucose uptake in vivo. Am J Physiol. 1999;276(1):E70-7.
64. Hansen PA, Gulve EA, Holloszy JO. Suitability of 2-deoxyglucose for in vitro measurement of glucose transport activity in skeletal muscle. J Appl Physiol (1985). 1994;76(2):979-85.
65. Chi MM, Manchester JK, Carter JG, Pusateri ME, McDougal DB, Lowry OH. A refinement of the Akabayashi-Saito-Kato modification of the enzymatic methods for 2-deoxyglucose and 2-deoxyglucose 6-phosphate. Anal Biochem. 1993;209(2):335-8.
66. Olson AL, Pessin JE. Structure, function, and regulation of the mammalian facilitative glucose transporter gene family. Annu Rev Nutr. 1996;16:235-56.
67. Nedachi T, Kanzaki M. Regulation of glucose transporters by insulin and extracellular glucose in C2C12 myotubes. Am J Physiol Endocrinol Metab. 2006;291(4):E817-28.
68. Rauch C, Loughna P. C2C12 skeletal muscle cells exposure to phosphatidylcholine triggers IGF-1 like-responses. Cell Physiol Biochem. 2005;15(5):211-24.
69. Al-Khalili L, Chibalin AV, Kannisto K, Zhang BB, Permert J, Holman GD, et al. Insulin action in cultured human skeletal muscle cells during differentiation: assessment of cell surface GLUT4 and GLUT1 content. Cell Mol Life Sci. 2003;60(5):991-8.
70. Nishitani S, Takehana K, Fujitani S, Sonaka I. Branched-chain amino acids improve glucose metabolism in rats with liver cirrhosis. Am J Physiol Gastrointest Liver Physiol. 2005;288(6):G1292-300.
71. Doi M, Yamaoka I, Nakayama M, Sugahara K, Yoshizawa F. Hypoglycemic effect of isoleucine involves increased muscle glucose uptake and whole body glucose oxidation and decreased hepatic gluconeogenesis. Am J Physiol Endocrinol Metab. 2007;292(6):E1683-93.
72. Doi M, Yamaoka I, Fukunaga T, Nakayama M. Isoleucine, a potent plasma glucose-lowering amino acid, stimulates glucose uptake in C2C12 myotubes. Biochem Biophys Res Commun. 2003;312(4):1111-7.

73. Gannon NP, Schnuck JK, Vaughan RA. BCAA Metabolism and Insulin Sensitivity - Dysregulated by Metabolic Status? Mol Nutr Food Res. 2018;62(6):e1700756.
74. Patti ME, Brambilla E, Luzi L, Landaker EJ, Kahn CR. Bidirectional modulation of insulin action by amino acids. J Clin Invest. 1998;101(7):1519-29.
75. Anthony JC, Yoshizawa F, Anthony TG, Vary TC, Jefferson LS, Kimball SR. Leucine stimulates translation initiation in skeletal muscle of postabsorptive rats via a rapamycin-sensitive pathway. J Nutr. 2000;130(10):2413-9.
76. Kobayashi H, Kato H, Hirabayashi Y, Murakami H, Suzuki H. Modulations of muscle protein metabolism by branched-chain amino acids in normal and muscle-atrophying rats. J Nutr. 2006;136(1 Suppl):234S-6S.
77. Buse MG, Reid SS. Leucine. A possible regulator of protein turnover in muscle. J Clin Invest. 1975;56(5):1250-61.
78. Alvestrand A, Hagenfeldt L, Merli M, Oureshi A, Eriksson LS. Influence of leucine infusion on intracellular amino acids in humans. Eur J Clin Invest. 1990;20(3):293-8.
79. Wolff C, Shipman P, Peelor III F, Hamilton K, Miller B. Assessment of Muscle Protein Synthesis Rates are Dependent upon Individual Protein Synthetic Rates, Content, and Duration of Label Incorporation. The FASEB Journal. 2015;29(S1):824.3.
80. Stansfield BN, Brown AD, Stewart CE, Burniston JG. Dynamic Profiling of Protein Mole Synthesis Rates during C2C12 Myoblast Differentiation. Proteomics. 2021;21(1):e2000071.
81. Yoneshiro T, Wang Q, Tajima K, Matsushita M, Maki H, Igarashi K, et al. BCAA catabolism in brown fat controls energy homeostasis through SLC25A44. Nature. 2019;572(7771):614-9.
82. Diaz-Moralli S, Ramos-Montoya A, Marin S, Fernandez-Alvarez A, Casado M, Cascante M. Target metabolomics revealed complementary roles of hexose- and pentose-phosphates in the regulation of carbohydrate-dependent gene expression. Am J Physiol Endocrinol Metab. 2012;303(2):E234-42.
83. Biswas D, Duffley L, Pulinilkunnil T. Role of branched-chain amino acid-catabolizing enzymes in intertissue signaling, metabolic remodeling, and energy homeostasis. Faseb J. 2019;33(8):8711-31.
84. Biswas D, Dao KT, Mercer A, Cowie AM, Duffley L, El Hiani Y, et al. Branched-chain ketoacid overload inhibits insulin action in the muscle. J Biol Chem. 2020;295(46):15597-621.

7

The association between circulating branched-chain amino acids and the temporal risk of developing type 2 diabetes mellitus: a systematic review & meta-analysis

Imran Ramzan [1,2], Arash Ardavani [1,2], Froukje Vanweert [3], Aisling Mellett [1,4], Philip J Atherton [1,2] and Iskandar Idris [1,2,]

[1] *Clinical, Metabolic and Molecular Physiology Research Group, MRC-Versus Arthritis Centre for Musculo-skeletal Ageing Research, University of Nottingham, Royal Derby Hospital Centre, Derby, DE22 6DT, UK*

[2] *National Institute for Health Research (NIHR) Nottingham Biomedical Research Centre, Nottingham, NG7 2UH, UK*

[3] *Department of Nutrition and Movement Sciences, NUTRIM, School of Nutrition and Translational Re-search in Metabolism, Maastricht University, Maastricht, 6229 ER, the Netherlands*

[4] *School of Nutrition, School of Agriculture and Food Science, Agriculture and Food Science Centre, University College Dublin, Belfield, Dublin, Ireland.*

This article has been published in: Nutrients. 2022 Oct 20;14(20):4411

ABSTRACT

Introduction: Recent studies have concluded that elevated circulating branched chain amino ac-ids (BCAA) are associated with the pathogenesis of type 2 diabetes mellitus (T2D) and obesity. However, the development of this association over time and the quantification of the strength of this association for individual BCAAs prior to T2D diagnosis remains unexplored.

Methods: A systematic search was conducted using the Healthcare Databases Advance Search (HDAS) via the National Institute for Health and Care Excellence (NICE) website. The data sources included EMBASE, MEDLINE and PubMed for all papers from inception until November 2021. Nine studies were identified in this systematic review and meta-analysis. Stratification was based on follow-up times (0–6, 6–12 and 12 or more years) and controlling of body mass index (BMI) through the specific assessment of overweight cohorts was also undertaken.

Results: The meta-analysis revealed a statistically significant positive association between BCAA concentrations and the development of T2D, with valine OR = 2.08 (95% CI = 2.04–2.12, $p < 0.00001$), leucine OR = 2.25 (95% CI = 1.76–2.87, $p < 0.00001$) and isoleucine OR = 2.12, 95% CI = 2.00–2.25, $p < 0.00001$. In addition, we demonstrated a positive consistent temporal association between circulating BCAA levels and the risk of developing T2D with differentials in the respective follow-up times of 0–6 years, 6–12 years and ≥12 years follow-up for valine (OR = 2.08, 1.86 and 2.14, $p<0.05$ each), leucine (OR = 2.10, 2.25 and 2.16, $p<0.05$ each) and isoleucine (OR = 2.12, 1.90 and 2.16, $p<0.05$ each) demonstrated.

Conclusion: Plasma BCAA concentrations are associated with T2D incidence across all temporal subgroups. We suggest the potential utility of BCAAs as an early biomarker for T2D irrespective of follow-up time.

INTRODUCTION

Type 2 diabetes mellitus (T2D) and obesity are major causes of morbidity and mortality worldwide (1). The World Health Organisation (WHO) estimates that, by the year 2035, 592 million patients with diabetes will exist worldwide (2). The discovery of novel metabolites and biomarkers that reflect early changes in T2D and obesity are crucial for both early diagnosis and the prevention of long-term complications. Indeed, a recent study determined that early detection and treatment of T2D reduces the risk of cardiovascular morbidity and mortality (3).

Metabolomics has increasingly been implemented as an analytical approach to identify novel metabolites within a biological specimen (4,5) potentially facilitating their utilisation as biomarkers and novel drug therapy targets (6). The utilisation of metabolomics in large epidemiological studies has also increased exponentially over the last decade. Specifically, the metabolic alterations associated with or preceding the development of T2D makes this an attractive area of research (7). Almost 50 metabolites have demonstrated an association with T2D, but the most extensively researched group of metabolites are the branched chain amino acids (BCAAs) (8,9).

BCAAs consist of the amino acids isoleucine, leucine and valine. BCAAs are widely present in human food sources containing protein, such as meat and dairy food items, in addition to being supplemented by recreational and professional athletes in an isolated, supplement form (10–13). Recent prospective studies have highlighted an association between elevated circulating levels of BCAAs and the development of T2D (14–16). In addition, studies that have successfully reduced circulating BCAA levels in humans have also demonstrated improvements in insulin sensitivity (17,18). Consequently, elevated circulating levels of BCAAs could potentially serve as biomarkers for the development of T2D (19–21). However, systematic reviews that have investigated this association using cohort studies have provided conflicting results (16,22). Furthermore, systematic reviews which analysed an association between individual (rather than total) BCAAs and risks of T2D did not reach statistical significance (22). Analysis derived from case–control studies would produce more robust findings due to the inherent reduction in the potential for confounding (23). Further interpretation of these associations will also be strengthened by analysing total and individual BCAA levels at baseline and follow-up. (24,25). A further ubiquitous issue within the literature is the presence of confounding variables, including body mass index (BMI) and ethnicity (26–28), with various studies implementing differing statistical procedures or variable lists for covariate adjustment (29–31). Thus, any summative analysis of the relationship

between BCAAs and T2D should address these variables through a sufficiently structured study design.

Multiple *in vitro* and epidemiological studies have revealed variations in the effect of leucine, isoleucine and valine with various components of glucose metabolism (32–34). Additionally, the relationship between BCAAs and their contribution to the development of T2D phenotype with time has not been investigated previously (35). The determination of temporality as an additional environmental contributor to the resulting T2D phenotype, within the context of BCAA metabolism, is therefore of novel epidemiological and clinical interest. The identification of specific BCAA-temporal patterns may provide healthcare professionals with an evidence-based rationale to undertake timeframe and BCAA-specific assessments for the development of T2D in at-risk patient groups, in order to facilitate early lifestyle and dietary interventions.

In this systematic review, an appraisal of the relationship between BCAAs and T2D development is undertaken through case–control study data of patients with an overweight BMI status. In addition, we also aim to appraise this relationship with the effect of temporality, based on the duration of follow-up in the incorporated studies. To the best of our knowledge, no review has attempted to determine if the association between BCAAs and T2D exhibits any variation based on follow-up time to diagnosis of T2D. Finally, we also aimed to assess any differences in the effect size of each individual BCAA for each of the studied timeframes.

MATERIALS AND METHODS

Protocol and registration

The study protocol is registered in the International prospective register of systematic reviews (PROSPERO 2022; registration number CRD42022297132), with the entry registration occurring after the finalization of our research question and the implementation of our search strategy. The Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) reporting guidelines are provided (Supplementary S1) (36).

Eligibility criteria

We exclusively sourced prospective case–control studies possessing pre- and post-follow up period individual plasma BCAA levels of at least one year in duration. The inclusion criteria, with respect to samples from within the case

groups of the study cohorts, is patients that eventually developed non-insulin-dependent type 2 diabetes mellitus (T2D) and a body mass index (BMI) of between 25 and 30 kg/m^2. The control groups were matched for BMI. A full enumeration of our criteria for study inclusion and exclusion, with additional justifications, are provided through the population, intervention, comparison, and studies (PICOS) format (Supplementary S2).

Information sources and searches

MEDLINE and EMBASE databases were accessed through the NICE Healthcare Databases Advanced Search (HDAS). An additional search inquiry was performed through PubMed. Grey area literature sources came through Google Scholar and Scopus. These five databases constituted our primary sources. Studies that did not meet our inclusion criteria based on design, but nonetheless addressed the research question, were included into our secondary sources list, and were reviewed for additional studies through their references. The primary sources were all searched on 3 November 2021 and the secondary sources were reviewed on 13 January 2022. The full electronic search strategy preformed for MEDLINE, EMBASE, Pubmed, Scopus and Google Scholar are presented in (Supplementary S5).

Study selection

Assessment of studies from primary sources performed by two independent researchers (I.R., F.V.) with a third (A.A.) serving as consensus former in the event of disagreement. The titles, abstracts and full texts of the included studies were evaluated after removal of any duplicates. Inter-observer agreement was found to be substantial (88.41%, Cohen's κ 67%). Secondary sources were assessed by one independent researcher (A.A.). Study screening (assessment of manuscript suitability for inclusion based on our eligibility criteria) and eligibility (assessment for the presence of statistical data that met our eligibility criteria) were performed in parallel by two researchers (I.R., A.A.), and no disagreement was observed at either stage.

Data collection process

A single independent researcher (A.A.) created a data extraction form (Microsoft Excel spreadsheet). Thereafter, another independent researcher (A.M.) extracted the following information: first author, publication year, sample size, patient demographics, diabetic and BMI status, and main outcomes. In addition, any correlation statistics present in the studies that are present at the eligibility stage of screening were included for qualitative assessment and extracted into a separate excel sheet.

Intra-study risk of bias assessment

Intra-study bias: The Newcastle Ottawa tool (NOS) was used to assess the overall quality and reliability of the case–control studies that reached synthesis (37). Assessment was performed by two independent researchers (I.R., F.V.) with a third (A.A.) providing consensus. The overall quality and extent of bias was ranked based on a summative count and assessed within the context of three domains: comparability of cases and controls, selection of cases and controls and exposure. These three domains were separated into 8 individual questions each receiving a single star except comparability which received two stars. Thus, each study was given a score of bias ranging from 7–9 stars "High quality", 4–7 stars as "Fair quality" and 1–3 stars "Low quality".

Inter-study bias: Should sufficient studies (n = 10) be encountered, publication bias will be assessed using Egger's regression test through the Copenhagen Trial Unit's Trial Sequential Analysis (TSA) software, with the generation of a funnel plot for visual inspection of distribution skew.

Summary measures

The summary measure per data-point is the log–odds ratio (+-standard error), with the measure of effect reported as the inverse variance (IV) with 95% confidence intervals (CI) and a measure of significance ($p < 0.05$).

Synthesis of results

The inverse-variance method and random effects model was selected due to anticipated imprecision. The effect size (reported as a Z-score) and study heterogeneity (reported as an I^2 and Tau statistic) are reported. Heterogeneity is interpreted as advocated by Cochrane (38). Should the original manuscripts present numeric correlation data, these were incorporated without further transformation or synthesis and are reported with their associated measurements of statistical significance ($p < 0.05$). Temporal groupings were defined on a continuous scale (follow-up duration in years) based on the requirement for at least three datapoints to be represented in each grouping, with the duration represented by each temporal grouping defined post hoc following allocation.

Risk of bias across studies

Inter-study bias: Should a sufficient number of studies (n = 10) be encountered, publication bias will be assessed using Egger's regression test through the Copenhagen Trial Unit's Trial Sequential Analysis (TSA) software, with the generation of a funnel plot (39).

Addition al Analyses

In order to appraise the decision to include datapoints from studies with estimations of effect size through differing covariates (Table 1), sensitivity analysis was performed through all available datapoints for each of the amino acids and irrespective of follow-up duration. Herein, only those studies presenting effect size estimations through the inclusion of less than five covariates (Table 1) were retained for pooled estimation of effect size for each assessed amino acid and follow-up duration. In accordance with Cochrane guidelines (40), the resulting estimates are presented in tabulated form.

Table 1. Study characteristics of the manuscripts, which reached synthesis.

Lead Author	Publication Date	Study Design	Cases (n)	Control (n)	Patient Demographics (Intervention(s))
F Ottosson	2018	Case-control	204	496	Mean of 69.5 years, predominantly male (69%), Swedish nationality
Y Lu	2016	Case-control	197	197	55.15 ± 2.8 years, predominantly female (59.4%), Chinese nationality
L Shi	2018	Case-control	503	503	50.1 ± 8 years, predominantly female (55.5%), Swedish nationality
T Wang (Framingham)	2011	Case-control	189	189	56.5 ± 8.5 years, predominantly male (58%), USA nationality
T Wang (Malmo)	2011	Case-control	163	163	Mean of 58 years, predominantly female (55%), Swedish nationality
A Floegel	2013	Case-control	800	2282	52.1 ± 8.1 years, predominantly women (52.1%), German nationality
R Wang-Sattler	2012	Case-control	91	866	64.7 ± 5.45 years, predominantly male (53%), German nationality
A Stancáková	2012	Case-control	646	3026	57 ± 7 years, all male, Finnish nationality
T Tillin (European)	2015	Case-control	643	1007	50.6 ± 7.0 years, all male, South Asian origin
T Tillin (South Asian)	2015	Case-control	801	1279	52.75 ± 7.25 years, all male, European origin
ND Palmer	2015	Case-control	76	70	56 ± 8 years, predominantly female (63%), European-American, Hispanic, and African American ethnicity

T2D, type 2 diabetes mellitus; BCAAs, branched chain amino acids; C2, Acetylcarnitine; C3, Propionylcarnitine; C4, Butyrylcarnitine; C5, Isovalerylcarnitine; C10, Decenoylcarnitine; KMV, 3-methyl-2-oxovaleric acid; BMI, body mass index; FBC, fasting blood glucose; PA, physical acitivity; WHR, waist hip ratio; IR, insulin resistance; BP, blood pressure; HDL, high-density lipid.

Follow-Up Period (Y)	Covariates	Cases Group Status	Control Group Status	Individual BCAAs	BCAA Metabolites
6.3	Age and Sex.	T2D	Non-diabetic	Leucine, Isoleucine, Valine	C5, C4 and C3
6	BMI, smoking, history of hypertension	T2D	Non-diabetic	Leucine, Isoleucine, Valine	C10
10	BMI, FBC, PA, education, smoking, consumption of alcohol, dietary fibre, red and processed meat, and coffee, plasma total cholesterol, triglycerols, and systolic and diastolic BP.	T2D (no medication)	Non-diabetic	Leucine, Isoleucine, Valine	C3 and KMV
12	Age, sex, BMI, fasting glucose, and parental history.	T2D	Non-diabetic	Leucine, Isoleucine, Valine	-
12.6	Age, sex, BMI, and fasting glucose.	T2D	Non-diabetic	Leucine, Isoleucine, Valine	-
7	Age, sex, alcohol intake, smoking, physical activity, education, coffee intake, red meat intake, prevalent hypertension, BMI, and waist circumference (cm)	T2D	Non-diabetic	Isoleucine, Valine	-
7	Age, sex, BMI, physical activity, alcohol intake, smoking, systolic BP, HDL cholesterol, HbA1c, fasting glucose and fasting insulin	T2D (no medication)	Non-diabetic	Leucine, Isoleucine, Valine	C2
4.7	Age and BMI	T2D (no medication)	Non-diabetic	Leucine, Isoleucine, Valine	-
19	Age, WHR, truncal skinfold thickness, Matsuda-IR, HDL cholesterol level, current smoking, and alcohol consumption.	T2D (no medication)	Non-diabetic	Leucine, Isoleucine, Valine	-
19	Age, WHR, truncal skinfold thickness, Matsuda-IR, HDL cholesterol level, current smoking, and alcohol consumption.	T2D (no medication)	Non-diabetic	Leucine, Isoleucine, Valine	-
5	Age, sex, and BMI	T2D (no medication)	Non-diabetic	Leucine or isoleucine, valine	C2, C5 and C10

RESULTS

Study selection

During the identification phase, three hundred and five studies were discovered; after systematically appraising the studies, nine reached synthesis (Supplementary S3) (30–32,41–46). A total of 29 studies at the eligibility stage were excluded based on inappropriate study design or an absence of usable data (Supplementary S3).

Study characteristics

The study characteristics can be found in Table 1, they comprised of nine independent studies reporting data from 4313 T2D patients and 10078 healthy controls *(29–31,41–45,47)*. All the included studies were case–controls studies, the control group comprised of non-diabetic volunteers. In the case group, all volunteers had laboratory-confirmed (either fasting glucose or glycated haemoglobin) T2D and were not currently undertaking any T2D medication. The average profile of the case cohort consists of participants with an age of 56.25 years, a BMI of 28.37 and a bias towards male biological sex (with 63.15% of participants across the synthesized studies being represented by men). The included studies have a publication date of between 2011 and 2018 representing the most recent and up-to date BCAA research *(29–31,41–45,47)*. The outcome of interest was reported as an odds ratio (OR) in eight of the included studies with a single study reporting as a β coefficient. The patient demographics across the cases and the controls ranged from 48.5 ± 13 to 69.5 ± 2.1 for age and were predominantly female, with mixed ethnicity or nationality status. Follow up time ranged from 4.7 to 20 years (Table 1).

Risk of bias within studies

All of the nine included studies received a 'high quality' score after assessment of the studies using the NOS scale. As per the NOS guidelines studies rewarded with 7–9 stars are considered as low risk of bias, high quality studies. All the included studies received this status with two of the studies receiving the maximum nine stars (47,43). (Supplementary S4).

Results of individual studies

For this systematic review and meta-analysis, we only focused on the BCAAs and their metabolites. Since their discovery in the development of T2D, the BCAAs are the most extensively and most consistently researched set of amino acids. The results of our meta-analysis performed for the three individual BCAAs are provided below (Table 2).

Table 2. Calculated log(OR) for individual serum BCAA levels per each data-point incorporated into the meta assessment.

Lead Author	Publication Date	Valine outcome measure			Leucine outcome measure			Isoleucine outcome measure		
		Log(OR)	SE	*p*-value	Log(OR)	SE	*p*-value	Log(OR)	SE	*p*-value
F Ottosson	2018	0.73	0.01	0.94	0.74	0.02	0.197	0.75	0.03	0.064
Y Lu	2016	0.94	0.84	0.0003*	0.92	0.83	0.0023	0.92	0.83	0.4*
L Shi	2018	0.84	0.27	<0.001*	0.81	0.22	0.002	0.81	0.22	0.002*
T Wang	2011	0.75	0.14	0.34	0.79	0.18	0.034	0.80	0.17	0.01*
T Wang	2011	0.24	0.05	5.89×10^{-5}*	-	-	-	0.26	0.05	3.04×10^{-5}*
A Floegel	2013	0.81	0.28	0.03*	0.81	0.28	0.06	0.85	0.37	0.008*
R Wang-Sattler	2012	0.79	0.15	0.016*	0.84	0.22	0.006	0.85	0.23	0.001*
A Stancáková	2012	0.82	0.29	0.02*	0.84	0.32	0.006	0.85	0.32	0.004*
T Tillin	2015	0.88	0.57	0.01*	0.83	0.29	0.009	0.80	0.26	0.09
T Tillin	2015	0.78	0.14	0.044*	0.77	0.14	0.074	0.77	0.14	0.13
ND Palmer	2015	0.73	0.12	0.9	0.75	0.12	0.4	0.75	0.13	0.4

* = Statistically significant p<0.05. *OR, Odds Ratio; SE, Standard Error.*

Valine

Within the zero-to-six year subgroup, three cohorts were incorporated for assessment (Palmer et al., 2015, Stancáková et al., 2012, Lu et al., 2016), with all demonstrating a positive association (OR = 2.08–2.56) (Figure 1) (29,30,44). The overall OR was calculated to be 2.08 (95% CI = 2.04–2.12, $p < 0.00001$) (Figure 1). Although an assessment of overall heterogeneity was equivocal (I^2= 0%, $p = 0.89$) (Figure 1), a substantially wide 95% CI was observed with one cohort (Palmer et al., 2015, 0.49–13.28) (Figure 1) (29).

Four cohorts (Floegel et al., 2013, Ottosson et al., 2018, Shi et al., 2018, Wang-Sattler et al., 2012) were sourced for data in the subgroup with a follow-up of more-than-six, but less-than-twelve year follow-up period (Figure 1) (31,42,43,47). A positive association was observed in all (OR = 1.27–2.25), with an overall OR of 1.86 (95% CI = 1.28–2.68, $p = 0.001$) (Figure 1). Subgroup heterogeneity was found to be considerable ($I^2 = 0\%$, $p = 0.89$) (Figure 1).

Four cohorts sourced from two separate studies (Tillin et al., 2015; European and South Asian, Wang et al., 2011; Framingham and Malmo datasets) represented the subgroup consisting of at least twelve years of follow-up, with each demonstrating demonstrated positive associations (OR = 2.08–2.41), resulting in an overall OR of 2.14 (95% CI = 1.81–2.53, $p < 0.00001$) (Figure 1) (41,45). An overall heterogeneity assessment was also equivocal ($I^2 = 0\%$, $p = 0.98$), with substantially wide 95% CI observed in the Malmo cohort data from Wang et al., 2011 (Figure 1).

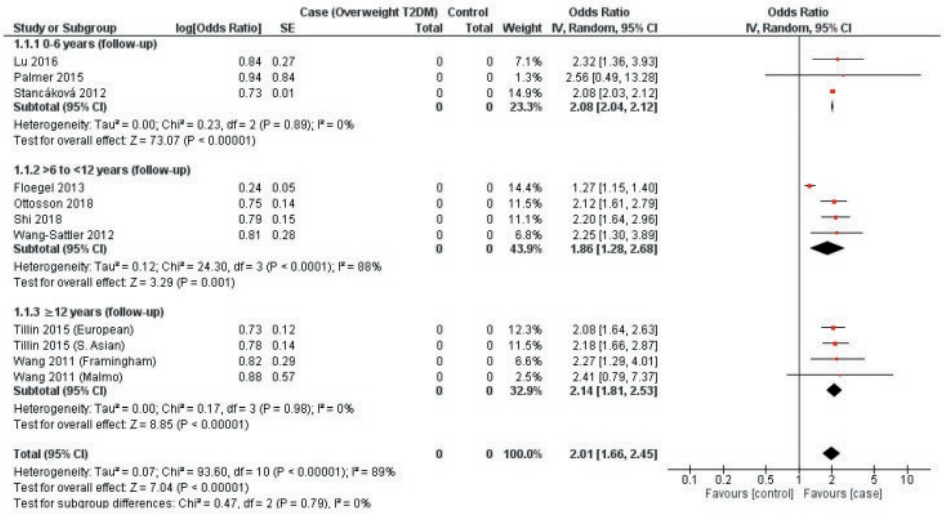


Figure 1. Forest plot depicting the effect sizes of the individual studies for valine in each of the designated temporal subgroups, (29–31,41,42,44–46).

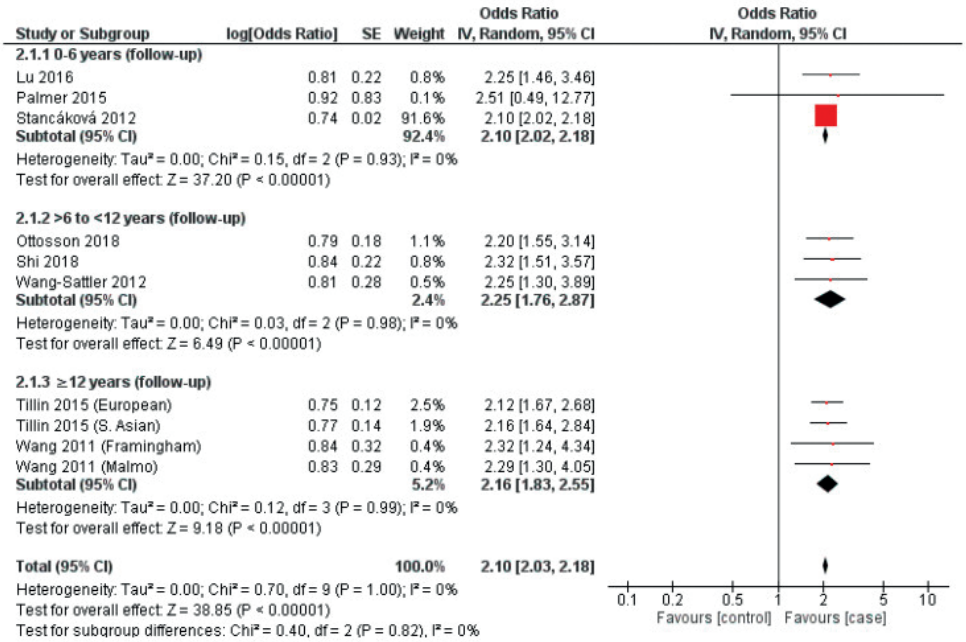


Figure 2. Forest plot depicting the effect sizes of the individual studies for leucine in each of the designated temporal subgroups (29–31,41,42,44–46)

Leucine

Three cohorts (Palmer et al., 2015, Stancáková et al., 2012, Lu et al., 2016) represented the zero-to-six year subgroup, where all presented a positive association (2.10–2.51) and a resulting overall OR (2.10, 95% CI = 2.02–2.18, $p < 0.00001$) (Figure 2) (30,31,45). A further three cohorts (Ottosson et al., 2018, Shi et al., 2018, Wang-Sattler et al., 2012) were present in the more-than-six, but less-than-twelve year follow-up subgroup (Figure 2) (31,43,47). Similarly, each cohort presented a positive association (OR = 2.20–2.32) and a combined OR of 2.25 (95% CI = 1.76–2.87, $p < 0.00001$) (Figure 2).

Within the subgroup consisting of at least twelve years' follow-up, the same four cohorts were incorporated (Figure 1), with all exhibiting a positive association (OR = 2.12–2.32) (Figure 2). This result was reflected in the overall OR (2.25; 95% CI = 1.76–2.87, $p < 0.00001$) (Figure 2). Heterogeneity assessments for each of the above-described three temporal subgroups was equivocal ($I^2 = 0$, $p > 0.05$ in each) (Figure 2).

Isoleucine

Once more, Palmer et al., 2015, Stancáková et al., 2012 and Lu et al., 2016 provided datapoints for the zero-to-six year follow-up period subgroup, with all demonstrating a positive association (OR = 2.12–2.51), as reflected in the overall subgroup value (OR = 2.12, 95% CI = 2.00–2.25, $p < 0.00001$) (Figure 3) (29,30,44). As observed with the same subgroup present within the analysis for valine (Figure 3), an assessment of overall heterogeneity was equivocal ($I^2 = 0\%$, $p = 0.94$) (Figure 3), with Palmer et al., 2015 producing a substantially wide 95% CI (0.49–12.47) (Figure 3) (29). Four cohorts from the same studies that were present in the analysis of the valine output were present for isoleucine (Figures 1 and 3). Furthermore, all revealed a positive association (OR = 1.30–2.34), as reflected in the resulting overall subgroup OR (1.90, 95% CI = 1.27–2.84, $p = 0.002$) (Figure 3) (31,42,43,47). An assessment for heterogeneity demonstrated a considerable estimate ($I^2 = 82\%$, $p = 0.002$) (Figure 3).

As present in valine and leucine, four data-points from two studies (Tillin et al., 2015 and Wang et al., 2011) were present in the subgroup with at least twelve years follow-up, where all demonstrated a positive association (OR = 2.12–2.34) and overall subgroup OR (2.16, 95% CI = 1.82–2.56, $p < 0.00001$) (Figure 3) (41,45). Heterogeneity assessment was, once more, equivocal ($I^2 = 0\%$, $p = 0.99$) (Figure 3).

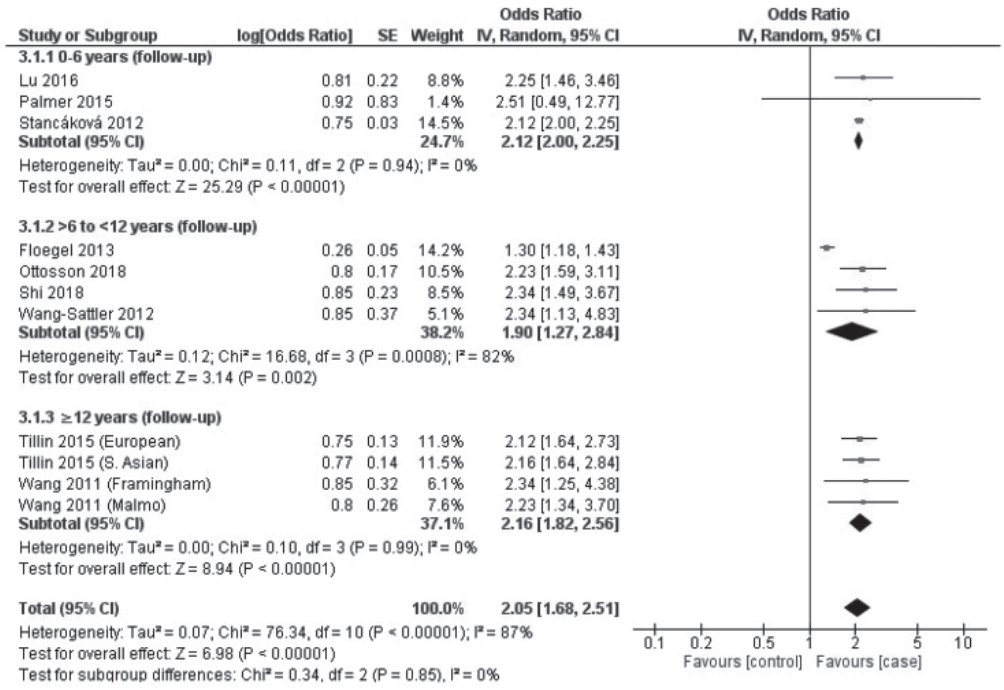


Figure 3. Forest plot depicting the effect sizes of the individual studies for isoleucine in each of the designated temporal subgroups (29–31,41,42,44–46).

Synthesis of Results

Risk of Bias across Studies

Unfortunately, as only nine studies reached the synthesis stage, an assessment of publication bias was not possible in this instance.

Sensitivity

Palmer et al., 2015**,** Lu et al., 2016, Ottosson et al., 2018 and Wang et al., 2011 were found to have published association estimates with models adjusting for less than five covariates for each of the BCAAs, resulting in their incorporation for sensitivity analysis (Table 1). The resultant output validates the rationale to include all published association estimates, irrespective of their model adjustment status (Table 3).

Table 3. Sensitivity analysis of all available datapoints, per temporal subgroup, where model adjustment with less than five covariates was undertaken by the publishing team**.**

Temporal Subgroup (Years Follow-Up)	Data Sources (Lead Author; Year of Publication; BCAAs)	Covariates (Lead Author; Variables)	OR	95% CI (Lower–Upper)	*p*-value
0 to 6	Palmer; 2015 (iso, leu, val) Lu; 2016 (iso, leu, val)	Palmer; Age, sex, BMI, and AIR Lu; BMI, smoking, history of hypertension	2.28	1.77–2.94	$p < 0.00001$
>6 to <12	Ottosson; 2018 (iso, leu, val)	Ottosson; age and sex	2.17	1.81–2.60	$p < 0.00001$
≥12	Wang (Malmo dataset); 2011 (iso, leu, val)	Wang (Malmo dataset); age, sex, BMI, and fasting glucose	2.27	1.59–3.25	$p < 0.00001$

Iso, Isoleucine; leu, leucine; val, valine; BMI, body mass index; AIR, acute insulin response.

DISCUSSION

Summary of evidence

Numerous prospective studies have consistently reported an association between circulating BCAA concentrations and development of T2D (21,28,46). Our meta-analysis demonstrated all three BCAAs exhibit a positive association with the development of T2D (OR = 2.01–2.10, $p < 0.00001$). Our findings are in accordance with those of Guasch-Ferre 2016 et al., who also demonstrated a positive association between BCAAs and incident T2D, with a pooled risk ratio (RR) for isoleucine, leucine and valine of 1.36 (95% CI 1.24–1.48), 1.36 (1.17–1.58), and 1.35 (1.19–1.53), respectively (16).

In addition, this is the first meta-analysis to evaluate the temporal association between BCAAs and risk of developing T2D. The findings of our meta-analysis confirm that, across each individual BCAA, a significant and consistent risk for development of T2D was observed at each time frame, prior to incident T2D (Figures 1–3). These positive associations between BCAAs and T2D at variable period prior to incident T2D suggests that elevated BCAA concentrations could potentially predict the onset of T2D years before the clinical T2D symptoms or sequelae of T2D may manifest. The findings of our systematic review and their potential utility as biomarkers for T2D is in agreement with multiple metabolomic studies that have confirmed BCAAs exhibit sufficient statistical association with T2D to serve as risk biomarkers (16,20,41).

In addition, we observed a variation in association between the BCAAs and T2D with valine providing the lowest overall OR (2.01, $p < 0.00001$) and leucine the highest (2.10, $p < 0.00001$) (Figures 1–3). Similarly, this association was also observed in a meta-analysis by Y Sun, 2019 et al., who demonstrated an increased association between leucine and T2D, with valine producing the weakest association of the BCAAs, where their RRs were 1.40 (95% CI 1.27–1.44) and 1.26 (95% CI 1.18–1.34), respectively (22). Individually, the strength of each BCAA for T2D varies between studies. *In vitro* studies have demonstrated leucine to exhibit a greater affinity in activating mTORc1 compared to either isoleucine or valine which is a pathway suggested to be involved in the development of insulin resistance (48,49) Indeed, the studies included in this review reported differing strengths in association for T2D for each individual BCAA. Y Lu, 2016 et al., and ND Palmer 2015 et al., reported a higher OR for Valine (1.66 and 2.81) when compared to leucine (1.44 and 2.4) (Figures 1and 2) (29,30). What is consistent throughout literature is the strength of the collective BCAAs for T2D.

A consistent observation between valine, leucine and isoleucine is a deviation from the above-described trend in association in the intermediary temporal follow-up subgroup assessed (>6 to <12 years follow-up) (Figures 1–3). Statistical significance was achieved in all assessed subgroups specific to each BCAA, potentially reflecting a non-linear relationship between T2D incidence and temporality with respect to the mechanistic involvement of BCAAs. A temporally stratified assessment of BCAA plasma levels are seldom reported in either case–control or cohort studies, limiting comparisons with the existing literature (15,25). Specifically, the majority of studies only report initial and final individual or total results, dependent on the duration of follow-up. As a result, a summative appraisal of the temporal trends observed in each of the BCAAs and their association with T2D requires further investigation.

Recent studies have suggested that defects in the catabolic pathway of BCAAs may also be responsible for the accumulation of BCAAs in the plasma (50,51). We therefore aimed to also appraise downstream BCAA metabolites and their association with T2D over time. Unfortunately, insufficient data were available to statistically appraise BCAA metabolites, as four of the included studies did not report these, while the remaining studies all reported on different metabolites (29,31,44,47). As evidenced by the robust and consistent association between BCAAs and T2D, further research into determining the role of BCAA downstream metabolites in association with T2D, and in conjunction with their upstream sources, is warranted.

Limitations

Despite the statistically significant and consistent results generated through the described approach, this study is not without limitations. Substantial inter-study heterogeneity was observed in the statistical analysis, which were likely due to demographic differences between the assessed populations. This likely also would explain the unusual trend observed in the 6–12-year sub-group. Due to a paucity in the available data, this current analysis was constrained by the inclusion of outcomes from different ethnic backgrounds, age distributions and follow-up durations. In addition, both targeted and untargeted metabolomic approaches were utilised by studies, which may also contribute to the heterogeneity, observed. Further, sources of heterogeneity were also observed per study with Palmer et al., 2015 yielded consistently for each individual BCAA analysis (29). Equivocal result for analysis in the majority of temporal subgroups (Figures 1–3) was observed. In addition, potential confounding factors were present in the included studies, despite attempt to control for BMI, diabetic status and subgroup per study design. Although, our sensitivity analysis confirmed the feasibility in including studies with a reduced independent variable adjustment count, the differences in covariate adjustment implemented by each of the incorporated studies may explain the substantial variation in confidence intervals observed across several included studies. This may be resolved in future studies through the obtaining of crude plasma BCAA levels for the assessed populations, with post hoc adjustment undertaken thereafter. Moreover, the extracted correlation data was insufficient for either qualitatively or quantitatively appraisal in relation to the presented findings (Table 4).

Through this systematic review and meta-analysis, we demonstrated consistent associations between each BCAA and the eventual development of T2D irrespective of follow-up period duration. Due to the implemented study design and the absence of individual-level participant data, we were unable to explore any relationships between each of the BCAAs with disease severity, the trait-pharmacology interactions, or any clinically recognized subtypes, such as gestational or monogenic diabetes. Indeed, a recent metabolomic study (Del Coco et al., 2019) validated the existence of relationship heterogeneity between BCAAs and manifestations of diabetes mellitus beyond a recent-onset, metabolic syndrome implicated clinical presentation. (52) We therefore recommend further investigation into the specific associations between the recognized subtypes of diabetes mellitus, with the consideration of time as a separate variable, in future work.

Table 4. Presented correlation statistics between serum BCAA levels with either of body mass index, glycated haemoglobin, insulin resistance and/or fasting glucose in the assessed studies at baseline.

Lead Author	Valine						Leucine						Isoleucine					
	BMI		HOMA-IR		HbA1c		BMI		HOMA-IR		HbA1c		BMI		HOMA-IR		HbA1c	
	Outcome	*p*-value	Outcome	*p*-value	Outcome	*p*-value	Outcome	*p*-value	Outcome	*p*-value	Outcome	*p*-value	Outcome	*p*-value	Outcome	*p*-value	Outcome	*p*-value
F Ottosson	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Y Lu	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
L Shi (S)	0.24	-	0.2	0.01	-	-	0.26	-	0.25	<0.001	-	-	0.26	-	0.29	<0.001	-	-
T Wang (P) (Framingham)	-	-	0.24	0.0008	-	-	-	-	0.24	0.0009	-	-	-	-	0.24	0.0007	-	-
T Wang (Malmo)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
A Floegel	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
R Wang-Sattler (P)	0.27	-	-	-	0.08	-	0.19	-	-	-	0.09	-	0.19	-	-	-	0.09	-
A Stancáková	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
T Tillin (S) (European)	0.22	<0.05	0.27	<0.05	-	-	0.18	<0.05	0.26	<0.05	-	-	0.23	<0.05	0.34	<0.05	-	-
T Tillin (S) (South. Asian)	0.29	<0.05	0.33	<0.05	-	-	0.27	<0.05	0.31	<0.05	-	-	0.31	<0.05	0.35	<0.05	-	-
ND Palmer	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

S, Spearmans correlation test; P, Pearsons correlation test; BMI, body mass index

CONCLUSIONS

In summation, the findings from this meta-analysis demonstrate that alterations in Plasma BCAA concentrations are associated with T2D incidence, independent of the baseline plasma BCAA levels. We suggest the potential utility of BCAAs as biomarkers, which reflect early changes in T2D. Detection of these changes years before the onset of physiological symptoms may be crucial for the early diagnosis and development of novel interventions.

AUTHOR CONTRIBUTIONS

One researcher (R.I.) is the first author for this study. Study conception—R.I.; study lead—R.I.; logistical planning, allocation and implementation—R.I. and A.A.; literature search—R.I.; data extraction—M.A.; risk of bias assessment—R.I., V.F. and A.A.; statistical contribution—A.A., manuscript composition—R.I. and A.A.; preliminary draft review—R.I. and A.A.; senior review and revision—I.I. and P.J.A; approval of final manuscript for submission—I.I. All authors have read and agreed to the published version of the manuscript.

FUNDING

No direct funding towards this study was received. One contributing author (Arash Ardavani) is the recipient of the Novo Nordisk UK Research Foundation (Registered Charity number 1056410), Research Fellow Scholarship; we thank them for providing their support. A second author (Froukje Vanweert) was financially supported by the Foundation "De Drie Lichten" in The Netherlands and by the EMBO Scientific Exchange Grant (number 9142).

DATA AVAILABILITY STATEMENT

The data presented in this study are available on request from the corresponding author.

ACKNOWLEDGMENTS

This work was supported by the Medical Research Council (grant number MR/P021220/1) as part of the MRC-Versus Arthritis Centre for Musculoskeletal Ageing Research awarded to the Universities of Nottingham and Birmingham, and the National Institute for Health Research, United Kingdom, Nottingham Biomedical Research Centre.

CONFLICTS OF INTEREST

Ramzan I: No disclosures. Ardavani A: No disclosures. Vanweert F: No disclosures. Mellett A: No disclosures. Atherton PJ: No disclosures. Idris I: No disclosures.

SUPPLEMENTARY MATERIAL FOR CHAPTER 7

Supplementary S1. Table 5. PRISMA checklist

Section/topic	#	Checklist item	Reported on page #
TITLE			
Title	1	Identify the report as a systematic review, meta-analysis, or both.	1
ABSTRACT			
Structured summary	2	Provide a structured summary including, as applicable: background; objectives; data sources; study eligibility criteria, participants, and interventions; study appraisal and synthesis methods; results; limitations; conclusions and implications of key findings; systematic review registration number.	2
INTRODUCTION			
Rationale	3	Describe the rationale for the review in the context of what is already known.	2
Objectives	4	Provide an explicit statement of questions being addressed with reference to participants, interventions, comparisons, outcomes, and study design (PICOS).	3
METHODS			
Protocol and registration	5	Indicate if a review protocol exists, if and where it can be accessed (e.g., Web address), and, if available, provide registration information including registration number.	3
Eligibility criteria	6	Specify study characteristics (e.g., PICOS, length of follow-up) and report characteristics (e.g., years considered, language, publication status) used as criteria for eligibility, giving rationale.	4
Information sources	7	Describe all information sources (e.g., databases with dates of coverage, contact with study authors to identify additional studies) in the search and date last searched.	4
Search	8	Present full electronic search strategy for at least one database, including any limits used, such that it could be repeated.	4
Study selection	9	State the process for selecting studies (i.e., screening, eligibility, included in systematic review, and, if applicable, included in the meta-analysis).	4
Data collection process	10	Describe method of data extraction from reports (e.g., piloted forms, independently, in duplicate) and any processes for obtaining and confirming data from investigators.	4
Data items	11	List and define all variables for which data were sought (e.g., PICOS, funding sources) and any assumptions and simplifications made.	4
Risk of bias in individual studies	12	Describe methods used for assessing risk of bias of individual studies (including specification of whether this was done at the study or outcome level), and how this information is to be used in any data synthesis.	5

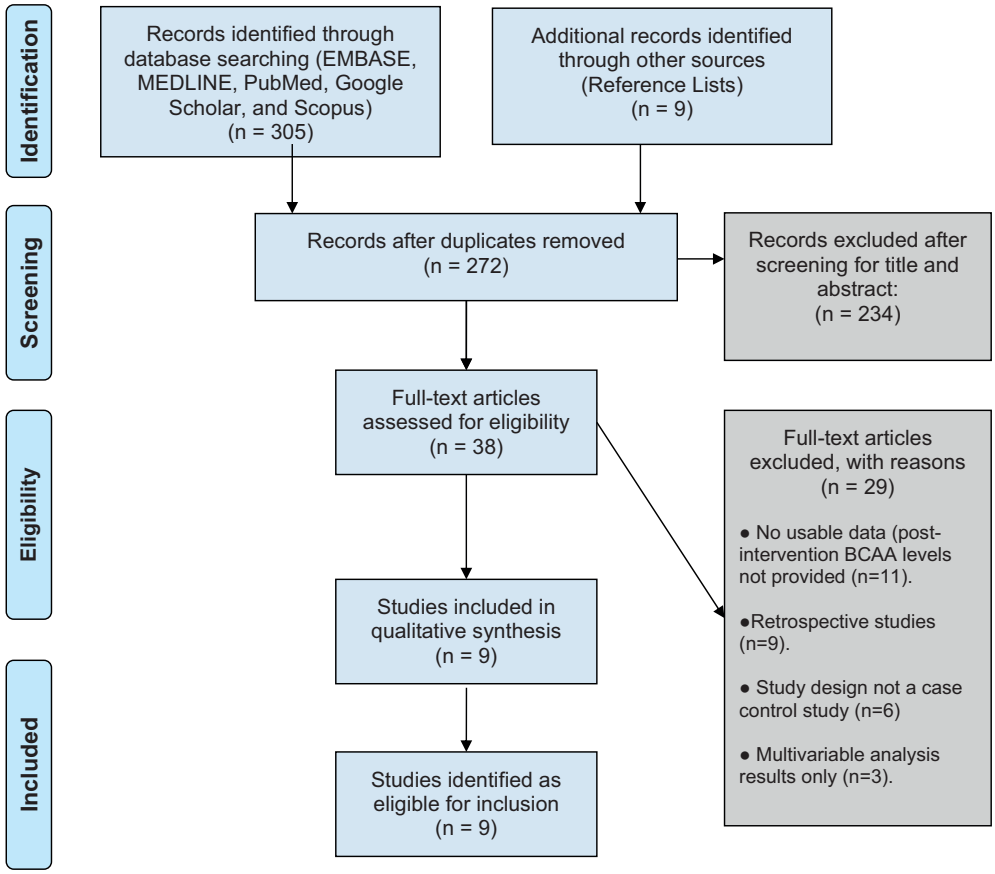
Section/topic	#	Checklist item	Reported on page #
Summary measures	13	State the principal summary measures (e.g., risk ratio, difference in means).	5
Synthesis of results	14	Describe the methods of handling data and combining results of studies, if done, including measures of consistency (e.g., I2) for each meta-analysis.	5
Risk of bias across studies	15	Specify any assessment of risk of bias that may affect the cumulative evidence (e.g., publication bias, selective reporting within studies).	5
Additional analyses	16	Describe methods of additional analyses (e.g., sensitivity or subgroup analyses, meta-regression), if done, indicating which were pre-specified.	5
RESULTS			
Study selection	17	Give numbers of studies screened, assessed for eligibility, and included in the review, with reasons for exclusions at each stage, ideally with a flow diagram.	6
Study characteristics	18	For each study, present characteristics for which data were extracted (e.g., study size, PICOS, follow-up period) and provide the citations.	6
Risk of bias within studies	19	Present data on risk of bias of each study and, if available, any outcome level assessment (see item 12).	7
Results of individual studies	20	For all outcomes considered (benefits or harms), present, for each study: (a) simple summary data for each intervention group (b) effect estimates and confidence intervals, ideally with a forest plot.	7
Synthesis of results	21	Present results of each meta-analysis done, including confidence intervals and measures of consistency.	7
Risk of bias across studies	22	Present results of any assessment of risk of bias across studies (see Item 15).	8
Additional analysis	23	Give results of additional analyses, if done (e.g., sensitivity or subgroup analyses, meta-regression [see Item 16]).	10
DISCUSSION			
Summary of evidence	24	Summarize the main findings including the strength of evidence for each main outcome; consider their relevance to key groups (e.g., healthcare providers, users, and policymakers).	11
Limitations	25	Discuss limitations at study and outcome level (e.g., risk of bias), and at review-level (e.g., incomplete retrieval of identified research, reporting bias).	12
Conclusions	26	Provide a general interpretation of the results in the context of other evidence, and implications for future research.	12
FUNDING			
Funding	27	Describe sources of funding for the systematic review and other support (e.g., supply of data); role of funders for the systematic review.	13

PRISMA, Preferred Reporting Items for Systematic Reviews and Meta-Analyses; PICOS, population, intervention, comparison, and studies. Adapted from From Moher D. et al. (36)

Supplementary S2. Table 2. Eligibility criteria with justification provided with PICOS format

Domain & Inclusion/Exclusion	Justification
Patients: *Inclusion:* Humans only - Adult participants (18+ years of age), irrespective of ethnicity, biological sex, or age; confirmed type 2 diabetes mellitus (T2D) with a body mass index (BMI) of between 25- 30. *Exclusion:* Pregnant human participants, human participants under the age of 18, any non-human organism, patients with type 1 diabetes mellitus, patients with a BMI of less than 24 or more than 31.	Participants with T2D and are overweight represent the population of interest. We attempted to reduce the potential for confounding by controlling for BMI, diabetic status and sub group per study design. We chose a BMI range of 25-30 as this is considered overweight range by the CDC and NHS
Intervention: Not applicable.	No intervention was assessed, as only prospective case-control studies are included.
Comparison: *Inclusion:* With respect to the case group: Inclusion: Overweight (BMI=25-30) population with laboratory-confirmed (either fasting glucose or glycated haemoglobin) T2D. Exclusion: The development of any significant co-morbidity across the group (e.g., cardiovascular, renal, and gastrointestinal), gestational diabetes, insulin-dependent type 2 diabetes mellitus.	Control groups must consist of BMI-matched individuals who otherwise match the baseline characteristics of the case group (those who develop type 2 diabetes mellitus), to limit confounding. Development of co morbidities could potentially affect circulating serum BCAA levels as shown by (9).
Observation: *Inclusion:* Serum branch chain amino acid (BCAA), either for the individual amino acids or the total count, reported as mmol/L, or an equivalent measurement that may be converted to mmol/L, to be reported as serum total count and reported as mmol/L. Any correlation data available will be used for qualitative analysis. *Exclusion:* Any studies not reporting on an association between circulating BCAA levels and T2D.	For this systematic review and meta-analysis, we only focused on the BCAAs and their metabolites. Since their discovery in the development of T2D, the BCAAs are the most extensively researched set of amino acids (8). In addition, BCAAs have consistently demonstrated an association between alterations in circulating BCAAs and T2D (41), (42). The associations between the remaining amino acids and T2D and obesity are not as robust and not as widely researched as the BCAAs, they were the sole focus of this systematic review and meta-analysis (8).
Study: *Inclusion:* Prospective case-control studies (only), with at least one full year of follow-up. *Exclusion:* Randomised-control studies, cohort studies, review articles, meta-analyses, pre-print or non-peer reviewed materials, surveys, qualitative studies, a follow-up time of less than one year.	Follow up time of a year minimum was important to limit confounding. Case-control studies were utilised in this systematic review for the following reasons: 1. Prior systematic reviews had used cohort trials - These yielded conflicting results, with some cohort studies demonstrating an inverse correlation between BCAAs with diabetes and obesity (22). 2. Further, case-control studies are preferred to cohort studies due to the inherent reduction in confounding. 3. The utilisation of cohort studies presupposes an exposure, which is independent of our research questions. Due to our study considerations with respect to temporality, weight status, diabetic status and follow-up period, we address the majority of the recognised sources of confounding within the literature pertaining to this topic. Our systematic review and meta-analysis is, therefore, a confounder-limiting reconsideration of the association between BCAAs with the overweight and diabetic disease state.

PICOS, population, intervention, comparison and studies



Supplementary S3. 1. PRISMA flow diagram

Supplementary S4. Table 3. Risk of Bias assessment by NOS

		Selection			
STUDYTITLE	**AUTHOR**	**Case Definition adequate?**	**Repre-sentatives of the cases?**	**Selec-tion of controls**	**Defini-tion of controls**
Altered asparagine and glutamate homeostasis precede coronary artery disease and type 2 diabetes	F Ottosson (31)	★	-	★	★
Metabolic signatures and risk of type 2 diabetes in a Chinese population: an untargeted metabolomics study using both LC- MS and GC-MS	A Stancáková (44)	★	-	★	★
Plasma metabolites associated with type 2 diabetes in a Swedish population: a case–control study nested in a prospective cohort	L Shi (47)	★	★	★	★
Metabolite profiles and the risk of developing diabetes (Framingham Heart Study)	T Wang (41)	★	-	★	★
Metabolite Profiles and the Risk of Developing Diabetes (Malmö Heart Study)	T Wang (41)	★	-	★	★
Identification of Serum Metabolites Associated With Risk of Type 2 Diabetes Using a Targeted Metabolomic Approach	A Floegel (42)	★	★	★	★
Novel biomarkers for prediabetes identified by metabolomics	R Wang-Sattler (43)	★	★	★	★
Hyperglycemia and a common variant of GCKR are associated with the levels of eight amino acids in 9,369 Finnish men	A Stancáková (44)	★	-	★	★
Diabetes risk and amino acid profiles: cross-sectional and prospective analyses of ethnicity, amino acids and diabetes in a South Asian and European cohort from the SABRE (Southall And Brent REvisited) Study (South Asian Male Patients)	T Tillin (45)	★		★	★
Diabetes risk and amino acid profiles: cross-sectional and prospective analyses of ethnicity, amino acids and diabetes in a South Asian and European cohort from the SABRE (Southall And Brent REvisited) Study (European Male Patients)	T Tillin (45)	★	-	★	★
Metabolomic Profile Associated With Insulin Resistance and Conversion to Diabetes in the Insulin Resistance Atherosclerosis Study	ND Palmer (29)	★	★	★	★

NOS, Newcastle-Ottawa Scale

Comparability		Outcome		
Comparability of cases and controls on the basis of the design or analysis	**Ascertainment of exposure**	**Same method of ascertainment for cases and controls?**	**Non-response rate**	**Overall Score**
★★	★	★	-	7
★★	★	★	★	8
★★	★	★	★	9
★★	★	★	★	8
★★	★	★	★	8
★★	★	★	-	8
★★	★	★	★	9
★★	★	★	-	7
★★	★	★	★	8
★★	★	★	★	8
★★	★	★	-	8

Supplementary S5. The full electronic search strategy

The full electronic search strategy performed for MEDLINE, EMBASE and PubMed are provided below:
Embase: (((obes* OR BMI OR overweight OR diabet* OR T2D*) AND (Branch* amino acid OR BCAA)) AND (prospective OR observation* OR case*)).ti,ab [English language] [Human age groups Adult 18 to 64 years OR Aged 65+ years] [Humans]
Medline: (((obes* OR BMI OR overweight OR diabet* OR T2D*) AND (Branch* amino acid OR BCAA)) AND (prospective OR observation* OR case*))

PubMed: (CASE REPORTS META-ANALYSIS MULTICENTRE STUDY OBSERVATIONAL STUDY REVIEW SYSTEMATIC REVIEW, HUMAN, ADULT: 19+ YEARS)

Google Scholar: (branched chain amino acid obesity diabetes observational study).

Scopus: TITLE-ABS-KEY ((((obes* OR bmi OR overweight OR diabet* OR t2d*) AND (branch* AND amino AND acid OR bcaa)) AND (prospective OR observation* OR case*

))) AND (LIMIT-TO (PUBSTAGE , “final”)) AND (LIMIT-TO (DOCTYPE , “ar”) OR LIMIT-TO (DOCTYPE , “re”)) AND (LIMIT-TO (SUBJAREA , “MEDI”) OR LIMIT-TO (SUBJAREA , “BIOC”)) AND (LIMIT-TO (EXACTKEYWORD , “Human”)) AND (LIMIT- TO (LANGUAGE , “English”)) AND (LIMIT-TO (EXACTKEYWORD , “Humans”))

REFERENCES

1. Abdelaal, M.; le Roux, C.W.; Docherty, N.G. Morbidity and mortality associated with obesity. *Ann. Transl. Med.* **2017**, *5*, 161. https://doi.org/10.21037/atm.2017.03.107.
2. World Health Organization. Obesity and Overweight Facts : How Do We Define Obesity and Global Strategy on. World Health. 2003 https://www.who.int/news-room/fact-sheets/detail/obesity-and-overweight (1st September 2022).
3. Herman, W.H.; Ye, W.; Griffin, S.J.; Simmons, R.K.; Davies, M.J.; Khunti, K.; Rutten, G.E.; Sandbaek, A.; Lauritzen, T.; Borch-Johnsen, K.; et al. Early Detection and Treatment of Type 2 Diabetes Reduce Cardiovascular Morbidity and Mortality: A Simulation of the Results of the Anglo-Danish-Dutch Study of Intensive Treatment in People With Screen-Detected Diabetes in Primary Care (ADDITION-Europe). *Diabetes Care* **2015**, *38*, 1449–1455. https://doi.org/10.2337/dc14-2459.
4. Clish, C.B. Metabolomics: An emerging but powerful tool for precision medicine. *Mol. Case Stud.* **2015**, *1*, a000588. https://doi.org/10.1101/mcs.a000588.
5. Wang, J.H.; Byun, J.; Pennathur, S. Analytical Approaches to Metabolomics and Applications to Systems Biology. *Semin. Nephrol.* **2010**, *30*, 500–511. https://doi.org/10.1016/j.semnephrol.2010.07.007.
6. Johnson, C.H.; Ivanisevic, J.; Siuzdak, G. Metabolomics: Beyond biomarkers and towards mechanisms. *Nat. Rev. Mol. Cell Biol.* **2016**, *17*, 451–459.
7. Jin, Q.; Ma, R.C.W. Metabolomics in Diabetes and Diabetic Complications: Insights from Epidemiological Studies. *Cells* **2021**, *10*, 2832. https://doi.org/10.3390/cells10112832.
8. Pallares-Méndez, R.; Aguilar-Salinas, C.A.; Cruz-Bautista, I.; del Bosque-Plata, L. Metabolomics in diabetes, a review. *Ann. Med.* **2016**, *8*, 89–102. Available online: http://search.ebscohost.com/login.aspx?direct=true&scope=site&site=ehost-live&db=mdc&AN=26883715 (1st September 2022).
9. Cuomo, P.; Capparelli, R.; Iannelli, A.; Iannelli, D. Role of Branched-Chain Amino Acid Metabolism in Type 2 Diabetes, Obesity, Cardiovascular Disease and Non-Alcoholic Fatty Liver Disease. *Int. J. Mol. Sci*. **2022**, *23*, 4325. https://doi.org/10.3390/ijms23084325.
10. Holeček, M. Branched-chain amino acids in health and disease: Metabolism, alterations in blood plasma, and as supplements. *Nutr. Metab.* **2018**, *15*, 33. https://doi.org/10.1186/s12986-018-0271-1.
11. Merz, B.; Frommherz, L.; Rist, M.J.; Kulling, S.E.; Bub, A.; Watzl, B. Dietary Pattern and Plasma BCAA-Variations in Healthy Men and Women—Results from the KarMeN Study. *Nutrients* **2018**, *10*, 623. https://doi.org/10.3390/nu10050623.
12. Rousseau, M.; Guénard, F.; Garneau, V.; Allam-Ndoul, B.; Lemieux, S.; Pérusse, L.; Vohl, M.-C. Associations Between Dietary Protein Sources, Plasma BCAA and Short-Chain Acylcarnitine Levels in Adults. *Nutrients* **2019**, *11*, 173. https://doi.org/10.3390/nu11010173.
13. Williams, M. Dietary Supplements and Sports Performance: Amino Acids. *J. Int. Soc. Sports Nutr.* **2005**, *2*, 63–67. https://doi.org/10.1186/1550-2783-2-2-63.
14. Isanejad, M.; LaCroix, A.Z.; Thomson, C.A.; Tinker, L.; Larson, J.C.; Qi, Q.; Qi, L.; Cooper-DeHoff, R.M.; Phillips, L.S.; Prentice, R.L.; et al. Branched-chain amino acid, meat intake and risk of type 2 diabetes in the Women's Health Initiative. *Br. J. Nutr.* **2017**, *117*, 1523–1530. Available online: http://gateway.proquest.com/openurl?ctx_ver=Z39.88-2004&res_id=xri:pqm&req_dat=xri:pqil:pq_clntid=145298&rft_val_fmt=ori/fmt:kev:mtx:journal&genre=article&issn=0007-1145&volume=117&issue=11&spage=1523 ((1st September 2022).

15. Würtz, P.; Soininen, P.; Kangas, A.J.; Rönnemaa, T.; Lehtimäki, T.; Kähönen, M.; Viikari, J.S.; Raitakari, O.T.; Ala-Korpela, M. Branched-Chain and Aromatic Amino Acids Are Predictors of Insulin Resistance in Young Adults. *Diabetes Care* **2013**, *36*, 648–655. https://doi.org/10.2337/dc12-0895.
16. Guasch-Ferré, M.; Hruby, A.; Toledo, E.; Clish, C.B.; Martínez-González, M.A.; Salas-Salvadó, J.; Hu, F.B. Metabolomics in Prediabetes and Diabetes: A Systematic Review and Meta-analysis. *Diabetes Care* **2016**, *39*, 833–846. https://doi.org/10.2337/dc15-2251.
17. Ramzan, I.; Taylor, M.; Phillips, B.; Wilkinson, D.; Smith, K.; Hession, K.; Idris, I.; Atherton, P. A Novel Dietary Intervention Reduces Circulatory Branched-Chain Amino Acids by 50%: A Pilot Study of Relevance for Obesity and Diabetes. *Nutrients* **2020**, *13*, 95. https://doi.org/10.3390/nu13010095.
18. Karusheva, Y.; Koessler, T.; Strassburger, K.; Markgraf, D.; Mastrototaro, L.; Jelenik, T.; Simon, M.-C.; Pesta, D.; Zaharia, O.-P.; Bódis, K.; et al. Short-term dietary reduction of branched-chain amino acids reduces meal-induced insulin secretion and modifies microbiome composition in type 2 diabetes: A randomized controlled crossover trial. *Am. J. Clin. Nutr.* **2019**, *110*, 1098–1107. https://doi.org/10.1093/ajcn/nqz191.
19. Kramer, N.B.; Evers, S.S.; Shin, J.H.; Silverwood, S.; Wang, Y.; Burant, C.F.; Sandoval, D.A.; Seeley, R.J. The Role of Elevated Branched-Chain Amino Acids in the Effects of Vertical Sleeve Gastrectomy to Reduce Weight and Improve Glucose Regulation. *Cell Rep*. **2020**, *33*, 108239. https://doi.org/10.1016/j.celrep.2020.108239.
20. Long, J.; Yang, Z.; Wang, L.; Han, Y.; Peng, C.; Yan, C.; Yan, D. Metabolite biomarkers of type 2 diabetes mellitus and pre-diabetes: A systematic review and meta-analysis. *BMC Endocr. Disord*. **2020**, *20*, 174. https://doi.org/10.1186/s12902-020-00653-x.
21. Flores-Guerrero, J.L.; Osté, M.C.J.; Kieneker, L.M.; Gruppen, E.G.; Wolak-Dinsmore, J.; Otvos, J.D.; Connelly, M.A.; Bakker, S.J.L.; Dullaart, R.P.F. Plasma Branched-Chain Amino Acids and Risk of Incident Type 2 Diabetes: Results from the PREVEND Prospective Cohort Study. *J. Clin. Med*. **2018**, *7*, 513. https://doi.org/10.3390/jcm7120513.
22. Sun, Y.; Gao, H.-Y.; Fan, Z.-Y.; He, Y.; Yan, Y.-X. Metabolomics Signatures in Type 2 Diabetes: A Systematic Review and Integrative Analysis. *J. Clin. Endocrinol. Metab*. **2019**, *105*, 1000–1008. https://doi.org/10.1210/clinem/dgz240.
23. Song, J.W.; Chung, K.C. Observational Studies: Cohort and Case-Control Studies. *Plast. Reconstr. Surg*. **2010**, *126*, 2234–2242. https://doi.org/10.1097/prs.0b013e3181f44abc.
24. Mirmiran, P.; Bahadoran, Z.; Esfandyari, S.; Azizi, F. Dietary Protein and Amino Acid Profiles in Relation to Risk of Dysglycemia: Findings from a Prospective Population-Based Study. *Nutrients* **2017**, *9*, 971. https://doi.org/10.3390/nu9090971.
25. Asghari, G.; Farhadnejad, H.; Teymoori, F.; Mirmiran, P.; Tohidi, M.; Azizi, F. High dietary intake of branched-chain amino acids is associated with an increased risk of insulin resistance in adults. *J. Diabetes* **2018**, *10*, 357–364. https://doi.org/10.1111/1753-0407.12639.
26. Lee, C.C.; Watkins, S.M.; Lorenzo, C.; Wagenknecht, L.E.; Il'Yasova, D.; Chen, Y.-D.I.; Haffner, S.M.; Hanley, A.J. Branched-Chain Amino Acids and Insulin Metabolism: The Insulin Resistance Atherosclerosis Study (IRAS). *Diabetes Care* **2016**, *39*, 582–588. https://doi.org/10.2337/dc15-2284.
27. Chen, T.; Ni, Y.; Ma, X.; Bao, Y.; Liu, J.; Huang, F.; Hu, C.; Xie, G.; Zhao, A.; Jia, W.; et al. Branched-chain and aromatic amino acid profiles and diabetes risk in Chinese populations. *Sci. Rep.* **2016**, *6*, 20594. https://doi.org/10.1038/srep20594.
28. Geidenstam, N.; Al-Majdoub, M.; Ekman, M.; Spégel, P.; Ridderstråle, M. Metabolite profiling of obese individuals before and after a one year weight loss program. *Int. J. Obes.* **2017**, *41*, 1369–1378. https://doi.org/10.1038/ijo.2017.124.

29. Palmer, N.D.; Stevens, R.D.; Antinozzi, P.A.; Anderson, A.; Bergman, R.N.; Wagenknecht, L.E.; Newgard, C.B.; Bowden, D.W. Metabolomic Profile Associated With Insulin Resistance and Conversion to Diabetes in the Insulin Resistance Atherosclerosis Study. *J. Clin. Endocrinol. Metab.* **2015**, *100*, E463–E468. https://doi.org/10.1210/jc.2014-2357.
30. Lu, Y.; Wang, Y.; Ong, C.-N.; Subramaniam, T.; Choi, H.W.; Yuan, J.-M.; Koh, W.-P.; Pan, A. Metabolic signatures and risk of type 2 diabetes in a Chinese population: An untargeted metabolomics study using both LC-MS and GC-MS. *Diabetologia* **2016**, *59*, 2349–2359. https://doi.org/10.1007/s00125-016-4069-2.
31. Ottosson, F.; Smith, E.; Melander, O.; Fernandez, C. Altered Asparagine and Glutamate Homeostasis Precede Coronary Artery Disease and Type 2 Diabetes. *J. Clin. Endocrinol. Metab*. **2018**, *103*, 3060–3069. https://doi.org/10.1210/jc.2018-00546.
32. Yoon, M.-S. The Role of Mammalian Target of Rapamycin (mTOR) in Insulin Signaling. *Nutrients* **2017**, *9*, 1176. https://doi.org/10.3390/nu9111176.
33. Lee, S.; Gulseth, H.L.; Langleite, T.M.; Norheim, F.; Olsen, T.; Refsum, H.; Jensen, J.; Birkeland, K.I.; Drevon, C.A. Branched-chain amino acid metabolism, insulin sensitivity and liver fat response to exercise training in sedentary dysglycaemic and normoglycaemic men. *Diabetologia* **2020**, *64*, 410–423. https://doi.org/10.1007/s00125-020-05296-0.
34. Wada, E.; Kobayashi, M.; Kohno, D.; Kikuchi, O.; Suga, T.; Matsui, S.; Yokota-Hashimoto, H.; Honzawa, N.; Ikeuchi, Y.; Tsuneoka, H.; et al. Disordered branched chain amino acid catabolism in pancreatic islets is associated with postprandial hypersecretion of glucagon in diabetic mice. *J. Nutr. Biochem.* **2021**, *97*, 108811. https://doi.org/10.1016/j.jnutbio.2021.108811.
35. Diabetes Branch Chain Amino Acid Time Progression-Search Results-PubMed. Available online: https://pubmed.ncbi.nlm.nih.gov/?term=diabetes branch chain amino acid time progression&page=4 (accessed on 25 July 2022).
36. Moher, D.; Liberati, A.; Tetzlaff, J.; Altman, D.G.; the PRISMA Group. Preferred reporting items for systematic reviews and meta-analyses: The PRISMA statement. *BMJ* **2009**, *339*, b2535. https://doi.org/10.1136/bmj.b2535.
37. The Newcastle-Ottawa Scale (NOS) for Assessing the Quality if Nonrandomized Studies in Meta-Analyses-ScienceOpen. Available online: https://www.scienceopen.com/document?vid=54b48470-4655-4081-b5d4-e8ebe8d1792e (accessed on 2 December 2020).
38. What Is Heterogeneity? Available online: https://handbook-5-1.cochrane.org/chapter_9/9_5_1_what_is_heterogeneity.htm (accessed on 2 December 2020).
39. Identifying Publication Bias in Meta-Analyses of Continuous Outcomes|Cochrane Training. Available online: https://training.cochrane.org/resource/identifying-publication-bias-meta-analyses-continuous-outcomes (accessed on 26 January 2021).
40. Chapter 10: Analysing Data and Undertaking Meta-Analyses|Cochrane Training. Available online: https://training.cochrane.org/handbook/current/chapter-10 (accessed on 27 January 2021).
41. Wang, T.J.; Larson, M.G.; Vasan, R.S.; Cheng, S.; Rhee, E.P.; McCabe, E.; Lewis, G.D.; Fox, C.S.; Jacques, P.F.; Fernandez, C.; et al. Metabolite profiles and the risk of developing diabetes. *Nat. Med.* **2011**, *17*, 448–453. https://doi.org/10.1038/nm.2307.
42. Floegel, A.; Stefan, N.; Yu, Z.; Mühlenbruch, K.; Drogan, D.; Joost, H.-G.; Fritsche, A.; Häring, H.-U.; De Angelis, M.H.; Peters, A.; et al. Identification of Serum Metabolites Associated With Risk of Type 2 Diabetes Using a Targeted Metabolomic Approach. *Diabetes* **2013**, *62*, 639–648. https://doi.org/10.2337/db12-0495.
43. Wang-Sattler, R.; Yu, Z.; Herder, C.; Messias, A.; Floegel, A.; He, Y.; Heim, K.; Campillos, M.; Holzapfel, C.; Thorand, B.; et al. Novel biomarkers for pre-diabetes identified by metabolomics. *Mol. Syst. Biol.* **2012**, *8*, 615. https://doi.org/10.1038/msb.2012.43.

44. Stancáková, A.; Civelek, M.; Saleem, N.K.; Soininen, P.; Kangas, A.J.; Cederberg, H.; Paananen, J.; Pihlajamäki, J.; Bonnycastle, L.L.; Morken, M.A.; et al. Hyperglycemia and a common variant of GCKR are associated with the levels of eight amino acids in 9,369 Finnish men. *Diabetes* **2012**, *61*, 1895–1902. Available online: http://www.ncbi.nlm.nih.gov/pmc/articles/PMC3379649 (1st September 2022).
45. Tillin, T.; Hughes, A.; Wang, Q.; Würtz, P.; Ala-Korpela, M.; Sattar, N.; Forouhi, N.; Godsland, I.F.; Eastwood, S.; McKeigue, P.M.; et al. Diabetes risk and amino acid profiles: Cross-sectional and prospective analyses of ethnicity, amino acids and diabetes in a South Asian and European cohort from the SABRE (Southall And Brent REvisited) Study. *Diabetologia* **2015**, *58*, 968–979. https://doi.org/10.1007/s00125-015-3517-8.
46. Chen, S.; Akter, S.; Kuwahara, K.; Matsushita, Y.; Nakagawa, T.; Konishi, M.; Honda, T.; Yamamoto, S.; Hayashi, T.; Noda, M.; et al. Serum amino acid profiles and risk of type 2 diabetes among Japanese adults in the Hitachi Health Study. *Sci. Rep.* **2019**, *9*, 7010. https://doi.org/10.1038/s41598-019-43431-z.
47. Shi, L.; Brunius, C.; Lehtonen, M.; Auriola, S.; Bergdahl, I.A.; Rolandsson, O.; Hanhineva, K.; Landberg, R. Plasma metabolites associated with type 2 diabetes in a Swedish population: A case-control study nested in a prospective cohort. *Diabetologia* **2018**, *61*, 849–861. https://doi.org/10.1007/s00125-017-4521-y.
48. Efeyan, A.; Zoncu, R.; Sabatini, D.M. Amino acids and mTORC1: From lysosomes to disease. *Trends Mol. Med.* **2012**, *18*, 524–533. https://doi.org/10.1016/j.molmed.2012.05.007.
49. Yoon, M.-S. The Emerging Role of Branched-Chain Amino Acids in Insulin Resistance and Metabolism. *Nutrients* **2016**, *8*, 405. https://doi.org/10.3390/nu8070405.
50. White, P.J.; Lapworth, A.L.; An, J.; Wang, L.; McGarrah, R.W.; Stevens, R.D.; Ilkayeva, O.; George, T.; Muehlbauer, M.J.; Bain, J.R.; et al. Branched-chain amino acid restriction in Zucker-fatty rats improves muscle insulin sensitivity by enhancing efficiency of fatty acid oxidation and acyl-glycine export. *Mol. Metab.* **2016**, *5*, 538–551. https://doi.org/10.1016/j.molmet.2016.04.006.
51. Lynch, C.J.; Adams, S.H. Branched-chain amino acids in metabolic signalling and insulin resistance. *Nat. Rev. Endocrinol.* **2014**, *10*, 723–736. https://doi.org/10.1038/nrendo.2014.171.
52. del Coco, L.; Vergara, D.; de Matteis, S.; Mensà, E.; Sabbatinelli, J.; Prattichizzo, F.; Bonfigli, A.R.; Storci, G.; Bravaccini, S.; Pirini, F.; et al. NMR-Based Metabolomic Approach Tracks Potential Serum Biomarkers of Disease Progression in Patients with Type 2 Diabetes Mellitus. *J. Clin. Med.* **2019**, *8*, 720

8

General discussion and conclusion

Insulin resistance is the main risk factor for type 2 diabetes (T2D) (1), and includes a wide range of metabolic disturbances (2). Many studies have provided important information on the pathogenesis of insulin resistance and T2D. Traditionally, research mostly focuses on disturbances in carbohydrate and fat metabolism (3), and only little research has been conducted into amino acid metabolism, especially the branched-chain amino acids (BCAA). Moreover, evidence accumulates that disturbances in BCAA metabolism play a role in the progression from obesity towards the development of T2D. Therefore, it is important to gain further insights into the role of BCAA metabolism in the pathophysiology of T2D.

The high prevalence of T2D worldwide continues to rise despite significant investment in pharmaceutical research and public health interventions (4). Even with a broad gamut of antidiabetic medication, hyperglycemia in patients with T2D fails to normalize and it even increases with the development of the disease. Our current approach to diabetes management is mostly focusing on symptoms of the disease, hyperglycemia, but not on causes of the development of T2D. Since patients with T2D comprises a broad scale of metabolic disturbances closely related to insulin resistance, new intervention strategies should not only focus on hyperglycemia, but also target these metabolic perturbations. For example, patients with T2D feature low mitochondrial function (5-7), are less capable in switching between fat and glucose oxidation under insulin-stimulated conditions, termed as metabolic inflexibility, and are characterized by elevated fat content in liver and muscle (2). Therefore, this epidemic will require an urgent need for new strategies to treat diabetes, and more importantly, reduce the progression from prediabetes to diabetes. The search for a treatment strategy targeting several metabolic derangements at the same time should be encouraged, likely to increase the change for the actual improvement of metabolic health in T2D.

Within this thesis, I investigated the role of BCAA metabolism in insulin resistance and T2D and examined whether stimulating BCAA catabolism improves insulin sensitivity and metabolic health in patients with T2D.

DO PATIENTS WITH T2D FEATURE DISTURBED BCAA CATABOLISM?

The catabolism of all three individual BCAA, isoleucine, leucine and valine, is located inside the mitochondria (8, 9) as outlined in **chapter 2**. Tissue-specific BCAA catabolism has been rigorously investigated in rodent models, however, data in humans is limited. Suryawan et al. (10) was the first to report that in humans both

skeletal muscle and liver are key tissues in which BCAA catabolism finds place, with skeletal muscle as the major player (10, 11). Furthermore, BCAA catabolism prominently takes place in human heart (12-14) and adipose tissue (15-22). Although it is still not well understood why BCAA levels are elevated in individuals with insulin resistance and patients with T2D, a reduced BCAA-catabolic flux is a plausible explanation underlying elevated BCCA in the circulation.

In **chapter 3**, we aimed to test if patients with T2D feature disturbed BCAA catabolism by measuring BCAA oxidation with use of ^{13}C-leucine tracer during a 2-step hyperinsulinemic-euglycemic clamp. We found that after an overnight fast, *in vivo* whole-body leucine oxidation rates were lower in patients with T2D compared to controls. This strengthens the hypothesis that reduced BCAA catabolism in patients with T2D contribute to higher fasting plasma BCAA levels, which was also recognized by others (23) and confirmed in FDR (24). In contrast, another report (25) did not find differences in baseline leucine oxidation rates between patients with T2D and controls. This discrepancy can be explained by the notion that the obese control participants in this study (25) had a higher BMI (>30 kg/m2) compared to the controls of the other studies. Severe obesity might be associated with disturbances in protein metabolism, and therefore no difference could be picked up between the two groups. In line with our hypothesis, we observed lower *ex vivo* muscle mitochondrial respiratory rates in patients with T2D, who had the highest BCAA levels in plasma, compared to controls. Together, these findings could indicate that low mitochondrial oxidation of BCAA may contribute to higher plasma BCAA levels in T2D. Despite the limited availability of human studies, accumulating evidence supports this notion. It has been shown that activity (26, 27) and expression (19, 28, 29) of BCAA-catabolic enzymes is reduced in muscle and adipose tissue of insulin resistant patients resulting in a lower BCAA catabolism.

Insulin resistance itself has been speculated to drive higher plasma BCAA levels. Normally, insulin stimulates BCAA uptake from plasma into skeletal muscle and inhibits protein breakdown in muscle tissues. Together, this results in lower amino acid content in plasma, with the most marked decline observed for BCAA (30-33). Some studies show that individuals with insulin resistance feature blunted inhibitory action of insulin on muscle protein breakdown (34), and therefore, another hypothesis postulates that higher plasma BCAA levels in insulin resistance, is the consequence of insulin resistance state itself (35, 36). This hypothesis can be correct, nevertheless, controverse exists if patients with T2D truly feature elevated protein breakdown (37, 38). The studies included in this thesis did not measure muscle protein breakdown but aimed to measure amino acid oxidation, especially leucine. In our hands, insulin stimulated the leucine oxidation rates during the

clamp similarly in patients with T2D as in the control group, which was confirmed by others (23, 24, 38-41). Nevertheless, we observed lower BCAA oxidation rates in the fasted state in the patients with T2D (**chapter 3**). Importantly, only a limited number of participants underwent our leucine infusion protocol, and therefore, it would be valuable to reproduce this experiment in a larger population. In contrast, others found that insulin-mediated increase in leucine oxidation was blunted in T2D compared to BMI-matched controls (25), possibly reflecting a greater insulin resistance of leucine metabolism. In line, in **chapter 4**, we found that suppression of plasma BCAA levels under insulin stimulation was reduced with 15% in obese individuals with T2D and/or NAFL if compared to an obese control group. This indeed suggests insulin resistance of the BCAA-catabolic pathway. Whether or not the resistance towards insulin-suppression of plasma BCAA levels indeed contributes to the elevated plasma levels seen in the fasted state, cannot be deduced from the current study.

Despite the finding of an increase in leucine oxidation upon insulin stimulation, many reports show insulin-stimulated decrease in leucine oxidation rates during the clamp (40, 42). This discrepancy could potentially be explained by the co-infusion of amino acids during the clamp, which possibly affects BCAA oxidation rates, protein breakdown and synthesis, majorly changing the amino acid turnover. Nevertheless, in some studies the co-infusion prevents the drop of amino acids during the clamp, which could explain the differences found (43, 44). Characteristics of study population as well can explain conflicting results between studies. For instance, the anabolic action of insulin differs between males and females. Also, chronic adaptations to hyperglycemia and hyperinsulinemia, as well the oral glucose-lowering medication may explain differences between outcomes (38).

In summary, multiple lines of evidence point towards dysregulated BCAA metabolism in insulin resistant states. This emphasizes the importance of studying (tissue-specific) catabolic defects of BCAA and whether they occur in individuals with insulin resistance and T2D. The results presented in **chapter 3 and 4** indicate that patients with T2D feature compromised whole-body BCAA catabolism, which may partly underly the elevated plasma BCAA levels. Future studies including clamp methodology combined with other isotope tracers, like 13C-BCAA, in a larger population would be valuable.

DOES BCAA CATABOLISM LINK TO INSULIN RESISTANCE? WHAT IS THE EVIDENCE?

Although it has been suggested that increased BCAA levels could merely be a consequence of impaired insulin action, as discussed in previous paragraph, emerging evidence points towards a causal role of a compromised BCAA catabolism on insulin resistance, which will be discussed in the paragraphs below.

The results of **chapter 3 and 4** of this thesis indicate that BCAA catabolism is linked to insulin resistance. We demonstrated that patients with T2D, who had lower leucine oxidation rates, also displayed elevated BCAA plasma levels compared to BMI-matched controls (**chapter 3**). In another study, elevated plasma BCAA levels were observed in people with NAFL compared to a control group (**chapter 4**). Both groups (patients with T2D and NAFL) were characterized as insulin resistant, compared to BMI-matched control participants, thus these data are in line with previous studies (45-49). Several reports show that obese, insulin resistant individuals possess increased plasma BCAA levels compared to healthy lean individuals (49-53). Since these obese individuals were insulin resistant, it could be hypothesized that compromised BCAA catabolism develops with the progression of the insulin resistant state. A strong association of increased BCAA levels in plasma and insulin resistance has been repeatedly recognized in humans (49, 52). We not only confirmed this association (**chapter 3** and **4**), but also showed for the first time that elevated BCAA levels correlate with other diabetes-related metabolic disturbances, like mitochondrial dysfunction, metabolic inflexibility and high intrahepatic lipid (IHL) content. Although, an association between BCAA levels and insulin resistance was noted in **chapter 3** and **4**, causal conclusions cannot be made and was not the scope of these chapters.

To investigate causality, a systematic review and meta-analysis was performed, which demonstrated that elevated BCAA levels in people with obesity were related to a higher risk of the development of T2D (**chapter 7**). This finding has been supported by a genome-wide association study (GWAS) by Lotta et al. (26), who provided genetic evidence for a causal role of diminished BCAA catabolism in the development of insulin resistance, although others (54, 55) showed increased BCAA levels as consequence of insulin resistance. GWAS provides a great opportunity to make inference about causality, and therefore, genetic variants associated with BCAA levels can be used to study the aetiologic link between the BCAA-catabolic pathway and T2D (56). Moreover, the findings of **chapter 7** suggested that elevated BCAA levels could potentially predict the onset of T2D before the clinical T2D-symptoms manifest, and therefore, could be used as new

prognostic biomarkers to identify people with prediabetes, a population at risk to develop T2D. This was also confirmed in multiple metabolomic studies (57, 58). In FDR, however, with at least one first-line family member diagnosed with T2D and supposed to be at increased risk to develop T2D (57-59), we did not observe elevated plasma BCAA levels (**chapter 3**). It is important to mention that these individuals had a similar degree of insulin sensitivity as the control group, which indicate that they were rather metabolically healthy, and their risk to develop T2D may not be as high as compared to a group defined with prediabetes.

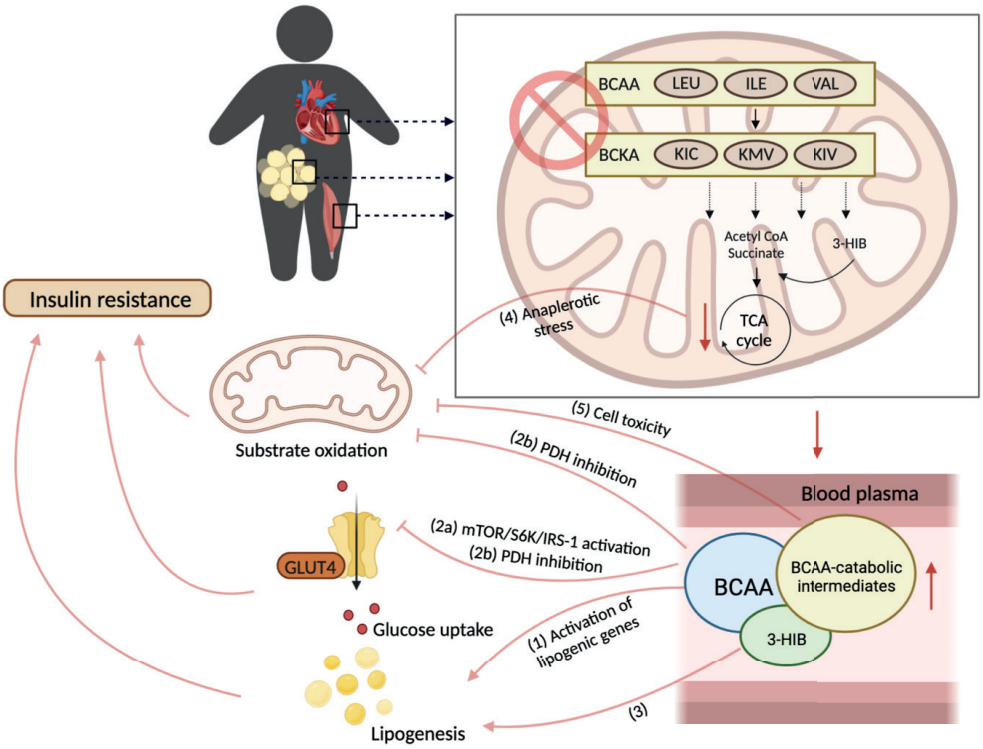


Figure 1. Metabolic routes linking compromised BCAA catabolism with insulin resistance.

BCAA levels contribute to insulin resistance via (1) activation of lipogenic genes resulting in dyslipidaemia, and (2) direct inhibition of the insulin signaling pathway via activation of mTOR/S6K/IRS-1 or PDH inhibition, (3) 3-HIB act as a paracrine factor regulating transendothelial transport resulting in lipid accumulation, (4) Anaplerotic stress exerts detrimental effects on substrate oxidation and glucose uptake, (5) Accumulation of toxic BCAA-catabolic intermediates leads to mitochondrial dysfunction and dysfunctional substrate oxidation. *3-HIB, 3-hydroxyisobutyrate; BCAA, branched-chain amino acids; GLUT4, glucose transporter type 4; ILE; isoleucine; IRS-1, insulin receptor substrate-1; KIC, ketoisocaproate; KIV, ketoisovalerate; KMV, keto-ß-methylvalerate; LEU, leucine; mTOR, mammalian target of rapamycin complex; PDH, pyruvate dehydrogenase complex; S6K, ribosomal S6 kinase; TCA, tricarboxylic acid*

In line with our hypothesis, the studies in this thesis indicate a compromised BCAA catabolism in patients with T2D, that associates with insulin resistance. To further test this hypothesis, as outlined in Figure 1, several studies were performed to investigate the link between BCAA levels and insulin resistance.

One potential mechanism explaining the link between BCAA and insulin resistance is the activation of lipogenic genes resulting in dyslipidaemia, as displayed in Figure 1 (mechanism 1). The results of **chapter 4** demonstrate that individuals with a high IHL content and those with low peripheral and hepatic insulin sensitivity feature highest plasma BCAA levels. A positive association between BCAA levels and IHL content was also found in other reports (60-62). A recent study concluded that elevated plasma BCAA levels play a role in the progression from NAFL towards the development of T2D (47). Several mechanisms have been hypothesized linking BCAA, insulin resistance and IHL content. Elevated BCAA levels have been shown to activate lipogenic genes, such as SREBP-1c, FAS and ACC in rodents (63, 64). In addition, inactivation of the rate-limiting BCAA-catabolic enzyme (BCKD) links to the activation of the ATP citrate lyase (ACL) (65), which may result in increased cytosolic citrate concentrations which in turn may promote de novo lipogenesis and inhibition of fatty acid oxidation via increased levels of malonyl-CoA, thus contributing to dyslipidaemia and contribute development of insulin resistance (65). This concept of BCAA-mediated increased lipogenesis has not been investigated in human tissue yet. Nevertheless, it could be speculated that disturbed BCAA catabolism and its elevated plasma BCAA levels modulate liver fat content. Whether elevated plasma BCAA levels contribute to the development of a fatty liver and insulin resistance cannot be deduced from the study performed in **chapter 4**. Given the latter, it would be highly interesting for future research to investigate whether infusion of BCAA in humans would activate lipogenic genes contributing to insulin resistance.

A second potential route linking BCAA with insulin sensitivity is that BCAA in plasma, as well accumulating levels in tissue, function as signaling molecules, directly interfering with the insulin signaling (Figure 1, mechanism 2a). The accumulation of plasma BCAA is thought to impair insulin signaling by phosphorylating the insulin receptor substrate-1 (IRS-1) via activation of S6 kinase (p70S6K) through mammalian target of rapamycin complex (mTOR) (66-68). IRS-1 regulates insulin-stimulated glucose uptake in muscle tissue by promoting glucose transporter type 4 (GLUT4) translocation to the plasma membrane and further glucose uptake (69). Therefore, higher plasma levels of BCAA could contribute to a decreased glucose uptake and insulin sensitivity in the muscle by diminished GLUT4 translocation. Conversely, it could be postulated that lowering plasma

BCAA levels may alleviate the activation of mTOR signaling and promotes GLUT4 translocation, and consequently improves insulin sensitivity in patients with T2D. In line, in an *in vitro* skeletal muscle cell model, it was found that treatment with sodium-phenylbutyrate (NaPB) resulted in lower BCAA concentrations together with an improved insulin-stimulated glucose uptake (70, 71) via better insulin signaling (71). This effect of NaPB on the insulin signaling pathway has never been investigated in patients with T2D. However, the NaPB-induced lower BCAA levels in our clinical trial (**chapter 5**) could indeed be responsible for the alleviation on insulin-inhibited signaling underlying the improved glucose uptake. Investigating these postulated molecular links were not the primary objectives of the present study, however, are currently being examined in the samples obtained from the study. In **chapter 6**, the *in vitro* mechanistic links between BCAA, insulin signaling and glucose metabolism in skeletal muscle were explored. It was investigated whether elevated BCAA levels, as seen in plasma of patients with T2D, could underlie disturbed insulin-stimulated glucose uptake in the muscle cells. We observed that exposing mice myotubes to sustained increases in extracellular BCAA caused impairments in intracellular insulin signaling, via increased S6K phosphorylation resulting in Akt inactivation. Therefore, the elevated BCAA levels in plasma seen in patients with T2D, could plausibly be responsible for the inhibition of the insulin signaling resulting in the insulin resistance. Nevertheless, it is important to highlight that an *in vitro* skeletal muscle model with continuous exposure of BCAA makes it difficult to extrapolate findings *in vivo*. In addition, as mice myotubes were exposed to supraphysiological levels of BCAA, it is not established whether small physiological changes in BCAA levels are sufficient to induce insulin resistance.

Besides activation of mTOR/S6K pathway, chronic accumulation of BCAA could decrease mitochondrial pyruvate utilization via direct inhibition of pyruvate dehydrogenase complex (PDH) (Figure 1, mechanism 2b) (12). PDH is critical in glucose oxidation (72) and links glycolysis to the TCA cycle by converting pyruvate to acetyl-coenzyme A (CoA) (12). The notion of BCAA- and its catabolic metabolite levels-induced inhibition of PDH (73-76), so far, has not been investigated in humans, also not in our clinical trial (**chapter 5**). NaPB treatment in patients with T2D effectively lowered BCAA plasma levels, and improved the *ex vivo* oxidative capacity on pyruvate in the muscle mitochondria. In line, insulin-stimulated carbohydrate oxidation *in vivo* significantly improved. A common manifestation in patients with T2D is the inability to shift from fatty acid oxidation in the fasted state to glucose oxidation in the fed state, also called metabolic inflexibility (77). These findings are in line with results obtained in a diabetic mouse model, showing that treatment with NaPB was capable to improve glucose metabolism

(78). Concomitantly, we showed that metabolic flexibility, expressed as the change in respiratory exchange ratio (RER) from fasted to insulin-stimulated state tended to improve. As PDH is an important enzyme regulating metabolic flexibility (79), it could be speculated that increased PDH activity is one of the underlying mechanisms of the NaPB-induced improved glucose metabolism in patients with T2D in our clinical trial.

A third mechanistic route which links BCAA catabolism with insulin resistance, is the accumulation of toxic BCAA-catabolic metabolites in plasma. For example, an intermediate of valine catabolism downstream of the BCKD complex, 3-hydroxyisobutyrate (3-HIB), has been found to be elevated in plasma of people with insulin resistance (80, 81) (Figure 1, mechanism 3). Moreover, several reports provided evidence that 3-HIB is associated with insulin resistance and risk of incident T2D in humans (82-86). A previous study by Jang et al. (80) suggested that 3-HIB might contribute to insulin resistance via a stimulated lipid accumulation since 3-HIB act as a paracrine factor regulating transendothelial transport and muscle cell uptake of fatty acids in mice. Until now, this concept has not been investigated in humans. In our clinical trial (**chapter 5**), we found that 3-HIB significantly decreased after NaPB compared to placebo. Interestingly, 3-HIB concentration associated with whole-body carbohydrate oxidation, but not with peripheral insulin sensitivity. To continue, it seems contradictory that 3-HIB levels in plasma are lower despite an NaPB-induced boost of BCAA catabolism. Indeed, end-products of BCAA catabolism enter the TCA cycle and are important anaplerotic substrates fuelling the TCA cycle. Therefore, an incomplete BCAA catabolism may cause so-called anaplerotic stress, which is a reduced BCAA-derived carbon flux to TCA cycle intermediates, and underlie low mitochondrial respiratory rates resulting in disturbed substrate oxidation and subsequent insulin resistance (Figure 1, mechanism 4) (87, 88). From that notion, it could be speculated that an NaPB-induced improvement in BCAA catabolism results in a better mitochondrial TCA flux, which might explain the improvement in insulin sensitivity. These assumptions are, however, speculative since our trial did not investigate whether the patients with T2D had a higher BCAA catabolism after treatment with NaPB. Also, other BCAA-derived metabolites, including BCAA-derived acylcarnitines (C3 and C5), 3-HIB and 2-hydroxbutyric acid (2-HB) and 2-ketobutyric acid (2-KB) (18, 28, 49, 89-93), which can have toxic effects on mitochondrial function and consequently insulin resistance, are not measured in this study (Figure 1, mechanism 5). Future untargeted and targeted metabolomic analysis in plasma and skeletal muscle could help reveal whether there is an actual improved TCA flux and whether toxic intermediates were decreased.

CAN BCAA CATABOLISM BE MODULATED IN HUMANS?

In chapter 2, several pharmaceutical and alternative strategies which effectively boost BCAA catabolism were reviewed. Several rodent studies (65, 94, 95) show the strength of boosting BCAA catabolism as potential strategy to improve glucose homeostasis, however, with the limited number of tools available in humans, this concept has not been extensively investigated in humans. Recent reports in rodents showed promising effects of 3,6-dichlorobenzo(b)thiopene-2-carboxylic acid (BT2), able to directly accelerate BCAA catabolism (96, 97), on glucose metabolism and insulin sensitivity (65, 94), and very interestingly also on cardiometabolic health (96, 98-100). As BT2 is not suitable for human use yet, we used an FDA-approved pharmacological agent, known to boost BCAA catabolism as side effect, as tool to test this concept in humans (**chapter 5**). Other pharmaceutical compounds, like fibrates (101-103) and new T2D therapies targeting incretin (GLP-1 and GIP (104)) and glucagon (105) receptors, seem to indirectly modulate BCAA catabolism as well. Also, alternative strategies including physical activity and exercise, diet low in BCAA and cold acclimatisation seem to indirectly modulate BCAA catabolism, which was discussed in **chapter 2**. So far, we conclude that there are available tools to modulate catabolism and levels of BCAA which improve metabolic health, however it needs to be rigorously investigated whether this improvement is attributed to changes in BCAA metabolism per se.

The effect of physical activity and exercise on BCAA metabolism was explored in more detail (**chapter 4**) since reports suggest that endurance exercise may result in promotion of BCAA catabolism (106-110). It was shown that physically active people of the Netherlands Epidemiology of Obesity Study (NEO) cohort had slightly, but significant lower plasma BCAA levels compared to less active people. In line with our results, another observational study showed an association between high physical activity level and low plasma BCAA levels, which may assume that chronically high physical activity results in lower BCAA levels (111). Nevertheless, we showed that an endurance-exercise training program did not affect BCAA levels in obese insulin resistant individuals with T2D and/or NAFL. In line with observations by others (112), the blunted inhibitory effect of insulin on BCAA levels was not rescued by physical activity of exercise training. Interestingly, the training program lowered IHL content and improved hepatic insulin sensitivity in people with NAFL. Therefore, we assume that with the absent changes in BCAA plasma levels, the observed positive effects could not have been attributable to a changed BCAA catabolism. Controversy does exist on the effect of physical activity and exercise on BCAA catabolism and the inconsistent results of the different studies could be explained by different work load, duration of physical activity

and exercise training, and individuals' training status. Our findings indicate that exercise training and physical activity differently affect plasma BCAA levels, and suggest that BCAA do not play a role in mediating the beneficial metabolic effects of exercise training on, among others, IHL content. The mechanism of long-term physical activity leading to lower plasma BCAA levels is, however, unknown and needs further research. In addition, changes in plasma BCAA levels upon exercise are not a good reflection of BCAA catabolism since exercise promotes protein turnover, and therefore triggers fluctuations in BCAA levels. Exercise training studies combined with stable isotope would elucidate the impact of exercise on BCAA catabolism. Recently, it has been suggested that BCKA, more so than BCAA, are associated with high IHL (113) Therefore, in future, BCKA, as the immediate substrate of the BCKD complex, will be measured as well.

In **chapter 5**, we aimed to address the effectiveness of stimulating BCAA catabolism with use of NaPB on metabolic health in patients with T2D. In more detail, NaPB inhibits the kinase regulating the BCKD complex activity via phosphorylation of E1α subunit. The phosphorylation of this kinase will alleviate inhibition of the BCKD complex, thereby stimulating BCAA catabolism (114, 115). We found that NaPB treatment for two weeks significantly reduced BCAA and BCKA levels in plasma of patients with T2D, which was accompanied by a robust improvement of glucose metabolism. Treatment with NaPB tended to decrease fasting glucose values with 0.5 mM. This might be clinically relevant since patients with T2D feature increased hepatic gluconeogenesis (116), to which BCAA may contribute directly as gluconeogenic precursors (9) or via the glucose-alanine cycle, also called Cahill cycle (117). Therefore, if NaPB induces lower BCAA levels in plasma, less substrates are available for gluconeogenesis, and fasting glucose values in plasma can drop. In addition, one could speculate that during the night, considered fasted, the energy expenditure is more dependent on amino acids, including BCAA. In contrast, no significant effect of BCAA on basal and sleeping energy expenditure and substrate utilization during the night was observed. In this study, we did not specifically measure gluconeogenesis, but would be interesting to examine (for example with deuterium (D_2O) tracer) in the future. Also, metabolic flexibility tended to improve after treatment with NaPB. Since metabolic flexibility is particularly linked to the capacity of mitochondria (118), it could be postulated that this improvement is due to the effect of NaPB-induced higher mitochondrial oxidative capacity for glycolysis. The effect of NaPB on glycolysis can be explained by several mechanisms. An indirect mechanism is that NaPB induced a decrease in plasma BCAA levels, which may alleviate the inhibition of the PDH complex promoting glycolysis. Conversely, NaPB has been proven to increase PDH directly by inhibiting the kinase of this complex (119). As with the activation of the BCKD complex, the activation of PDH

also occurs via phosphorylation of the E1α subunit since they share the same basic enzyme structure (120), although the exact mechanism has not been investigated in this thesis. Therefore, our data strongly suggest that pharmacologically stimulating BCAA catabolism results in beneficial outcomes on patients' glucose metabolism, which is in line with previous findings in rodents (78). It is also important to highlight that despite the rather small decrease (8%) in BCAA, relevant clinical beneficial effects on metabolic health were observed and therefore it could be stated that only small improvements in BCAA levels are needed to exert these relevant effects. Although, the small decrease could also indicate that other mechanisms, besides BCAA levels itself, are related to an improved glucose metabolism. This is possible as it is known that, next to improving of BCAA catabolism, NaPB is able to reduce endoplasmic reticulum stress and inhibit histone deacetylase expression (121, 122), which could also be related to the improvements observed in this study. Therefore, further investigations should focus on the development of strategies targeting specifically BCAA catabolism, without off-target effects, to evaluate the direct impact of BCAA catabolism on glucose metabolism. Furthermore, with this short duration of time and low dose of NaPB, we observed significant improvements, as well as tendencies, and therefore, it is strongly recommended that follow-up studies are performed to assess the effect of long-term stimulation of BCAA catabolism in patients with T2D. No effects on IHL, and hepatic and adipose insulin sensitivity were observed. Taken together, this could indicate that promoting BCAA catabolism affects the skeletal muscle more profoundly than hepatic or adipose tissue. This matches the observation that skeletal muscle has the highest capacity for BCAA catabolism in humans (10, 35) and is considered to be the predominant site of glucose uptake representing a critical site of insulin resistance (123). It has been assumed that skeletal muscle plays an important role in the transamination step, catalyzed by the BCAT, however data on BCKD complex is limited. It would be interesting to specifically study BCKD complex activity in the skeletal muscle, since NaPB is supposed to activate BCAA oxidation via the BCKD complex.

To summarize, the current findings indicate that patients with T2D are characterized by a compromised BCAA catabolism. We demonstrate that boosting BCAA catabolism exerts beneficial effects on glucose metabolism in patients with T2D. It would be valuable to explore more strategies, besides NaPB and exercise, to stimulate BCAA catabolism and its effect on metabolic health in T2D and to develop medications directly targeting the BCAA-catabolic pathway.

FUTURE PERSPECTIVES

We showed that the BCAA-catabolic pathway might be compromised in insulin resistant states by comparing BCAA oxidation and mitochondrial respiratory capacity in individuals with and without insulin resistance (**chapter 3**). This might partly explain the elevated plasma BCAA levels seen in these patients, which may hamper insulin sensitivity. It is, however, important to keep in mind that BCAA catabolism is tissue-specific and may act differently in several tissues. So far, BCAA catabolism has never been investigated in humans, and therefore, future research is needed to develop non-invasive methods to measure tissue-specific BCAA catabolism in humans.

Our data in patients with T2D translate the findings from the pioneering animal studies that (pharmacologically) promoting BCAA oxidation improves insulin sensitivity and glucose homeostasis (**chapter 5**). Treatment with NaPB decreased BCAA levels in plasma, which may be underlying the beneficial effects on glucose metabolism. The question, however, which mechanistic routes underlie these beneficial effects remains unanswered and should be further explored. In this study, BCAA oxidation itself was not investigated, and therefore, tracer studies with a mixture of 13C- leucine, 13C- isoleucine, 13C-valine would help to understand the actual effect of modulating BCAA oxidation on metabolic health. Since NaPB was administered for a relative short period, 2 weeks, future research should investigate the metabolic effects of long-term NaPB administration in T2D. Besides patients with T2D, measuring the effects of NaPB on metabolic health in individuals with prediabetes may tell us whether this would be effective in preventing the development towards T2D. In addition, we hope that soon a pharmacological compound which specifically affect BCAA catabolism, like BT2 as used in rodent models, becomes available for human use to further reveal the role of BCAA catabolism in metabolic health and T2D.

Not only there is a necessity to understand the exact mechanisms behind the putative tissue-specific effect of elevated BCAA levels on insulin resistance, it is also important to find alternative ways to directly manipulate BCAA metabolism in humans. Within this thesis, we provided evidence that elevated BCAA levels in plasma is linked with insulin resistance and other T2D-related metabolic disturbances. Our data suggested that patients with T2D are characterized by a compromised BCAA catabolism, which might explain the elevation in BCAA levels measured in these patients. Furthermore, the aim of this thesis was to investigate a new strategy, promoting BCAA catabolism, thereby exerting sustained improvements of metabolic health in patients with T2D. NaPB can be

used to modulate BCAA metabolism, however, has 'off-target' effects. Therefore, other pharmaceutical or alternative strategies boosting BCAA catabolism should be explored to prevent accumulation of BCAA levels in plasma and thereby possibly prevent detrimental effects on metabolic health in T2D.

Long-term investigations should focus whether targeting BCAA catabolism is able to slow down the progression of the disease. One of the questions that remains to be answered is whether T2D can be prevented by targeting BCAA catabolism and therefore, future research is needed whether this novel treatment strategy would be effective to prevent the progression towards T2D people with prediabetes. Before this promising new treatment can be sued clinically or commercialized as novel therapeutic approach to combat T2D, more-in-depth molecular research and reproducibility studies are needed.

REFERENCES

1. DeFronzo RA, Simonson D, Ferrannini E. Hepatic and peripheral insulin resistance: a common feature of type 2 (non-insulin-dependent) and type 1 (insulin-dependent) diabetes mellitus. Diabetologia. 1982;23(4):313-9.
2. Szendroedi J, Phielix E, Roden M. The role of mitochondria in insulin resistance and type 2 diabetes mellitus. Nat Rev Endocrinol. 2011;8(2):92-103.
3. Chen X, Yang W. Branched-chain amino acids and the association with type 2 diabetes. J Diabetes Investig. 2015;6(4):369-70.
4. Khan MAB, Hashim MJ, King JK, Govender RD, Mustafa H, Al Kaabi J. Epidemiology of Type 2 Diabetes - Global Burden of Disease and Forecasted Trends. Journal of epidemiology and global health. 2020;10(1):107-11.
5. Phielix E, Jelenik T, Nowotny P, Szendroedi J, Roden M. Reduction of non-esterified fatty acids improves insulin sensitivity and lowers oxidative stress, but fails to restore oxidative capacity in type 2 diabetes: a randomised clinical trial. Diabetologia. 2014;57(3):572-81.
6. Phielix E, Meex R, Moonen-Kornips E, Hesselink MK, Schrauwen P. Exercise training increases mitochondrial content and ex vivo mitochondrial function similarly in patients with type 2 diabetes and in control individuals. Diabetologia. 2010;53(8):1714-21.
7. Phielix E, Schrauwen-Hinderling VB, Mensink M, Lenaers E, Meex R, Hoeks J, et al. Lower intrinsic ADP-stimulated mitochondrial respiration underlies in vivo mitochondrial dysfunction in muscle of male type 2 diabetic patients. Diabetes. 2008;57(11):2943-9.
8. Holecek M. Branched-chain amino acids in health and disease: metabolism, alterations in blood plasma, and as supplements. Nutr Metab (Lond). 2018;15:33.
9. Neinast M, Murashige D, Arany Z. Branched Chain Amino Acids. Annu Rev Physiol. 2019;81:139-64.
10. Suryawan A, Hawes JW, Harris RA, Shimomura Y, Jenkins AE, Hutson SM. A molecular model of human branched-chain amino acid metabolism. Am J Clin Nutr. 1998;68(1):72-81.
11. Wahren J, Felig P, Hagenfeldt L. Effect of protein ingestion on splanchnic and leg metabolism in normal man and in patients with diabetes mellitus. J Clin Invest. 1976;57(4):987-99.
12. Li T, Zhang Z, Kolwicz SC, Jr., Abell L, Roe ND, Kim M, et al. Defective Branched-Chain Amino Acid Catabolism Disrupts Glucose Metabolism and Sensitizes the Heart to Ischemia-Reperfusion Injury. Cell Metab. 2017;25(2):374-85.
13. Uddin GM, Zhang L, Shah S, Fukushima A, Wagg CS, Gopal K, et al. Impaired branched chain amino acid oxidation contributes to cardiac insulin resistance in heart failure. Cardiovasc Diabetol. 2019;18(1):86.
14. Buse MG, Biggers JF, Friderici KH, Buse JF. Oxidation of branched chain amino acids by isolated hearts and diaphragms of the rat. The effect of fatty acids, glucose, and pyruvate respiration. J Biol Chem. 1972;247(24):8085-96.
15. Lackey DE, Lynch CJ, Olson KC, Mostaedi R, Ali M, Smith WH, et al. Regulation of adipose branched-chain amino acid catabolism enzyme expression and cross-adipose amino acid flux in human obesity. Am J Physiol Endocrinol Metab. 2013;304(11):E1175-87.
16. She P, Van Horn C, Reid T, Hutson SM, Cooney RN, Lynch CJ. Obesity-related elevations in plasma leucine are associated with alterations in enzymes involved in branched-chain amino acid metabolism. Am J Physiol Endocrinol Metab. 2007;293(6):E1552-63.
17. Herman MA, She P, Peroni OD, Lynch CJ, Kahn BB. Adipose tissue branched chain amino acid (BCAA) metabolism modulates circulating BCAA levels. J Biol Chem. 2010;285(15):11348-56.

18. Adams SH, Hoppel CL, Lok KH, Zhao L, Wong SW, Minkler PE, et al. Plasma acylcarnitine profiles suggest incomplete long-chain fatty acid beta-oxidation and altered tricarboxylic acid cycle activity in type 2 diabetic African-American women. J Nutr. 2009;139(6):1073-81.
19. Pietilainen KH, Naukkarinen J, Rissanen A, Saharinen J, Ellonen P, Keranen H, et al. Global transcript profiles of fat in monozygotic twins discordant for BMI: pathways behind acquired obesity. PLoS Med. 2008;5(3):e51.
20. Leskinen T, Rinnankoski-Tuikka R, Rintala M, Seppanen-Laakso T, Pollanen E, Alen M, et al. Differences in muscle and adipose tissue gene expression and cardio-metabolic risk factors in the members of physical activity discordant twin pairs. PLoS One. 2010;5(9).
21. Klimcakova E, Roussel B, Marquez-Quinones A, Kovacova Z, Kovacikova M, Combes M, et al. Worsening of obesity and metabolic status yields similar molecular adaptations in human subcutaneous and visceral adipose tissue: decreased metabolism and increased immune response. J Clin Endocrinol Metab. 2011;96(1):E73-82.
22. Stancakova A, Civelek M, Saleem NK, Soininen P, Kangas AJ, Cederberg H, et al. Hyperglycemia and a common variant of GCKR are associated with the levels of eight amino acids in 9,369 Finnish men. Diabetes. 2012;61(7):1895-902.
23. Burgos SA, Chandurkar V, Tsoukas MA, Chevalier S, Morais JA, Lamarche M, et al. Insulin resistance of protein anabolism accompanies that of glucose metabolism in lean, glucose-tolerant offspring of persons with type 2 diabetes. BMJ Open Diabetes Res Care. 2016;4(1):e000312.
24. Lattuada G, Sereni LP, Ruggieri D, Scollo A, Benedini S, Ragogna F, et al. Postabsorptive and insulin-stimulated energy homeostasis and leucine turnover in offspring of type 2 diabetic patients. Diabetes Care. 2004;27(11):2716-22.
25. Pereira S, Marliss EB, Morais JA, Chevalier S, Gougeon R. Insulin resistance of protein metabolism in type 2 diabetes. Diabetes. 2008;57(1):56-63.
26. Lotta LA, Scott RA, Sharp SJ, Burgess S, Luan J, Tillin T, et al. Genetic Predisposition to an Impaired Metabolism of the Branched-Chain Amino Acids and Risk of Type 2 Diabetes: A Mendelian Randomisation Analysis. PLoS Med. 2016;13(11):e1002179.
27. Hernandez-Alvarez MI, Diaz-Ramos A, Berdasco M, Cobb J, Planet E, Cooper D, et al. Early-onset and classical forms of type 2 diabetes show impaired expression of genes involved in muscle branched-chain amino acids metabolism. Sci Rep. 2017;7(1):13850.
28. Lerin C, Goldfine AB, Boes T, Liu M, Kasif S, Dreyfuss JM, et al. Defects in muscle branched-chain amino acid oxidation contribute to impaired lipid metabolism. Mol Metab. 2016;5(10):926-36.
29. Lefort N, Glancy B, Bowen B, Willis WT, Bailowitz Z, De Filippis EA, et al. Increased reactive oxygen species production and lower abundance of complex I subunits and carnitine palmitoyltransferase 1B protein despite normal mitochondrial respiration in insulin-resistant human skeletal muscle. Diabetes. 2010;59(10):2444-52.
30. Felig P. Amino acid metabolism in man. Annu Rev Biochem. 1975;44:933-55.
31. Aoki TT, Brennan MF, Muller WA, Soeldner JS, Alpert JS, Saltz SB, et al. Amino acid levels across normal forearm muscle and splanchnic bed after a protein meal. Am J Clin Nutr. 1976;29(4):340-50.
32. Elia M, Livesey G. Effects of ingested steak and infused leucine on forelimb metabolism in man and the fate of the carbon skeletons and amino groups of branched-chain amino acids. Clin Sci (Lond). 1983;64(5):517-26.
33. Hagenfeldt L, Eriksson LS, Wahren J. Amino acids in liver disease. Proc Nutr Soc. 1983;42(3):497-506.
34. Abdulla H, Smith K, Atherton PJ, Idris I. Role of insulin in the regulation of human skeletal muscle protein synthesis and breakdown: a systematic review and meta-analysis. Diabetologia. 2016;59(1):44-55.

35. Adeva MM, Calvino J, Souto G, Donapetry C. Insulin resistance and the metabolism of branched-chain amino acids in humans. Amino Acids. 2012;43(1):171-81.
36. Shaham O, Wei R, Wang TJ, Ricciardi C, Lewis GD, Vasan RS, et al. Metabolic profiling of the human response to a glucose challenge reveals distinct axes of insulin sensitivity. Mol Syst Biol. 2008;4:214.
37. Halvatsiotis P, Short KR, Bigelow M, Nair KS. Synthesis rate of muscle proteins, muscle functions, and amino acid kinetics in type 2 diabetes. Diabetes. 2002;51(8):2395-404.
38. Halvatsiotis PG, Turk D, Alzaid A, Dinneen S, Rizza RA, Nair KS. Insulin effect on leucine kinetics in type 2 diabetes mellitus. Diabetes Nutr Metab. 2002;15(3):136-42.
39. Fukagawa NK, Minaker KL, Rowe JW, Goodman MN, Matthews DE, Bier DM, et al. Insulin-mediated reduction of whole body protein breakdown. Dose-response effects on leucine metabolism in postabsorptive men. J Clin Invest. 1985;76(6):2306-11.
40. Luzi L, Petrides AS, De Fronzo RA. Different sensitivity of glucose and amino acid metabolism to insulin in NIDDM. Diabetes. 1993;42(12):1868-77.
41. Staten MA, Matthews DE, Bier DM. Leucine metabolism in type II diabetes mellitus. Diabetes. 1986;35(11):1249-53.
42. Luzi L, Giordano M, Caloni, Castellino P. Effects of insulin and amino acids on leucine metabolism in young and middle-aged humans. Eur J Nutr. 2001;40(3):106-12.
43. Bell JA, Fujita S, Volpi E, Cadenas JG, Rasmussen BB. Short-term insulin and nutritional energy provision do not stimulate muscle protein synthesis if blood amino acid availability decreases. Am J Physiol Endocrinol Metab. 2005;289(6):E999-1006.
44. Chevalier S, Gougeon R, Kreisman SH, Cassis C, Morais JA. The hyperinsulinemic amino acid clamp increases whole-body protein synthesis in young subjects. Metabolism. 2004;53(3):388-96.
45. Xu F, Tavintharan S, Sum CF, Woon K, Lim SC, Ong CN. Metabolic signature shift in type 2 diabetes mellitus revealed by mass spectrometry-based metabolomics. J Clin Endocrinol Metab. 2013;98(6):E1060-5.
46. Palmer ND, Stevens RD, Antinozzi PA, Anderson A, Bergman RN, Wagenknecht LE, et al. Metabolomic profile associated with insulin resistance and conversion to diabetes in the Insulin Resistance Atherosclerosis Study. J Clin Endocrinol Metab. 2015;100(3):E463-8.
47. van den Berg EH, Flores-Guerrero JL, Gruppen EG, de Borst MH, Wolak-Dinsmore J, Connelly MA, et al. Non-Alcoholic Fatty Liver Disease and Risk of Incident Type 2 Diabetes: Role of Circulating Branched-Chain Amino Acids. Nutrients. 2019;11(3).
48. Kaikkonen JE, Wurtz P, Suomela E, Lehtovirta M, Kangas AJ, Jula A, et al. Metabolic profiling of fatty liver in young and middle-aged adults: Cross-sectional and prospective analyses of the Young Finns Study. Hepatology. 2017;65(2):491-500.
49. Newgard CB, An J, Bain JR, Muehlbauer MJ, Stevens RD, Lien LF, et al. A branched-chain amino acid-related metabolic signature that differentiates obese and lean humans and contributes to insulin resistance. Cell Metab. 2009;9(4):311-26.
50. Tai ES, Tan ML, Stevens RD, Low YL, Muehlbauer MJ, Goh DL, et al. Insulin resistance is associated with a metabolic profile of altered protein metabolism in Chinese and Asian-Indian men. Diabetologia. 2010;53(4):757-67.
51. Krebs M, Krssak M, Bernroider E, Anderwald C, Brehm A, Meyerspeer M, et al. Mechanism of amino acid-induced skeletal muscle insulin resistance in humans. Diabetes. 2002;51(3):599-605.
52. Huffman KM, Shah SH, Stevens RD, Bain JR, Muehlbauer M, Slentz CA, et al. Relationships Between Circulating Metabolic Intermediates and Insulin Action in Overweight to Obese, Inactive Men and Women. Diabetes Care. 2009;32(9):1678-83.
53. Perng W, Gillman MW, Fleisch AF, Michalek RD, Watkins SM, Isganaitis E, et al. Metabolomic profiles and childhood obesity. Obesity (Silver Spring). 2014;22(12):2570-8.

54. Mahendran Y, Jonsson A, Have CT, Allin KH, Witte DR, Jorgensen ME, et al. Genetic evidence of a causal effect of insulin resistance on branched-chain amino acid levels. Diabetologia. 2017;60(5):873-8.
55. Wang Q, Holmes MV, Davey Smith G, Ala-Korpela M. Genetic Support for a Causal Role of Insulin Resistance on Circulating Branched-Chain Amino Acids and Inflammation. Diabetes care. 2017;40(12):1779-86.
56. Zhu Z, Zheng Z, Zhang F, Wu Y, Trzaskowski M, Maier R, et al. Causal associations between risk factors and common diseases inferred from GWAS summary data. Nat Commun. 2018;9(1):224.
57. Guasch-Ferre M, Hruby A, Toledo E, Clish CB, Martinez-Gonzalez MA, Salas-Salvado J, et al. Metabolomics in Prediabetes and Diabetes: A Systematic Review and Meta-analysis. Diabetes Care. 2016;39(5):833-46.
58. Wang TJ, Larson MG, Vasan RS, Cheng S, Rhee EP, McCabe E, et al. Metabolite profiles and the risk of developing diabetes. Nat Med. 2011;17(4):448-53.
59. Gar C, Rottenkolber M, Prehn C, Adamski J, Seissler J, Lechner A. Serum and plasma amino acids as markers of prediabetes, insulin resistance, and incident diabetes. Crit Rev Clin Lab Sci. 2018;55(1):21-32.
60. Greenhaff PL, Karagounis LG, Peirce N, Simpson EJ, Hazell M, Layfield R, et al. Disassociation between the effects of amino acids and insulin on signaling, ubiquitin ligases, and protein turnover in human muscle. Am J Physiol Endocrinol Metab. 2008;295(3):E595-604.
61. Iwasa M, Ishihara T, Mifuji-Moroka R, Fujita N, Kobayashi Y, Hasegawa H, et al. Elevation of branched-chain amino acid levels in diabetes and NAFL and changes with antidiabetic drug treatment. Obes Res Clin Pract. 2015;9(3):293-7.
62. Sliz E, Sebert S, Wurtz P, Kangas AJ, Soininen P, Lehtimaki T, et al. NAFLD risk alleles in PNPLA3, TM6SF2, GCKR and LYPLAL1 show divergent metabolic effects. Hum Mol Genet. 2018;27(12):2214-23.
63. Cheng Y, Meng Q, Wang C, Li H, Huang Z, Chen S, et al. Leucine deprivation decreases fat mass by stimulation of lipolysis in white adipose tissue and upregulation of uncoupling protein 1 (UCP1) in brown adipose tissue. Diabetes. 2010;59(1):17-25.
64. Du Y, Meng Q, Zhang Q, Guo F. Isoleucine or valine deprivation stimulates fat loss via increasing energy expenditure and regulating lipid metabolism in WAT. Amino Acids. 2012;43(2):725-34.
65. White PJ, McGarrah RW, Grimsrud PA, Tso SC, Yang WH, Haldeman JM, et al. The BCKDH Kinase and Phosphatase Integrate BCAA and Lipid Metabolism via Regulation of ATP-Citrate Lyase. Cell Metabolism. 2018;27(6):1281-+.
66. Krebs M, Brunmair B, Brehm A, Artwohl M, Szendroedi J, Nowotny P, et al. The Mammalian target of rapamycin pathway regulates nutrient-sensitive glucose uptake in man. Diabetes. 2007;56(6):1600-7.
67. Um SH, D'Alessio D, Thomas G. Nutrient overload, insulin resistance, and ribosomal protein S6 kinase 1, S6K1. Cell Metab. 2006;3(6):393-402.
68. Tremblay F, Brule S, Hee Um S, Li Y, Masuda K, Roden M, et al. Identification of IRS-1 Ser-1101 as a target of S6K1 in nutrient- and obesity-induced insulin resistance. Proc Natl Acad Sci U S A. 2007;104(35):14056-61.
69. Thirone AC, Huang C, Klip A. Tissue-specific roles of IRS proteins in insulin signaling and glucose transport. Trends Endocrinol Metab. 2006;17(2):72-8.
70. Hu H, Li L, Wang C, He H, Mao K, Ma X, et al. 4-Phenylbutyric acid increases GLUT4 gene expression through suppression of HDAC5 but not endoplasmic reticulum stress. Cell Physiol Biochem. 2014;33(6):1899-910.
71. Crossland H, Smith K, Idris I, Phillips BE, Atherton PJ, Wilkinson DJ. Exploring mechanistic links between extracellular branched-chain amino acids and muscle insulin resistance: an in vitro approach. Am J Physiol Cell Physiol. 2020;319(6):C1151-C7.

72. Lee IK. The role of pyruvate dehydrogenase kinase in diabetes and obesity. Diabetes Metab J. 2014;38(3):181-6.
73. Jackson RH, Singer TP. Inactivation of the 2-ketoglutarate and pyruvate dehydrogenase complexes of beef heart by branched chain keto acids. J Biol Chem. 1983;258(3):1857-65.
74. Randle PJ, Garland PB, Hales CN, Newsholme EA. The glucose fatty-acid cycle. Its role in insulin sensitivity and the metabolic disturbances of diabetes mellitus. Lancet. 1963;1(7285):785-9.
75. Randle PJ, Newsholme EA, Garland PB. Regulation of glucose uptake by muscle. 8. Effects of fatty acids, ketone bodies and pyruvate, and of alloxan-diabetes and starvation, on the uptake and metabolic fate of glucose in rat heart and diaphragm muscles. Biochem J. 1964;93(3):652-65.
76. Williamson JR, Walajtys-Rode E, Coll KE. Effects of branched chain alpha-ketoacids on the metabolism of isolated rat liver cells. I. Regulation of branched chain alpha-ketoacid metabolism. J Biol Chem. 1979;254(22):11511-20.
77. Kelley DE, Mandarino LJ. Fuel selection in human skeletal muscle in insulin resistance: a reexamination. Diabetes. 2000;49(5):677-83.
78. Ozcan U, Yilmaz E, Ozcan L, Furuhashi M, Vaillancourt E, Smith RO, et al. Chemical chaperones reduce ER stress and restore glucose homeostasis in a mouse model of type 2 diabetes. Science. 2006;313(5790):1137-40.
79. Zhang S, Hulver MW, McMillan RP, Cline MA, Gilbert ER. The pivotal role of pyruvate dehydrogenase kinases in metabolic flexibility. Nutr Metab (Lond). 2014;11(1):10.
80. Jang C, Oh SF, Wada S, Rowe GC, Liu L, Chan MC, et al. A branched-chain amino acid metabolite drives vascular fatty acid transport and causes insulin resistance. Nature medicine. 2016;22(4):421-6.
81. Zhang Y, Cao J, Zhang S, Lee AJ, Sun G, Larsen CN, et al. Genetic changes found in a distinct clade of Enterovirus D68 associated with paralysis during the 2014 outbreak. Virus Evol. 2016;2(1):vew015.
82. Harris LLS, Smith GI, Patterson BW, Ramaswamy RS, Okunade AL, Kelly SC, et al. Alterations in 3-Hydroxyisobutyrate and FGF21 Metabolism Are Associated With Protein Ingestion-Induced Insulin Resistance. Diabetes. 2017;66(7):1871-8.
83. Nilsen MS, Jersin RA, Ulvik A, Madsen A, McCann A, Svensson PA, et al. 3-Hydroxyisobutyrate, A Strong Marker of Insulin Resistance in Type 2 Diabetes and Obesity That Modulates White and Brown Adipocyte Metabolism. Diabetes. 2020;69(9):1903-16.
84. Anderson EJ, Lustig ME, Boyle KE, Woodlief TL, Kane DA, Lin CT, et al. Mitochondrial H2O2 emission and cellular redox state link excess fat intake to insulin resistance in both rodents and humans. J Clin Invest. 2009;119(3):573-81.
85. Andersson-Hall U, Gustavsson C, Pedersen A, Malmodin D, Joelsson L, Holmang A. Higher Concentrations of BCAAs and 3-HIB Are Associated with Insulin Resistance in the Transition from Gestational Diabetes to Type 2 Diabetes. Journal of Diabetes Research. 2018;2018.
86. Mardinoglu A, Gogg S, Lotta LA, Stancakova A, Nerstedt A, Boren J, et al. Elevated Plasma Levels of 3-Hydroxyisobutyric Acid Are Associated With Incident Type 2 Diabetes. EBioMedicine. 2018;27:151-5.
87. Lynch CJ, Adams SH. Branched-chain amino acids in metabolic signalling and insulin resistance. Nat Rev Endocrinol. 2014;10(12):723-36.
88. Olson KC, Chen G, Xu Y, Hajnal A, Lynch CJ. Alloisoleucine differentiates the branched-chain aminoacidemia of Zucker and dietary obese rats. Obesity (Silver Spring). 2014;22(5):1212-5.
89. Newgard CB. Interplay between Lipids and Branched-Chain Amino Acids in Development of Insulin Resistance. Cell Metabolism. 2012;15(5):606-14.

90. She P, Olson KC, Kadota Y, Inukai A, Shimomura Y, Hoppel CL, et al. Leucine and protein metabolism in obese Zucker rats. PLoS One. 2013;8(3):e59443.
91. Magkos F, Bradley D, Schweitzer GG, Finck BN, Eagon JC, Ilkayeva O, et al. Effect of Roux-en-Y gastric bypass and laparoscopic adjustable gastric banding on branched-chain amino acid metabolism. Diabetes. 2013;62(8):2757-61.
92. Shah SH, Crosslin DR, Haynes CS, Nelson S, Turer CB, Stevens RD, et al. Branched-chain amino acid levels are associated with improvement in insulin resistance with weight loss. Diabetologia. 2012;55(2):321-30.
93. Fiehn O, Garvey WT, Newman JW, Lok KH, Hoppel CL, Adams SH. Plasma metabolomic profiles reflective of glucose homeostasis in non-diabetic and type 2 diabetic obese African-American women. PLoS One. 2010;5(12):e15234.
94. Zhou M, Shao J, Wu CY, Shu L, Dong W, Liu Y, et al. Targeting BCAA Catabolism to Treat Obesity-Associated Insulin Resistance. Diabetes. 2019;68(9):1730-46.
95. She P, Reid TM, Bronson SK, Vary TC, Hajnal A, Lynch CJ, et al. Disruption of BCATm in mice leads to increased energy expenditure associated with the activation of a futile protein turnover cycle. Cell Metab. 2007;6(3):181-94.
96. Lian K, Guo X, Wang Q, Liu Y, Wang RT, Gao C, et al. PP2Cm overexpression alleviates MI/R injury mediated by a BCAA catabolism defect and oxidative stress in diabetic mice. Eur J Pharmacol. 2020;866:172796.
97. Neinast MD, Jang C, Hui S, Murashige DS, Chu Q, Morscher RJ, et al. Quantitative Analysis of the Whole-Body Metabolic Fate of Branched-Chain Amino Acids. Cell Metab. 2019;29(2):417-29 e4.
98. Sun H, Olson KC, Gao C, Prosdocimo DA, Zhou M, Wang Z, et al. Catabolic Defect of Branched-Chain Amino Acids Promotes Heart Failure. Circulation. 2016;133(21):2038-49.
99. Wang W, Zhang F, Xia Y, Zhao S, Yan W, Wang H, et al. Defective branched chain amino acid catabolism contributes to cardiac dysfunction and remodeling following myocardial infarction. Am J Physiol Heart Circ Physiol. 2016;311(5):H1160-H9.
100. Chen M, Gao C, Yu J, Ren S, Wang M, Wynn RM, et al. Therapeutic Effect of Targeting Branched-Chain Amino Acid Catabolic Flux in Pressure-Overload Induced Heart Failure. J Am Heart Assoc. 2019;8(11):e011625.
101. Paul HS, Adibi SA. Paradoxical effects of clofibrate on liver and muscle metabolism in rats. Induction of myotonia and alteration of fatty acid and glucose oxidation. J Clin Invest. 1979;64(2):405-12.
102. Kadota Y, Kazama S, Bajotto G, Kitaura Y, Shimomura Y. Clofibrate-induced reduction of plasma branched-chain amino acid concentrations impairs glucose tolerance in rats. JPEN J Parenter Enteral Nutr. 2012;36(3):337-43.
103. Ishiguro H, Katano Y, Nakano I, Ishigami M, Hayashi K, Honda T, et al. Clofibrate treatment promotes branched-chain amino acid catabolism and decreases the phosphorylation state of mTOR, eIF4E-BP1, and S6K1 in rat liver. Life Sci. 2006;79(8):737-43.
104. Pirro V, Roth KD, Lin Y, Willency JA, Milligan PL, Wilson JM, et al. Effects of Tirzepatide, a Dual GIP and GLP-1 RA, on Lipid and Metabolite Profiles in Subjects with Type 2 Diabetes. J Clin Endocrinol Metab. 2021.
105. Karwi QG, Zhang L, Wagg CS, Wang W, Ghandi M, Thai D, et al. Targeting the glucagon receptor improves cardiac function and enhances insulin sensitivity following a myocardial infarction. Cardiovasc Diabetol. 2019;18(1):1.
106. Shimomura Y, Murakami T, Nakai N, Nagasaki M, Harris RA. Exercise promotes BCAA catabolism: effects of BCAA supplementation on skeletal muscle during exercise. J Nutr. 2004;134(6 Suppl):1583S-7S.
107. Shimomura Y, Fujii H, Suzuki M, Murakami T, Fujitsuka N, Nakai N. Branched-chain alpha-keto acid dehydrogenase complex in rat skeletal muscle: regulation of the activity and gene expression by nutrition and physical exercise. J Nutr. 1995;125(6 Suppl):1762S-5S.

108. Xu M, Nagasaki M, Obayashi M, Sato Y, Tamura T, Shimomura Y. Mechanism of activation of branched-chain alpha-keto acid dehydrogenase complex by exercise. Biochem Bioph Res Co. 2001;287(3):752-6.

109. Kobayashi R, Shimomura Y, Murakami T, Nakai N, Otsuka M, Arakawa N, et al. Hepatic branched-chain alpha-keto acid dehydrogenase complex in female rats: activation by exercise and starvation. J Nutr Sci Vitaminol (Tokyo). 1999;45(3):303-9.

110. Rennie MJ. Influence of exercise on protein and amino acid metabolism. Compr Physiol. 2011;25(1):995-1035.

111. Kujala UM, Peltonen M, Laine MK, Kaprio J, Heinonen OJ, Sundvall J, et al. Branched-Chain Amino Acid Levels Are Related with Surrogates of Disturbed Lipid Metabolism among Older Men. Front Med (Lausanne). 2016;3:57.

112. Glynn EL, Piner LW, Huffman KM, Slentz CA, Elliot-Penry L, AbouAssi H, et al. Impact of combined resistance and aerobic exercise training on branched-chain amino acid turnover, glycine metabolism and insulin sensitivity in overweight humans. Diabetologia. 2015;58(10):2324-35.

113. Grenier-Larouche T, Coulter Kwee L, Deleye Y, Leon-Mimila P, Walejko JM, McGarrah RW, et al. Altered branched-chain alpha-keto acid metabolism is a feature of NAFLD in individuals with severe obesity. JCI Insight. 2022;7(15).

114. Brunetti-Pierri N, Lanpher B, Erez A, Ananieva EA, Islam M, Marini JC, et al. Phenylbutyrate therapy for maple syrup urine disease. Hum Mol Genet. 2011;20(4):631-40.

115. Holecek M, Vodenicarovova M, Siman P. Acute effects of phenylbutyrate on glutamine, branched-chain amino acid and protein metabolism in skeletal muscles of rats. Int J Exp Pathol. 2017;98(3):127-33.

116. Turk D, Alzaid A, Dinneen S, Nair KS, Rizza R. The effects of non-insulin-dependent diabetes mellitus on the kinetics of onset of insulin action in hepatic and extrahepatic tissues. J Clin Invest. 1995;95(2):755-62.

117. Nurjhan N, Bucci A, Perriello G, Stumvoll M, Dailey G, Bier DM, et al. Glutamine: a major gluconeogenic precursor and vehicle for interorgan carbon transport in man. J Clin Invest. 1995;95(1):272-7.

118. Muoio DM. Metabolic inflexibility: when mitochondrial indecision leads to metabolic gridlock. Cell. 2014;159(6):1253-62.

119. Zhang W, Zhang SL, Hu X, Tam KY. Phenyl butyrate inhibits pyruvate dehydrogenase kinase 1 and contributes to its anti-cancer effect. Eur J Pharm Sci. 2017;110:93-100.

120. Sugden MC, Holness MJ. Recent advances in mechanisms regulating glucose oxidation at the level of the pyruvate dehydrogenase complex by PDKs. Am J Physiol Endocrinol Metab. 2003;284(5):E855-62.

121. Cuadrado-Tejedor M, Ricobaraza AL, Torrijo R, Franco R, Garcia-Osta A. Phenylbutyrate is a multifaceted drug that exerts neuroprotective effects and reverses the Alzheimer s disease-like phenotype of a commonly used mouse model. Curr Pharm Des. 2013;19(28):5076-84.

122. Tanis RM, Piroli GG, Day SD, Frizzell N. The effect of glucose concentration and sodium phenylbutyrate treatment on mitochondrial bioenergetics and ER stress in 3T3-L1 adipocytes. Biochim Biophys Acta. 2015;1853(1):213-21.

123. DeFronzo RA, Tripathy D. Skeletal muscle insulin resistance is the primary defect in type 2 diabetes. Diabetes Care. 2009;32 Suppl 2:S157-63.

A

Appendices

Impact
Samenvatting
Summary
Dankwoord
About the author
List of publications

IMPACT

(Research) What is the main objective of the research described in the thesis and what are the most important results and conclusions?

The aim of this thesis is to investigate the role of branched-chain amino acid (BCAA) catabolism in insulin resistance and type 2 diabetes (T2D) and if BCAA catabolism can be pharmacologically modulated in patients with T2D to beneficially effect metabolic health.

In **chapter 2**, a review of literature was performed investigating BCAA catabolism in insulin resistance and T2D, and strategies to boost BCAA catabolism. We conclude from this review that dysregulation of BCAA catabolism is closely related to obesity- and T2D related metabolic disturbances. Dysfunctional BCAA catabolism in several tissues could be a plausible explanation for the elevated plasma BCAA levels seen in obesity and T2D, although, an integrated understanding of tissue-specific BCAA catabolism remains largely unknown in humans. Therefore, exploring intervention strategies to increase BCAA oxidation and lower BCAA levels is important to investigate whether this could be a new potential strategy in the treatment of T2D.

We further explored the relation between BCAA metabolism and T2D-related complications in **chapter 3 and 4**. To this end, we observed that individuals with insulin resistance, including patients with T2D and NAFL, featured elevated BCAA levels compared to controls with obesity. We showed for the first time that elevated BCAA levels correlate with other type 2-diabetes related metabolic disturbances. We furthermore showed that patients with T2D have a lower whole-body leucine oxidation compared to healthy obese individuals, and therefore, a low mitochondrial BCAA oxidation may partly explain higher BCAA levels seen in these patients. These results underlined the importance to address BCAA oxidation in the treatment of T2D.

This challenged us to investigate whether BCAA oxidation can be improved by use of several strategies. To specify, we first investigated the potential effect of physical activity and exercise since it is well now for its role in metabolic health and might be an important player in BCAA catabolism. In **chapter 4**, it was tested if physical activity or an exercise training program would affect liver fat content via BCAA levels. We showed that BCAA levels were lower in more active individuals compared to less active individuals. Furthermore, it appeared that by doing

exercise liver fat decreased in people with NAFL, however this was not paralleled by a reduction in BCAA levels. Therefore, we can conclude that physical activity and exercise training differently affect BCAA levels in plasma, and that BCAA do not play a role in mediating the beneficial metabolic effects of exercise training. More research is needed to explore the amount and intensity of exercise that is needed to boost BCAA oxidation and therefore can contribute to the prevention and treatment of obesity and T2D.

In **chapter 5**, we investigated the potential effect of sodium-phenylbutyrate (NaPB), a booster of BCAA catabolism on metabolic health in patients with T2D as novel approach. In rodents, it has been shown that boosting BCAA oxidation has many metabolic benefits including improved glucose metabolism. In this thesis, these effects could be translated to humans for the first time. Although a relative short intervention period was applied in this thesis, the observed effects on metabolic health are promising. This finding may contribute to developing alternative novel strategies to boost BCAA oxidation, and whether they can prevent or treat T2D on the long-term.

In addition to these results, it was shown that exposing mice skeletal muscle cells to elevated extracellular BCAA levels caused impairments in intracellular insulin signaling via the mTOR/S6K pathway, however, insulin-mediated glucose uptake was not affected (**chapter 6**). We used 2-deoxyglucose (2DG) to study insulin-stimulated glucose uptake, which is a powerful tool to investigate the insulin resistant state *in vitro*. Performing cell culture experiments gives us more knowledge on the molecular level to better understand how insulin resistance can be caused by supplementation of BCAA. The results of this study are proof-of-concept the elevated BCAA levels can be a causal factor in insulin resistance and forms the lead to investigate this further in human skeletal muscle cells.

The association between BCAA levels and incident T2D was assessed in a systematic review and meta-analysis in **chapter 7**. In addition, the development of this association over time prior to T2D diagnosis was investigated. Positive associations between BCAA levels and development of T2D were found, irrespective of follow-up durations. These findings suggest the potential utility of BCAA as biomarkers, which reflect early changes in T2D. Detection of changes in BCAA levels years before the onset of physiological symptoms may be crucial for the early diagnosis and development of novel interventions.

(Relevance) What is the (potential contribution) of the results from this research to science, and, if applicable, to social sectors and social challenges?

Worldwide, 4.3 million deaths per year can be attributed to the consequences of T2D, making it the ninth leading cause of mortality (1). T2D is characterized by insulin resistance and metabolic dysfunction in several tissues including skeletal muscle, liver and adipose tissue resulting in hyperglycaemia (2), which can progress to severe complications and comorbidities. These affects patients' functional capacities, leading to significant morbidity and mortality (3). With the rising prevalence of T2D, the rising burden of diabetes forms a major public health problem with great impact on quality of life (4). Therefore, the social impact of T2D is enormous and emphasize the relevance of good treatment strategies. Current classic interventions mostly target one specific mechanism in a specific tissue to lower hyperglycaemia, but is not sufficient to slow down T2D-related comorbidities, that occur with the progression of the disease. This highlights the importance to developing effective treatment strategies working on multiple tissues since they all contribute to hyperglycaemia. In that way, T2D can be prevented or treated, which in turn improves the social burden caused by this disease.

Given the extraordinary healthcare costs to treat T2D and occurring comorbidities, T2D takes a significant part of the health budget around the world. With the increasing prevalence, this health budget is set to increase further (5). Again, this shows the importance of implementing more feasible strategies to prevent the development of T2D and related comorbidities. The knowledge in this thesis contribute considerably to the existing understanding of the pathology and treatment of T2D. This knowledge is important for further development of treatment in the end to delay the onset of diabetes-related comorbidities, thereby reducing health care costs. Even more, prevention of T2D will become increasingly important as this is a cost-effective way to reduce the major economic impact. Since BCAA levels are considered to be a risk factor of T2D, the development of novel potential BCAA-related biomarkers in diabetes can be exploited to intervene at an early stage in the development or even before the onset of T2D.

The research presented in this thesis shows the effects of a potential novel strategy targeting BCAA catabolism, which so far, has not been investigated in patients with T2D. At present, knowledge on BCAA catabolism in patients with insulin resistance and T2D and the health consequences of disturbed BCAA catabolism are limited. The results of the present thesis contribute to better understanding of BCAA catabolism and its importance for metabolic health in humans. Based on the results of this research, development of new treatment strategies can be found to prevent and improve T2D. Because boosting BCAA catabolism act upon several

mechanisms in multiple tissues to improve insulin sensitivity, this treatment is a proposing novel strategy to combat T2D. Taken together, the results obtained in this thesis are of great social and economic relevance since they could contribute to the development of new treatment options and treatment strategies, which are feasible and can be implemented in daily life, for people with prediabetes and T2D. Ultimately, this will contribute to better quality of life, less healthcare costs and relieving the pressure on the health care system.

(Target group) To whom are the research results interesting and/or relevant? And why?

The results of the studies described in this thesis add an extra piece to the complex puzzle of BCAA metabolism. This can be of great interest for other researchers in this field to design new studies to fill the current knowledge gap in human physiology regarding BCAA metabolism and insulin resistance. In the end, this will result in a better understanding of the aetiology of T2D and development of better treatment strategies. Next to this, future studies can extend the findings demonstrated in this thesis by exploring if BCAA catabolism can be stimulated by several approaches, including diet, exercise, medication or change in microbiota and the consequences on metabolic health in people with insulin resistance and T2D on the long-term. Therefore, knowledge from future studies based on our results could lead to the development of new drugs for the treatment of T2D, thereby reducing the risk of progressed diabetes-related complications or related diseases.

Next, the knowledge obtained in this thesis is beneficial for patients with T2D. If we understand how BCAA is compromised and can be modulated, we could develop strategies to boost BCAA catabolism in patients with T2D. Since BCAA catabolism is involved in multiple tissues, metabolic health can be targeted by multiple mechanisms, which might reduce their symptoms and improve the quality of life. In terms of prevention, the results of the studies described in this thesis might also be relevant for the increasing number of people at risk of T2D and classified as having prediabetes. If we can identify people with a compromised BCAA catabolism before the onset of T2D with specific markers, it is possible prevent the onset of the disease. To translate those results to a broader population, e.g., people with prediabetes, more research is needed.

Furthermore, health care professionals involved in treating patients with T2D, including general practitioners, endocrinologist en dieticians could use the findings in this thesis to understand the consequence of a compromised BCAA catabolism in the development of T2D. The results of this thesis support the development

of strategies to boost BCAA catabolism as potential novel treatment of diabetes. Additionally, lack of knowledge is a huge barrier to self-care for patients with T2D (6), and therefore, they can help to inform and aware patients about the aetiology of their disease and the advantage of using this new strategy to improve their disease and delay T2D-related symptoms.

(Activity) In what way can these target groups be involved in and informed about the research results, so that the knowledge gained can be used in the future?

The conducted research and obtained results in this thesis have been communicated to other researchers and health care professionals through oral presentations and posters at national and international conferences. Additionally, most of the results presented in this thesis are and other results will be published in the future as original scientific articles in international well-recognized peer-reviewed journals. The scientific articles are available online and are shared on websites and social media. In this way, the acquired knowledge in this thesis was assessable worldwide for researchers, health care professionals and other people interested.

Next to this, this thesis has strengthened the collaboration between our lab and other external labs. The lab of Prof. Z. Arany at the Perelman School of Medicine (Pennsylvania, US) provided us to use metabolomics to obtain more insight in our valuable plasma samples. The partnership with the research group at the University of Nottingham (UK), headed by Prof. P. Atherton, shared their expertise in several methodologies in cell culture studies, which has given a lot of new opportunities to our lab. Future metabolic analysis on plasma and human skeletal muscle samples already obtained from patients with T2D following interventions known to boost BCAA catabolism will be established by Lilly Corporate Centre (Indianapolis, US). This cooperation will contribute to a molecular understanding of the link between BCAA catabolism and insulin sensitivity and a further evaluation of a potential new treatment strategy to combat T2D. Together, these collaborations make it possible to exchange ideas and share knowledge, which will stimulate future research.

All study participants received a document including their individual and group study results in layman terms. To transfer the knowledge obtained in this thesis to the society, the results were also communicated at participant information events. In that way, it was possible to inform patient with T2D and people at risk for the development of T2D or related diseases.

REFERENCES

1. Khan MAB, Hashim MJ, King JK, Govender RD, Mustafa H, Al Kaabi J. Epidemiology of Type 2 Diabetes - Global Burden of Disease and Forecasted Trends. Journal of epidemiology and global health. 2020;10(1):107-11.
2. Daryabor G, Atashzar MR, Kabelitz D, Meri S, Kalantar K. The Effects of Type 2 Diabetes Mellitus on Organ Metabolism and the Immune System. Front Immunol. 2020;11:1582.
3. Ramtahal R, Khan C, Maharaj-Khan K, Nallamothu S, Hinds A, Dhanoo A, et al. Prevalence of self-reported sleep duration and sleep habits in type 2 diabetes patients in South Trinidad. J Epidemiol Glob Health. 2015;5(4 Suppl 1):S35-43.
4. Organization WH. Global Report on Diabetes. France; 2016.
5. Nowakowska M, Zghebi SS, Ashcroft DM, Buchan I, Chew-Graham C, Holt T, et al. The comorbidity burden of type 2 diabetes mellitus: patterns, clusters and predictions from a large English primary care cohort. BMC Med. 2019;17(1):145.
6. Bayliss EA, Steiner JF, Fernald DH, Crane LA, Main DS. Descriptions of barriers to self-care by persons with comorbid chronic diseases. Ann Fam Med. 2003;1(1):15-21.

SAMENVATTING

Het stijgend aantal mensen gediagnosticeerd met suikerziekte type 2 is wereldwijd een groot probleem en gaat gepaard met een grote gezondheidslast met aanzienlijke toenemende kosten in de gezondheidszorg. Een grote risicofactor van suikerziekte type 2 is een verminderde insulinegevoeligheid en gaat altijd vooraf aan de ontwikkeling van deze ziekte. Uiteindelijk leidt dit tot verhoogde suikerwaarden in het bloed, ook wel hyperglycemie genoemd. Verhoogde suikerwaarden hebben ernstige, schadelijke gevolgen en leiden tot ernstige complicaties, zoals hart- en vaatziekten, chronische nierziekten en zenuwschade. Daarom zijn strategieën die insulinegevoeligheid verbeteren als doel om de suikerwaarden te normaliseren van essentieel belang om de progressie van suikerziekte type 2, en de daarmee samenhangende complicaties, te voorkomen. Ondanks de huidige beschikbare medicatie en adviezen voor een gezonde leefstijl, blijft hyperglycemie moeilijk onder controle te houden bij patiënten met suikerziekte type 2, en dus is de ontwikkeling van nieuwe behandelingsstrategieën van cruciaal belang.

In de afgelopen 30 jaar is aangetoond dat de stofwisseling van de vertakte-keten aminozuren, leucine, isoleucine en valine, ook wel BCAA genoemd, een steeds grotere rol speelt bij de ontwikkeling van suikerziekte type 2. Er is aangetoond dat de BCAA-waarden verhoogd zijn in het bloed en in sommige organen, zoals de spier en het hart, bij mensen met obesitas en suikerziekte type 2. Bovendien is er een sterk verband tussen verhoogde BCAA-waarden in bloed en insulinegevoeligheid. Het is nog niet duidelijk wat de precieze onderliggende oorzaken zijn van de verhoogde BCAA-waarden in deze mensen, en waarom deze een verband hebben met een verminderde insulinegevoeligheid. Een verminderde werking van de BCAA-verbranding, d.w.z. een verminderde afbraak van BCAA, kan logischerwijze een van de onderliggende factoren zijn. Een belangrijk aanknopingspunt is dat meerdere studies bij knaagdieren hebben aangetoond dat het farmacologisch stimuleren van de BCAA-verbranding de insulinegevoeligheid en de suikerhuishouding verbetert. Deze en andere proefdierstudies, leveren het bewijs dat de BCAA-verbranding een belangrijke rol speelt bij suikerziekte type 2, en dat het stimuleren van BCAA-verbranding een potentiële strategie zou kunnen zijn bij de behandeling van suikerziekte type 2. Tot nu toe is de BCAA-stofwisseling nauwkeurig bestudeerd in knaagdiermodellen, maar kennis in mensen is vrij beperkt. In dit proefschrift staat de rol van de BCAA-verbranding in de pathogenese van obesitas en suikerziekte type 2-gerelateerde metabole stoornissen, en het stimuleren van BCAA-verbranding als nieuwe potentiële behandeling voor suikerziekte type 2, centraal.

In **hoofdstuk 2** heb ik middels een literatuurstudie meer inzicht gekregen in de mechanismen waarom BCAA-waarden verhoogd zijn in het bloed bij mensen met suikerziekte type 2 en de rol daarvan op insulinegevoeligheid. Verder onderzocht ik welke farmaceutische en leefstijlinterventies in staat zijn om de BCAA-waarden in het bloed te verlagen en wat de effecten daarvan zijn op de metabole gezondheid. Eén van de bevindingen in dit hoofdstuk waren dat het merendeel van de studies met knaagdieren aantoonden dat verhoogde BCAA-waarden in bloed het gevolg zouden kunnen zijn van een verstoorde BCAA-verbranding in verschillende weefsels. Bovendien bleek uit deze studies dat het stimuleren van de BCAA-verbranding een goede strategie is om de suikerhuishouding te verbeteren, maar deze bevindingen waren nog niet bevestigd in de mens, aangezien er weinig middelen beschikbaar zijn om dit concept te testen. De literatuur laat tevens zien dat de verhoogde BCAA-waarden in het bloed en organen, alsook de gerelateerde tussenafbraakproducten, de insuline signalering belemmeren. Dit zorgt uiteindelijk voor een verstoorde glucose opname uit het bloed. Het is daarom belangrijk om nieuwe interventies te onderzoeken die de BCAA-verbranding verhogen en/of de BCAA-waarden verlagen om na te gaan of dit een nieuwe potentiële strategie zou kunnen zijn bij de behandeling van suikerziekte type 2.

In **hoofdstuk 3** werden BCAA-waarden en diabetes-gerelateerde metabole verstoringen onderzocht bij patiënten met suikerziekte type 2, eerstegraads familieleden van patiënten met suikerziekte type 2 en deelnemers zonder suikerziekte type 2, allen met dezelfde BMI en leeftijd. In deze studie bevestigden wij de bevinding dat patiënten met suikerziekte type 2 hogere BCAA-waarden in het bloed hebben in vergelijking met mensen zonder suikerziekte type 2. De waardes in de eerstegraads familieleden van patiënten met suikerziekte type 2 waren niet verschillend van de mensen zonder suikerziekte. Verder toonden wij voor het eerst aan dat de BCAA-waarden in het bloed een significant verband houden met diabetes-gerelateerde verstoringen, zoals *ex vivo* mitochondriële functie en *in vivo* metabole flexibiliteit; dit zijn respectievelijk de energieproductie door substraatverbranding op niveau van de spier (*ex vivo*) en het hele lichaam (*in vivo*). Vervolgens werd *in vivo* leucine-verbranding, als maat van BCAA-verbranding, gemeten en was deze significant lager bij patiënten met suikerziekte type 2 in vergelijking met mensen zonder suikerziekte. Deze resultaten suggereren dat een lage mitochondriële BCAA-verbranding kan bijdragen aan verhoogde BCAA-waarden en invloed kan hebben op de metabole gezondheid in mensen met suikerziekte type 2. De resultaten bevestigen de relatie tussen de BCAA-verbranding, insulinegevoeligheid en metabole gezondheid, en laten tevens zien dat verhoogde BCAA-waarden samengaan met belangrijke metabole verstoringen in patiënten met suikerziekte type 2.

De relatie tussen verhoogde BCAA-waarden in bloed en levervet werd onderzocht in **hoofdstuk 4**. In deze studie werd gebruik gemaakt van het Nederlandse Epidemiologie van Obesitas (NEO) cohort en werd aangetoond dat de BCAA-waarden een positief verband hadden met de hoeveelheid levervet. De cohortdata toonde ook aan dat mensen die meer lichamelijk actief zijn, lagere BCAA-waarden in het bloed hadden, hoewel de mate van lichamelijke activiteit geen invloed had op het verband tussen de bloed BCAA-waarden en hoeveelheid levervet. In **hoofdstuk 4** werd tevens onderzocht of de afname van levervet na een 12 weken trainingsprogramma relateerde met een verlaging van BCAA-waarden in het bloed. Ik concludeerde dat de gecombineerde weerstands- en duurtraining effectief was in het verlagen van de hoeveelheid levervet bij mensen met een niet-alcoholische leververvetting (met of zonder suikerziekte type 2), en in deelnemers zonder een niet-alcoholische leververvetting (met of zonder suikerziekte type 2). Deze daling in de hoeveelheid levervet ging niet gepaard met een daling in de bloed BCAA-waarden. Deze resultaten wijzen erop dat lichamelijke activiteit en training een verschillend effect hebben op de BCAA-waarden in het bloed. De resultaten tonen ook aan dat BCAA geen rol spelen bij het mediëren van de gunstige metabole effecten op de hoeveelheid levervet na een training. Het mechanisme en effect van fysieke activiteit op de BCAA-waarden in bloed is echter onbekend en moet verder onderzocht worden.

In **hoofdstuk 5** heb ik de bevindingen van **hoofdstuk 2** vertaalt naar de mens. In dit hoofdstuk heb ik onderzocht of het stimuleren van de BCAA-verbranding een nieuwe potentiële behandelingsstrategie kan zijn voor patiënten met suikerziekte type 2. In deze studie werd fenylboterzuur gebruikt als middel om de BCAA-verbranding te stimuleren met als doel de BCAA-waarden in het bloed te verlagen. Fenylboterzuur, een medicatie bedoeld voor mensen met een nierfunctie stoornis, werd voor de eerste keer voorgeschreven bij patiënten met suikerziekte type 2. Ik heb laten zien dat 2 weken behandeling met fenylboterzuur de BCAA-waarden in het bloed effectief verlaagde bij zestien patiënten met suikerziekte type 2. Deze verlaging ging gepaard met een 27% verbetering van de insulinegevoeligheid, voornamelijk toe te schrijven aan een verhoogde insuline-gestimuleerde glucose verbranding. Bovendien verhoogde de behandeling met fenylboterzuur de *ex vivo* mitochondriële functie in de spier met 10%. De behandeling met fenylboterzuur liet geen effecten zien in de lever (lever insulinegevoeligheid, levervet gehalte en -samenstelling), wat suggereert dat de lever minder gevoelig is voor deze behandeling. Samen tonen deze resultaten aan dat de effecten van de behandeling met fenylboterzuur bij patiënten met suikerziekte type 2 voor een groot deel plaatsvinden in perifere weefsels, voornamelijk spieren. Dit komt overeen met de waarneming dat de skeletspier bij de mens de grootste capaciteit heeft voor de BCAA-verbranding.

Uit **hoofdstuk 5** kunnen we concluderen dat het farmacologisch stimuleren van BCAA-verbranding de BCAA-waarden in het bloed bij patiënten met suikerziekte type 2 verlaagt, hetgeen gunstige resultaten oplevert voor de suikerhuishouding van deze patiënten.

De gunstige effecten van fenylboterzuur op de suikerhuishouding uit **hoofdstuk 5**, heb ik in **hoofdstuk 6** verder mechanistisch onderzocht. In dit hoofdstuk heb ik onderzocht of het blootstellen van skeletspiercellen aan verhoogde BCAA-waarden zorgt voor een verstoorde insuline-gestimuleerde glucose opname, als reflectie van een verminderde insulinegevoeligheid, via een remming van de insuline signaleringsroute. De resultaten van het onderzoek toonden aan dat het toevoegen van BCAA aan spiercellen van muizen de insuline signaleringsroute belemmerde via stimulatie van de zogenaamde 'mTOR/S6K pathway', hoewel er geen veranderingen werden gemeten in de insuline-gestimuleerde glucose-opname. Het model voor het meten van insuline-gestimuleerde glucose-opname wordt nu verder geoptimaliseerd. **Hoofdstuk 6** bevestigt een modulerende rol van de BCAA op de insuline signalering. Er moet echter meer onderzoek uitgevoerd worden in (spier)cellen van mensen om beter inzicht te krijgen in de link tussen verhoogde BCAA-waarde, en de remming van de insuline-gestimuleerde glucose opname in de spier.

De rol van verschillende BCAA-waarden en de ontwikkeling van suikerziekte type 2 werd bestudeerd in **hoofdstuk 7**, in de vorm van een systematische review en meta-analyse. Hier wilden wij het verband tussen de BCAA-waarden en de pathogenese van suikerziekte type 2 onderzoeken aan de hand van gegevens uit case-control studies van mensen met overgewicht. Bovendien werd gekeken naar de ontwikkeling van dit verband in de tijd voorafgaand aan de diagnose suikerziekte type 2. De resultaten in dit hoofdstuk laten consistente, positieve verbanden zien tussen de individuele BCAA-waarden en de uiteindelijke ontwikkeling van suikerziekte type 2, ongeacht de follow-up duur. Deze positieve associaties tussen BCAA en incidenteel suikerziekte type 2 suggereren dat verhoogde BCAA-waarden mogelijk het ontstaan van suikerziekte type 2 kunnen voorspellen jaren voordat de klinische symptomen van suikerziekte type 2 tot uiting komen.

Van de studies beschreven in dit proefschrift kunnen we concluderen dat de verhoogde BCAA-waarden een verband houden met insulinegevoeligheid en andere suikerziekte type 2-gerelateerde metabole verstoringen. De resultaten tonen aan dat patiënten met suikerziekte type 2 een verstoorde BCAA-verbranding hebben, wat een mogelijke verklaring zou zijn voor de verhoogde BCAA-waarden in deze patiënten. Ook laat dit proefschrift zien dat het stimuleren van de

BCAA-verbranding een nieuwe potentiële strategie zou kunnen zijn om suikerziekte type 2 te behandelen. Dit proefschrift vormt de basis voor toekomstig onderzoek waarin de effecten van het moduleren van BCAA metabolisme op de metabole gezondheid verder worden onderzocht.

SUMMARY

The trend for increasing type 2 diabetes (T2D) has become a major problem worldwide as T2D comes with a profound health burden and possesses significant impact on health expenses. A hallmark of T2D is insulin resistance, a decreased tissue response to insulin stimulations, with disturbed glucose homeostasis as a consequence. Eventually, this will result in elevated glucose levels circulating in the bloodstream, also called hyperglycaemia. Chronic hyperglycaemia has severe, detrimental effects and lead to serious complications, like cardiovascular dysfunction, chronic kidney disease and nerve damage. Therefore, strategies to improve insulin resistance to normalize hyperglycaemia are key to prevent the progression of T2D and its related complications. Despite the availability of current lifestyle and pharmacological therapies, hyperglycaemia remains difficult to manage in patients with T2D, and thus, development of novel treatment strategies is crucial.

Over the last 30 years, branched-chain amino acid (BCAA; leucine, isoleucine and valine) metabolism has been shown to play an emerging role in the development of insulin resistance and T2D. It has been shown that BCAA levels are considerably elevated in plasma and tissues in people with obesity and T2D. Furthermore, elevated BCAA levels in plasma strongly associates with insulin resistance in people with obesity and T2D. Although it is still unknow why BCAA levels are elevated and why BCAA levels correlate positively with insulin resistance, it is clear that dysfunctional BCAA catabolism may be one of the underlying factors. Interestingly, multiple rodent studies have shown that pharmacologically boosting BCAA oxidation improved insulin sensitivity and glucose homeostasis. These studies provide evidence that BCAA catabolism plays an important role in T2D, and that boosting BCAA catabolism could be a potential strategy in the treatment of T2D. Thus far, BCAA metabolism has been rigorously studied in rodent models. Information in humans, however, is rather limited. In this thesis, the role of BCAA catabolism in the pathogenesis of obesity and T2D-related metabolic disturbances and the potential of boosting BCAA catabolism in the treatment of T2D was explored.

In **chapter 2**, we aimed to provide insight into the mechanisms behind elevated plasma BCAA levels in people with insulin resistance and T2D and its role in insulin resistance. Furthermore, we reviewed pharmaceutical and alternative lifestyle intervention strategies in order to lower plasma BCAA levels and its effects on metabolic health. Main findings described in this thesis were that studies in rodents showed that increased levels of BCAA in plasma might be

the results of a dysregulated BCAA catabolism in several tissues. Although only limited knowledge derives from human studies, large differences were observed compared to the results found in rodent tissues. In addition, several rodent studies showed the strength of boosting BCAA catabolism as potential strategy to improve glucose homeostasis, but so far, these findings have not yet been confirmed in humans since a limited number of tools is available to test this concept. The main conclusions of **chapter 2** were that insulin resistance could occur via dysregulated BCAA catabolism or BCAA levels *per se* acting a signaling molecules hampering the insulin signaling pathways. Therefore, exploring intervention strategies to increase BCAA catabolism and/or lower BCAA levels is important to investigate whether this could be a new potential strategy in the treatment of T2D.

In **chapter 3**, BCAA levels and diabetes-related metabolic parameters were investigated in fifteen patients with T2D, thirteen first-degree relatives of patients with T2D (FDR) and seventeen BMI- and age-matched control participants. In the present study, we confirmed the finding of higher plasma BCAA levels in patients with T2D compared to control participants and showed for the first time that plasma BCAA levels significantly correlated with diabetes-related disturbances, like *ex vivo* mitochondrial oxidative capacity and metabolic flexibility. Subsequently, *in vivo* leucine oxidation was measured as reflection of BCAA oxidation and was significantly lower in patients with T2D compared to control participants. Together, these results suggest that a low mitochondrial oxidation of BCAA may contribute to higher plasma BCAA levels and affect metabolic health in people with T2D.

The relationship between elevated BCAA levels and diabetes-related disturbances was further explored in **chapter 4**. In this study, it was shown that BCAA levels were positively associated with intrahepatic lipid (IHL) content, based on data from 1983 individuals from the Netherlands Epidemiology of Obesity (NEO) cohort and a 12-week exercise intervention program, performed in seven patients with T2D, seven individuals with non-alcoholic fatty liver (NAFL) and seven BMI-matched control participants. Together, the results described within this thesis underline that BCAA catabolism relates to insulin resistance and energy metabolism, and that elevated BCAA levels associate with key metabolic disturbances in patients with T2D.

We investigated the effect of boosting BCAA oxidation, by use of exercise intervention (**chapter 4**) and one pharmaceutical strategy (**chapter 5**), on metabolic health in humans. Physical activity and exercise training is well known to improve insulin sensitivity and reduce liver fat content, however, it has never

been investigated whether this effect relates to a reduction in plasma BCAA levels. Based on the strong association between plasma BCAA and liver fat content and considering that physical activity level or exercise training may affects liver fat content, we aimed to investigate whether physical activity and exercise training was able to lower plasma BCAA levels along with alterations in liver fat (**chapter 4**). We found that physically active people had slightly, but significant lower plasma BCAA levels, although the level of physical activity did not alter the positive association between plasma BCAA levels and liver fat content. In addition, we found that a conventional exercise program, including both resistance and endurance training was effective in lowering liver fat content in people with NAFL and control participants. This, however, this drop in liver fat did not coincide with lower plasma BCAA levels. These results indicate that chronic physical activity and exercise training differently affect plasma BCAA levels. Results also show that BCAA do not play a role in mediating the beneficial metabolic effects of exercise training on liver fat content. The mechanism of physical activity leading to lower plasma BCAA levels is, however, unknown and needs further research.

In **chapter 5**, the therapeutic value of boosting BCAA catabolism in type 2 diabetes was considered. We investigated the potential effect of sodium-phenylbutyrate ((NaPB), an inhibitor of the BCKD kinase, and stimulant of BCAA oxidation) on metabolic health in patients with T2D as novel approach. Sixteen participants were administered daily with 4.8 g/m^2/day NaPB or placebo for 2 weeks, whereafter they underwent a comprehensive metabolic evaluation. NaPB was for the first time prescribed 'off-label' to patients with T2D to stimulate the oxidation of BCAA aiming to lower the levels in plasma. In the present study, we showed that 2-weeks treatment of NaPB effectively reduced plasma BCAA levels. This reduction was accompanied by a 27% improved peripheral insulin sensitivity, mainly exerted by enhanced insulin-stimulated glucose oxidation. In addition, NaPB treatment increased *ex vivo* mitochondrial oxidative capacity upon a glycolytic-like substrate in muscle by 10%. We did not find major effects of NaPB treatment on the level of hepatic tissue (hepatic insulin sensitivity, liver fat content and composition), which suggests that insulin resistance in the liver is less responsive to NaPB treatment. Together, these results show that effects of NaPB treatment in patients with T2D for a great part take place in peripheral tissues, mainly muscle, which matches with the observation that skeletal muscle in humans has the highest capacity for BCAA catabolism. From **chapter 5,** we can conclude that pharmacologically boosting BCAA oxidation is able to lower BCAA plasma levels in patients with T2D resulting in beneficial outcomes on patients' glucose metabolism.

The molecular link of BCAA levels with insulin resistance was studied in **chapter 6**. Here, we aimed to investigate whether exposing skeletal muscle cells to elevated BCAA levels could underlie disturbed insulin-stimulated glucose uptake, reflecting insulin resistance, via an inhibition of the insulin signaling pathway. The results of the study showed that supplementation of BCAA to mice skeletal muscle cells impeded the insulin signaling pathway via stimulation of mTORC1 and S6K, although no effects were found on insulin-stimulated glucose uptake. This chapter supports the use of targeting BCAA levels in condition of insulin resistance. Future work in human cells will be required to further increase the insight about the underlying mechanisms of elevated BCAA levels in insulin resistant states, such as T2D.

The role of systemic BCAA levels and T2D development was studied in **chapter 7,** a systematic review and meta-analysis. Here, we aimed to examine the association between BCAA levels and the pathogenesis of T2D through case-control study data of people with overweight, by using systematic search. Furthermore, development of this association over time prior to T2D diagnosis was assessed. The results described in this chapter revealed consistent positive associations between each BCAA level and the eventual development of T2D, irrespective of follow-up period duration. These positive associations between BCAA and T2D at a variable period prior to incident T2D suggest that elevated BCAA levels could potentially predict the onset of T2D years before the clinical T2D symptoms manifest.

In conclusion, the studies outlined in this thesis demonstrate that elevated BCAA levels are linked to insulin resistance and other T2D-related metabolic disturbances. However, the underlying mechanisms of how BCAA links to insulin resistance still require further study. Our data shows that patients with T2D feature compromised BCAA catabolism, which may explain the elevation in BCAA levels measured in these patients. We furthermore, provided evidence that boosting BCAA catabolism could be a new potential strategy to treat T2D. Further evaluations of this potential treatment strategy are needed aiming to prevent the progression towards T2D.

DANKWOORD

Ik wil graag mijn oprechte dank uitspreken aan ieder van jullie die een onschatbare bijdrage heeft geleverd aan mijn promotietraject en het afwerken van mijn proefschrift. Jullie hebben dit avontuur tot een onvergetelijke reis gemaakt, en het is fijn om te kunnen terugkijken op alle herinneringen en steun die ik van jullie heb ontvangen.

Graag wil ik beginnen met **Esther**, **Mathijs** en **Patrick**, mijn promotieteam. Jullie hebben mijn reis mogelijk gemaakt. Esther, na lang wachten kreeg ik van jou, in juli 2018, het mailtje met het goede nieuws dat ik in augustus van start kon gaan als PhD in de DMRG-groep. Je had snel door dat ik als 'Belg' wat meer assertiviteit kon gebruiken tussen al die Nederlandse collega's en stuurde me daarom al meteen op assertiviteitscursus. Je motiveerde me niet alleen om te groeien als goede onderzoekster, maar ook als persoon. Jouw vertrouwen en geloof in mij was een grote stimulans om mezelf voortdurend te verbeteren en grenzen te verleggen, zoals mijn trip naar de UK voor 6 maanden. Ook was je een grote steun bij het oefenen van belangrijke presentaties waarin tranen van frustratie en emotie bij te pas kwamen. Matthijs en Patrick, jullie toewijding is een grote bijdrage geweest gedurende mijn promotietraject. Jullie kritische kijk op data en wetenschappelijke inzichten hebben een onschatbare waarde gehad en heeft me geholpen bij het ontwikkelijken van mijn eigen kritische denkvermogen. Ook aan de andere leden van de DMRG-board **Vera**, **Joris**, **Mijke** en **Anne**, ook jullie bedankt voor de begeleiding en ondersteuning gedurende deze reis.

I would like to thank **Prof. Dr. B.E. de Galan**, **Prof. Dr. S.W.M. Olde Damink**, **Prof. Dr. C. B. Newgard**, **Dr. R. Meex**, and **Prof. Dr. C. Matthys**, the reading committee members. Thank you for taking the time to read and review my thesis and to be present during my dissertation.

Bedankt, lieve **Désirée** en **Yolanda**, voor de hartelijke ontvangst op mijn allereerste werkdag en voor jullie steun gedurende mijn PhD tijd. Vanaf het allereerste moment voelde ik me welkom en gewaardeerd. Ik ben heel erg dankbaar voor jullie betrokkenheid en de gezellige babbeltjes.

Anna, samen zijn we onze reis als PhD-studenten begonnen. Al snel werden we vaste partners wanneer er een kamer gedeeld moest worden tijdens de leuke congressen. Wanneer ik mijn presentatie wou oefenen was jij er altijd bij (ook al was dit om 2 uur 's nachts na een paar rode wijntjes). Bij het afronden van ons boekje, hebben we ook veel steun aan elkaar gehad, want er waren momenten dat het schrijven niet wilde vlotten (vooral die ene vrijdagmiddag). Wat deden we dan?

We lieten de woorden voor wat ze waren en besloten om te ontspannen met een heerlijke zelfgemaakte Limoncello Spritz. Bedankt Anna, om samen de PhD-reis te maken met mooie en leuke herinneringen.

Manon and **Edmundo**, I want to thank you for your invaluable assistance throughout the NaPB study. Having both of you on board of the BCAA train was an absolute pleasure. Manon, your help and excellent preparation ensured smooth sailing of the test days. Edmundo (or Snelle Eddy), you brought good energy to the team with your touch of humour and your biopsy techniques. I (and the participants) will never forget the words 'een klein priekje, druuk druuk'.

Esther K., ik wil jou van harte bedanken voor je inzet bij het verwerken van talloze biopten en plasma monsters voor onze analyse, en natuurlijk voor de mooie oxygraafmetingen. **Johanna**, bedankt voor het nemen van de tijd om me de tips and tricks van het oxygraven bij te brengen. Jouw expertise en begeleiding zijn van onschatbare waarde geweest, en ik ben dankbaar voor je geduldige instructies. **Gert**, jouw hulp bij de celkweek is werkelijk onmisbaar. Jouw bereidheid om me de fijne kneepjes van het kweken te delen heeft me ongetwijfeld doen groeien als onervaren celkweker.
Naast het onderzoek ben ik ook dankbaar voor de leuke gesprekken. Jullie toewijding, expertise en bereidheid om te helpen, maken een groot verschil in deze onderzoeksgroep en wordt erg gewaardeerd.

Yvonne, een herinnering die ik nooit zal vergeten gaat over mijn allereerste ervaring met clampen. Zelfs nog voordat ik mijn officiële dag bij DMRG begon, kreeg ik de kans om met jou en Yvo mee te lopen tijdens een clamp bij een atleet. Laat me je vertellen, het was behoorlijk overweldigend voor me. Later zou jij degene zijn die me geduldig de kunst van het clampen zou aanleren. Ik heb ontzettend veel van jou geleerd, van het tot in de puntjes voorbereiden van testdagen, tot het onder de knie krijgen van de clamptechniek. **Yvo**, ook jij bedankt voor het afnemen van biopten en dat ik altijd bij je terecht kon met 'vreemde' medische vragen (zoals een fout geprikt infuus of een lage glucosewaarde tijdens te clamp). **Jeremy**, **Kay** en **Yvonne**, ik ben zo blij om een kantoor te delen met jullie. Deze plek is echt een centrum van samenwerking, lachmomentjes en veel eten geworden. Ook een speciaal bedankje aan **Evi.** Ik ben oprecht dankbaar voor jullie positieve impact en steun (zeker tijdens mijn klaag en zaagmomentjes). Het is juist deze warme sfeer die ervoor zorgt dat ik elke dag met plezier naar mijn werk kom.

Axel en **Kim**, heel erg bedankt voor jullie hulp bij de MR-scans. Jullie hebben niet altijd de scans zorgvuldig uitgevoerd, maar ook de sfeer altijd gezellig

gehouden. **Julian**, **Pandi** en **Vera**, dank voor jullie waardevolle ondersteuning bij de MR-analyses. Jullie hebben echt het buitengewone talent om zelfs de meeste complexe methoden begrijpelijk uit te leggen. Dit heeft ervoor gezorgd dat ik een beetje inzicht kreeg in de moeilijke MR-wereld. Ik ben er dankbaar voor dat ik met al mijn vragen - hoe dom ze ook leken- bij jullie terecht kon.

Vera de Wit, ik vond het heerlijk om jouw PET-clampbuddy te zijn tijdens de FIT-studie. Samen vormden we een dynamisch duo dat goed op elkaar aansloot. Jouw kalme 'het komt wel goed'-mindset vulde perfect mijn neiging tot stress en zorgen aan. Ik heb ontzettend veel van je geleerd zoals jouw altijd aanwezige positiviteit en gezellige houding tegenover proefpersonen (kan ook niet anders met dat gezellig Limburgs). Jouw vermogen om vrolijk te blijven in elke situatie (zelfs tijdens je zwangerschap bij een biopt om 6u in de ochtend) is werkelijk opmerkelijk.

Tineke, ik wil je van harte bedanken voor jouw medisch advies tijdens de studies. Ik waardeer ook heel erg de positieve en vrolijke sfeer die je met je meebrengt tijdens de PET-clamps, waardoor de erg vroege ochtenden toch iets minder zwaar worden.

Nynke, graag wil ik terugblikken op mijn eerste dag in de DMRG groep. Ik herinner me nog goed hoe ik me meteen welkom voelde toen ik bij jou op kantoor mocht zitten. Het was fijn om naast iemand te zitten die al ver stond in haar PhD traject en ik kon daarom ook altijd op je rekenen voor advies en een luisterend oor. Bedankt voor de steun en gezelligheid die je hebt toegevoegd aan mijn begin als PhD.

Lieve **Lotte**, het was een waar genoegen om mijn dagen naast een mede-Belg door te brengen en in die eerste week voelde ik me meteen op *mijn gemak*. Al snel ontdekten we nog andere overeenkomsten tussen ons, van onze gedeelde achtergrond aan de Universiteit Hasselt tot onze gezamenlijke liefde voor Chiro en Aperol. Maar we deelden niet alleen onze gelijkenissen, maar ook de grappige situaties waarin we belandden: van verloren sleutels en vergeten pasjes tot zelfs vastzitten in het MRUM. We hebben elkaar vaak uit de nood moeten helpen. De late avonden waren niet enkel productief door onze Belgische efficiëntie, maar waren ook gevuld met gezelligheid en gelach. Lotte, bedankt dat je deel hebt uitgemaakt van mijn reis hier, en ik ben blij dat we elkaar blijven zien tijdens onze loopjes met de Trail Tantes. Zo leuk dat je mijn paranimf wilt zijn!

Anne, **Carlijn** en **Lotte**, oftewel de 'Trail Tantes'. Jullie staan altijd klaar met een luisterend oor en waardevol advies tijdens onze wekelijkse hardloopsessies na het werk en zaterdagochtend avonturen, die ondertussen onmisbaar voor mij

zijn geworden. Ik ben oprecht dankbaar dat we dit blijven voortzetten, ook al kan het soms een uitdaging zijn om met zijn vieren af te spreken. Fijn dat ik deel mag uitmaken van deze leuke, geweldige groep.

Kay, ik kijk met veel plezier terug op de leuke en grappige momenten die we hebben beleefd en hopelijk nog mogen gaan beleven. Van de gezellige avondjes uit (met als gevolg hoofdpijn de volgende ochtend), de leuke congressen tot ontspannen koffiekletsen op kantoor. En laten we zeker niet je inzet om Vlaams te leren vergeten. Ook bedankt voor het organiseren van de BBQ-feestjes. Ik vind het erg fijn om je kamergenoot te zijn en hoop oprecht dit nog lang te mogen zijn (er is immers nog veel werk aan je Vlaams 😉). *Mercikes dat gij mijn paranimf wilt zijn*.

Ook wil ik natuurlijk andere (ex-)collega's van DMRG bedanken (**Evelyn**, **Jakob**, **Jeroen**, **Pandi**, **Emmani**, **Rodrigo**, **Marlies**, **Elena**, **Stephanie**, **Bas**, **Ciaran**, **Daniel**, **Frederieke**, **Maaike**, **Sten**, **Charlotte**, **Pip, Marit**, **Evi**, **Soraya**, **Dzhansel**, **Frieder**, **Ivo**, **Florian**, **Elnaz**). Jullie inzet, hulp en enthousiasme hebben een aanzienlijke bijdrage geleverd aan dit boekje. Jullie gezelligheid zorgt ervoor dat werken altijd plezierig is en dat waardeer ik enorm.

Aan alle leden van **VBW** en **HB**, dank je wel voor de hulp en samenwerking van ons onderzoek. Daarnaast wil ik jullie ook bedanken voor de gezellige uitjes en het jaarlijkse carnaval, die niet alleen ons teamgevoel versterken maar ook voor memorabele momenten zorgen.

Lenore, begin september 2017 ben ik samen met Ines, mijn masterstage begonnen bij de ENHANCe studie. Toen deze studie (EINDELIJK) groen licht kreeg, mochten Ines en ik al snel meedraaien met de testdagen. En dat vond ik erg plezant. Ik ben je enorm dankbaar voor de begeleiding die je ons hebt geboden en voor het duwtje in de rug dat me motiveerde om te solliciteren voor de PhD positie, die ik net heb afgerond. Dankzij jou begon ik te geloven dat ik de capaciteiten had, en dat vertrouwen heeft me geholpen. **Ines**, jouw gezelschap tijdens de testdagen was eveneens onvergetelijk. Van het rekruteren van deelnemers tijdens een dans-middag tot het wachten bij de DEXA-scan, hebben we samen gelachen en geleerd. Ook jij hebt je weg gevonden als PhD binnen de Universiteit Maastricht en ik wens je veel succes toe.

Aan alle **proefpersonen** die hebben deelgenomen aan mijn onderzoek, jullie enthousiasme en inzet hebben dit werk tot een succes gemaakt. Ik ben dankbaar voor jullie deelname en inspirerende gesprekken. Zonder jullie is ons onderzoek niet mogelijk. Ook mijn oprechte dank aan de receptie van UNS 50. Altijd worden

de deelnemers warm ontvangen. **Hans**, dank je wel voor je hulp met het vinden van proefpersonen en verloren chauffeurs van Albert Heijn.

Also many thanks to the people of the University of Nottingham. **Phil Atherton**, **Ken Smith,** and **Dan Wilkinson**, I am genuinely grateful for the opportunity to visit your research group in Derby. Your guidance and support have been invaluable in enhancing my expertise in cell culture and deepening my understanding of mass spectrometry principles. **Nima**, **Hannah**, **Joe**, **Debbie,** and **Paula**, thank you for guiding me through the lab. With limited lab experience, it was challenging at the beginning, but thanks to all of you, I've learned a lot and managed to finish the experiments. **Eleanor**, **Celia**, **Shelby**, **Izzy**, **Tom**, **Jake**, **Seun**, **Weiland**, **Isabelle**, **Nishadi**, **Imran**, **Ash** and **Abdulmajeed**. thank you for making me feel welcome in Derby, and for the memorable moments shared during karaoke nights, pub drinks, fish and chips, and the festive Christmas party. As you journey towards the completion of your PhD, I wish you all the best of luck.

Ik wil mijn **allerliefste vrienden en vriendinnen** uit het prachtige dorp Vlijtingen (en omstreken) van harte bedanken. Dank jullie wel voor de geweldige momenten, de leuke (verrassings)feestjes, brunches, festivals, de onvergetelijke citytripjes en zo veel meer. We kennen elkaar al sinds onze jonge jaren bij Chiro Vlijtingen en hebben samen veel meegemaakt. Het doet me zo goed te zien dat onze vriendschap nog steeds even sterk is. Ik ben ontzettend blij om onderdeel te zijn van zo een hechte vriendengroep. Ik zie jullie graag!

Lieve **mama** en **papa**, **oma** en **make**, ik wil jullie bedanken voor de onschatbare steun die jullie me hebben geboden. Zonder jullie onvoorwaardelijke liefde zou ik niet de persoon zijn die ik vandaag ben. Jullie hebben me altijd gesteund, ongeacht de uitdagingen die ik ben tegengekomen. Julie warmte, zorgzaamheid en betrokkenheid hebben me geholpen om te groeien. En Lotte, het is verbazingwekkend hoe onze band is gegroeid van ruziënde kinderen tot de sterke band die we nu delen. Ik wil je laten weten hoe diep ik dit waardeer.

En, last but not least, **Roel**. Ik geloof dat ik jou nog het hardste moet bedanken. Vaak ben jij de eerste die mijn frustraties ontvangt na een moeilijke testdag of wanneer het schrijven niet lukt. Bij drukke en stressvolle periodes is jouw vermogen om alles te relativeren en rust te brengen in de chaos een zegen voor mij. Ik waardeer enorm dat je altijd voor me klaarstaat. Ik hou van jou en hoop dat we nog veel leuke reisjes met de *camionet* (of het busje voor de collega's) mogen maken.

ABOUT THE AUTHOR

Froukje Vanweert was born on February 2nd 1994 in Bilzen, Belgium. She started her bachelor Biomedical Sciences at Hasselt University in 2012. During her bachelor's thesis, she worked on the detection of autoantibodies in cerebrospinal fluid in patients with multiple sclerosis.

After completing her Bachelor's degree in 2016, she continued her education with the master Biomedical Sciences at the Katholieke Universiteit Leuven. She performed her master's thesis at the department of Chronic Diseases, Metabolism and Ageing (CHROMETA), the division of Gerontology and Geriatrics investigating the effect of a combined exercise and nutritional intervention in (pre-)sarcopenic elderly. In 2018, Froukje graduated with her master's degree in Biomedical Sciences with the specialization Clinical Biomedical Sciences.

Subsequently, Froukje started her PhD at Maastricht University at the department of Nutrition and Movement Sciences under the supervision of dr. Esther Phielix, prof. dr. Matthijs Hesselink and prof. dr. Patrick Schrauwen. The research conducted during this period, as described in this PhD thesis, was focused on branched-chain amino acid metabolism in insulin resistance and type 2 diabetes. She will continue her research as postdoctoral researcher.

LIST OF PUBLICATIONS

Vanweert F., de Ligt M., Hoeks J., Hesselink M.K.C., Schrauwen P., Phielix E. Elevated plasma branched-chain amino acid levels correlate with type 2 diabetes-related metabolic disturbances. J Clin Endocrinol Metab. 2021 Mar 25;106(4):e1827-e1836. doi: 10.1210/clinem/dgaa751

Vanweert F., Boone S.C., Brouwers B., Mook-Kanamori D.O., de Mutsert R., Rosendaal F.R., Lamb H.J., Schrauwen-Hinderling V.B., Schrauwen P., Hesselink M.K.C., Phielix E. The effect of physical activity level and exercise training on the association between plasma branched-chain amino acids and intrahepatic lipid content in participants with obesity. Int J Obes (Lond). 2021
Jul;45(7):1510-1520. doi: 10.1038/s41366-021-00815-4

Vanweert F., Neinast M., Tapia, E.E., Weijer van de T., Hoeks J., Schrauwen-Hinderling V.B., Blair M.C., Bornstein M.R., Hesselink M.K.C., Schrauwen P., Arany Z., Phielix E. A randomized placebo-controlled clinical trial for pharmacological activation of BCAA catabolism in patients with type 2 diabetes. Nat Commun 13, 3508 (2022). doi: 10.1038/s41467-022-31249-9

Vanweert F., Schrauwen P., Phielix E. Role of branched-chain amino acid metabolism in the pathogenesis of obesity and type 2 diabetes-related metabolic disturbances BCAA metabolism in type 2 diabetes. Nutr Diabetes. 2022 Aug 5;12(1):35. doi: 10.1038/s41387- 022-00213-3

Ramzan I., Ardavani A., **Vanweert F.**, Mellet A., Atherton P.J., Idris I. The association between circulating branched chain amino acids and the temporal risk of developing type 2 diabetes Mellius: a systematic review & meta-analysis. Nutrients. 2022 Oct 20;14(20):4411. doi: 10.3390/nu14204411.

Beek van S.M.M., Bruls Y.M.H., **Vanweert F**., Fealy C.E., Connell N.J., Schaart G., Moonen-Kornips E., Jörgensen J.A., Vaz F.M., Smeets E.T.H.C., Joris P.J., Gemmink A., Houtkooper R.H., Hesselink M.K.C., Bengtsson T. Havekes B., Schrauwen P., Hoeks J.. Effect of β2-agonist treatment on insulin-stimulated peripheral glucose disposal in healthy men in a randomised placebo-controlled trial. Nat Commun 14, 173 (2023). doi.org/10.1038/s41467-023-35798-5

de Wit-Verheggen V.H.W., **Vanweert F.**, Raiko J., Liénard V., Schaart G., Gemmink A., Nascimento E.B.M., Hesselink M.K.C., Wildberger J.E., Wierts R., Joris P.J., Haas J., Montaigne D., Staels B., Phielix E., Schrauwen P., Schrauwen-Hinderling V.B., van de Weijer T. The tissue-specific metabolic effects of the PPARα agonist ciprofibrate in insulin-resistant male individuals: a double-blind, randomized, placebo-controlled crossover study. Obesity (Silver Spring). 2023 Sep 5. doi: 10.1002/oby.23874.