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Branched-chain amino acids and trimethylamine N-oxide as biomarkers of cardiometabolic outcomes

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José Luis Flores Guerrero

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Branched-Chain Amino Acids and Trimethylamine N-oxide as Biomarkers of Cardiometabolic Outcomes

PhD thesis

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and in accordance with
the decision by the College of Deans.

This thesis will be defended in public on

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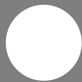

Chapter 1

General introduction and aims of this thesis

Introduction

Non-communicable diseases represented the leading cause of death worldwide over the last decades [1]. Even the recent global emergency caused by the severe acute respiratory syndrome coronavirus 2, has been catalogued as a syndemic, given the fact that people with hypertension, obesity, diabetes, and cardiovascular diseases are those with higher burden disease; being several biological and social interactions responsible of such an increased risk [2]. According to the last report of the Global Burden of Diseases, Injuries, and Risk Factors Study, the total number of deaths attributed to this group of diseases (41 million people each year) signifies 71% of the global death toll. Due to their global rise in incidence and prevalence, cardiovascular disease (CVD) and type 2 diabetes (T2D) are considered the top killers, accounting for 17.9 and 1.6 million deaths annually [1]. Over the last decade, high plasma glucose, was among the top three risk factors with the largest incidence increase in the world, along with smoking and high systolic blood pressure [1].

T2D itself had been considered the greatest pandemic in human history [3]. According to the International Diabetes Federation, the latest global diabetes prevalence (2019) is estimated at 9.3%, accounting for 463 million people [4]. In most parts of the world, the incidence of T2D has increased over the last decades and it is expected that the incidence will keep growing in the upcoming years [1]. These numbers become even more compelling if one realizes that it has been argued that such figures underestimate the real number of T2D prevalence by at least 25% [3]. Currently, the global burden associated with T2D is estimated to be 67.9 million disability-adjusted life-years (DALYs); the projections for the next lustrum, point to an increment of 11.4 million, resulting in 79.3 million DALYs by 2025 [5].

Given the fact that T2D could potentially be reverted at an early stage [6–10], the identification of novel, early biomarkers, particularly those that are potentially involved in the development of the disease, and may represent modifiable risk factors, is highly relevant. Among these, the potential impact of branched chain amino acids and trimethylamine N-oxide is increasingly attaining interest.

Branched chain amino acids

The branched chain amino acids (BCAA) are comprised of valine, leucine, and isoleucine. They are essential amino acids, with a chemical structure consisting of an aliphatic side-chain with a central carbon atom bound to three carbon atoms [11]. The potential role of BCAA in glucose metabolism has been investigated for more than 50 years [12]. Early research proposed that among all the essential amino acids, the BCAA could promote insulin secretion in healthy individuals [12] and provides a rationale for the potential role as biomarkers for T2D and its complications. After the secretagogue capabilities of the BCAA were discovered, further investigation was conducted to understand BCAA metabolism and its crosstalk within different organs and tissues under physiological conditions [13]. Nevertheless, only the use of high throughput metabolomic screening allowed the identification of a strong BCAA signature in individuals at higher risk of developing T2D [14].

Several mechanisms have been proposed as a link between BCAA and the risk of T2D (Figure 1) [15–23]. The biochemical mechanism underlying the association of BCAA with insulin resistance has been studied in different tissues. Newgard et al. have reported that murine models, in which the animals were fed with a high-fat diet supplemented with BCAA resulted in an accumulation of mitochondrial acylcarnitines, leading to insulin resistance in skeletal muscle. They demonstrated that BCAA play a particular role in the chronic activation of the mammalian target of rapamycin (mTOR) protein kinase, which was not wholly explained by the high-fat diet itself [24]. It has been further described that the persistent activation of the mTOR pathway promotes the uncoupling of the insulin receptor from the insulin signaling mediator (IRS-1), leading to insulin resistance in the hepatic and muscle tissue [25]. It is well known that most of the essential amino acids are metabolized in the liver, whereas the BCAA are catabolized under the joint control of both skeletal muscle and liver [26].

Remarkably, it has recently been appreciated that adipose tissue also plays an important role in the catabolism of BCAA and therefore, it can modulate circulating BCAA concentrations [27]. Of note, the proportion of BCAA catabolism is predominantly high in the brown adipose tissue and it is linked to the whole-body energy homeostasis [28]. This finding, when put in the context of the well-known relationship between the loss of brown adipose tissue and increased risk of T2D [29], suggests that the higher risk of T2D associated with

increased circulating concentrations of BCAA could be mediated by reduced catabolism of the BCAA in brown adipose tissue [30]. In addition, a recent study has further investigated the role of BCAA in T2D and obesity, via a different mechanism: hyperphagia. For this mechanism it has been proposed that an increased ratio of BCAA/ non BCAA could disrupt the metabolism of tryptophan, resulting in a downregulation of 5-hydroxytryptamine and 5-hydroxyindo-leacetic acid, which are key neuropeptides involved in the appetite control [22]. Finally, novel research venues have also found a role of the microbiome in the regulation of circulating BCAA concentrations. Gut microbiota species such as *Prevotella copri* and *B. vulgatus* are able to produce BCAA. It has been found that BCAA production by microbiota may affect circulating BCAA concentrations and insulin resistance in the host [31]. Moreover, it has been reported that subjects with insulin resistance have gut microbiota dysbiosis characterized by a reduced capacity of BCAA catabolism in the microbiota [32].

This evidence (summarized in Figure 1) makes BCAA a prime biomarker candidate for assessing the risk of T2D in the general population. Therefore, in **Chapter 2**, we longitudinally investigated the association of the circulating concentrations of BCAA with the risk of developing T2D in a population based-cohort.

Insulin resistance of glucose metabolism in liver and skeletal muscle is a common factor that is present in non-communicable disease comorbidities [33–36]. Among interrelated comorbidities, non-alcoholic fatty liver disease (NAFLD), recently renamed to metabolic-associated liver disease (MAFLD) [37], and T2D have a complex relationship and may increase each other incidence, perhaps synergistically resulting in unfavorable clinical sequalae [38]. According to the latest reports, NAFLD has a global prevalence of about 25%, being even more prevalent in countries with a concomitantly high prevalence of obesity and T2D [39].

NAFLD is characterized by hepatic steatosis and is closely related to insulin resistance [38]. While T2D may precede NAFLD [2,4], there is also evidence that NAFLD, may precede T2D [3,5]. It has been reported that circulating BCAA are elevated in subjects with NAFLD [24,40], at least, in part due to a disturbance in BCAA catabolism in hepatic and adipose tissue [41]. As a consequence of the defects in BCAA metabolism, in liver an oxidative stress process is triggered, which could worsen progression of both NAFLD and impaired glucose

metabolism [42]. Considering that the prevalence of NAFLD is in general higher than the prevalence of T2D [39,43], and given the possibility that NAFLD and impaired BCAA metabolism could jointly impact on glucose tolerance deterioration, in **Chapter 3** we investigated the extent to which BCAA influence T2D development in the context of NAFLD.

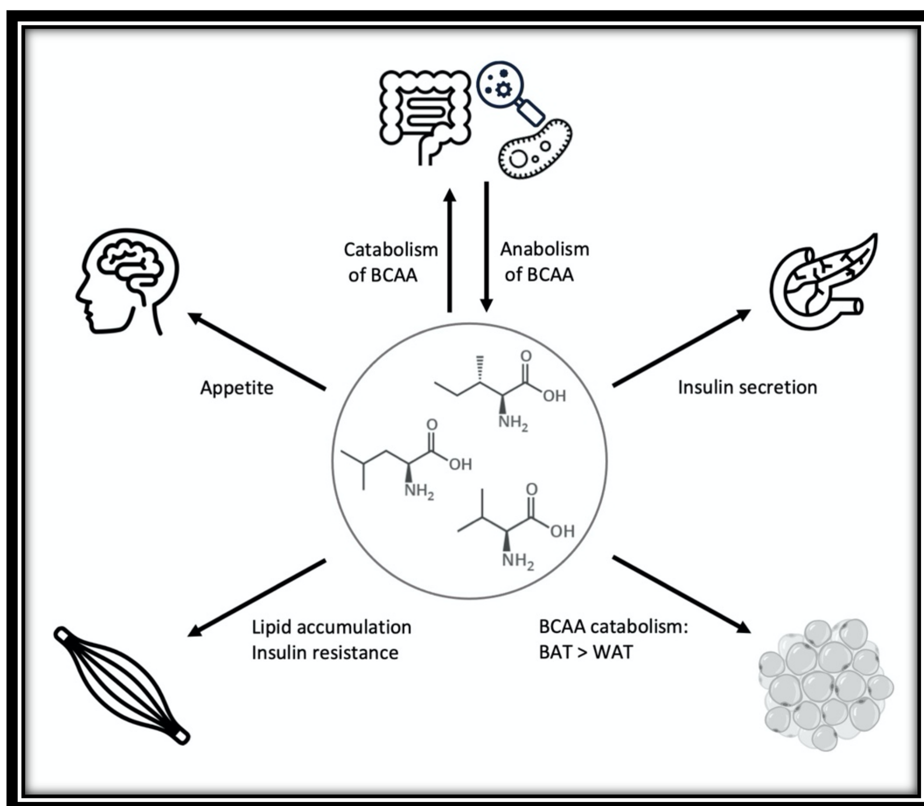

Figure 1. Schematic representation of the proposed mechanisms between BCAA (from top to bottom isoleucine, leucine and valine) and T2D, including microbiota co-host BCAA metabolism, secretagogue effects of BCAA, BCAA catabolism in adipocytes, lipid accumulation and insulin resistance in skeletal muscle and role of BCAA in the appetite regulation. Abbreviations: BAT, brown adipose tissue; WAT, white adipose tissue.

Insulin resistance precedes the development of several non-communicable diseases, including T2D [35]. Therefore, discovery of insulin resistance-related biomarkers that could help to identify subjects at risk of developing metabolic disease has received a lot of attention over the last years [44–47].

The use of high throughput metabolomic screening technologies has permitted the identification of novel molecules that are prospectively associated with a higher risk of developing T2D [14,46]. For instance, the lipoprotein insulin resistance index (LP-IR), is a high-throughput multimarker that combines the information of six lipoprotein particle parameters: the weighted average sizes of very-low-density lipoprotein, low-density lipoprotein and high-density lipoprotein (HDL), along with concentrations of large very-low-density lipoprotein, small low-density lipoprotein, and large HDL particles. It has been reported that LP-IR has a strong association with insulin resistance [48,49].

Recently, a novel Diabetes Risk Index (DRI) was developed with the goal of stratifying T2D risk among individuals with similar glycemic status. DRI is a nuclear magnetic resonance spectroscopy (NMR)-based multimarker algorithm that includes measurements of LP-IR and the BCAA in plasma. Hence, in **Chapter 4**, we examined for the first time the prospective association of the DRI and the risk of T2D in a population-based cohort.

It is well described that T2D and hypertension are two highly prevalent non-communicable diseases, that commonly overlap in the population [50]. The overlap of these pathologies is partly due at least in part to common risk underlying pathogenetic factors, such as obesity [51], sedentarism [52,53], social inequality [54–56] and unhealthy diet (e.g., reduced vegetable consumption) [57,58]. T2D and hypertension also share biological pathways, including low-grade systemic inflammation [59–61] and insulin resistance [48,62], which are key factors that underlie the development of these two entities [51].

Considering such shared pathways, the potential role of BCAA in the development of hypertension has gained attention [63,64]. Importantly, basic research reports have investigated the influence of circulating BCAA on human endothelial cells. *Ex vivo* and *in vitro* experiments showed that elevated concentrations of BCAA can induce a pro-inflammatory response and trigger endothelial dysfunction, mediated by the mTOR signaling pathway [65,66], which was previously described as a potential mediator of the T2D risk [24].

Despite these novel findings, there is a lack of epidemiological reports describing whether or not BCAA are associated with the development of hypertension. On the one hand, Batch and colleagues reported a positive cross-sectional

association between circulating BCAA and prevalent hypertension [63]. On the other hand, Yamaguchi and colleagues investigated this association in a Japanese population and found that such a cross-sectionally demonstrated association was no longer present after adjustment for age, sex and plasma lipids [67]. In extension of these cross-sectional studies, in **Chapter 5**, we, evaluated the potential prospective association between BCAA and risk of hypertension in the middle-aged and elderly participants initially free of hypertension from a large general population-based Dutch cohort. In further extension thereof, in **Chapter 6**, we prospectively investigated whether the DRI, a multimarker that combines LP-IR and BCAA, would provide an enhanced clinical ability to identify individuals at higher risk of developing primary hypertension.

The interplay of metabolic and cardiovascular disease.

The first reports about the potential link between CVD and diabetes date from 1883 [68]. Nevertheless, it took almost a century to have the first long-term prospective studies demonstrating the presence of this association in a population-based cohort [69]. Very few countries so far accomplished to stop the rise of the T2D incidence [70], and the global prevalence of T2D is anticipated to keep growing, with rise in life expectancy as major contributor [71,72]. Therefore, it is desirable to further investigate the risk markers that are associated with the development of T2D complications.

Two major milestones in research had shaped the direction of the biomarker discovery in cardiometabolic disease: the development of high throughput technologies [14,46], making it possible to e.g. measure BCAA and lipoprotein particles in large numbers of samples, which formed the basis of the first part of this thesis, and the microbiome revolution [73–75], which led to the discovery of new markers that can also be measured with the new high throughput technologies, of which trimethylamine-N-oxide (TMAO) is an important one, and the basis for the second part of this thesis.

TMAO as a biomarker of cardiometabolic disease.

Over the last years, the connection between the microbiota (the collection of all bacteria, viruses, and fungi that live in and on the human body) and a great many of physiological and pathophysiological pathways have been recognized [73–75].

TMAO is a microbiota-derived metabolite [76,77] that has recently gained attention due to its potential role in the development and progression of T2D complications [78], CVD [79], and kidney disease [80–82], and its association with increased mortality risk in the general population [83,84]. Trimethylamine (TMA) is a by-product of microbial fermentation of dietary components such as choline, phosphatidylcholine and L-carnitine. Subsequently, TMA is oxidized to TMAO by the liver enzyme flavin monooxygenase 3, whereas circulating TMAO is cleared by the kidneys [76] (Figure 2).

To date, there are several *in vivo* and *in vitro* studies that have identified platelet activation and aggregation as potential mechanisms responsible for the association of TMAO with CVD [76,85,86]. Briefly, the TMAO meta-organismal pathway in platelet hyperresponsiveness and atherosclerosis seems to act via distinct agonists including ADP, thrombin, collagen and arachidonic acid, which display TMAO-dependent enhancement in Ca^{2+} release from platelet intracellular stores. These pathways appear to be hyper-active in subjects exposed to high concentrations of TMAO [85].

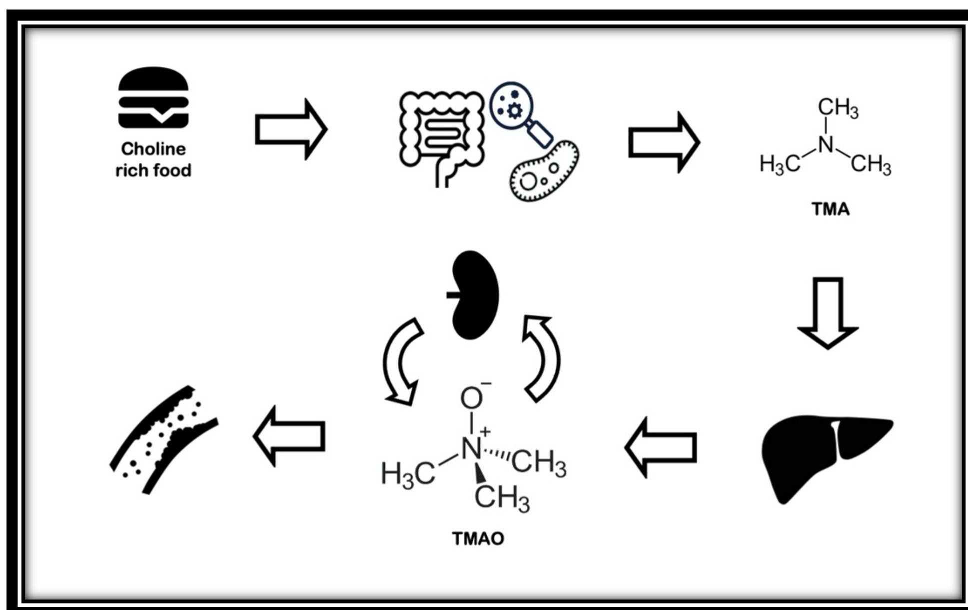

Figure 2. Schematic representation of the TMAO metabolism and the proposed mechanism underlying the association between TMAO and cardiovascular disease.

Even though some studies have identified an association of plasma concentrations of TMAO with adverse cardiovascular (CV) outcomes in the general population [84], studies in individuals with latent comorbidities are not always consistent [80]. Moreover, the association of TMAO with CV mortality in individuals with T2D has recently been identified to be present in high-risk individuals [87]. Thus, in **Chapter 7** we investigated the prospective association of the plasma concentration of TMAO with the risk of cardiovascular mortality in people with T2D.

It has been recently reported that the gut microbiome may play an important role in the development and progression of NAFLD [88,89]. One of the proposed mechanisms relies on the fact the liver is the “first pass” organ of gut microbiota-derived metabolites, exposing the liver tissue to the highest concentrations of such metabolites and therefore being more vulnerable to their deleterious effects. Likewise, hepatic tissue that already is affected by inadvertent lipid accumulation is more susceptible to such effects, possibly worsening the clinical prognosis of patients with NAFLD [90].

Clinical studies have revealed an association between TMAO, NAFLD and non-alcoholic steatohepatitis [91–93]. In addition to the above-mentioned mechanisms of TMAO on the development of CVD, it has been reported that TMAO inhibits cholesterol conversion into bile acids, promoting steatosis and worsening the progression of NAFLD [93]. In **Chapter 8**, we investigated the association of circulating concentrations of TMAO with cardiovascular and all-cause mortality in individuals with suspected NAFLD, using the internationally recommended fatty liver index (FLI) as proxy.

The relationship between TMAO and renal function is bidirectional, as the kidneys are responsible for the clearance of TMAO and therefore a reduced kidney function may result in increased concentrations of TMAO [76,94], high concentrations of TMAO may also exert a deleterious effect on kidney function via vascular dysfunction, leading to deterioration of glomerular filtration rate [95,96]. To date, T2D remains the main cause of end-stage kidney disease world-wide [97]. For patients with end-stage kidney disease, kidney transplantation is the treatment of choice, given its superiority in terms of quality of life, long-term survival, and healthcare cost, compared to dialysis [98]. In view of the rise in the prevalence of T2D, it is expected that its complications will become more prevalent [4], challenging the shortage of available organs for transplantation.

Therefore, it is clinically relevant to investigate biomarkers that reflect the interplay of the kidney with other organs, such as the gut microbiota, the vasculature and the kidney, which could be of utility in the assessment of graft survival. Although some studies have identified an association between plasma concentrations of TMAO with adverse cardiovascular outcomes in patients with chronic kidney disease [95,99] and hemodialysis patients [81], the potential role of TMAO in graft survival in renal transplant recipients remains unknown. Hence, in **Chapter 9** we longitudinally investigated the potential association of TMAO with the risk of graft failure in renal transplant recipients.

Integrating the information of multiple biomarkers

The potential role of individual plasma biomarkers in the pathogenesis of cardiometabolic pathologies, i.e. T2D has been broadly studied. Nonetheless, most of the studies investigated such biomarkers separately, and the impact of biomarkers interaction remains underexplored. In this thesis, in chapters 4 and 6, we evaluated the performance of a multimarker that integrated the information of BCAA and six lipoprotein particle parameters; still, the information of other relevant biomarkers was not taken into consideration.

Recently, it has been proposed that the Mahalanobis distance (MD) of circulating biomarkers, can assess the simultaneous variation of such biomarkers among themselves and could be used as a proxy of homeostasis loss that occurs with ageing [100]. Previous investigations had shown that a higher MD is associated with ageing-related outcomes [100]. Yet, the suitability of MD to further assess the risk of T2D has not been considered before. Therefore, in **Chapter 10**, we investigated whether the MD calculated from conventional and novel circulating biomarkers is prospectively associated with the development of T2D among individuals from the general population.

General hypothesis

The general hypothesis of this thesis is that circulating plasma concentrations of novel biomarkers that are driven by insulin resistance (BCAA) are prospectively associated with the incidence of non-communicable diseases such as T2D and hypertension. Meanwhile other biomarkers that are closely related to atherosclerotic processes (TMAO) may be prospectively associated with development of cardiovascular and kidney disease, as well as all-cause and cardiovascular mortality in high-risk individuals.

Aims and outline of the thesis

The aim of this thesis is to investigate the prospective associations of BCAA and TMAO with the onset and progression of cardiometabolic disorders, in particular T2D, hypertension and NAFLD, and CVD in three different populations: the general population (individuals who develop T2D, individuals who develop hypertension and individuals with (suspected) NAFLD), outclinic patients with T2D, and renal transplant recipients (RTRs). In **Chapter 2** we investigated whether high concentrations of BCAA in plasma, measured by means of NMR, are prospectively associated with a higher risk of incidence of T2D in the Prevention of Renal and Vascular End-stage Disease (PREVEND) population-based cohort. In **Chapter 3**, the association of plasma concentration of BCAA with the incidence of hypertension was investigated in the same population-based cohort. In **Chapter 4**, the extent to which BCAA influence T2D development in participants with NAFLD was investigated in the same population-based cohort. Under the light of the results obtained in chapter 2 and 4, we hypothesized that a multimarker that contains the information of BCAA and independent biomarkers could also be strongly associated with a higher incidence of T2D. In this context, we evaluate the prospective association of the diabetes risk index (DRI), a multimarker that comprehends the concentrations of valine and leucine, as well as 6 lipoprotein subfractions with the incidence of T2D, again in the same population-based cohort. The results of this study are presented in **Chapter 5**. Additionally, in **Chapter 6**, we further investigated the association of the multimarker DRI with the risk of hypertension incidence in this cohort. In **Chapter 7**, we assessed the association of TMAO concentrations in plasma with the risk of cardiovascular mortality in a prospective cohort of patients with T2D from the Zwolle Outpatient Diabetes project Integrating Available Care (ZODIAC). In **Chapter 8**, the association of plasma concentrations of TMAO with all-cause mortality was investigated in the context of NAFLD. For this study we again used the PREVEND cohort. In **Chapter 9**, the prospective association of the plasma concentrations of TMAO, with the risk of kidney graft failure was evaluated in TransplantLines, a cohort of RTRs. Finally, in **Chapter 10**, we investigated whether the Mahalanobis distance, a novel statistical proxy of homeostasis loss, which integrates information of several circulating biomarkers, including BCAA and TMAO, is longitudinally associated with risk of T2D in the general population.

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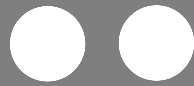

Chapter 2

Plasma branched-chain amino acids and risk of incident type 2 diabetes: results from the PREVEND prospective cohort study.

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Abstract

Plasma branched-chain amino acids (BCAA) are linked to metabolic disease, but their relevance for prediction of type 2 diabetes development is unclear. We determined the association of plasma BCAA with type 2 diabetes risk in the PREVEND cohort. BCAA were measured by means of nuclear magnetic resonance spectroscopy. We evaluated the prospective associations of BCAA with type 2 diabetes in 6244 subjects. BCAA were positively associated with HOMA-IR after multivariable adjustment P<0.0001). During median follow-up for 7.5 years, 301 cases of type 2 diabetes were ascertained. The Kaplan–Meier plot demonstrated that patients in the highest BCAA quartile presented higher risk (P log-rank <0.001). Cox regression analyses revealed a positive association between BCAA and type 2 diabetes; HR for the highest quartile was 6.15 (95% CI: 4.08, 9.24, P<0.0001). After adjustment for multiple clinical and laboratory variables, the association remained (HR 2.80 [95% CI: 1.72, 4.53], P<0.0001). C-statistics, NRI and -2 log likelihood were better after adding BCAA to the traditional risk model (P=0.01 to <0.001). In conclusion, high concentrations of BCAA associate with insulin resistance and with increased risk of type 2 diabetes. This association is independent of multiple risk factors, HOMA-IR and β cell function.

Introduction

Amino acids have an important function in addition to building proteins; they are also critical intermediaries of intracellular signaling [1]. Branched-chain amino acids (BCAA) are amino acids that have non-linear aliphatic side-chains, and include the essential amino acids leucine, valine and isoleucine. Most of the essential amino acids are metabolized in the liver, whereas BCAA are catabolized under the joint control of both skeletal muscle and liver [2].

During the last years, the association of BCAA with obesity, insulin resistance and diabetes risk had received more attention, as it is reflected in the increased number of publications. From experimental studies in murine models to clinical reports based on food frequency questionnaires and metabolomics approach, the evidence points to BCAA as a relevant factor in the pathogenenis of dysglycemia and the metabolic syndrome [3].

Oxidation of BCAA in muscle has been linked to glucose homeostasis, but there is equivocal evidence regarding the role of BCAA on insulin sensitivity. Some studies suggest that BCAA may improve muscle glucose uptake by enhancing glucose recycling via the glucose-alanine cycle and that they may contribute to the regulation of insulin signaling [4]. However, other studies in humans and in animal models have reported that increased plasma concentrations of leucine have no effect [5] or may even increase insulin resistance via the inhibitory serine phosphorylation of insulin receptor substrate-1 [6]. In addition, it has been demonstrated that leucine deprivation increases hepatic insulin sensitivity[7].

The biochemical mechanism underlying the association of BCAA with insulin resistance had been approached in several studies. Newgard et al have reported that in murine models fed with BCAA and a high fat diet presented accumulation of mitochondrial acylcarnitines, which lead to insulin resistance. They demonstrated that the BCAA plays a particular role in the chronic activation of mTOR, which was not explained only by the high fat diet [8].

There is also epidemiological evidence to suggest a positive cross-sectional association of circulating concentrations of BCAA with insulin resistance, and it had been suggested that BCAA are relevant or type 2 diabetes development [9, 10]. In line, using data from two independent cohorts we have

recently reported that high circulating concentrations of BCAA are associated with the presence of type 2 diabetes and metabolic syndrome [11].

Some studies have also explored the prospective association of BCAA with glycemia [12] and incidence of type 2 diabetes. Wang et al. reported the association of individual BCAA with type 2 diabetes incidence in two nested case-control studies with 704 participants in total [13]. In another study with 526 participants being followed for 4.7 years, the association of BCAA and incident type 2 diabetes did not remain significant after adjustment for insulin resistance [14]. A positive association of circulating BCAA with incident type 2 diabetes has also been reported for an Asian population [15]. Recently, Ruiz-Canela et al. reported a positive association between plasma BCAA with type 2 diabetes incidence in a case-cohort study among European subjects followed for 3.8 years [16]. However, given the fact that in a case-cohort study cases are overrepresented, there are limitations in the assessment of prediction measures in such studies [17 ,18].

Previous studies have limitations in terms of sample size, design and follow-up. For that reason, it is unclear whether circulating concentrations of BCAA have the ability of actually improving prediction of an established type 2 diabetes risk model. We, therefore, determined the extent to which BCAA plasma concentrations, i.e the sum of valine, leucine and isoleucine, can improve risk prediction of type 2 diabetes incidence, in the Prevention of Renal and Vascular End-stage Disease (PREVEND), a prospective population-based cohort study.

Materials and Methods

Study Population

The Prevention of Renal and Vascular End-stage Disease (PREVEND) Study is a prospective population-based cohort study in Groningen, the Netherlands. The design of the PREVEND Study has been described in detail elsewhere [19],[20]. Briefly, from 1997 to 1998, all residents from Groningen aged 28–75 years were invited to participate. Pregnant women, type 1 diabetic subjects and type 2 diabetic subjects using insulin were not allowed to participate. All participants with a urinary albumin concentration ≥ 10 mg/L were invited to our clinic together with randomly selected subjects with a urinary albumin concentration < 10 mg/L. 8592 individuals completed an extensive examination.

For the present analysis, we conducted a post-hoc analysis, using data of participants who completed the second screening round, excluding those with missing values of BCAA concentrations (n=1901) or pre-existing type 2 diabetes (n=447), leaving a cohort of 6244 participants with complete information for analysis. The protocol for the PREVEND study was approved by the local ethics committee of the University Medical Center Groningen. All participants in the present analysis provided written informed consent to participate and all study procedures were conducted according to the Declaration of Helsinki.

Baseline assessment of BCAA

During two outpatient visits, baseline data were collected on demographics, lifestyle factors, anthropometric measurements, medical history as well as prevalent medical conditions, and use of medication. Plasma samples were taken from participants after an overnight fast and 15 minutes of rest prior to sample collection. All blood samples were taken between 8:00 and 10:00 AM. Plasma samples were prepared by centrifugation at 4 ºC and were stored at -80 ºC until analysis.

Plasma valine, leucine, isoleucine concentrations were measured using a Vantera Clinical Analyzer (LabCorp, Morrisville, NC), a fully automated, high-throughput, 400 MHz proton (1H) nuclear magnetic resonance (NMR) spectroscopy platform. Plasma samples were prepared on board the instrument, and automatically delivered to the flow probe in the NMR spectrometer's magnetic field. The validation of the use of NMR for quantification of BCAA has been described by our group [10],[11]. Data acquisition on the Vantera and the spectra data processing have been reported in greater detail elsewhere [21].

Clinical and laboratory measures

Height and weight were measured with the participants standing without shoes and heavy outer garments. Body mass index (BMI) was calculated by dividing weight in kilograms by height in meters squared. Systolic and diastolic blood pressure values were recorded as the means of the last two recordings of the second visit. Total cholesterol, triglycerides, insulin, serum creatinine, and serum cystatin C were measured using standard protocols, which have been previously described [22–25]. Urinary albumin excretion (UAE) was measured as described in two 24-hour urine collections and the results were averaged for

analysis [23–25]. Fasting plasma glucose was measured by dry chemistry (Eastman Kodak, Rochester, New York). HOMA-IR was calculated as fasting plasma insulin (mU/l) x fasting plasma glucose (mmol/l)/22.5. HOMA-β was calculated using the equation: 20 × fasting plasma insulin (mU/l)/[fasting plasma glucose (mmol/l) - 3.5]. HOMA-β represents the relative β-cell function of an individual and is expressed as a percentage. Estimated glomerular filtration rate (eGFR) was calculated using the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) combined creatinine-cystatin C equation [26].

End Point of the Study

Participants were followed from the date of the baseline center visit until end of follow-up. Incident type 2 diabetes was established if one or more of the four criteria were met during follow-up: 1. Blood glucose ≥ 7.0 mmol/l (126 mg/dl), 2. Random sample plasma glucose ≥ 11.1 mmol/l (200 mg/dl); 3. Self-report of a physician diagnosis; and 4. Initiation of glucose lowering medication according to the central pharmacy registry follow-up data was complete as of 1 January 2011.

Statistical Analysis

Variables with a nonlinear distribution were natural log transformed. Data are presented as the mean (standard deviation, SD) or median (interquartile range, IQR) for continuous variables and percentages for categorical variables. Cross-sectional associations at baseline were assessed by multivariable linear regression for continuous variables and by $\chi 2$ test for categorical variables. Results of cross-sectional associations of BCAA with insulin resistance and pancreatic β-cell function are presented as unstandardized regression coefficients and 95% confidence intervals (CI).

For the prospective analysis, we plotted cumulative Kaplan-Meier curves for type 2 diabetes development during follow-up according to quartiles of BCAA. Time-to-event Cox proportional hazards models were used to assess the hazard ratio (HR) and 95% CI of incident type 2 diabetes among 6244 participants free of type 2 diabetes at baseline. HRs were calculated in 5 adjusted models: for (1) age and sex; (2) plus family history of type 2 diabetes and BMI; (3) plus alcohol consumption and smoking status; (4) plus triglycerides; (5a) plus HOMA-IR; (5b) and HOMA-β; (5c) plus HOMA-IR and HOMA-β. Possible effect modification was explored by including the interaction terms between BCAA and age or sex in the multivariable adjusted models. These analyses were

conducted using valine, leucine, isoleucine separately and its sum (BCAA) as independent variables.

In order to determine whether BCAA values can improve the predictive ability of a conventional model [27], we calculated measures of discrimination for censored time-to-event data (Harrell's C-index) [28] and reclassification. In order to evaluate the change in C-index in addition of BCAA, two type 2 diabetes risk prediction models were fitted: first, a model using clinical and laboratory variables (age, sex, family history of type 2 diabetes, BMI, insulin, triglycerides, and fasting plasma glucose), as used by Wilson et al. in the Framingham Offspring Study [29]; second, a model with the variables mentioned above plus BCAA. Subsequently, we tested the ability of the combined model with BCAA concentrations to correctly reclassify participants into categories of predicted type 2 diabetes risk. Using predefined risk categories of type 2 diabetes development (<10%), intermediate (10% to 20%), and high (≥20%) [27], reclassification was assessed using the categorical net reclassification improvement (NRI) approach [30].

Considering that Harrell's C-index may not be able to detect differences in risk prediction of potential biomarkers because its calculation is not based on continuous data, but ranks [31], we decided to use the -2 log likelihood test as another sensitive risk discrimination methods [32]. For that reason, in addition to Harrell's C-index, we tested differences in the -2 log likelihood of prediction models with and without inclusion of BCAA values. All statistical analyses were conducted in R version 3.4.2 (Boston, MA). Two-sided p-values <0.05 were considered significant.

Results

Baseline characteristics

Baseline characteristics of the 6244 subjects included in the current study are shown in Table 1 (in sex-stratified quartiles). Among them, 50.6% were women and 14.2% reported to have positive family history of type 2 diabetes. Mean BCAA concentration for all participants was 370.3 ± 88.6 µM, valine was 203.08 ± 46.5 µM, leucine was 124.9 ± 32.5 µM and isoleucine was 42.9 ± 16.1 µM (Table 1). In men, the mean BCAA concentration was 405.40 ± 90.00 µM, which was 366.11 ± 72.43 µM in women (P<0.001). Subjects with the highest quartile of BCAA concentrations were more likely to be, older, have higher BMI, blood

Table 1. Participant characteristics according sex-stratified quartiles of BCAA in participants free of type 2 diabetes at baseline (n=6244)

	Quartiles of BCAA				
	Q1	Q2	Q3	Q4	
	♂ < 365.31	♂ 365.32 - 408.34	♂ 408.35 - 454.02	♂ > 454.023	
Variables	♀ < 299.38	♀ 299.39 - 336.23	♀ 336.24 - 377.35	♀ > 377.36	P-value*
Participants, N	1562	1560	1560	1562	
Sex, men, %	49.4	49.4	49.3	49.4	0.99
Age, y	51.7 ±1 3.2	52.7 ± 12.3	53.7 ± 12.3	54.3 ± 11.4	<0.001
Race, white, %	96.3	96.2	95.8	93.2	<0.001
High Education, %	39.1	41.4	37.8	33.8	<0.01
BMI, kg/m2	24.7 ± 3.6	25.8 ± 3.7	26.7 ± 3.9	28.6 ± 4.4	<0.001
SBP, mm Hg	123.0 ±18.6	123.6 ±17.5	125.8 ±18.5	130.3 ±18.6	<0.001
DBP, mm Hg	71.9 ± 9.4	72.5 ± 8.8	73.4 ± 9.0	75.0 ± 8.7	<0.001
Parental history of CKD, %	0.6	0.3	0.8	0.3	0.18
Parental history of T2D, %	12.8	12.5	13.5	18.1	<0.001
Current smoking status, no %	65.8	70.8	73.7	72.4	<0.001
Alcohol intake, never, %	23.7	22.4	24.5	25.6	0.23
Antihypertensive drugs, %	14.7	14.5	18.2	24.5	<0.001
Lipid-lowering drugs, %	5.1	5.5	7.5	9.8	<0.001
Total BCAA, µM	< 365.31	365.32 - 408.34	408.35 - 454.02	> 454.02	
Valine, µM	156.32 ± 50.40	194.59 ± 20.71	215.14 ± 21.63	246.30 ± 31.10	<0.001
Leucine, µM	92.85 ± 31.17	118.95 ± 16.50	132.01 ± 18.48	154.90 ± 25.31	<0.001
Isoleucine, µM	30.01 ± 12.73	39.45 ± 10.35	45.02 ± 11.37	56.67 ± 16.43	<0.001
TC, mmol/l	5.22 ± 0.95	5.36 ± 1.02	5.51 ± 1.04	5.67 ± 1.06	<0.001
HDL-C, mmol/l	1.13 ± 0.37	1.12 ± 0.30	1.25 ± 0.29	1.17 ± 0.28	<0.001
TG, mmol/l	0.88(0.65 - 1.20)	1.01(0.75 - 1.38)	1.13(0.84 - 1.61)	1.47(1.06 - 2.08)	<0.001
Glucose, mmol/l	4.87 ± 0.60	4.75 ± 0.59	4.85 ± 0.60	5.03 ± 0.70	<0.001
Insulin, mU/l	6.50(4.80-8.72)	7.20(5.20 - 10.25)	8.30(6.00 - 11.70)	11.84(7.70-16.62)	<0.001
Serum creatinine, µmol/l	83.4 ±30.6	83.9 ±16.3	85.1 ±15.3	85.6 ±16.1	0.13
eGFR, ml/min/1.73m^2	95.4 ±17.6	93.5 ±16.5	91.7 ±16.3	90.3 ±17.0	<0.001
UAE, mg/24h	8.15 (5.94-13.82)	8.23 (5.88-13.62)	8.41 (5.98-14.85)	9.65 (6.50-18.20)	0.101
HOMA-IR	1.40 (1.02-1.93)	1.54 (1.10 - 2.24)	1.78 (1.25 - 2.60)	2.53 (1.66 - 3.85)	<0.001
HOMA-β	116.9 (82.5 - 178.6)	128.5 (90.0 - 190.0)	133.3 (96.0 - 193.5)	160 (110.5 -237.1)	<0.001
HOMA-β/ HOMA-IR	79.79 (55.1 - 113.6)	79.79 (55.1 - 113.6)	79.79 (50.9 - 113.6)	60.0 (40.9 - 88.9)	<0.001

Continuous variables are reported as mean ± SD, median (interquartile range) and categorical variables are reported as percentage. *Determined by linear-by-linear association chi-square test (categorical variables) and linear regression (continuous variables).
Abbreviations: BCAA, branched chain amino acids; BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; CKD, chronic kidney disease; T2D, type 2 diabetes ; TC, total cholesterol; HDL-C, high density lipoprotein cholesterol; TG, triglycerides; eGFR, estimated glomerular filtration rate; UAE, Urinary albumin excretion; PREVEND, Prevention of Renal and Vascular End-stage Disease.

pressure, and used tobacco more frequently. Additionally, those subjects also presented higher concentrations of total cholesterol, triglycerides, glucose, insulin, HOMA-IR, HOMA-β and creatinine. The percentages of a positive family history of chronic kidney disease (CKD) and alcohol consumption, as well as the urinary albumin excretion rate were similar among the different quartiles of BCAA (Table 1).

Associations at baseline

BCAA were associated with HOMA-IR and HOMA-β in crude as well as in age- and sex- adjusted analyses (Table 2). The positive association of BCAA with HOMA-IR remained after additional adjustment for HOMA-β. However, after adjustment for HOMA-IR, there was an inverse relationship of BCAA with HOMA-β. The associations of BCAA concentrations and other variables of interest were further evaluated with univariable and multivariable regression (Table 3). In univariable analyses, sex, age, race, BMI, systolic and diastolic blood pressure, parental history of type 2 diabetes, alcohol consumption, use of antihypertensive and lipid-lowering drugs, cholesterol, triglycerides, glucose, insulin, creatinine, and UAE were positively associated with BCAA, whereas smoking status, HDL cholesterol and eGFR were inversely associated.

In a multivariable analysis, taking account of all these variables together, the positive associations with BCAA that remained at a $P<0.05$ were: sex, race, BMI, parental history of type 2 diabetes, alcohol consumption, total cholesterol, triglycerides, and HOMA-IR. HDL cholesterol remained inversely associated (Table 3). Of note, in fully adjusted analysis, BCAA was associated with HOMA-IR but was unrelated to HOMA-β.

Longitudinal analysis

During a median follow-up of 7.5 years (IQR, 7.2–8.0.), 301 participants (4.8%) developed type 2 diabetes (Table 4). The Kaplan–Meier curves for incident type 2 diabetes according to quartiles of BCAA concentrations are presented in Figure 1. The graph revealed an increased risk of type 2 diabetes in the top quartile of BCAA concentrations (P-value for log-rank test < 0.001).

Table 2. Cross-sectional associations of BCAA with Insulin Resistance and β-cell function

	HOMA-IR, (mU mmol/l^2)/22.5		HOMA-β, %	
	β (95% C.I.)	P-value	β (95% C.I.)	P-value
Crude Model	28.92 (27.16, 30.67)	<0.0001	20.46 (18.67, 22.25)	<0.0001
Model 1	26.80 (25.18, 28.43)	<0.0001	21.01 (19.38, 22.64)	<0.0001
Model 2	30.95 (27.83, 34.07)	<0.0001	-4.73 (-7.77, -1.70)	0.002

Unstandardized regression coefficients are shown.
Model 1. Adjustment for age and sex.
Model 2. Model 1 + HOMA- β (for HOMA-IR) and HOMA-IR (for HOMA- β)

Table 3. Uni- and multivariable linear regression analyses with BCAA as dependent variable

Variables	**Univariable**		**Multivariable**	
	β (95% C.I.)	P-value	β (95% C.I.)	P-value
Sex, female vs. male	69.29 (65.24, 73.33)	<0.0001	55.31 (48.32, 62.30)	<0.0001
Age, years/10	0.58 (0.39, 0.76)	<0.0001	-0.04 (-0.38, 0.31)	0.82
Caucasian, yes vs. no	7.61 (2.36, 12.86)	0.0004	5.73 (0.01, 11.44)	0.05
BMI, kg/m2	5.17 (4.67, 5.68)	<0.0001	2.44 (1.76, 3.13)	<0.0001
High education, yes vs. no	0.20 (-0.87, 1.28)	0.7107	0.40 (-0.67, 1.48)	0.46
SBP, mm Hg	0.81 (0.70, 0.93)	<0.0001	-0.09 (-0.31, 0.12)	0.89
DBP, mm Hg	1.92 (1.69, 2.16)	<0.0001	0.15 (-0.27, 0.57)	0.39
Parental history of CKD, yes vs. no	9.50 (-20.38, 39.38)	0.533	8.44 (-24.63, 41.52)	0.61
Parental history of T2D, yes vs. no	10.22 (3.94, 16.51)	0.0001	7.91 (1.03, 14.80)	0.02
Current smoking, yes vs. no	-7.94 (-12.85, -3.03)	0.0001	-4.76 (-10.52, 1.01)	0.10
Alcohol consumption, yes vs. no	11.51 (6.36, 16.66)	<0.0001	9.01 (3.06, 14.96)	0.003
Antihypertensive drugs, yes vs. no	24.35 (18.55, 30.14)	<0.0001	3.78 (-3.19, 10.75)	0.28
Lipid-lowering drugs, yes vs. no	24.87 (16.22, 33.52)	<0.0001	8.16 (-1.48, 17.80)	0.09
TC, mmol/l	7.04 (4.92, 9.15)	<0.0001	4.38 (1.58, 7.19)	0.002
HDL-C, mmol/l	-60.34(-67.05, -53.63)	<0.0001	-20.54 (-30.64,-10.44)	<0.0001
TG, mmol/l	25.51 (23.23, 27.78)	<0.0001	3.61 (0.61, 6.62)	0.02

Serum creatinine, μmol/l	71.16 (61.64, 80.68)	<0.0001	12.23 (-6.78, 31.24)	0.20
eGFR, ml/min/1.73 m²	-0.44 (-0.57, -0.31)	<0.0001	-0.01 (-0.29, 0.27)	0.95
UAE, mg/24h	0.01 (-0.00, 0.03)	0.095	-0.02 (-0.04, -0.01)	0.005
HOMA-IR, (mU mmol/l²)/22.5	28.92 (27.16, 30.67)	<0.0001	22.21 (17.82, 26.59)	<0.0001
HOMA-β , %	20.46 (18.67, 22.25)	<0.0001	-0.89 (-5.01, 3.24)	0.67

Unstandardized regression coefficients are shown. Abbreviations: BCAA, branched-chain amino acids; BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; CKD, chronic kidney disease; T2D, type 2 diabetes ; TC, total cholesterol; HDL-C, high density lipoprotein cholesterol; TG, triglycerides; eGFR, estimated glomerular filtration rate; UAE, Urinary albumin excretion.

In Cox regression analysis that compared the highest with the lowest quartiles of the distribution of BCAA concentrations adjusted for age and sex, high BCAA concentrations were associated with increased risk of incident type 2 diabetes, showing a HR of 6.15 (95% CI: 4.08, 9.24) (Table 4). The association of type 2 diabetes risk with BCAA remained significant after adjustment for HOMA-IR (HR: 2.80; 95% CI: 1.72, 4.53). Likewise, when BCAA was analyzed as HR per 1 SD increase, the risk of newly developing type 2 diabetes was significant (HR 1.28 (95% CI: 1.13, 1.46)) after adjustment for HOMA-IR (Table 4). There was no statistically significant interaction between BCAA and age or sex on T2D incidence (interactions: P= 0.37 and P=0.11, respectively).

Cox regression analysis were also performed with the individual BCAA in the same models. This analysis essentially showed a similar pattern with diabetes risk. When valine was analyzed as HR per 1 SD increase, the risk of newly developing type 2 diabetes was close to significance (HR 1.13 (95% CI: 0.98, 1.29) per 1 SD increment, P=0.07) in the fully adjusted model (Supplemental Table 1). In the same model, leucine presented a HR similar to BCAA (HR 1.18 (95% CI: 1.03, 1.34) per 1 SD increment, P=0.011) (Supplemental Table 2). Finally, isoleucine showed a significant but marginally weaker association with incident type 2 diabetes HR (1.11 95%, CI: 1.00, 1.24, per 1 SD increment, P=0.043) (Supplemental Table 3).

Stratified analyses were performed for fasting plasma glucose concentrations (using two cut points: 4.7 mmol/l (median) and 5.6 mmol/l (prediabetes cutoff value). The results of the stratified analyses were essentially similar compared to the main results (data not shown). The HRs adjusted for traditional risk factors in the subset of patients with baseline glucose

concentrations <4.7 mmol/l and ≥4.7 mmol/l were 1.47 (95% CI: 1.02, 2.17) and 1.36 (95% CI: 1.20, 1.54), respectively. Using the cutoff values of <5.6 mmol/l and ≥5.6 mmol/l, the HRs were 1.35 (95% CI: 1.10, 1.66) and 1.31 (95% CI: 1.11, 1.54), respectively; (P-value <0.001 for all comparisons).

Effect of inclusion of BCAA on type 2 diabetes risk prediction

A type 2 diabetes risk prediction model containing established risk factors yielded a C-index of 0.8034 (95% CI: 0.8005, 0.8063). After addition of information on BCAA concentrations, the C-index increased to 0.8057 (95% CI: 0.8028, 0.8086) (P<0.01). The differences of the -2 log likelihood of the type 2 diabetes predictive model with addition of total BCAA, also showed a significant improvement (P=0.001). The NRI assessment of the participants that remained free of type 2 diabetes revealed that 27% were correctly reclassified to a lower risk category and 10% were reclassified to a higher risk category. There was a significant improvement in the classification of participants into predicted type 2 diabetes risk categories with a NRI of 0.43 (95% CI: 0.31-0.54, P<0.0001).

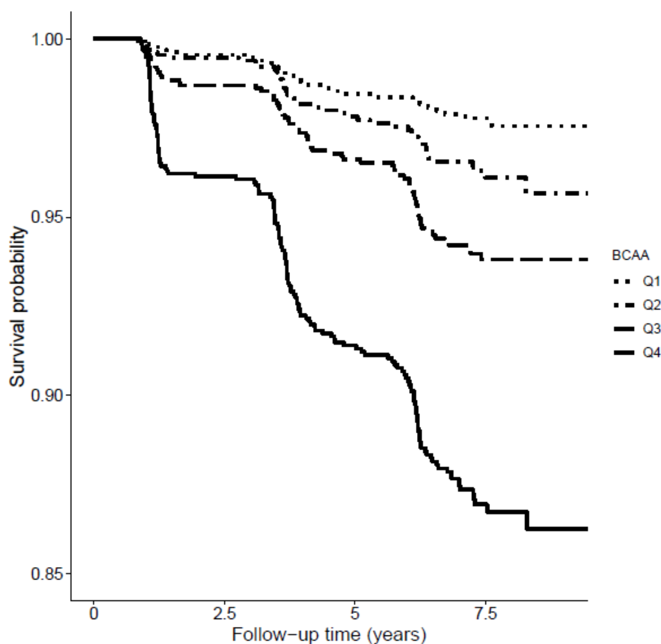

Figure 1. Kaplan Meier curves for incident type 2 diabetes survival according to quartiles of BCAA, by log-rank test (P<0.001).

Table 4. Prospective associations of BCAA quartiles with risk of Type 2 Diabetes.

	Q1	Q2	P- value	Q3	P -value	Q4	P- value	BCAA per 1 SD increment	P- value
Participants, n	1561	1561		1561		1561		6244	
Events, n	27	44		72		158		301	
		HR (95 % CI)		HR (95 % CI)		HR (95 % CI)		HR (95 % CI)	
Crude Model	(ref)	1.65 (1.01, 2.66)	0.04	2.67 (1.71, 4.14)	<0.0001	6.15 (4.08, 9.24)	<0.0001	1.80 (1.64, 1.98)	<0.0001
Model 1	(ref)	1.58 (0.97, 2.56)	0.06	2.56 (1.62, 4.05)	<0.0001	6.12 (3.92, 9.55)	<0.0001	1.76 (1.59, 1.96)	<0.0001
Model 2	(ref)	1.41 (0.85, 2.32)	0.17	1.87 (1.17, 3.00)	0.009	3.49 (2.19, 5.55)	<0.0001	1.46 (1.29, 1.65)	<0.0001
Model 3	(ref)	1.41 (0.85, 2.33)	0.17	1.87 (1.17, 3.01)	0.008	3.56 (2.24, 5.65)	<0.0001	1.48 (1.31, 1.68)	<0.0001
Model 4	(ref)	1.45 (0.88, 2.41)	0.14	1.84 (1.15, 2.94)	0.01	3.14 (1.99, 4.97)	<0.0001	1.39 (1.23, 1.57)	<0.0001
Model 5a	(ref)	1.50 (0.89, 2.53)	0.12	1.91 (1.17, 3.10)	0.009	2.80 (1.72, 4.53)	<0.0001	1.28 (1.13, 1.46)	0.0001
Model 5b	(ref)	1.59 (0.94, 2.68)	0.07	2.12 (1.30, 3.44)	0.002	3.64 (2.26, 5.87)	<0.0001	1.41 (1.25, 1.60)	<0.0001
Model 5c	(ref)	1.46 (0.87, 2.46)	0.15	1.70 (1.04, 2.77)	0.03	2.32 (1.42, 3.78)	0.0007	1.19 (1.04, 1.35)	0.008

Data are presented as hazard ratios (HR) with 95 % confidence intervals (CI).

Model 1: Model adjusted for Age and Sex. Model 2: Model 1 + family history of type 2 diabetes and BMI. Model 3: Model 2 + alcohol intake and smoking status.

Model 4: Model 3 + TG. Model 5a: Model 4 + HOMA-IR. Model 5b: Model 4 + HOMA-β. Model 5c: Model 4 + HOMA-IR and HOMA-β

Abbreviations: BMI, body mass index; TG, triglycerides.

Discussion

In this large-scale prospective population-based cohort study, we investigated the associations of plasma concentrations of total BCAA (valine, leucine, isoleucine) with the risk of type 2 diabetes. Baseline characteristics such as male sex, older age and high BMI were positive associated with high concentrations of BCAA, coinciding with the findings of other cross sectional [13], and longitudinal studies [33]. Moreover, BCAA concentrations were positively associated with insulin resistance but not with β cell function in fully adjusted analysis. We found that subjects with high circulating BCAA concentrations presented a significantly higher risk for type 2 diabetes. The association remained significant after adjustment for established risk factors, including age, sex, BMI, parental history of type 2 diabetes, hypertension, alcohol consumption as well as HOMA-IR and HOMA-β. Addition of BCAA to the traditional predictive model improved its type 2 diabetes predictive ability. Furthermore, the BCAA enriched model improved reclassification of participants across clinical risk categories for type 2 diabetes.

In our study, men had higher BCAA concentrations compared to women. The most recent study about plasma BCAA and type 2 diabetes was conducted in women with a history of gestational diabetes and demonstrated that the positive association of circulating BCAA and type 2 diabetes is also be presented in women [34]. We also found that older participants had the highest values of BCAA at baseline, however the association with incident type 2 diabetes was independent of age. Previous studies suggest that the association of BCAA with metabolic disorders can also be present in young people from 8 to 18 years old [35].

Insulin resistance is one of the main factors in the development of type 2 diabetes. In this study it was estimated by HOMA-IR, which fairly correlates with glucose disposal as determined by the hyperinsulinemic euglycemic clamp technique [35]. We found a cross-sectional association of BCAA with insulin resistance (β= 28.92, $P<0.0001$) which agrees with our previous smaller studies, that showed insulin resistance to be associated with BCAA, independent of sex, age, type 2 diabetes status and BMI [11]. Similarly, Shah and colleagues also reported a correlation between BCAA and HOMA-IR [36].

Notably, we found no association of BCAA with HOMA-β in the fully adjusted analysis (Table 2). Previous studies did not evaluate the association of

βcell function with BCAA taking account of insulin resistance [11,12,37]. Others studies only evaluated insulin resistance or fasting plasma insulin [14,36,38,39]. The influence of BCAA on glucose metabolism has been intensively investigated in animal models and humans [9]. Wang-Sattler et al reported a lack of association of the baseline concentrations of leucine, isoleucine and valine with glucose tolerance status 7 years later in the KORA cohort [38]. Earlier studies have shown that circulating concentrations of BCAA are positively associated with incident type 2 diabetes in an Asian population [15]; nonetheless such association was not significant among the European population of the SABRE cohort [40]. On the other hand, Ferrannini et al reported that 130 representative subjects from Botnia who developed type 2 diabetes after a follow-up of 9.5 years presented increased concentrations of leucine, isoleucine, valine at baseline, in comparison with the 412 subjects that remained free from type 2 diabetes, but no assessment for confounding factors was conducted [37].

This study has certain strengths. To the best of our knowledge, this prospective, population-based cohort study involves many more participants and incident type 2 diabetes cases than the previous studies conducted in a general population. Moreover, this is the first study to investigate the association between human plasma concentrations of BCAA with incident type 2 diabetes using sensitive measures such as the -2 log likelihood, and testing the robustness of the findings using several sensitivity analyses. We are also aware of the limitations of the study. The PREVEND population mainly comprises individuals of European ancestry, which could be translated in an inability to generalize the findings to different ethnicities. We did not have measurements of insulin beyond it baseline assessment, which impedes us to evaluate the evolution of insulin resistance and its association with BCAA. This fact limits our capacity to describe the biological phenomenon. Finally, because of the absence of repeated BCAA measurements we are not able to correct for regression dilution, which could have underestimated the BCAA-incident type 2 diabetes associations.

In conclusion in a population-based cohort, we found that BCAA was associated with insulin resistance at baseline and with an increased risk of incident type 2 diabetes over 7.5 years of follow-up. Additionally, our results show that BCAA can improve the predictive ability of a conventional risk model. More data are needed to elucidate the interaction between BCAA and other risk factors during the progression of impaired glucose tolerance.

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Supplemental Table 1. Prospective associations of Valine with risk of Type 2 Diabetes.

	Q1	Q2	P value	Q3	P value	Q4	P value	Valine per 1 SD increment	P value
	<=179.80	179.81- 204.17		204.18- 230.02		> 230.02			
Participants, n	1561	1561		1561		1561		6244	
Events, n	30	46		70		155		301	
		HR (95 % CI)		**HR (95 % CI)**		**HR (95 % CI)**		**HR (95 % CI)**	
Crude Model	(ref)	1.52 (0.96, 2.42)	0.072	2.31 (1.50, 3.54)	0.0001	5.34 (3.61, 7.90)	<0.0001	1.71 (1.55, 1.88)	<0.0001
Model 1	(ref)	1.39 (0.87, 2.22)	0.161	2.06 (1.33, 3.21)	0.0001	4.68 (3.07, 7.14)	<0.0001	1.63 (1.46, 1.88)	<0.0001
Model 2	(ref)	0.98 (0.60, 1.60)	0.958	1.40 (0.89, 2.21)	0.137	2.42 (1.56, 3.75)	<0.0001	1.33 (1.17, 1.51)	<0.0001
Model 3	(ref)	0.99 (0.61, 1.61)	0.975	1.42 (0.90, 2.23)	0.126	2.48 (1.60, 3.84)	<0.0001	1.35 (1.19, 1.54)	<0.0001
Model 4	(ref)	1.01 (0.62, 1.64)	0.957	1.42 (0.90, 2.23)	0.126	2.35 (1.52, 3.63)	0.0001	1.34 (1.18, 1.51)	<0.0001
Model 5a	(ref)	1.14 (0.69, 1.91)	0.592	1.46 (0.90, 2.35)	0.12	1.88 (1.17, 3.01)	0.008	1.16 (1.01, 1.32)	0.026
Model 5b	(ref)	1.11 (0.66, 1.87)	0.672	1.62 (1.00, 2.63)	0.047	2.71 (1.70, 4.31)	<0.0001	1.40 (1.23, 1.59)	<0.0001
Model 5c	(ref)	1.08 (0.64, 1.80)	0.757	1.41 (0.87, 2.28)	0.158	1.68 (1.04, 2.71)	0.033	1.13 (0.98, 1.29)	0.07

Data are presented as hazard ratios (HR) with 95 % confidence intervals (CI).
Model 1: Model adjusted for Age and Sex. Model 2: Model 1 + family history of type 2 diabetes and BMI. Model 3: Model 2 + alcohol intake and smoking status. Model 4: Model 3 + TG. Model 5a: Model 4 + HOMA-IR. Model 5b: Model 4 + HOMA-β. Model 5c: Model 4 + HOMA-IR and HOMA-β

Supplemental Table 2. Prospective associations of Leucine with risk of Type 2 Diabetes.

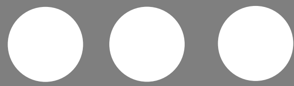
	Q1	Q2	P value	Q3	P value	Q4	P value	Leucine per 1 SD increment	P value
	<=106.36	106.37- 124.31		124.32- 143.32		> 143.32			
Participants, n	1561	1561		1561		1561		6244	
Events, n	29	48		72		152		301	
		HR (95 % CI)		**HR (95 % CI)**		**HR (95 % CI)**		**HR (95 % CI)**	
Crude Model	(ref)	1.63 (1.02, 2.59)	0.038	2.47 (1.60, 3.80)	<0.0001	5.40 (3.63, 8.04)	<0.0001	1.80 (1.62, 2.01)	<0.0001
Model 1	(ref)	1.55 (0.97, 2.47)	0.062	2.28 (1.46, 3.55)	0.0002	4.97 (3.25, 7.55)	<0.0001	1.74 (1.54, 1.95)	<0.0001
Model 2	(ref)	1.29 (0.80, 2.09)	0.284	1.68 (1.07, 2.64)	0.023	3.05 (1.98, 4.71)	<0.0001	1.47 (1.30, 1.67)	<0.0001
Model 3	(ref)	1.31 (0.81, 2.11)	0.261	1.72 (1.09, 2.69)	0.018	3.07 (1.99, 4.74)	<0.0001	1.48 (1.30, 1.68)	<0.0001
Model 4	(ref)	1.32 (0.82, 2.13)	0.248	1.65 (1.05, 2.59)	0.027	2.69 (1.75, 4.13)	<0.0001	1.35 (1.20, 1.53)	<0.0001
Model 5a	(ref)	1.46 (0.88, 2.42)	0.136	1.67 (1.04, 2.69)	0.033	2.14 (1.34, 3.42)	0.001	1.21 (1.07, 1.38)	0.002
Model 5b	(ref)	1.44 (0.87, 2.42)	0.152	1.93 (1.19, 3.14)	0.006	2.99 (1.88, 4.73)	<0.0001	1.39 (1.23, 1.58)	<0.0001
Model 5c	(ref)	1.35 (0.81, 2.23)	0.242	1.66 (1.03, 2.67)	0.036	1.90 (1.18, 3.05)	0.007	1.18 (1.03, 1.34)	0.011

Data are presented as hazard ratios (HR) with 95 % confidence intervals (CI).
Model 1: Model adjusted for Age and Sex. Model 2: Model 1 + family history of type 2 diabetes and BMI. Model 3: Model 2 + alcohol intake and smoking status. Model 4: Model 3 + TG. Model 5a: Model 4 + HOMA-IR. Model 5b: Model 4 + HOMA-β. Model 5c: Model 4 + HOMA-IR and HOMA-β

Supplemental Table 3. Prospective associations of Isoleucine with risk of Type 2 Diabetes.

	Q1	Q2	P value	Q3	P value	Q4	P value	Isoleucine per 1 SD increment	P value
	<= 32.54	32.55- 41.98		41.99- 52.00		> 52.01			
Participants, n	1543	1543		1543		1543		6172	
Events, n	38	40		84		137		299	
		HR (95 % CI)		**HR (95 % CI)**		**HR (95 % CI)**		**HR (95 % CI)**	
Crude Model	(ref)	1.02 (0.65, 1.59)	0.928	2.23 (1.52, 3.27)	<0.0001	3.71 (2.60, 5.34)	<0.0001	1.64 (1.50, 1.78)	<0.0001
Model 1	(ref)	1.01 (0.68, 1.59)	0.945	2.16 (1.45, 3.21)	0.0001	3.70 (2.49, 5.48)	<0.0001	1.64 (1.49, 1.81)	<0.0001
Model 2	(ref)	0.81 (0.51, 1.30)	0.397	1.68 (1.12, 2.52)	0.012	2.29 (1.53, 3.44)	<0.0001	1.39 (1.25, 1.54)	<0.0001
Model 3	(ref)	0.81 (0.51, 1.29)	0.387	1.69 (1.13, 2.54)	0.01	2.34 (1.56, 3.51)	<0.0001	1.41 (1.27, 1.56)	<0.0001
Model 4	(ref)	0.85 (0.53, 1.36)	0.517	1.73 (1.15, 2.60)	0.007	2.09 (1.40, 3.11)	0.0002	1.27 (1.15, 1.41)	<0.0001
Model 5a	(ref)	0.85 (0.52, 1.38)	0.522	1.45 (0.94, 2.22)	0.085	1.57 (1.02, 2.40)	0.037	1.14 (1.02, 1.27)	0.017
Model 5b	(ref)	0.84 (0.52, 1.38)	0.513	1.83 (1.20, 2.81)	0.004	2.43 (1.59, 3.69)	<0.0001	1.32 (1.19, 1.47)	<0.0001
Model 5c	(ref)	0.81 (0.50, 1.32)	0.411	1.42 (0.93, 2.18)	0.103	1.59 (1.03, 2.45)	0.033	1.11 (1.00, 1.24)	0.043

Data are presented as hazard ratios (HR) with 95 % confidence intervals (CI).
Model 1: Model adjusted for Age and Sex. Model 2: Model 1 + family history of type 2 diabetes and BMI. Model 3: Model 2 + alcohol intake and smoking status. Model 4: Model 3 + TG. Model 5a: Model 4 + HOMA-IR. Model 5b: Model 4 + HOMA-β. Model 5c: Model 4 + HOMA-IR and HOMA-β



Chapter 3

Non-Alcoholic Fatty Liver Disease and Risk of Incident Type 2 Diabetes: Role of Circulating Branched-Chain Amino Acids.

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Abstract

Non-alcoholic fatty liver disease (NAFLD) is likely to be associated with elevated plasma branched-chain amino acids (BCAAs) and may precede the development of type 2 diabetes (T2D). We hypothesized that BCAAs may be involved in the pathogenesis of T2D attributable to NAFLD and determined the extent to which plasma BCAAs influence T2D development in NAFLD. We evaluated cross-sectional associations of NAFLD with fasting plasma BCAAs (nuclear magnetic resonance spectroscopy), and prospectively determined the extent to which the influence of NAFLD on incident T2D is attributable to BCAA elevations. In the current study, 5791 Prevention of REnal and Vascular ENd-stage Disease (PREVEND) cohort participants without T2D at baseline were included. Elevated fatty liver index (FLI) ≥60, an algorithm based on triglycerides, gamma-glutamyltransferase, body mass index (BMI) and waist circumference, was used as proxy of NAFLD. Elevated FLI ≥ 60 was present in 1671 (28.9%) participants. Cross-sectionally, BCAAs were positively associated with FLI ≥ 60 ($\beta = 0.208$, $p < 0.001$). During a median follow-up of 7.3 years, 276 participants developed T2D, of which 194 (70.2%) had an FLI ≥ 60 (log-rank test, $p < 0.001$). Cox regression analyses revealed that both FLI ≥60 (hazard ratio (HR) 3.46, 95% CI 2.45–4.87, $p < 0.001$) and higher BCAA levels (HR 1.19, 95% CI 1.03–1.37, $p = 0.01$) were positively associated with incident T2D. Mediation analysis showed that the association of FLI with incident T2D was in part attributable to elevated BCAAs (proportion mediated 19.6%). In conclusion, both elevated FLI and elevated plasma BCAA levels are associated with risk of incident T2D. The association of NAFLD with T2D development seems partly mediated by elevated BCAAs.

Introduction

Non-alcoholic fatty liver disease (NAFLD) is emerging as the most common cause of chronic liver disease in the Western world [1]. NAFLD is characterized by hepatic steatosis in the absence of alcohol abuse and its spectrum ranges from simple steatosis to non-alcoholic steatohepatitis (NASH), fibrosis and cirrhosis [1]. NAFLD and insulin resistance are closely related [2]. NAFLD frequently coincides with the metabolic syndrome (MetS) and type 2 diabetes (T2D). While MetS and T2D may precede NAFLD [2,4], two recent meta-analyses have demonstrated that NAFLD, irrespective of whether it is diagnosed by elevated liver enzymes, by radiological abnormalities or by histological abnormalities, may in fact precede T2D [3,5]. Moreover, an elevated fatty liver index (FLI), as proxy of NAFLD and assessed by an algorithm based on obesity measures, plasma triglycerides and gamma-glutamyltransferase (GGT), has been shown to predict T2D in two independent European populations [6,7]. Taken together, these findings [2,3,5–7] point to an intricate relationship between NAFLD, MetS and T2D, which are indeed considered as manifestations of a common cardio-metabolic multisystem disorder [8–11].

Branched-chain amino acids (BCAAs) are amino acids with non-linear aliphatic side-chains, and include the essential amino acids leucine, valine and isoleucine [12]. Although not fully understood, BCAAs may contribute to the development of obesity-associated insulin resistance [13,14]. Moreover, in the clinical setting, disturbances in BCAA metabolism have been described in insulin-resistant states, including MetS and T2D [15–21]. Conversely, a decrease in BCAA levels may result in an improvement in glucose metabolism [22]. Additionally, high plasma BCAA levels have been shown to be associated with an increased risk of T2D development [15].

Fasting plasma BCAAs may be elevated in (obesity-associated) NAFLD [13,23–26], and may coincide with abnormalities in BCAA catabolic enzymes in liver and adipose tissue [27]. Such abnormalities in (hepatic) BCAA metabolism conceivably affect carbon substrate oxidation that, together with impairment of antioxidant defence, may contribute to the generation of reactive oxygen species [13,19]. In turn, mitochondrial dysfunction in NAFLD, in particular in the context of hepatic inflammation, is likely to convey impaired BCAA metabolism conceivably resulting in plasma BCAA elevations [28–30]. Thus, it seems

plausible to hypothesize that NAFLD and impaired hepatic BCAA metabolism jointly impact on deteriorating glucose tolerance.

Despite continued clinical interest concerning the impact of NAFLD and increased circulating BCAAs on the development of T2D, no large-scale population-based studies have yet reported on the relative contributions of NAFLD and plasma BCAAs on incident T2D in initially diabetes-naïve NAFLD subjects. Therefore, we initiated the present study to determine the extent to which BCAAs influence T2D development in the context of NAFLD. To this end, we carried out cross-sectional and prospective analyses among 5791 subjects participating in the Prevention of REnal and Vascular ENd-stage Disease (PREVEND) cohort, comprising a large and well-characterized population from the North of the Netherlands.

Materials and Methods

Study Population

The study was performed among participants of the Prevention of REnal and Vascular ENd-stage Disease (PREVEND) cohort study [31,32]. The PREVEND study was approved by the Medical Ethics Committee of the University Medical Center Groningen and performed in accordance with the Declaration of Helsinki guidelines [31,32]. All participants gave written informed consent. PREVEND is a large prospective general population-based study that was initiated to investigate cardiovascular and renal disease with a focus on albuminuria. All inhabitants (28–75 years old) of Groningen, The Netherlands were sent a questionnaire on demographics and cardiovascular morbidity and were asked to supply an early morning urine specimen. Pregnant women, type 1 diabetic subjects and T2D subjects using insulin were not allowed to participate. All participants with a urinary albumin concentration of ≥10 mg/L were invited to our clinic together with randomly selected subjects with a urinary albumin concentration of <10 mg/L. The initial study population of the PREVEND study was comprised of 8592 subjects who completed the total study screening program.

For the present study, we conducted a post-hoc analysis, using data of participants who completed the second screening round (n = 6893). We excluded all subjects with missing values of BCAA concentrations, pre-existing T2D, non-fasting subjects, and subjects in which the clinical and biochemical

variables required to calculate the fatty liver index (FLI), a proxy of NAFLD, were not available, leaving a study population of 5791 participants with complete information for analysis.

Measurements and Definitions

During two outpatient visits, baseline data were collected on demographics, lifestyle factors, anthropometric measurements, medical history, parental history of T2D and medication use. Information on medication use was combined with information from a pharmacy-dispensing registry, which had complete information on the drug usage of >95% of subjects in the PREVEND study. Height and weight were measured in standing position without shoes and heavy outer garments. Body mass index (BMI) was calculated as weight (kg) divided by height squared (meter). Waist circumference was measured as the smallest girth between the rib cage and iliac crest. The waist/hip ratio was determined as the waist circumference divided by the largest girth between waist and thigh [31]. Blood pressure was measured using an automatic device, where the last two recordings of the second outpatient visit were averaged for analysis. Alcohol consumption was recorded with one alcoholic drink being assumed to contain 10 g of alcohol. Smoking was categorized into current and never/former smokers. Past cardiovascular history included hospitalization for myocardial ischemia, obstructive coronary artery disease or revascularization procedures. Homeostasis Model Assessment (HOMA)-IR was calculated as follows: Fasting plasma insulin (mU/L) × fasting plasma glucose (mmol/L)/22.5. HOMA-β was calculated as follows: 20 × Fasting plasma insulin (mU/L)/(fasting plasma glucose (mmol/L) − 3.5, where HOMA-β represents the relative β-cell function (expressed as a percentage).

Urinary albumin excretion (UAE) was measured as described in two 24 h urine collections and the results were averaged for analysis [31]. Estimated glomerular filtration rate (eGFR) was calculated applying the combined creatinine cystatin C-based Chronic Kidney Disease Epidemiology Collaboration equation.

For the diagnosis of NAFLD, the algorithm of the FLI was used [33]. The FLI was calculated according to the following formula: [e (0.953 × loge (triglycerides + 0.139 × BMI + 0.718 × loge (GGT) + 0.053 × waist circumference − 15.745)]/[1 + e (0.953 × loge (triglycerides) + 0.139 × BMI + 0.718 × loge (GGT) + 0.053 × waist circumference − 15.745)] × 100.

The optimal cut-off value for the FLI is documented to be 60 with an accuracy of 84%, a sensitivity of 61% and a specificity of 86% for detecting NAFLD as determined by ultrasonography [33]. FLI ≥60 was therefore used as proxy of NAFLD. The FLI is currently considered as one of the best-validated steatosis scores for larger scale screening studies [34]. Alternatively, we used the hepatic steatosis index (HSI) [35]. The HSI is defined as follows: HSI = 8 × ALT/AST ratio + BMI (+2, if diabetes; +2, if female), where ALT is alanine aminotransferase and AST is aspartate aminotransferase. The cut-off value of the HSI for detecting NAFLD is 36 [35].

The MetS was defined according to the revised National Cholesterol Education Program Adult Treatment Panel (NCEP-ATP) III criteria [36]. Participants were categorized with MetS when at least three out of the following five criteria were present: waist circumference >102 cm for men and >88 cm for women; plasma triglycerides ≥1.7 mmol/L; high-density lipoprotein (HDL) cholesterol <1.0 mmol/L for men and <1.3 mmol/L for women; hypertension (blood pressure ≥130/85 mm Hg or the use of antihypertensive medication); hyperglycaemia (fasting glucose ≥5.6 mmol/L or the use of glucose-lowering drugs).

Laboratory Methods

Venous blood samples were drawn after an overnight fast while participants rested for 15 min. Heparinized plasma and serum samples were obtained by centrifugation at 1400× *g* for 15 min at 4 °C. Plasma and serum samples were stored at −80 °C until analysis. EDTA plasma valine, leucine and isoleucine concentrations were measured using a Vantera Clinical Analyzer (LabCorp., Morrisville, NC, USA), a fully automated, high-throughput, 400 MHz proton (1H) nuclear magnetic resonance (NMR) spectroscopy platform. Plasma samples were prepared on board the instrument, and automatically delivered to the flow probe in the NMR spectrometer's magnetic field. The validation of the use of NMR for quantification of BCAAs has been previously described [20,21]. Data acquisition on the Vantera and the spectra data processing have been reported in greater detail elsewhere [37].

Fasting plasma glucose was measured by dry chemistry (Eastman Kodak, Rochester, NY, USA) directly after blood collection. Insulin was measured with an immunoturbidometric assay (Diazyme Laboratories, Poway, CA, USA). Plasma total cholesterol, triglycerides and HDL cholesterol were measured as previously

described [31,32]. Non-HDL cholesterol was calculated as the difference between total cholesterol and HDL cholesterol. Low-density lipoprotein (LDL) cholesterol was calculated by the Friedewald formula if triglycerides were <4.5 mmol/L. Serum ALT and AST were measured using the standardized kinetic method with pyridoxal phosphate activation (Roche Modular P, Roche Diagnostics, Mannheim, Germany). Serum GGT was assayed by an enzymatic colorimetric method (Roche Modular P, Roche Diagnostics, Mannheim, Germany). Standardization of ALT, AST and GGT was performed according to the International Federation of Clinical Chemistry guidelines [38–40]. High sensitivity C-reactive protein (hsCRP) was assayed by nephelometry (Dade Behring Diagnostic, Marburg, Germany). Serum creatinine was measured by an enzymatic method on a Roche Modular analyzer (Roche Diagnostics, Mannheim, Germany). Serum cystatin C was measured using Gentian Cystatin C Immunoassay (Gentian AS, Moss, Norway) reagents on a modular analyzer (Roche Diagnostics). Urinary albumin was measured by nephelometry (Dade Behring Diagnostic, Marburg, Germany).

T2D Development

Participants were followed from baseline outpatient visit until end of the follow-up period. Incident T2D was established if one or more of the four criteria were met during follow-up:

(1) Blood glucose ≥7.0 mmol/L (126 mg/dL); (2) Random sample plasma glucose ≥11.1 mmol/L (200 mg/dL); (3) Self-report of a physician diagnosis; (4) Initiation of glucose-lowering medication according to the central pharmacy registry follow-up data, which was completed on January 1, 2011.

Statistical Analysis

IBM SPSS software (version 23.0, IBM Corp., Armonk, NY, USA) was used for cross-sectional data analysis. R version 3.4.2 (Boston, MA, USA) and STATA version 13.1 (StataCorp LP, College Station, TX, USA) were used for prospective analyses. Cross-sectional data are expressed as mean ± standard deviation (SD), median with interquartile range (IQR) or as numbers (percentages). HOMA-IR and HOMA-β were $\log_e$ transformed for multivariable regression analyses.

Normality of distribution was assessed and checked for skewness. Between-group differences in variables were determined by unpaired *t*-tests for

normally distributed and $\log_e$ transformed variables, by Mann–Whitney U tests for non-normally distributed variables or by chi-squared tests for categorical variables where appropriate. Multivariable linear regression analyses were carried out to disclose the independent associations of BCAA levels with an elevated FLI or HSI while taking into account clinical covariates and laboratory parameters. Results from multivariable linear regression analyses are presented as standardized regression coefficients.

For the prospective analyses, we plotted cumulative Kaplan–Meier curves for T2D development during follow-up according to quartiles of plasma total BCAAs. Time-to-event Cox proportional hazards models were used to assess the hazard ratio (HR) with 95% confidence intervals (CI) of incident T2D.

A mediation analysis was performed to disclose the extent to which the plasma total BCAA concentration was a possible mediator between an elevated FLI and HSI, as proxies of NAFLD, and incident T2D, following the procedures as advocated by Preacher and Hayes [41,42]. The significance of the mediation effect was tested by computing bias-corrected bootstrap CIs with 2000 repetitions. Finally, the magnitude of mediation was calculated by dividing the coefficient of the indirect effect by the total effect. Significance of mediation was proved with $p < 0.05$ if zero was not between the lower and upper bound of the 95% CI of the indirect effect. Interaction terms were considered to be statistically significant at two-sided *p*-values <0.10 [43]. Otherwise, two-sided *p*-values <0.05 were considered significant.

Results

Baseline Clinical and Laboratory Characteristics of the Study Population

The study population consisted of 5791 participants free of T2D at baseline. There were 1671 participants (28.9%) with an FLI ≥60. Table 1 shows the clinical characteristics and laboratory data of the study population according to FLI categorization. Subjects with an FLI ≥60 were older, more likely to be men (67.8% vs. 32.2%) and more likely to be classified with MetS, history of cardiovascular disease and parental history of T2D. Consequently, subjects with an FLI ≥60 used antihypertensive medication and lipid-lowering drugs more frequently. Alcohol consumption ≥10 g/day was recorded in subjects with an elevated FLI more frequently, but cigarette smoking was not significantly different. BMI, waist circumference, waist/hip ratio, systolic and diastolic blood pressure, glucose, insulin, HOMA-IR, HOMA-β, hsCRP, transaminases, ALP, GGT,

UAE, total cholesterol, non-HDL cholesterol, LDL cholesterol and triglycerides were higher in subjects with FLI ≥60, whereas eGFR and HDL cholesterol were lower in subjects with an elevated FLI (Table 1). Plasma total BCAAs as well as valine, leucine and isoleucine concentrations were all higher in subjects with an FLI ≥60 (Table 1).

Table 1. Clinical and laboratory characteristics including plasma branched-chain amino acids in 4120 subjects with a fatty liver index (FLI) < 60 and 1671 subjects with an FLI ≥60.

	FLI < 60, n = 4120 (71.1%)	FLI ≥ 60, n = 1671 (28.9%)	*P* Value
Age (years)	49.6 (41.9–59.3)	56.0 (47.7–65.6)	<0.001
Sex (men/women)	1707 (41.4)/2413 (58.6)	1133 (67.8)/538 (32.2)	<0.001
MetS	296 (7.2)	946 (56.6)	<0.001
History of cardiovascular disease	169 (4.1)	155 (9.3)	<0.001
Parental history of T2D	577 (14.0)	266 (15.9)	0.061
Current smokers	1167 (28.3)	457 (27.3)	0.454
Alcohol ≥10 g/day	137 (3.4)	109 (6.6)	<0.001
Antihypertensive medication	560 (13.6)	556 (33.3)	<0.001
Lipid-lowering drugs	249 (6.0)	234 (14.0)	<0.001
SBP (mm Hg)	121 ± 17	134 ± 18	<0.001
DBP (mm Hg)	71 ± 8	77 ± 9	<0.001
BMI (kg/m2)	24.7 ± 2.8	30.8 ± 4.0	<0.001
Waist circumference	85.8 ± 9.1	104.8 ± 8.9	<0.001
Waist/hip ratio	0.87 ± 0.07	0.96 ± 0.07	<0.001
Glucose (mmol/L)	4.71 ± 0.58	5.10 ± 0.67	<0.001
Insulin (mU/L)	6.80 (5.1–9.2)	12.50 (9.2–18.1)	<0.001
HOMA-IR (mU mmol/L2/22.5)	1.42 (1.04–1.98)	2.86 (2.00–4.17)	<0.001
HOMA-β (%)	25.55 (18.61–35.31)	46.92 (33.29–66.68)	<0.001
hsCRP (mg/L)	1.00 (0.48–2.26)	2.35 (1.17–4.25)	<0.001
ALT (U/L)	15 (12–20)	23 (17–32)	<0.001
AST (U/L)	21 (19–25)	25 (21–29)	<0.001
ALP (U/L)	63 ± 19	72 ± 22	<0.001
GGT (U/L)	19 (13–27)	39 (27–60)	<0.001
eGFR (mL/min/1.73 m^2)	95.9 (84.2–105.7)	88.4 (76.4–99.4)	<0.001
UAE (mg/24 h)	7.4 (5.6–11.0)	9.7 (6.6–17.3)	<0.001
TC (mmol/L)	5.31 ± 1.00	5.71 ± 1.03	<0.001
Non-HDLC (mmol/L)	3.96 ± 0.98	4.60 ± 1.00	<0.001
LDL cholesterol (mmol/L)	3.49 ± 0.90	3.79 ± 0.92	<0.001
HDL cholesterol (mmol/L)	1.35 ± 0.30	1.11 ± 0.24	<0.001
Triglycerides (mmol/L)	0.94 (0.71–1.26)	1.66 (1.26–2.19)	<0.001
Total BCAAs (μM)	356.90 ± 62.58	425.48 ± 67.78	<0.001
Valine (μM)	197.22 ± 33.20	229.74 ± 35.48	<0.001
Leucine (μM)	119.78 ± 23.64	144.48 ± 27.63	<0.001
Isoleucine (μM)	39.74 ± 13.47	51.19 ± 15.99	<0.001

Data are given in number with percentages (%), mean ± standard deviation (SD) for normally distributed data or median with interquartile ranges (IQR) for non-normally distributed data. Abbreviations: ALP, alkaline phosphatase; ALT, aminotransferase; AST, aspartate aminotransferase; BCAA, branched-chain amino acids; BMI, body mass index; DBP, diastolic blood pressure; eGFR, estimated glomerular filtration rate; FLI, fatty liver index; GGT, gamma-glutamyltransferase; HOMA, Homeostasis Model Assessment; HDL, high-density lipoproteins; hsCRP, high sensitivity C-reactive protein; IR, insulin resistance; LDL, low-density lipoproteins; MetS, metabolic syndrome; SBP, systolic blood pressure; T2D, type 2 diabetes; TC, total cholesterol; UAE, urinary albumin excretion. LDL cholesterol was calculated by the Friedewald formula.

Cross-Sectional Associations of BCAA with an Elevated FLI and HSI

Multivariable linear regression analyses were subsequently performed in order to establish the extent to which plasma BCAA concentrations were independently associated with an elevated FLI (Table 2). In an age- and sex-adjusted analysis, a positive association of plasma BCAA concentrations with an elevated FLI was found (Table 2, Model 1, $\beta = 0.326$, $p < 0.001$). This positive association of plasma BCAA concentrations with an elevated FLI was also demonstrated after further adjustment for family history of T2D (Table 2, Model 2, $\beta = 0.324$, $p < 0.001$), alcohol intake and current smoking (Table 2, Model 3, $\beta = 0.323$, $p < 0.001$), eGFR, UAE, use of antihypertensive medication and lipid-lowering drugs (Table 2, Model 4, $\beta = 0.318$, $p < 0.001$) and, finally, after additional adjustment for HOMA-IR and HOMA-β (Table 2, Model 5, $\beta = 0.208$, $p < 0.001$). In alternative analyses with an elevated HSI instead of an elevated FLI, similar independent positive associations of plasma BCAA concentrations with an elevated HSI were found (Table S1, all models $p < 0.001$).

Prospective Analyses of FLI and BCAA with Incident T2D

During a median follow-up of 7.3 years (IQR 5.7–7.7), a total of 276 participants developed T2D, of which 194 (70.2%) subjects had an elevated FLI (Table 3). Figure 1 shows the Kaplan–Meier curves for incident T2D survival according to non-elevated FLI (<60) and elevated FLI (≥60), which shows a significantly increased risk of incident T2D in subjects with an elevated FLI (log-rank test $p < 0.001$). Comparing associations of non-elevated and elevated FLI with incident T2D in Cox regression analyses showed an HR of 5.84 (95% CI 4.46–7.66, $p < 0.001$) when adjusted for age and sex (Table 3). This positive association remained present after further for family history of T2D, HR 5.72 (95% CI 4.37–7.50, $p < 0.001$); alcohol intake and smoking, HR 5.64 (95% CI 4.31–7.39, $p < 0.001$); eGFR, UAE, antihypertensive and lipid-lowering drugs, HR 5.09 (95% CI 3.77–6.88, $p < 0.001$); HOMA-IR and HOMA-β, HR 3.84 (95% CI 2.76–5.36, $p < 0.001$); and, finally, for BCAAs, HR 3.46 (95% CI 2.45–4.87, $p < 0.001$) (Table 3).

Table 2. Multivariable linear regression analysis demonstrating the positive association of plasma branched-chain amino acids with an elevated fatty liver index (FLI) (≥60) after adjustment for clinical and laboratory covariates in 5791 subjects.

	Model 1		Model 2		Model 3		Model 4		Model 5	
	β	*P* Value	β	*P* Value	β	*P* Value	β	*P* Value	β	*P* Value
Age	0.015	0.173	0.014	0.199	0.01	0.339	−0.032	0.035	−0.046	0.002
Sex (men vs. women)	0.424	<0.001	0.426	<0.001	0.426	<0.001	0.431	<0.001	0.431	<0.001
FLI ≥60 vs. <60	0.326	<0.001	0.324	<0.001	0.323	<0.001	0.318	<0.001	0.208	<0.001
Family history of T2D (yes/no)			0.045	<0.001	0.048	<0.001	0.046	<0.001	0.033	0.002
Alcohol intake (≥10 g/day)					0.007	0.495	0.011	0.325	0.014	0.207
Current smoking (yes/no)					−0.043	<0.001	−0.044	<0.001	−0.030	0.005
eGFR (mL/min/1.73 m^2)							−0.057	<0.001	−0.046	0.002
UAE (mg/24 h)							−0.015	0.165	−0.021	0.058
Use of antihypertensive medication							0.009	0.478	−0.011	0.376
Use of lipid-lowering drugs							0.026	0.026	0.01	0.395
HOMA-IR									0.271	<0.001
HOMA-β									−0.043	0.089

β: standardized regression coefficients. HOMA-IR and HOMA-β were $\log_e$ transformed for analyses. Model 1: adjusted for age and sex. Model 2: Model1 plus family history of type 2 diabetes. Model 3: Model 2 adjusted for alcohol intake and current smoking. Model 4: Model 3 adjusted for eGFR, urinary albumin excretion and use of antihypertensive medication and lipid-lowering drugs. Model 5: Model 4 adjusted for and lipid-lowering drugs, HOMA-IR and HOMA-β.

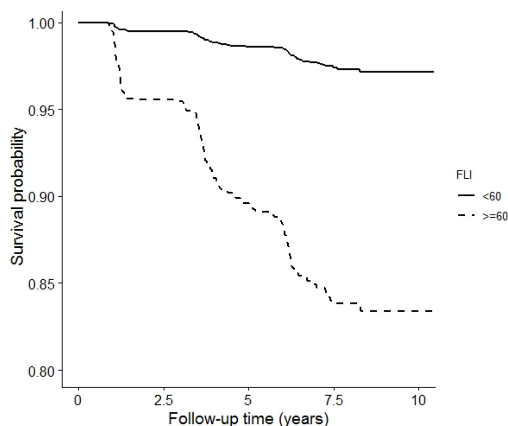

Figure 1. Kaplan–Meier curves for incident type 2 diabetes survival according to FLI score. Log-rank test (p < 0.001). FLI, fatty liver index.

Similar Cox regression analyses were performed to address the relationship between BCAA (per 1 SD increment) and incident T2D (Table 4), which showed a similar pattern with T2D risk. However, in the fully adjusted model 6, additional adjustment for an elevated FLI attenuated the hazard ratio to 1.19 (95% CI 1.03–1.37, p = 0.01) (Table 4).

Table 3. Prospective associations of FLI with incident type 2 diabetes.

	Non-Elevated FLI (<60)	**Elevated FLI (≥60)**	
Participants, *n*	4120	1671	
Incident T2D, *n* (%)	82 (2.0)	194 (11.6)	
	HR (95% CI)	**HR (95% CI)**	***P* Value**
Crude Model	(ref)	6.78 (5.23–8.79)	<0.001
Model 1	(ref)	5.84 (4.46–7.66)	<0.001
Model 2	(ref)	5.72 (4.37–7.50)	<0.001
Model 3	(ref)	5.64 (4.31–7.39)	<0.001
Model 4	(ref)	5.09 (3.77–6.88)	<0.001
Model 5	(ref)	3.84 (2.76–5.36)	<0.001
Model 6	(ref)	3.46 (2.45–4.87)	<0.001

Data are presented as hazard ratio (HR) with 95% confidence interval (CI). FLI, fatty liver index; BCAA, branched-chain amino acids; T2D, type 2 diabetes. Model 1: adjusted for age and sex. Model 2: Model 1 adjusted for family history of type 2 diabetes. Model 3: Model 2 adjusted for alcohol intake and current smoking. Model 4: Model 3 adjusted for eGFR, UAE, antihypertensive medication and lipid-lowering drugs. Model 5: Model 4 adjusted for HOMA-IR and HOMA-β. Model 6: Model 5 adjusted for BCAAs.

There was no significant interaction of elevated FLI with age (p = 0.11) and there was a marginally significant interaction with sex (p = 0.07). In Cox regression analyses in which an elevated HSI was used instead, an elevated HSI was similarly associated with incident T2D, which remained associated with incident T2D independent of adjustment for plasma total BCAAs, whereas in analyses with plasma total BCAAs as determinant of incident T2D, the association of plasma total BCAAs with incident T2D was reduced

after adjustment for an elevated HSI (Tables S2 and S3, Supplementary Materials). There was no significant interaction of sex with an elevated HSI on incident T2D (*p* = 0.36 for sex interaction) and there was a marginally significant interaction of elevated HSI with age (*p* = 0.07).

Table 4. Prospective associations of FLI with incident type 2 diabetes.

	BCAA Per 1 SD Increment	
Participants, *n*	5791	
Incident T2D, *n* (%)	276 (4.8)	
	HR (95% CI)	***P* Value**
Crude Model	1.68 (1.57–1.81)	<0.001
Model 1	1.65 (1.52–1.79)	<0.001
Model 2	1.63 (1.50–1.77)	<0.001
Model 3	1.64 (1.50–1.79)	<0.001
Model 4	1.64 (1.49–1.81)	<0.001
Model 5	1.35 (1.20–1.53)	<0.001
Model 6	1.19 (1.03–1.37)	0.01

Data are presented as hazard ratio (HR) with 95% confidence interval (CI). FLI, fatty liver index; BCAA, branched-chain amino acids; T2D, type 2 diabetes. Model 1: adjusted for age and sex. Model 2: Model 1 adjusted for family history of type 2 diabetes. Model 3: Model 2 adjusted for alcohol intake and current smoking. Model 4: Model 3 adjusted for eGFR, UAE, antihypertensive medication and lipid-lowering drugs. Model 5: Model 4 adjusted for HOMA-IR and HOMA-β. Model 6: Model 5 adjusted for FLI elevated (yes/no).

Additionally, mediation analyses were performed to disclose the extent to which the plasma total BCAA concentration was a possible mediator between elevated FLI or HSI and incident T2D. BCAAs appeared to be a mediator in the association of FLI with incident T2D (Figure 2). The indirect pathway was significant (B = 0.040, 95% CI 0.027–0.054, *p*-indirect < 0.001) and the magnitude of mediation was 19.6% (Table 5). In alternative analyses, BCAAs appeared to be a mediator in the association of HSI with incident T2D (Figure 3). The indirect pathway was significant (B = 0.033, 95% CI 0.026–0.041, *p*-indirect < 0.001) and the magnitude of mediation was 22.6% (Table 6).

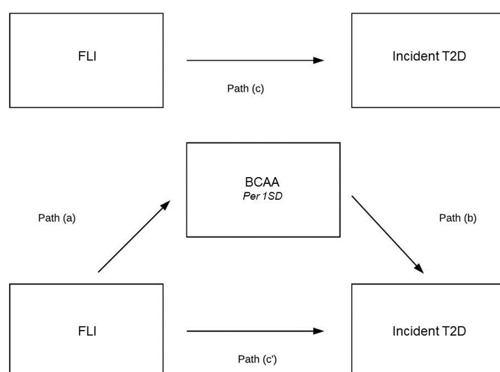

Figure 2. Mediation analysis on the association of FLI with incident type 2 diabetes. (a)–(c) are the regression coefficients between variables. The indirect effect is calculated as a × b. Total effect (c) is a × b + c′. Magnitude of mediation is calculated as indirect effect divided by total effect.

Table 5. Mediating effect of BCAAs on the association of FLI with incident type 2 diabetes.

	Coefficient (95% CI) *	Proportion Mediated
Indirect pathway (ab path)	B = 0.040 (95% CI 0.027–0.054)	19.6% **
Total effect (ab + c′ path)	B = 0.204 (95% CI 0.174–0.232)	

Analyses were performed according to Preacher and Hayes Procedure. B: unstandardized regression coefficient. Coefficients are adjusted for age and sex. * 95% CIs were bias-corrected confidence intervals after running 2000 bootstrap samples. ** The size of the significant mediated effect is calculated as the standardized indirect effect divided by the standardized total effect multiplied by 100.

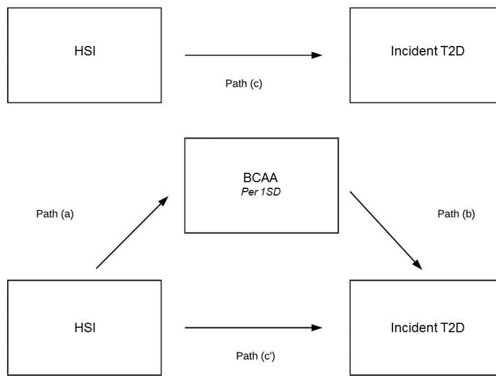

Figure 3. Mediation analysis on the association of HSI with incident type 2 diabetes. (a)–(c) are the regression coefficients between variables. The indirect effect is calculated as a × b. Total effect (c) is a × b + c′. Magnitude of mediation is calculated as indirect effect divided by total effect.

Table 6. Mediating effect of BCAAs on the association of HSI with incident type 2 diabetes.

	Coefficient (95% CI) *	Proportion mediated
Indirect pathway (ab path)	B = 0.033 (95% CI 0.026–0.041)	22.6% **
Total effect (ab + c′ path)	B = 0.146 (95% CI 0.115–0.176)	

Analyses were performed according to Preacher and Hayes Procedure. B: unstandardized regression coefficient. Coefficients are adjusted for age and sex. * 95% CIs were bias-corrected confidence intervals after running 2000 bootstrap samples. ** The size of the significant mediated effect is calculated as the standardized indirect effect divided by the standardized total effect multiplied by 100.

Discussion

In this large-scale study among a predominantly Caucasian population, who were free of T2D at the baseline evaluation, we have first cross-sectionally demonstrated that fasting total plasma BCAA levels are elevated in subjects with NAFLD and that this relationship of BCAAs with NAFLD is independent of a considerable number of clinical and laboratory covariates, including measures of insulin resistance and β-cell function. Second, we have longitudinally documented that an elevated FLI predicts the development of T2D, an association that was in part attributable to higher plasma total BCAAs as inferred from mediation analyses. Taken together, the current results not only indicate

that there is an intricate relationship between NAFLD and elevated plasma BCAA levels, but also suggest that NAFLD and elevated circulating BCAAs could act together on T2D development. In this study, we have used the FLI [33] as proxy of NAFLD, which is in line with recommendations of international guidelines [34], advocating the use of biomarker-derived algorithms in order to categorize subjects with probable NAFLD in large-scale studies. Alternative analyses using the HSI as proxy of NAFLD [35] confirmed the findings, supporting the validity of our results.

The cross-sectional part of our study demonstrated that plasma BCAA elevations were independently associated with suspected NAFLD, irrespective of whether an elevated FLI or an elevated HSI was used as a proxy of NAFLD. In comparison, recent studies have also shown that plasma BCAAs are elevated in the context of (obesity-associated) NAFLD, and that BCAAs may aggravate mitochondrial dysfunction in NAFLD [13,19]. Obesity, MetS and T2D are all related to plasma BCAA elevations [24], and elevated BCAA levels in NAFLD are likely linked to increased insulin resistance and protein catabolism [13,24].

Accumulating evidence points to a key role of mitochondrial dysfunction in the pathophysiology and progression of NAFLD, possibly caused by increased mitochondrial β-oxidation of fatty acids in insulin-resistant states [28,29] and by impaired adaptation of hepatic mitochondrial function in NAFLD [30]. A dysfunctional hepatic tricarboxylic acid (TCA) cycle is regarded as a central feature of hepatic insulin resistance. BCAAs are essential to mediate the transport of carbon substrates for oxidation through the mitochondrial TCA cycle, and an impaired upregulation of BCAA-mediated TCA is thought to be a significant contributor of mitochondrial dysfunction in NAFLD [19]. Moreover, the hepatic accumulation of BCAAs is mainly regulated by the activities of transporters including the SLC43A1 (LAT3), which are responsible for controlling the efflux of BCAAs from the liver to the circulation [45]. In addition to the accumulation of BCAAs in hepatocytes, hepatic BCAA-degrading enzymes are downregulated during worsening of NAFLD [46]. Taken together, it seems plausible that hepatic fat accumulation and altered BCAA metabolism have a negative impact on each other in worsening of NAFLD and further deteriorating BCAA metabolism and their accumulation in the circulation. Notably, the association of plasma BCAA elevations with suspected NAFLD, as cross-sectional documented in the current study, was found to be independent of HOMA-IR and β-cell function. We did not adjust for BMI or waist circumference in the

multivariable analysis because obesity indices comprise part of the FLI and HSI algorithms. Such an association of plasma BCAA elevations with suspected NAFLD would suggest that maladaptation in fatty liver may contribute to mitochondrial dysfunction [13], resulting in plasma BCAA elevations.

Our findings regarding the longitudinal association of an elevated FLI with incident T2D was anticipated. Of note, a similar association was found in alternative analysis using an elevated HSI instead. Two recent smaller-scaled studies among subjects of European ancestry, namely, the European Prospective Investigation into Cancer and Nutrition-Potsdam study and a cohort of Spanish adults with pre-diabetes, have demonstrated that an elevated FLI is associated with increased risk of T2D development [6,7]. We also anticipated that plasma BCAA elevations predicted T2D risk [15]. However, to the best of our knowledge, our study shows for the first time that the association of suspected NAFLD with incident T2D appears to be in part attributable to plasma BCAA elevations. In the current study, mediation analysis suggested that 19.6% of the association of an elevated FLI and, alternatively, 22.6% of the association of an elevated HSI with incident T2D was mediated by plasma BCAA concentrations. Hepatic fat accumulation has a deleterious role in T2D development [3,44], and recent evidence shows accumulation of BCAAs in the liver to take place in the context of progressive steatohepatitis [47]. Although our study supports the hypothesis that higher BCAA levels promote the development of T2D, it is clear that further research is needed regarding the mechanisms whereby altered BCAA metabolism and NAFLD may act together to deteriorate glucose tolerance, an effect which seems, at least in part, independent of insulin resistance as demonstrated here with respect to both elevated FLI- and BCAA-associations with incident T2D.

Our study has several strengths. To the best of our knowledge, this is the first study reporting on the joint contributions of NAFLD and higher total plasma BCAAs on the development of T2D. Furthermore, considering a sample size of over 5500 individuals, this is the largest study to date reporting on the association of both suspected NAFLD and plasma BCAA levels with T2D development, which enabled us to carry out sufficiently powered multivariable adjusted analyses. Furthermore, we also used a robust methodology, adjusting our results for relevant variables such as HOMA-IR to investigate the association between FLI and incident T2D. Several other methodological aspects and limitations also need to be addressed. First, the PREVEND cohort study mainly

comprises individuals of European ancestry, which could limit extrapolation of our findings to other ethnicities. Second, the FLI is not an absolute measure of hepatic fat accumulation and thus some over- and underestimation of NAFLD could have occurred. However, the FLI is considered to have sufficient accuracy for NAFLD assessment and has been validated against magnetic resonance spectroscopy with moderate diagnostic accuracy for NAFLD [48]. Indeed, the use of the FLI is in line with international guidelines to apply biomarker scores in order to characterize NAFLD in larger-sized cohorts [33,34]. Additionally, liver biopsy, with well-known limitations with respect to invasiveness and sampling variability, or liver ultrasound were not feasible in the PREVEND cohort study, which recruited individuals from the general population. Moreover, the positive associations of plasma BCAA levels with suspected NAFLD and prospective associations with incident T2D were confirmed using the HSI as an alternative algorithm for NAFLD categorization [35]. Third, we did not have measurements of insulin and BCAA levels beyond baseline assessment, which limited us to evaluate the evolution of insulin resistance and regression dilution of BCAAs could not be excluded. Therefore, underestimation of the BCAA–incident T2D associations could have occurred. Fourth, the proportion of subjects using alcohol in excess of 30 g per day in the PREVEND is low (5.2%) [49], and we adjusted for alcohol consumption in all analyses. Finally, people with micro-albuminuria preferentially participated in the PREVEND cohort and therefore multivariable and prospective analyses were adjusted for eGFR and UAE, showing positive and independent associations of plasma BCAA levels in NAFLD and incident T2D development.

Conclusions

This large-scale population study cross-sectionally demonstrated that elevated plasma BCAA levels are positively associated with an elevated FLI, as a proxy of NAFLD. Furthermore, it was longitudinally shown that the association of NAFLD with T2D development is likely to be attributable in part to plasma BCAA elevations, which likely reflects abnormalities in BCAA metabolism.

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Table S1. Multivariable linear regression analysis demonstrating the positive association of plasma branched-chain amino acids with an elevated hepatic steatosis index (HSI) (>36) after adjustment for clinical and laboratory covariates in 5791 subjects.

	Model 1		Model 2		Model 3		Model 4		Model 5	
	β	*P* Value	β	*P* Value	β	*P* Value	β	*P* Value	β	*P* Value
Age	0.05	<0.001	0.049	<0.001	0.047	<0.001	-0.019	0.217	-0.041	0.007
Sex (men vs. women)	0.503	<0.001	0.505	<0.001	0.502	<0.001	0.505	<0.001	0.474	<0.001
FLI ≥60 vs. <60	0.244	<0.001	0.242	<0.001	0.24	<0.001	0.231	<0.001	0.116	<0.001
Family history of T2D (yes/no)			0.044	<0.001	0.046	<0.001	0.045	<0.001	0.03	0.005
Alcohol intake (≥10 g/day)					0.018	0.107	0.021	0.06	0.022	0.046
Current smoking (yes/no)					-0.026	0.019	-0.029	0.011	-0.019	0.076
eGFR (mL/min/1.73 m2)							-0.078	<0.001	-0.057	<0.001
UAE (mg/24 h)							-0.007	0.557	-0.016	0.143
Use of antihypertensive medication							0.029	0.021	0	0.994
Use of lipid-lowering drugs							0.032	0.007	0.012	0.278
HOMA-IR									0.318	<0.001
HOMA-β									-0.045	0.083

β: standardized regression coefficients. HOMA-IR and HOMA-β were $\log_e$ transformed for analyses. Model 1: adjusted for age and sex. Model 2: Model1 plus family history of type 2 diabetes. Model 3: Model 2 adjusted for alcohol intake and current smoking. Model 4: Model 3 adjusted for eGFR, urinary albumin excretion and use of antihypertensive medication and lipid-lowering drugs. Model 5: Model 4 adjusted for and lipid-lowering drugs, HOMA-IR and HOMA-β.

Table S2. Prospective associations of HSI with incident type 2 diabetes.

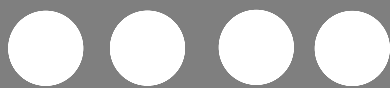
	Non-Elevated HSI	Elevated HSI	
	≤36	**>36**	
Participants, *n*	4328	1463	
Incident T2D, *n* (%)	128 (3.0)	148 (10.1)	
	HR (95% CI)	**HR (95% CI)**	***P* Value**
Crude Model	(ref)	3.69 (2.91–-4.68)	<0.001
Model 1	(ref)	3.57 (2.81–4.52)	<0.001
Model 2	(ref)	3.47 (2.73–4.40)	<0.001
Model 3	(ref)	3.53 (2.78–4.48)	<0.001
Model 4	(ref)	3.08 (2.36–4.00)	<0.001
Model 5	(ref)	2.20 (1.62–2.99)	<0.001
Model 6	(ref)	1.99 (1.45–2.17)	<0.001

Data are presented as hazard ratio (HR) with 95% confidence interval (CI). HSI, hepatic steatosis index; BCAA, branched-chain amino acids; T2D, type 2 diabetes. Model 1: adjusted for age and sex. Model 2: Model 1 adjusted for family history of type 2 diabetes. Model 3: Model 2 adjusted for alcohol intake and current smoking. Model 4: Model 3 adjusted for eGFR, UAE, antihypertensive medication and lipid-lowering drugs. Model 5: Model 4 adjusted for HOMA-IR and HOMA-β. Model 6: Model 5 adjusted for BCAAs.

Table S3. Prospective associations of HSI with incident type 2 diabetes.

	BCAA Per 1 SD Increment	
Participants, *n*	5791	
Incident T2D, *n* (%)	276 (4.8)	
	HR (95%CI)	***p*-Value**
Crude Model	1.68 (1.57–1.81)	<0.001
Model 1	1.65 (1.52–1.79)	<0.001
Model 2	1.63 (1.50–1.77)	<0.001
Model 3	1.64 (1.50–1.79)	<0.001
Model 4	1.64 (1.49–1.81)	<0.001
Model 5	1.35 (1.20–1.53)	<0.001
Model 6	1.29 (1.13–1.48)	<0.001

Data are presented as hazard ratio (HR) with 95% confidence interval (CI). HSI, hepatic steatosis index; BCAA, branched-chain amino acids; T2D, type 2 diabetes. Model 1: adjusted for age and sex. Model 2: Model 1 adjusted for family history of type 2 diabetes. Model 3: Model 2 adjusted for alcohol intake and current smoking. Model 4: Model 3 adjusted for eGFR, UAE, antihypertensive medication and lipid-lowering drugs. Model 5: Model 4 adjusted for HOMA-IR and HOMA-β. Model 6: Model 5 adjusted for HSI elevated (yes/no).



Chapter 4

A Newly Developed Diabetes Risk Index, Based on Lipoprotein Subfractions and Branched Chain Amino Acids, is Associated with Incident Type 2 Diabetes Mellitus in the PREVEND Cohort.

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Abstract

Objective: Evaluate the ability of a newly developed diabetes risk score, the Diabetes Risk Index (DRI), to predict incident type 2 diabetes mellitus (T2D) in a large adult population. Methods: The DRI was developed by combining the Lipoprotein Insulin Resistance Index (LP-IR), calculated from 6 lipoprotein subspecies and size parameters, and the branched chain amino acids, valine and leucine, all of which have been shown previously to be associated with future T2D. DRI scores were calculated in a total of 6134 nondiabetic men and women in the Prevention of Renal and Vascular End-Stage Disease (PREVEND) Study. Cox proportional hazards regression was used to evaluate the association of DRI scores with incident T2D. Results: During a median follow-up of 8.5 years, 306 new T2D cases were ascertained. In analyses adjusted for age and sex, there was a significant association between DRI scores and incident T2D with the hazard ratio (HR) for the highest versus lowest quartile being 12.07 (95% confidence interval: 6.97–20.89, $p < 0.001$). After additional adjustment for body mass index (BMI), family history of T2D, alcohol consumption, diastolic blood pressure, total cholesterol, triglycerides, HDL cholesterol and HOMA-IR, the HR was attenuated but remained significant (HR 3.20 [1.73–5.95], $p = 0.001$). Similar results were obtained when DRI was analyzed as HR per 1 SD increase (HR 1.37 [1.14–1.65], $p < 0.001$). The Kaplan–Meier plot demonstrated that patients in the highest quartile of DRI scores presented at higher risk (p-value for log-rank test <0.001). Conclusions: Higher DRI scores are associated with an increased risk of T2D. The association is independent of clinical risk factors for T2D including HOMA-IR, BMI and conventional lipids.

Introduction

In order to curtail the growing epidemic of obesity and type 2 diabetes mellitus (T2D), new clinical practice guidelines recommend structured lifestyle modification and/or pharmacological intervention for patients who are at high risk of developing T2D [1,2]. Over 80 million adults in the United States alone qualify as being high risk based on their glycemic status [3]. Early intervention in individuals who are insulin resistant but have not yet shown signs of impaired glucose tolerance or fasting dysglycemia, may prevent or delay the progression to T2D [4,5]. Multiple clinical studies have also shown that lifestyle interventions and pharmacological therapies are able to delay to an important extent the onset of T2D even in subjects who are already experiencing dysglycemia [6–9].

Genome-wide association studies have identified more than 200 genetic loci which are associated with development of T2D [10,11]. Currently, application of this genetic information is more likely to be used in support of studies on pathophysiological understanding of T2D rather than to be applied in actual clinical risk prediction, where biomarkers still are most promising [12]. However, disappointingly, few biomarker candidates provide improvement of T2D risk prediction over conventional measures of glycaemia and adiposity [12]. Therefore, there remains a clinical need for diagnostic tools that identify high risk patients in order to employ therapeutic measures early in the course of worsening dysglycemia.

The Lipoprotein Insulin Resistance Index (LP-IR) is a multimarker score derived from six lipoprotein subclass and size parameters measured clinically by the high-throughput NMR LipoProfile® test [13,14]. LP-IR scores (0–100) reflect the magnitude of insulin resistance in individual patients and exhibit strong associations with homeostatic model assessment of insulin resistance (HOMA-IR) and the glucose disposal rate (GDR) assessed by hyperinsulinemic-euglycemic clamp, and have been shown to reflect both peripheral and hepatic insulin resistance [15].

LP-IR scores have been shown recently to predict future T2D in several studies, namely the Multi-Ethnic Study of Atherosclerosis (MESA) [16], Women's Health Study [17], Justification for the Use of Statins in Prevention: an Intervention Trial Evaluating Rosuvastatin trial [18] and Prevention of Renal and Vascular End Stage Disease (PREVEND) [19]. In all four of these studies, LP-IR

scores were strongly associated with incident T2D even after adjustment for known T2D risk factors and in individuals at low risk for T2D based on their clinical profiles [16–19]. Importantly, lifestyle interventions have been shown to lower LP-IR scores, suggesting that LP-IR may be useful for monitoring treatments that may prevent or delay the onset of T2D [20–23].

Plasma levels of branched chain amino acids (BCAA), i.e., leucine, valine, and isoleucine, have also been shown to be higher in insulin resistant conditions and to be associated with development of T2D [24–29]. Moreover, it has been proposed that reducing elevated BCAA levels may provide a therapeutic approach for treating insulin resistance [30]. Since concentrations of BCAA and other metabolites are measured at no incremental analytic cost during NMR LipoProfile® testing [31], we hypothesized that a multimarker score combining LP-IR and BCAA would provide an enhanced clinical ability to stratify T2D risk in individuals with similar glucose levels. To this end, data from MESA [16] were used to develop a new multimarker called the Diabetes Risk Index (DRI).

The aim of this study was to assess the ability of DRI scores to predict future T2D in the PREVEND study, a large cohort of adults from the general population.

Materials and Methods

Study Design and Participants

The PREVEND study was approved by the local medical ethics committee at the University Medical Center Groningen (approval number: MEC96/01/022). All participants provided written informed consent and all procedures were conducted according to the Declaration of Helsinki. Details of the study design and recruitment have been described elsewhere [32]. Briefly, the PREVEND study is a Dutch cohort drawn from the general population of the city of Groningen in the northern part of the Netherlands. After exclusion of subjects with insulin-treated diabetes and pregnant women, all subjects with a urinary albumin concentration ≥10 mg/L were invited to participate (n = 7768), of whom 6000 accepted.

In addition, a random sample of 2592 individuals with a urinary albumin concentration <10 mg/L was included. These 8592 subjects (aged 28–75 years)

completed the baseline survey (1997–1998). The second screening, which was the starting point of the current study, took place between 2001 and 2003 (n = 6892). For the current study, subjects with T2D at baseline, missing data on diabetes or glucose at baseline, and those with missing NMR or covariate data at baseline and follow-up were excluded, leaving 6134 subjects for the present analyses.

Follow-up time was defined as the period between the second screening round (baseline) and the date of ascertainment of T2D. Follow-up time was censored at 8.5 years. In case a person moved to an unknown destination, census date was the date of removal from the municipal registry. Incident cases of diabetes were ascertained if one or more of the following criteria were met: (1) fasting plasma glucose (FPG) ≥ 7.0 mmol/L (126 mg/dL); (2) random sample plasma glucose ≥ 11.1 mmol/L (200 mg/dL); (3) self-report of a physician diagnosis of T2D and (4) initiation of glucose-lowering medication use, retrieved from a central pharmacy registry [33,34].

Laboratory Measurements

Venous blood was obtained after an overnight fast. EDTA plasma samples were prepared by centrifugation at 4 °C as per manufacturer's instructions. Total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C) and triglycerides (TG) were measured on a Beckman Coulter® AU680 Analyzer [35]. Fasting plasma glucose was measured by dry chemistry (Eastman Kodak, Rochester, NY, USA). HOMA-IR was calculated as fasting plasma insulin (µU/mL) × (FPG (mg/dL) × 0.055)/22.5 and values were log transformed for analysis.

EDTA plasma samples from the second screening were stored at <−70 °C until being shipped to LabCorp for NMR LipoProfile® testing. NMR spectra were collected on a Vantera® Clinical Analyzer and LP-IR scores were calculated and branched chain amino acids were quantified as previously described [13,14,31].

DRI Development

The DRI score was designed to improve upon the established performance of LP-IR as a clinical predictor of T2D by combining it with BCAA measures, elevated levels of which are linked to diabetes risk by possibly novel mechanisms [26,27]. To determine how best to combine LP-IR and BCAA to optimize T2D prediction, we employed logistic regression using the same dataset used for the

development of LP-IR [15], namely baseline NMR data from MESA comprised of 4982 U.S. adults (mean age 62 years; range 45–84) without cardiovascular disease or diabetes, of whom 595 developed T2D during a mean 7.8-year follow-up [16]. Initial development work used total BCAA (sum of valine, leucine, and isoleucine), but subsequently it was found that better performance was achieved without isoleucine, possibly because measurement precision for isoleucine (ranging from 8.8%–21.3% CV for within run and within lab imprecision) is not as good as that for valine (1.7%–5.4% CV) and leucine (4.4%–9.1% CV) [31]. In a logistic regression model including age, sex, race, and fasting glucose, both LP-IR and a BCAA parameter (valine + 2 × leucine) contributed independently to prediction of incident diabetes and the regression coefficients from this model were used as weighting factors for the following equation: DRI = 0.0167 [LP-IR] + 1.907 [ln (valine + 2 × leucine)]. For clinical use, the DRI values were transformed into a 1–100 score, using 1st and 99th percentile values to define the low and high limits of the range. The coefficients of variation for intra- and inter-assay precision ranged from 3.9%–6.4% and 2.7%–7.9% for LP-IR and DRI, respectively.

Statistical Analyses

All statistical analyses were performed with R language for statistical computing software [36], v. 3.6.2 and the integrated development environment (IDE) RStudio [37], v.1.2.5019. For all analyses, two-sided p values <0.05 were considered statistically significant, except for interaction terms for which in agreement with existing literature, the level of significance was set at $p < 0.10$ [38].TG and HOMA-IR were log transformed when used as a continuous variable in the analysis.

Baseline characteristics were calculated across sex-specific quartiles of DRI scores. p-values across quartiles of DRI were determined by linear regression for continuous variables or chi-square test for categorical variables. Cox proportional hazards regression analysis was performed to examine the associations of DRI across quartiles calculated in the whole study population with the risk of developing T2D. In addition, hazards were calculated per 1 standard deviation (SD) increment of DRI. Hazard ratios (HR) were expressed with 95% confidence intervals (CI). Harrell's c-index was calculated with and without the addition of DRI scores. To identify the best-fitting model, the Bayesian Information Criterion and the Akaike Information Criterion were

computed [39,40]. In addition, the performance of the enriched model with LPIR and DRI in terms of true positive rates and false positive rate was evaluated with the McNemar test [41].

The Net Reclassification Index (NRI) [42] was calculated using the Framingham Offspring Study T2D risk score [43] with and without the addition of DRI scores as a continuous variable, considering predefined risk categories of type 2 diabetes development (<10%), intermediate (10% to 20%), and high (≥20%) [44]

Results

Baseline characteristics

Of the PREVEND participants that completed the second round of screening, 6134 subjects who did not have T2D and had complete data available on DRI and covariates at the time of screening were included in this study. Baseline characteristics for these subjects, stratified by quartiles of DRI scores, can be found in Table 1. Participants with higher DRI scores were more likely to be men and were older in age. They were also more likely to have a parental history of T2D, be current smokers, consume more alcohol, and to be on lipid lowering or hypertensive medications. Those in the highest quartile of DRI scores had higher BMI, systolic and diastolic blood pressure, TC, TG, LDL-C, glucose, BCAA and LP-IR scores, and lower HDL-C. Baseline characteristics of sex-stratified quartiles of DRI show similar frequencies (Supplemental Table 1).

After a median (interquartile range) follow-up period of 8.5 (8.0–9.0) years, 306 new T2D cases were ascertained, 193 cases in men and 113 cases in women, 6.4% vs. 3.6%, respectively. Cox proportional hazards regression was used to evaluate the DRI scores with incident T2D (Table 2). The crude model revealed that DRI scores are associated with incident T2D with a hazard ratio (HR) for the highest quartile of 12.05 (95% confidence interval (CI): 7.12–20.41; $p < 0.001$). The association of DRI scores with future T2D remained significant even after adjustment for age, sex, BMI, family history of T2D, alcohol consumption, blood pressure, TC, TG, HDL-C and HOMA-IR. The HR for the highest quartile being 3.20 (1.73–5.95; $p < 0.001$). DRI per 1 standard deviation (SD) increment after full adjustment for clinical risk factors for T2D was 1.50 (1.25–1.79; $p = 0.001$).

Table 1. Baseline characteristics by quartile of DRI scores in PREVEND participants free of T2D at baseline (n = 6134).

Variables	Quartiles of DRI				p-value
	Q1	Q2	Q3	Q4	
Participants, *n*	1455	1596	1489	1594	
DRI score	11 (5–15)	25 (22–29)	39 (36–43)	58 (52–64)	<0.001
Sex, men, %	16.4	42.7	56.7	79.2	<0.001
Age, years	50.6 ± 11.7	53.0 ± 12.5	54.5 ± 11.9	54.5 ± 11.3	<0.001
BMI, kg/m2	24.2 ± 3.4	25.7 ± 3.8	27.2 ± 4.2	28.7 ± 4.0	<0.001
SBP, mm Hg	118.4 ±17.8	123.1 ±18.1	127.5 ± 17.9	132.7 ± 17.5	<0.001
DBP, mm Hg	69.5 ± 8.9	71.9 ± 8.7	74.3 ± 8.5	76.9 ± 8.4	<0.001
Parental history of T2DM, yes, %	11.9	13.7	15.1	16.7	<0.001
Smoking status					<0.001
Never, %	34.4	30.3	25.9	23.9	
Former, %	37.2	41.4	44.3	45.3	
Current, %	26.9	27.3	28.3	29.9	
Alcohol consumption					<0.001
<1 drinks/week, %	23.7	24.0	23.8	21.8	
1–7 drinks/week, %	50.0	51.9	46.5	46.1	
>7 drinks/week, %	21.7	23.4	28.0	31.4	
Antihypertensive drugs, %	10.3	15.9	20.1	25.8	<0.001
Lipid-lowering drugs, %	3.4	5.8	8.1	11.0	<0.001
TC, mmol/L	5.2 ± 1.0	5.3 ± 1.0	5.5 ± 1.0	5.7 ± 1.0	<0.001
HDL-C, mmol/L	1.5 ± 0.3	1.3 ± 0.3	1.2 ± 0.3	1.0 ± 0.2	<0.001
LDL-C, mmol/L	2.7 ± 0.7	2.9 ± 0.7	3.0 ± 0.7	3.0 ± 0.8	<0.001
TG, mmol/L	0.8 (0.6–0.9)	0.9 (0.7–1.2)	1.2 (1.0–1.5)	1.9 (1.4–2.4)	<0.001
Glucose, mmol/L	4.6 ± 0.5	4.8 ± 0.6	4.9 ± 0.6	5.0 ± 0.7	<0.001
Total BCAA, µM	301.7 ± 35.1	354.9 ± 38.8	393.8 ± 44.4	455.0 ± 61.5	<0.001
Valine, µM	171.0 ± 21.4	197.4 ± 24.1	216.0 ± 26.6	241.4 ± 33.7	<0.001
Leucine, µM	99.1 ±13.9	118.8 ± 15.1	131.7 ± 17.2	156.5 ± 24.6	<0.001
LP-IR score	15 (8–23)	29 (20–39)	47 (38–57)	73 (62–85)	<0.001
Large VLDL-P, nmol/L	1.4 (0.75–2.2)	2.3 (1.4–3.7)	4.1 (2.7–6.4)	9.2 (6.1–13.8)	<0.001
VLDL size, nm	45.7 ± 8.3	46.9 ± 7.5	49.6 ± 7.6	56.5 ± 8.7	<0.001
Small LDL-P, nmol/L	166 (1–274)	274 (166–289)	378 (251–522)	624 (434–848)	<0.001
LDL size, nm	21.2 ± 1.8	21.1 ± 1.9	21.0 ± 1.1	20.5 ± 1.1	<0.001
Large HDL-P, µmol/L	7.6 ± 4.7	5.8 ± 2.4	4.3 ± 2.3	2.8 ± 1.6	<0.001
HDL size, nm	9.7 ± 0.5	9.3 ± 0.5	9.0 ± 0.5	8.7± 0.4	<0.001

Continuous variables are reported as mean ± standard deviation, median (interquartile range) and categorical variables are reported as percentage. p values were determined using a one-way analysis of variance for normally distributed data, Kruskal–Wallis test for skewed distributed data, and chi-square test for categorical data and represent a significant difference across the quartiles of DRI score. Abbreviations: DRI, Diabetes Risk Index; PREVEND, Prevention of Renal and Vascular End-Stage Disease; T2D, type 2 diabetes mellitus; BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; TC, total cholesterol; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; TG, triglycerides; BCAA, branched chain amino acids; LP-IR, Lipoprotein Insulin Resistance Index; VLDL-P, very low-density lipoprotein particles; LDL-P, low-density lipoprotein particles; HDL-P, high-density lipoprotein particles.

Similarly, Cox proportional hazard regression analysis was performed using sex-stratified quartiles of DRI scores. The crude model again revealed that DRI scores are associated with incident T2D with a hazard ratio (HR) for the highest quartile of 7.27 (95% CI: 4.84–10.92; p < 0.001). The association of DRI scores with future T2D remained significant even after adjusting for clinical T2D risk factors with a HR for the highest quartile being 1.80 (1.07–3.02; p < 0.001). The Kaplan–Meier curves for sex-stratified quartiles of DRI scores are shown in Figure 1. The data show that increasing quartiles of DRI scores corresponded to higher T2D incidence (log rank p < 0.001) (Figure 1). To assess the performance of DRI, we calculated the Harrell's C-index (95% CI) for the Framingham Offspring risk score (a traditional T2D risk assessment tool that takes into account age, sex, family history of T2D, BMI, blood pressure, TG, and glucose) to be 0.870 (0.869–0.870), which increased to 0.876 (0.875–0.877) after addition of DRI, a statistically significant improvement (p < 0.001). The Net Reclassification Index (NRI) was 0.41 (0.30–0.52; p < 0.001), denoting that when DRI was added to the model, more subjects were correctly re-classified than when the Framingham Offspring Study risk score was used alone.

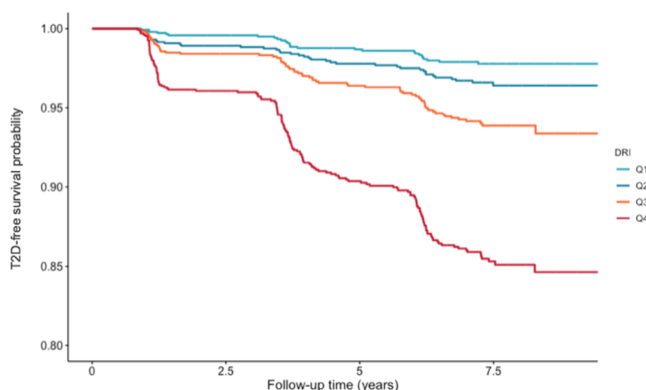

Figure 1. Kaplan–Meier survival curves for time to T2D diagnosis according to sex-stratified quartiles of DRI, by log-rank test (p < 0.001).

The addition of LP-IR to the Framingham Offspring Study risk score allowed the proper reclassification of 27% of subjects who developed T2D, from a lower to a higher risk. Importantly, the addition of DRI allowed for 42% of the participants who developed T2D during the follow-up to be properly reclassified from a lower to a higher risk category.

Table 2. Association of DRI scores with incident T2D by quartile in the PREVEND study (*n*= 6134).

	Q1	Q2		Q3		Q4		DRI Per 1 SD Increment	
	DRI < 19	**DRI 19–33**		**DRI 33–48**		**DRI > 48**			
Participants, *n*	1455	1596		1489		1594		6134	
Events, *n*	15	39		71		181		306	
		HR (95 % CI)	***p*-Value**	**HR (95% CI)**	***p*-Value**	**HR (95% CI)**	***p*-Value**	**HR (95% CI)**	***p*-Value**
Crude Model	(ref)	2.48 [1.37; 4.50]	0.002	4.89 [2.80; 8.53]	<0.001	12.05 [7.12; 20.41]	<0.001	2.34 [2.09; 2.62]	<0.001
Model 1	(ref)	2.42 [1.33; 4.40]	0.003	4.69 [2.66; 8.26]	<0.001	12.07 [6.97; 20.89]	<0.001	2.46 [2.17; 2.80]	<0.001
Model 2	(ref)	1.84 [1.01; 3.36]	0.04	2.83 [1.59; 5.04]	<0.001	6.01 [3.42; 10.58]	<0.001	2.02 [1.76; 2.31]	<0.001
Model 3	(ref)	1.71 [0.93; 3.14]	0.08	2.22 [1.23; 4.03]	0.008	3.20 [1.73; 5.95]	<0.001	1.50 [1.25; 1.79]	0.001

Data are presented as HRs with 95% CIs. Model 1: Model adjusted for age and sex. Model 2: Model 1 + BMI + family history of type 2 diabetes + alcohol consumption. Model 3: Model 2 + DBP + TC+ TG + HDL-C + HOMA-IR. Abbreviations: DRI, Diabetes Risk Index; T2D, type 2 diabetes mellitus; PREVEND, Prevention of Renal and Vascular End-Stage Disease; HR, hazard ratio; CI, confidence intervals; BMI, body mass index; DBP, diastolic blood pressure; TC, total cholesterol; TG, triglycerides; HDL-C, high-density lipoprotein cholesterol; HOMA-IR, Homeostasis Model

Table 3. Association of DRI scores with incident T2DM by sex-stratified quartiles in the PREVEND study. (*n*= 6134).

	Q1 ♀ DRI < 13 ♂ DRI < 30	Q2 ♀ DRI 13–23 ♂ DRI 30–43		Q3 ♀ DRI 23–36 ♂ DRI 43–56		Q4 ♀ DRI > 36 ♂ DRI > 56	
Participants, *n*	1628	1494		1534		1478	
Males, %	48.6	50.7		49.4		48.8	
Events, *n*	28	42		70		166	
		HR (95% CI)	***p*-Value**	**HR (95% CI)**	***p*-Value**	**HR (95% CI)**	***p*-Value**
Crude Model	(ref)	1.63 [1.00; 2.65]	0.05	2.87 [1.84; 4.47]	<0.001	7.27 [4.84; 10.92]	<0.001
Model 1	(ref)	1.55 [0.95; 2.52]	0.08	2.62 [1.68; 4.09]	<0.001	6.74 [4.49; 10.13]	<0.001
Model 2	(ref)	1.34 [0.81; 2.20]	0.25	1.75 [1.10; 2.78]	0.018	3.75 [2.44; 5.76]	<0.001
Model 3	(ref)	1.30 [0.77; 2.20]	0.32	1.35 [0.81; 2.24]	0.25	1.80 [1.07; 3.02]	0.02

Data are presented as HRs with 95% CIs. Model 1: Model adjusted for age and sex. Model 2: Model 1 + BMI + family history of type 2 diabetes + alcohol consumption. Model 3: Model 2 + DBP + TC+ TG + HDL-C + HOMA-IR. Abbreviations: DRI, Diabetes Risk Index; T2DM, type 2 diabetes mellitus; PREVEND, Prevention of Renal and Vascular End-Stage Disease; HR, hazard ratio; CI, confidence intervals; BMI, body mass index; DBP, diastolic blood pressure; TC, total cholesterol; TG, triglycerides; HDL-C, high-density lipoprotein cholesterol; HOMA-IR, Homeostasis Model Assessment Insulin Resistance.

Furthermore, when the LP-IR enriched model was compared against the DRI-enriched model, 24% of participants who developed T2D during the follow-up to be properly reclassified from a lower to a higher risk category, with a Net Reclassification Index (NRI) of 0.34 (0.22–0.45; $p < 0.001$). The Framingham Offspring Study models enriched with LPIR and DRI were compared. The true positive rate for the DRI-enriched model was superior to the LPIR-enriched model (0.20 vs. 0.16, respectively, $p < 0.05$). Although the true negative rates of the DRI- and LPIR-enriched models were similar (0.95 vs. 0.95, $p > 0.05$), the difference with respect to the negative predictive values (0.0015) was in favor to DRI-enriched model ($p < 0.05$). Based on the lowest Bayesian Information Criterion, the best-fit model for the T2D risk was the model enriched with DRI. The Bayesian Information Criterion for DRI-enriched model was 1863, while the LPIR-enriched model was 1879. Likewise, based on the lowest Akaike Information Criterion (AIC), the best-fit model for the T2D risk was the model enriched with DRI. The AIC for DRI-enriched model was 1802, while the LPIR-enriched model was 1818.

Considering that DRI values are higher in men, the association was analyzed separately in men and women using different cutoff values (55 for women and 65 for men) based on the MESA study [16]. The association was found to be stronger in women than in men when using these cutoff values in crude- and age-adjusted analysis. The age-adjusted HR for women was 5.65 (3.67–8.72; $p < 0.001$), and the HR for men was 3.92 (2.88–5.33; $p < 0.001$). Consistently, the HR of DRI per 1SD increment in women was higher than in men. The HR for women was 2.71 (2.29–3.22; $p <0.001$) and 1.99 (1.72; 2.31; $p <0.001$), for men in crude models. Furthermore, the difference between women and men persisted in age-adjusted models, HR for women was 2.50 (2.10–2.97; $p <0.001$) and HR for men 2.04 [(1.75–2.37; $p <0.001$) (Supplemental Tables 2 and 3).

Discussion

In this large prospective cohort, comprising 6134 participants, we report for the first time that higher values of DRI, a newly developed diabetes risk algorithm, are associated with incidences of T2D. In addition, sex-stratified analyses revealed that the positive association of DRI with T2D was present in both men and women, even after adjustment for multiple T2D risk factors, including insulin resistance and BMI. Addition of DRI to the traditional predictive model improved the predictive ability for T2D. Additionally, the DRI enhanced model improved reclassification of participants across clinical risk categories for T2D compared to the Framingham Offspring Study model, and also to the previous reported model that include LP-IR, but not BCAA [19].

DRI includes the information of six lipoprotein parameters: the weighted average sizes of very low-density lipoprotein, low-density lipoprotein and HDL, along with concentrations of large very low-density lipoprotein, small low-density lipoprotein, and large HDL particles; such parameters are integrated in the LP-IR score. Although this is the first study that investigated the association between DRI and incidence of T2D, previous studies have revealed an association between LP-IR and T2D. LP-IR has been consistently found to be associated with risk of T2D in four large prospective studies, some of them with a remarkably large number of participants, i.e., the Women's Health Study, comprising 25,925 participants free from T2D at baseline [17]. LP-IR has also been evaluated in subject of various ethnicities, i.e., the Multi-Ethnic Study of Atherosclerosis, comprising 5314 adults [16]. In addition to the lipoprotein parameters, DRI also includes information of plasma concentrations of BCAA, which have been associated with T2D risk in several recent studies [24–29].

Consistent with previous studies, we found in our study population that high values of DRI associate with increased risk of T2D. Interestingly, the highest

quartile of DRI was comprised predominantly of men (79.2%), in comparison with the first quartile (16.4%). Such a difference in the proportion of the men and women among the quartiles of DRI is consistent with the reported in percentages in our previous LP-IR study, on which 72.7% of the participants in the 4th quartile of LP-IR were men and 28.6% in the 1st quartile of LP-IR were men. The increased proportion of men in the highest quartile of DRI, compared to LP-IR, could be explained by the fact that both plasma concentrations of BCAA and dietary intake of BCAA-rich foods are higher in men [45,46]. There is evidence suggesting that such differences may at least in part be attributed to differences in dietary patterns between men and women [47].

The association of BCAA with risk of T2D has been previously reported in this cohort [24]. Despite the fact that due to the design of our study we were not able to evaluate a causal mechanism, mendelian randomization studies have provided evidence about the causal relationship between BCAA and T2D [48]. Additionally, it has been described that the association of BCAA with T2D is independent of insulin resistance at baseline [29]. In the present study we demonstrated that the association of DRI with T2D was also independent of baseline insulin resistance, as assessed by HOMA-IR.

Plasma concentrations of BCAA have been found as a suitable metabolic predictor of insulin sensitivity improvement in overweight individuals after a lifestyle intervention [49]. Considering that lifestyle interventions have important health benefits, including T2D postponement [50], DRI could be a potential tool to assess the effect of lifestyle intervention in order to improve its efficacy. Future research is needed to evaluate the utility of DRI on that regard. The present study demonstrated that DRI enhances the performance of a typical T2D predictive model based on clinical data. Clinical data-based risk scores for T2D offer several advantages, like affordability and a non-invasive nature; nevertheless, it has been reported that the probability estimates may be

compromised, due several methodological flaws [51]. Furthermore, it has been reported that the performance of non-invasive risk scores varies with age, sex, BMI and country [52]. Finally, despite the fact that T2D is highly prevalent in developing countries, there is a scarcity of T2D risk scores, and the few available risk scores present several methodological limitations [53].

Besides the development of non-invasive risk scores, advances in genetics have allowed the identification of hundreds of genes associated with risk of T2D development. Although the role of singles genes in the onset of T2D is small, the combination of several genes into polygenic risk scores enhances the ability to identify patterns of disease predisposition, which could help to improve the clinical management of patients. Nevertheless, at this time, the use of genetic risk scores still has several limitations such as the fact that the current genetic scores were developed mainly in European populations, as well as the increased costs associated with genetic data acquisition and the full cost of genetic screening implementation [54]. Instead, the two main components of DRI have been demonstrated to have applicability in participants with varied ethnic backgrounds. The incremental cost of calculating DRI from data acquired for the NMR LipoProfile Test (which includes the calculation of LP-IR) is small, lending itself to the ability to screen patients for T2D risk. Furthermore, DRI is able to stratify a patient's T2D risk, even when glucose levels are in the normoglycemic and prediabetic range, allowing for early identification and treatment with the goal of reducing progression to T2D.

We acknowledge several strengths of the present study. Our study included a large number of participants with a wide age range which allowed us to adjust our analysis with sufficient statistical power. Another strength of the present study is the implementation of a robust method of BCAA quantification by means of NMR. To the best of our knowledge this study explores for first time

the performance of a test comprising the dual factors of lipoprotein subspecies and BCAA in the context of T2D risk assessment.

We are also aware of the limitations of the study. The PREVEND population is mainly comprised of individuals with European ancestry, which limits the generalizability of our findings to persons with different ethnicities. We did not have measurements of insulin beyond baseline assessment, which impedes us from evaluating the evolution of insulin resistance and its association with DRI. This fact limits our capacity to describe the underlying biological mechanisms. Moreover, because of the absence of repeated BCAA and LP-IR measurements, we were not able to correct for regression dilution. Finally, it should be emphasized that the validity of the LP-IR score and, as presently described the DRI score, relies on a NMR-derived laboratory parameters obtained from a single plasma specimen but does not account for genetic influences on diabetes risk.

Conclusions

In this prospective cohort study, high values of DRI, a NMR spectroscopy-measured multimarker of lipoprotein subclasses and BCAA, are associated with an increased risk of developing T2D in both men and women in the general population during extended follow-up.

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Supplemental Table 1. Baseline characteristics by sex-stratified quartiles of DRI scores in PREVEND participants free of T2D at baseline (n=6134).

Variables	Quartiles of DRI				p-value
	Q1	Q2	Q3	Q4	
Participants, n	1628	1494	1534	1478	
DRI score	12 (6-22)	31 (18-37)	36 (29-50)	59 (46-65)	<0.001
Sex, men, %	48.6	50.7	49.1	48.4	0.97
Age, years	49.0 ± 12.5	51.5 ± 12.0	53.1 ± 12.1	53.8 ± 10.8	<0.001
BMI, kg/m2	24.2 ± 3.3	25.7 ± 3.4	27.2 ± 4.2	28.9 ± 4.3	<0.001
SBP, mm Hg	120.9 ±18.0	123.9 ±18.3	126.9 ± 18.4	130.9 ± 18.0	<0.001
DBP, mm Hg	70.9± 9.1	72.8 ± 9.1	73.8 ± 8.7	75.6 ± 8.6	<0.001
Parental history of T2DM, yes, %	11.2	13.6	14.9	18.2	<0.001
Smoking status					<0.001
Never, %	32.6	28.8	26.7	25.8	
Former, %	39.8	42.6	43.2	42.9	
Current, %	26.1	27.2	29.3	30.2	
Alcohol consumption					0.01
<1 drinks/week, %	23.2	23.2	23.7	26.7	
1-7 drinks/week, %	51.2	50.1	47.3	45.7	
>7 drinks/week, %	24.4	25.6	28.3	26.9	
Antihypertensive drugs, %	12.3	14.7	20.6	25.6	<0.001
Lipid-lowering drugs, %	3.6	6.5	8.8	10.0	<0.001
TC, mmol/L	5.2±1.0	5.3±1.0	5.5±1.0	5.8 ± 1.1	<0.001
HDL-C, mmol/L	1.4 ± 0.3	1.3 ± 0.3	1.8 ± 0.3	1.1 ± 0.2	<0.001
LDL-C, mmol/L	2.7 ± 0.7	2.9 ± 0.7	3.0 ± 0.8	3.1 ± 0.8	<0.001
TG, mmol/L	0.8 (0.6–0.8)	1.0 (0.8–1.2)	1.3 (1.0–1.6)	1.9 (1.4-2.5)	<0.001
Glucose, mmol/L	4.7 ± 0.6	4.8 ± 0.6	4.9 ± 0.7	5.0 ± 0.7	<0.001
Total BCAA, µM	315.2 ± 48.7	358.9 ± 50.1	389.2 ± 55.5	436.2 ± 70.2	<0.001
Valine, µM	179.2 ± 27.1	201.2 ± 28.6	215.1 ± 31.4	235.4 ± 36.7	<0.001
Leucine, µM	103.4 ±18.1	119.8 ± 18.7	131.8 ± 20.9	149.9 ± 27.1	<0.001
LP-IR score	17 (10-26)	32 (21-43)	48 (36-61)	72 (59-85)	<0.001

Continuous variables are reported as mean ± standard deviation, median (interquartile range) and categorical variables are reported as percentage. P values were determined using a one-way analysis of variance for normally distributed data, Kruskal-Wallis test for skewed distributed data, and chi-square test for categorical data and represent a significant difference across the quartiles of DRI score. Abbreviations: DRI, Diabetes Risk Index; PREVEND, Prevention of Renal and Vascular End-Stage Disease; T2D, type 2 diabetes mellitus; BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; TC, total cholesterol; HDL-C, high density lipoprotein cholesterol; LDL-C, low density lipoprotein cholesterol; TG, triglycerides; BCAA, branched chain amino acids; LP-IR, Lipoprotein Insulin Resistance Index.

Supplemental Table 2. Prospective associations of DRI with risk of T2D in females.

	DRI Per 1 SD Increment		DRI <55	DRI ≥ 55	
Participants, n	3106		2934	172	
Events, n	113		84	29	
	HR (95 % CI)	**P-Value**		**HR (95 % CI)**	**P-Value**
Crude Model	2.71 [2.29;3.22]	<0.001	(ref)	6.85 [4.46;10.54]	<0.001
Model 1	2.50 [2.10;2.97]	<0.001	(ref)	5.65 [3.67;8.72]	<0.001
Model 2	2.12 [1.75;2.55]	<0.001	(ref)	3.82 [2.45;5.96]	<0.001
Model 3	1.49 [1.12;1.98]	0.006	(ref)	1.16 [0.63;2.13]	0.63

Data are presented as hazard ratios (HRs) with 95% confidence intervals (CIs).
Model 1: Model adjusted for age.
Model 2: Model 1 + BMI + family history of type 2 diabetes + alcohol consumption.
Model 3: Model 2 + DBP + TC+ TG + HDL-C + HOMA-IR.

Abbreviations: DRI, Diabetes Risk Index; T2DM, type 2 diabetes mellitus; HR, hazard ratio; CI, confidence intervals; BMI, body mass index; DBP, diastolic blood pressure; TC, total cholesterol; TG, triglycerides; HDL-C, high density lipoprotein cholesterol; HOMA-IR, Homeostasis Model Assessment Insulin Resistance.

Supplemental Table 3. Prospective associations of DRI with risk of T2D in males.

	DRI Per 1 SD Increment		DRI <55	DRI ≥ 55	
Participants, n	3106		2684	344	
Events, n	113		134	59	
	HR (95 % CI)	**P-Value**		**HR (95 % CI)**	**P-Value**
Crude Model	1.99 [1.72;2.31]	<0.001	(ref)	3.73 [2.75;5.07]	<0.001
Model 1	2.04 [1.75;2.37]	<0.001	(ref)	3.92 [2.88;5.33]	<0.001
Model 2	1.59 [1.34;1.88]	<0.001	(ref)	2.45 [1.76;3.42]	<0.001
Model 3	1.19 [0.96;1.48]	0.11	(ref)	1.36 [0.91;2.03]	0.14

Data are presented as hazard ratios (HRs) with 95% confidence intervals (CIs).
Model 1: Model adjusted for age.
Model 2: Model 1 + BMI + family history of type 2 diabetes + alcohol consumption.
Model 3: Model 2 + DBP + TC+ TG + HDL-C + HOMA-IR.

Abbreviations: DRI, Diabetes Risk Index; T2DM, type 2 diabetes mellitus; HR, hazard ratio; CI, confidence intervals; BMI, body mass index; DBP, diastolic blood pressure; TC, total cholesterol; TG, triglycerides; HDL-C, high density lipoprotein cholesterol; HOMA-IR, Homeostasis Model Assessment Insulin Resistance.

Chapter 5

Concentration of Branched-Chain Amino Acids Is a Strong Risk Marker for Incident Hypertension.

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Abstract

The potential role of branched-chain amino acids (BCAA) in the pathogenesis of cardiometabolic diseases is increasingly recognized, but the association of BCAA with incident hypertension remains unknown. The aim of the present study was to explore the association of BCAA with incident hypertension in a prospective population-based cohort study.

We measured plasma concentrations of BCAA by means of nuclear magnetic resonance spectroscopy in 4169 participants from the Prevention of Renal and Vascular End-stage Disease (PREVEND) study. We estimated the risk of incident hypertension using multivariable-adjusted Cox regression models. After a median follow-up of 8.6 years, incident hypertension was ascertained in 924 subjects. Cox regression analyses revealed a significant association between BCAA and incident hypertension. The hazard ratio (HR) per one standard deviation of BCAA was 1.11 (95% confidence interval (CI): 1.02-1.20, P=0.01) after full adjustment for multiple clinical variables. Likewise, the fully adjusted association remained significant when evaluated as categorical variable (HR for upper quartile with lowest quartile as reference category: 1.36, 95% CI: 1.11-1.68, P=0.003). Furthermore, the net reclassification improvement assessment improved after addition of BCAA to a traditional risk model (P <0.001).

This prospective study revealed that high plasma concentrations of BCAA are associated with an increased risk of newly developed hypertension. The association remained after adjusting for age, sex, body mass index, and lipid profile.

Introduction

Branched-chain amino acids (BCAA) have non-linear aliphatic side-chains and include the essential amino acids leucine, valine, and isoleucine. Recently, it has been proposed that BCAA are critical metabolic intermediates involved in intracellular signaling [1]. It has been proposed that BCAA may impair the function of the mammalian target of rapamycin complex 1 (mTORC1), which subsequently leads to insulin resistance, as well as to oxidative stress [2,3]. Such mechanisms are key in the regulation of blood pressure and the development of hypertension.4–6 It has also been proposed that the gut microbiome affects the metabolism of diet-derived BCAA linking gut dysbiosis to blood pressure regulation [7–9]. The availability of high-throughput metabolomics has allowed for the comparison of the amino acid signatures of patients with the respective risk of cardiometabolic disease. Using such an approach, there are several recent epidemiological studies that provided evidence in support of an association between BCAA and cardiovascular risk [10–12].

Interestingly, elevated concentrations of plasma BCAA may be associated with prevalent hypertension,10 but this association between BCAA is blunted after adjustment for biochemical variables such as lipids and plasma glucose, even though subjects with hypertension have higher concentrations of BCAA [12]. Thus, although cross-sectional studies underscore the possible relationship between BCAA and hypertension [10–12], these reports do not provide further insight regarding the temporality of the events. In fact, no large-scale studies have prospectively assessed the association of plasma BCAA concentrations with incident hypertension in the general population.

Therefore, characterization of the potential association between BCAA and risk of hypertension in the middle-aged and elderly participants initially free of hypertension from the large general population-based PREVEND cohort study was investigated.

Materials and Methods

Study Population

The Prevention of Renal and Vascular End-stage Disease (PREVEND) Study is a prospective population-based cohort study from the city of Groningen, The Netherlands. The design of the PREVEND Study has been

described in detail elsewhere [13,14]. Briefly, from 1997 to 1998, all residents from Groningen aged 28–75 years were invited to participate. Pregnant women, subjects with type 1 diabetes and subjects with type 2 diabetes (T2D) using insulin were excluded. All participants with a urinary albumin concentration ≥ 10 mg/L were invited to the clinic together with randomly selected subjects with a urinary albumin concentration < 10 mg/L. A total of 8592 individuals completed an extensive initial examination.

For the present analysis, data was used from participants who completed the second screening (n=6893), excluding those with missing values of BCAA concentrations (n=221) or pre-existing hypertension (n=2503), leaving a cohort of 4169 participants with complete information for the present analysis (Figure 1). The protocol for the PREVEND study was approved by the local ethics committee of the University Medical Center Groningen. All participants included in the present analysis provided written informed consent to participate and all study procedures were conducted according to the Declaration of Helsinki.

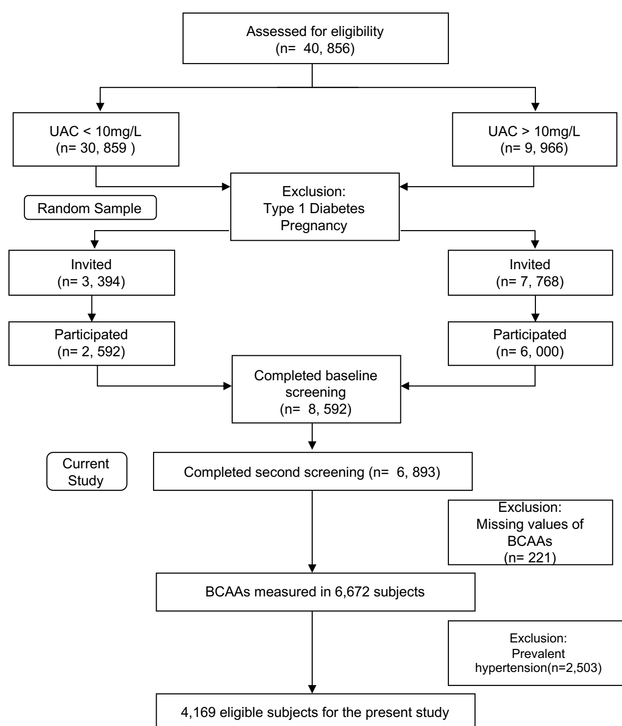

Figure 1. Flowchart of the Prevention of Renal and Vascular End-stage Disease (PREVEND) study participants included or excluded for the purposes of this study.

Biomarker Measurements

During two outpatient visits, baseline data were collected on demographics, lifestyle factors, anthropometric measurements, medical history as well as prevalent medical conditions and use of medication. Plasma samples were taken from participants after an overnight fast and 15 minutes of rest prior to sample collection. All blood samples were taken between 8:00 and 10:00 AM. Plasma samples were prepared by centrifugation at 4 ºC and were stored at -80 ºC until analysis.

Valine, leucine, isoleucine concentrations were measured in EDTA anticoagulated plasma samples using a Vantera Clinical Analyzer (LabCorp, Morrisville, NC), a fully automated, high-throughput, 400 MHz proton (1H) nuclear magnetic resonance (NMR) spectroscopy platform; in a standalone assay that has been optimized to quantify only the BCAA concentrations. Plasma samples were prepared on board the instrument and automatically delivered to the flow probe in the NMR spectrometer's magnetic field. The validation of the use of NMR for quantification of BCAA has been described by our group [15,16]. In brief, coefficients of variation for inter- and intra-assay precision ranged from 1.8-6.0, 1.7-5.4, 4.4-9.1, and 8.8-21.3%, for total BCAA, valine, leucine, and isoleucine, respectively. BCAA quantified from the same samples using NMR and LC-MS/MS were highly correlated (r2 = 0.97, 0.95 and 0.90 for valine, leucine and isoleucine) [15,16]. A detailed method description is provided in the Online Supplement.

Clinical and Laboratory Measurements

Height and weight were measured with the participants standing without shoes and heavy outer garments. Body-mass index (BMI) was calculated by dividing weight in kilograms by height in meters squared. Total cholesterol, high density lipoprotein cholesterol (HDL-C), triglycerides, insulin, serum creatinine, and serum cystatin C were measured using standard protocols, which have been previously described [17–19]. Urinary albumin excretion (UAE) was measured as described in two 24-hour urine collections and the results were averaged for analysis [17–19]. Fasting plasma glucose was measured by dry chemistry (Eastman Kodak, Rochester, New York). Estimated glomerular filtration rate (eGFR) was calculated using the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) combined creatinine-cystatin C equation [20].

Blood Pressure Measurement and Ascertainment of Hypertension

Participants were followed from the date of the baseline visit until end of follow-up. At both visits of each examination, blood pressure was assessed on the right arm in supine position, every minute for 10 and 8 minutes, respectively, with an automatic Dinamap XL Model 9300 series device. The mean of the last 2 recordings from each visit was used. The procedure has been previously described [21].

Use of antihypertensive medications was ascertained by a questionnaire at each examination and was complemented by information from a pharmacy-dispensing registry. For this study, incident hypertension was defined as hypertension that occurred after baseline, which included systolic blood pressure of ≥140 mm Hg, a diastolic blood pressure of ≥90 mm Hg, or the newly recorded use of antihypertensive drugs, in concordance with the recent Guidelines for the management of arterial hypertension from the European Society of Cardiology & European Society of Hypertension [22]. Antihypertensive medication use, for the definition of hypertension, included 5 second-level Anatomical Therapeutic Chemical codes: C02 (antihypertensives), C03 (diuretics), C07 (β-blockers), C08 (calcium channel blockers), and C09 (inhibitors). Initiation of blood pressure lowering medication according to the central pharmacy registry follow-up data was complete as of 1 January 2011.

Statistical Analyses

Data are presented as the mean (standard deviation, SD) or median (interquartile range, IQR) for continuous variables and percentages for categorical variables. Linear trends across sex-stratified quartiles were determined using analysis of covariance for normally distributed data, the Jonckheere-Terpstra test for skewed data, and the Mantel-Haenszel trend test for categorical data. Since total BCAA concentrations showed a normal distribution, the data was not transformed.

For the prospective analysis, we plotted cumulative Kaplan-Meier curves for incidence of hypertension during follow-up according to quartiles of BCAA. Time-to-event Cox proportional hazards models were used to compute hazard ratios (HRs) and 95% confidence interval (CI) of hypertension incidence among the 4169 participants with full information at baseline. HRs were calculated in models adjusted for age, sex, history of T2D, tobacco and alcohol consumption, BMI, systolic blood pressure, glucose, total cholesterol, HDL-C, triglycerides, insulin, UAE and eGFR. The proportionality of hazards assumption

was tested through the evaluation of independence between scaled Schoenfeld residuals with time for each variable and for every model as a whole [23]. Additionally, potential effect modification of the effect of BCAA on the risk of incident hypertension by sex interaction was explored by including product terms in the model. Two-sided P-values < 0.05 were considered to be significant.

In view of the positive association between T2D and concentrations of BCAA [8], we carried out sensitivity analyses in which we evaluated whether the association was present after the exclusion of participants with prevalent T2D at baseline. Additionally, considering the potential role of BCAA as regulator of the lipid metabolism [24], we conducted a sensitivity analysis after exclusion of participants under lipid-lowering medication. Likewise, given the importance of renal function impairment in the BCAA metabolism [25], we carried out a sensitivity analysis after exclusion of participants with impaired eGFR (<60 mL/min/1.73m^2).

In order to determine whether BCAA values can improve the predictive ability of a conventional model [26], measures of discrimination were calculated for censored time-to-event data (Harrell's C-index) [27] and reclassification. In order to evaluate the change in C-index with addition of BCAA, two hypertension risk prediction models were fitted: first, a model using clinical and laboratory variables (age, sex, parental history of hypertension, smoking behavior (current versus former or never smoker)), used in the Framingham Heart Study prediction model [28], as well as eGFR , being a strong predictor of incident hypertension in the general population [29], and insulin; second, a model with the variables mentioned above plus BCAA. Subsequently, we tested the ability of the combined model with BCAA concentrations to correctly reclassify participants into categories of predicted hypertension risk. Using predefined risk categories of hypertension development: low (<10%), intermediate (10% to 20%), and high (≥20%) [30], reclassification was assessed using the categorical net reclassification improvement (NRI) approach. The integrated discrimination improvement (IDI) was also calculated. According to the author of the method, a meaningful reclassification is achieved if >10% of people are reclassified with the new method compared with the old one [31]. Further, we calculated the optimal cut-point for BCAA as the sum of sensitivity and specificity minus one. All statistical analyses were conducted in R version 3.5.1 (Vienna, Austria).

Results

Baseline characteristics

Of the 6893 PREVEND participants that completed the second round of screening, 4169 subjects were included in the current study. Participant characteristics by sex-stratified quartiles of BCAA at baseline are shown in Table 1. Among the participants, 1903 (45.6%) were men and the mean age of the population was 49.2 ± 10.3 years. Subjects in the highest quartile of BCAA concentration were more likely to be older, have higher BMI, systolic and diastolic blood pressure, and had T2D more frequently. Additionally, subjects with higher concentrations of BCAA had a worse lipid profile (elevated total cholesterol and triglycerides, and low HDL-cholesterol) as well as higher concentrations of glucose, elevated UAE, and lower eGFR (Table 1). Mean baseline total BCAA concentration was 388.0±71.3 µM in the subjects who developed new-onset hypertension vs. 365.2±68.3 µM in the subjects who did not (P<0.001). This difference translated in a 0.3 SD (expressed as SD of the whole population) difference in BCAA in the subjects who developed hypertension. The calculated optimal cut-point for BCAA to predict incident hypertension was 372 µM.

Cross-sectional analysis

The associations of the concentration of BCAA with other variables of interest were evaluated with univariable and multivariable linear regression analyses. In a multivariable model, including all the variables presented (Table 1), BCAA remained positively associated with the following variables: age, BMI, diastolic blood pressure, current tobacco use, glucose, triglycerides and inversely with HDL-C, (P<0.05) (Table 2).

Longitudinal analysis

During a median follow-up period of 8.6 years (IQR, 8.0–8.2), 924 individuals were diagnosed with new-onset hypertension. The Kaplan–Meier curves for hypertension according to sex-specific quartiles of BCAA concentrations are presented in Figure 2. The graph depicts an increased risk of hypertension events in subjects in the top quartile of BCAA concentrations ($P_{log\ rank}$< 0.001).

Table 1. Baseline characteristics by sex-stratified quartiles of BCAA in PREVEND participants (n=4169).

	Quartiles of BCAA, μM				
	Q1	**Q2**	**Q3**	**Q4**	**p-value**
Male	<361.9	361.9–404.8	404.8-448.1	>448.1	
Female	<295.9	295.9-330.9	330.9-371.5	>371.5	
Participants, *n*	1043	1042	1041	1043	
Sex, male, %	45.6	45.6	45.6	45.6	0.99
Age, y	47.9 ± 10.3	49.3 ± 10.4	49.5 ± 10.4	50.3 ± 9.9	<0.001
BMI, kg/m^2	24.1 ± 3.6	25.0 ± 3.4	25.8 ± 3.6	27.6 ± 4.2	<0.001
SBP, mm Hg	115.3 ± 10.6	116.5 ± 11.0	116.9 ± 11.0	119.9 ± 10.9	<0.001
DBP, mm Hg	68.8 ± 7.1	69.9 ± 7.3	70.1 ± 7.2	71.7 ± 7.0	<0.001
T2D, yes, %	1.3	0.7	2.4	6.2	<0.001
Family history of HT, %	30.5	27.7	29.9	28.9	0.42
Current smoking, %	34.5	28.7	29.7	28.6	0.004
Alcohol, ≥10 g/day, %	23.3	23.5	25.9	29.2	0.31
Lipid-lowering drugs,%	1.8	2.0	3.3	4.2	<0.001
Glucose, mmol/L	4.6 (4.3–5.0)	4.6 (4.3–5.0)	4.7 (4.4–5.1)	4.8 (4.5–5.4)	<0.001
Insulin, mU/l	6.2 (4.7–7.0)	6.4 (5.0–7.4)	7.8 (5.6–9.1)	9.5 (6.7–14.2)	<0.001
TG, mmol/L	0.84 (0.63–1.15)	0.93 (0.72–1.26)	1.04 (0.77–1.48)	1.36 (0.97–1.93)	<0.001
TC, mmol/L	5.14 ± 0.94	5.31 ± 1.03	5.40 ± 1.05	5.61 ± 1.04	<0.001
HDL-C, mmol/L	1.34 ± 0.37	1.31 ± 0.30	1.27 ± 0.28	1.18 ± 0.28	<0.001
eGFR, mL/min/1.73m^2	99.7 ± 14.7	97.1 ± 13.9	95.9 ± 13.9	94.9 ± 14.4	<0.001
UAE, mg/24h	7.52 (5.7–11.4)	7.52 (5.6–10.8)	7.54 (5.7–12.4)	8.14 (5.8–13.6)	0.002

Abbreviations: BCAA, branched-chain amino acids; BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; T2D, type 2 diabetes; HT, hypertension; TG, triglycerides; TC, total cholesterol; HDL-C, high-density lipoprotein cholesterol; eGFR, estimated glomerular filtration rate; UAE, urinary albumin excretion; PREVEND, prevention of renal and vascular end-stage disease.

Cox regression analyses revealed that high concentrations of BCAA were associated with increased risk of hypertension in the crude model, with a HR per one SD increase of 1.30 (95% CI: 1.22,1.40; *P* <0.001) (Table 3). This association remained significant in the fully adjusted model (HR per one SD increase: 1.11, 95% CI: 1.02,1.19; *P*=0.011) (Table 3). The association with hypertension was stronger when comparing the highest with the lowest quartile of BCAA, in sex-stratified quartiles (Table 4). The crude model presented a HR of 1.85 (95% CI: 1.55,2.22; *P*<0.001). The association remained after adjustment for BMI, T2D, current smoking, alcohol consumption, family history of hypertension, total cholesterol/HDL-C ratio and triglycerides, eGFR, and UAE (HR: 1.36; 95% CI: 1.11, 1.68, *P*=0.003) (Table 4). The association of the individual BCAA, valine, leucine and isoleucine, with incident hypertension showed comparable results (Table S5).

Table 2. Uni- and multivariable linear regression analyses with BCAA as dependent variable.

	Univariable Regression		Multivariable Regression	
	β (95% CI)	*P*-value	β (95% CI)	*P*-value
Sex, male, %	-0.40 [-0.43, -0.37]	<0.0001	-0.34 [-0.38, -0.31]	<0.0001
Age, years/10	0.07 [0.04, 0.10]	<0.0001	-0.02 [-0.06, 0.02]	0.40
BMI, kg/m^2	0.25 [0.22, 0.28]	<0.0001	0.13 [0.10, 0.17]	<0.0001
SBP, mm Hg	0.22 [0.19, 0.25]	<0.0001	-0.04 [-0.08, 0.00]	0.06
DBP, mm Hg	0.22 [0.19, 0.25]	<0.0001	0.04 [-0.00, 0.08]	0.05
T2D, yes, %	0.12 [0.09, 0.15]	<0.0001	0.03 [-0.01, 0.07]	0.10
Family history of HT, yes, %	-0.05 [-0.08, -0.02]	0.003	-0.01 [-0.04, 0.02]	0.72
Current smoking, yes, %	-0.05 [-0.08, -0.02]	0.002	-0.06 [-0.09, -0.03]	0.0001
Alcohol, ≥10 g/day, %	0.03 [0.00, 0.06]	0.03	0.04 [0.01, 0.07]	0.22
Lipid-lowering drugs, %	0.05 [0.01, 0.08]	0.005	0.02 [-0.01, 0.05]	0.24
Glucose, mmol/L	0.21 [0.18, 0.24]	<0.0001	0.09 [0.05, 0.13]	<0.0001
Insulin, mU/l	0.27 [0.24, 0.30]	<0.0001	0.12 [0.08, 0.15]	<0.0001
TG, mmol/L	0.29 [0.26, 0.32]	<0.0001	0.09 [0.05, 0.12]	<0.0001
TC, mmol/L	0.10 [0.07, 0.13]	<0.0001	0.03 [-0.01, 0.06]	0.13
HDL-C, mmol/L	-0.23 [-0.26, -0.20]	<0.0001	-0.05 [-0.09, -0.01]	0.009
eGFR, mL/min/1.73m^2	-0.06 [-0.09, -0.03]	<0.0001	-0.03 [-0.07, 0.01]	0.10
UAE, mg/24h	0.05 [0.02, 0.08]	<0.0001	0.01 [-0.02, 0.04]	0.52

(Standardized) regression coefficients are shown. Abbreviations: BCAA, branched-chain amino acids; BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; T2D, type 2 diabetes; HT, hypertension; TG, triglycerides; TC, total cholesterol; HDL-C, high-density lipoprotein cholesterol; eGFR, estimated glomerular filtration rate; UAE, urinary albumin excretion.

Table 3. Prospective associations of plasma BCAA with risk of Hypertension.

	BCAA Per 1 SD Increment	
Incident Hypertension, n (%)	924/4169, (22.16)	
	HR [95 % CI],	***P*-value**
Crude Model	1.30 [1.22,1.40]	<0.001
Model 1	1.18 [1.10,1.28]	<0.001
Model 2	1.13 [1.04,1.22]	0.003
Model 3	1.11 [1.02,1.19]	0.011

Data are presented as hazard ratios (HRs) with 95% confidence intervals (CIs) and P-values.
Model 1: Model adjusted for age + sex
Model 2: Model 1 + BMI + T2D + smoking + alcohol consumption + Family history of hypertension + eGFR + UAE
Model 3: Model 2 + TC/HDL-C ratio + TG + insulin
Abbreviations: BMI, body mass index; T2D, type 2 diabetes; eGFR, estimated glomerular filtration rate; UAE, urine albumin excretion; TG, triglycerides; TC, total cholesterol; HDL-C, high density lipoprotein cholesterol.

Sensitivity Analyses

Excluding patients with reported T2D at baseline, or use of lipid lowering medication, as well as patients with reduced eGFR (<60 mL/min/1.73m^2) did not meaningfully change the results (Supplementary Tables S1-S3).

Effect of inclusion of BCAA on hypertension risk reclassification

The hypertension risk prediction model containing established risk factors (including eGFR and insulin) yielded a C-index of 0.809, (0.809, 0.810). After addition of the total BCAA concentration, the C-index discretely increased to 0.811 (95% CI: 0.810, 0.811) (P= 0.01). The results of the NRI assessment for hypertension risk were significant after the inclusion of BCAA on the traditional predictive model that includes age, sex, parental history of hypertension, systolic blood pressure, total cholesterol, triglycerides, HDL-cholesterol, BMI and smoking behavior. Thirteen percent of the participants that developed hypertension were correctly reclassified from a lower risk category to a higher risk category with the addition of BCAA to the risk model. The improvement in the classification of participants into predicted hypertension risk categories was statistically significant with a NRI of 0.123 (95% CI: 0.05, 0.19) (P<0.001). As well, the improvement in the classification of participants into predicted hypertension risk categories was statistically significant and meaningful according the parameters of the authors of the NRI method [31]. Likewise, the IDI showed a discrete improvement of 0.004 (0.001, 0.009) P=0.03.

Table 4. Prospective associations of plasma BCAA with risk of Hypertension in sex-stratified quartiles.

BCAA Quartiles	Q1	Q2	Q3	Q4
Incident Hypertension, n (%)	181/1043 (17.3)	203/1042 (19.4)	223/1041 (21.4)	317/1043 (30.4)
Males, %	45.6	45.6	45.6	45.6
		HR [95 % CI], *P*	**HR [95 % CI], *P***	**HR [95 % CI] , *P***
Crude Model	(ref)	1.10 [0.90,1.35], 0.35	1.33 [1.10,1.63], 0.004	1.85 [1.54,2.22], < 0.001
Model 1	(ref)	1.02 [0.83,1.25], 0.84	1.20 [0.99,1.47], 0.06	1.67 [1.39,2.00], <0.001
Model 2	(ref)	1.01 [0.81,1.25], 0.92	1.16 [0.94,1.43], 0.16	1.45 [1.18,1.77], <0.001
Model 3	(ref)	1.01 [0.81,1.25], 0.94	1.14 [0.92,1.41], 0.21	1.36 [1.11,1.68], 0.003

Data are presented as hazard ratios (HRs) with 95% confidence intervals (CIs) and P-values.
Model 1: Model adjusted for age. Model 2: Model 1 + BMI + T2D + smoking + alcohol consumption + Family history of hypertension + eGFR + UAE. Model 3: Model 2 + Total cholesterol/HDL-C ratio + triglycerides + insulin.

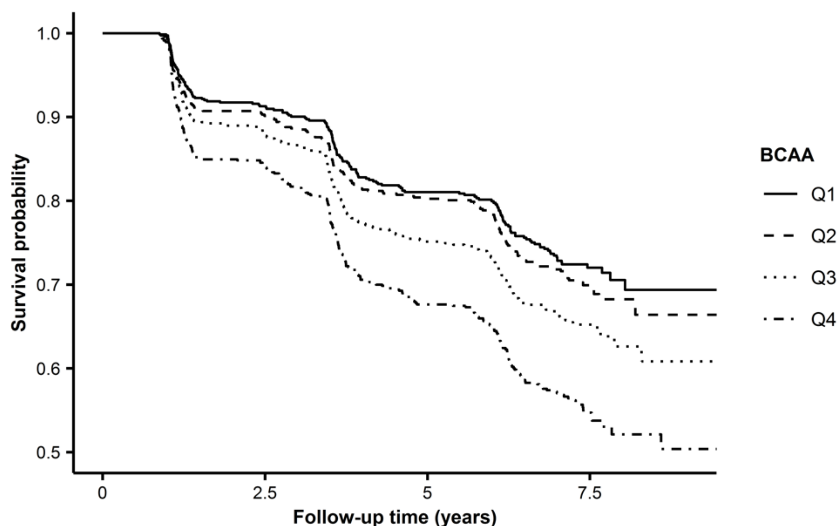

Figure 2. Kaplan-Meier plot for incident Hypertension comparing sex-stratified quartiles of BCAA. log-rank test, P<0.001.

Discussion

In this large-scale prospective population-based cohort study, plasma BCAA concentrations were associated with incident hypertension among adults of predominantly European ancestry. This association remained when taking into account important risk factors such as BMI and cigarette smoking. Clinical characteristics such as older age, high blood pressure, reduced eGFR, and elevated UAE were positively associated with high concentrations of BCAA at baseline. In both crude and fully-adjusted models, subjects with high BCAA concentrations had a significantly higher risk for new-onset hypertension. Of note, addition of circulating BCAA to a traditional predictive model of hypertension improved its predictive ability in terms of net reclassification improvement. In addition, statistically significant associations of the individual BCAA, valine, leucine and isoleucine, with incident hypertension were observed.

To the best of our knowledge, no evidence abounds on the prospective association of BCAA with incident hypertension in adults of the general population. There are few cross-sectional studies that have reported the association of high concentrations of BCAA with prevalent hypertension [10,12]. Nevertheless, those studies presented contradictory information about the role of BMI in the association. Yamaguchi et al. showed that the association was no

longer significant after the adjustment for BMI [12], whereas Batch et al. revealed that the association between BCAA and hypertension was independent of BMI [10]. Although subjects with hypertension at baseline were excluded, the results of our cross-sectional analysis, showing an independent positive association of BCAA with diastolic blood pressure despite the expected relationship with BMI, concur with the recent findings of Batch et al.10 Moreover, the recent observation that dietary BCAA intake is associated with increased risk of hypertension is in line with our current observations [32].

The role of BCAA on hypertension development has to be considered in the context of the study group age. A small study with 253 children, aged 11-15 revealed that concentrations of BCAA are associated with increasing SBP among pubertal, but not pre-pubertal boys [33]. In our study, the association of plasma BCAA with incident hypertension remained significant after the adjustment for age. Further studies of the association of circulating BCAA with changes in blood pressure that occur during early adulthood are needed.

Considering that there are sex differences in the molecular mechanisms regulating blood pressure [34], as well as the fact that the concentrations of BCAA are higher in men, we estimated the risk of incident hypertension using sex-stratified quartiles of BCAA. We reported that the association was present in both males and females, which is in line with the results of the Women's Health Study prospective cohort, where it was documented that high concentrations of BCAA are associated with prevalent hypertension [11].

The metabolism of BCAA is complex, making that our findings and its possible clinical implications have to be prudently interpreted. Circulating concentrations of BCAA are to a considerable extent derived from dietary intake, and the gut microbiome is important in determining their bioavailabilty.8 In turn, BCAA are not only required for protein synthesis and source of fuel, but may also affect cell signaling, which possibly results in impairment of insulin action and may aggravates oxidative stress [2,3,8]. In the gut, BCAA may serve as precursors of microbial-derived short chain fatty acids (SCFA), thereby enhancing SCFA bioavailability [7].

Interestingly, fecal SCFA excretion may be elevated in hypertensive subjects [35]. However, SCFA could exert insulin action promoting and anti-inflammatory properties [36]. Therefore, the precise role of BCAA as a source of SCFA in the interplay between alterations in the gut microbiome and development of hypertension [35,36] deserves further attention. Also, the

pathophysiological basis underlying the relationship between BCAA and cardiometabolic disease remains to be fully elucidated.

Strengths and Limitations

The present study has several strengths. The large population enrolled in the study enabled us to carry out sufficiently powered multivariable adjusted analyses and testing the robustness of the findings using several sensitivity analyses to provide solid evidence. As well, the long follow-up time of PREVEND allowed us to study biomarkers, with potentially subtle and cumulative effect, such as BCAA. Another strength of the present study is the implementation of a robust method of BCAA quantification by means of NMR. Finally, another important strength of the present study is the robustness of the association in several sensitivity analyses. Even though BCAA have been associated to T2D [8] and lipid metabolism [24] the association remained significant after the exclusion of participants with T2D or using lipid-lowering medication at baseline. Likewise, the reported association remained after the exclusion of participants with impaired eGFR (<60 mL/min/1.73m^2), which potentially extrapolate our conclusion to the general population with a normal renal function. Altogether, this would suggests that the association of BCAA with incident hypertension also holds in subjects with lower cardiometabolic risk.

Several limitations of the present study deserve mention. First, the PREVEND study, conducted in the north of The Netherlands, mainly comprises individuals of European ancestry, which could limit extrapolation of our findings. For instance, the calculated optimal cut-point for BCAA, may be different on other cohorts and other ethnicities. In addition, the present study lacks of BCAA measurements beyond baseline. Therefore, it is not possible to evaluate the regression dilution of BCAA. Additionally, the improvement of NRI and IDI were quite modest. Further, the design of PREVEND study did not include twenty-four hour ambulatory blood pressure monitoring, which remains the gold standard for diagnosing hypertension [37].

In conclusion, in this population-based cohort, we provide the first evidence that high concentrations of BCAA are associated with an increased risk of hypertension in middle-aged men and women. The documented prospective association was independent of other risk factors, and the addition of BCAA to traditional risk models improved the risk classification of individual from the general population. Further investigation is needed to unravel the complexity behind the circulating concentrations of BCAA.

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Supplementary Methods

NMR spectra acquisition and BCAA signal deconvolution analysis [1]

Nuclear Magnetic Resonance (NMR) spectra were collected on a Vantera Clinical Analyzer (LabCorp, Raleigh, NC), a fully automated, high-throughput, 400 MHz proton (1H) NMR platform. Plasma samples were set on board the instrument by mixing 1:1 sample: NMR diluent (50 mM sodium phosphate, 120 mM KCl, 5 mM Na2EDTA, 1 mM CaCl2, pH 7.4) and automatically delivered to the flow probe in the spectrometer's homogeneous magnetic field. The data acquisition on the Vantera Clinical Analyzer was accomplished with water suppression with the WET solvent suppression technique, reported before [2].

The NMR data was acquired as 3 blocks of 4 scans for a total acquisition time of 48 seconds. Spectra were acquired with a sweep width of 4496.4 Hz and 9024 data points. The data was processed by zerofilling to 32 K points and multiplied by a Gaussian function to provide resolution enhancement prior to Fourier Transformation. Two levels of controls were included at the beginning of each test for all experiments. The methyl signals from the three BCAA in the 1H NMR spectrum produce distinct patterns that can be used for quantification. These signals overlap with each other as well as with the methyl signals from the lipoprotein particles. Spiking experiments with pure BCAA in serum helped identify the positions of the signature peaks of valine, leucine and isoleucine. The relative positions of the various signature peaks of isoleucine (doublet and a triplet), leucine (multiplet), and valine (two doublets) are distinct even within the many NMR signals from the other components in serum.

Additionally, experiments with pH titration were conducted to identify how these signals move in relation to each other as well as the reference peak (calcium EDTA). With such information, mathematical equations were derived to relate pH to position of the signature peaks within the NMR spectrum in order to orient the particular patterns of each BCAA. Therefore, for BCAA analysis, an optimized deconvolution algorithm was developed which simultaneously mathematically models the methyl signals from the lipoproteins, proteins and BCAA (between 0.718 and 1.02 ppm) in each NMR spectrum and quantifies valine, leucine and isoleucine. The BCAA concentrations were determined using non-negative linear least squares by first determining the signal areas of their well-characterized distinctive pattern of peaks and then multiplying by the predetermined conversion factors.

Evaluation of the limits of detection

A Slide-A-Lyzer Dialysis 10 kDa molecular weight cutoff cassette (Thermo Scientific, Rockford, IL) was used to produce serum that was free of small molecules for determining the limits of blank (LOB). Five serum pools containing low concentrations

of valine, leucine or isoleucine were tested to determine the limits of detection (LOD) and quantitation (LOQ) according to Clinical and Laboratory Standards Institute (CLSI) guidelines. Mean concentration and coefficients of variation (CV%) were calculated for each pool. Within-run and within-laboratory imprecision were determined based on CLSI guidelines using serum pools targeted at low, intermediate and high ranges for each of the BCAA.

Within-run (intra-assay) imprecision was determined by analyzing each of the pools on one day with 20 replicates. The same pools were analyzed for 20 days with two replicates twice per day (total n = 80) to evaluate the within-laboratory (inter-assay) imprecision. Consistent with CLSI guidelines [3], linearity was evaluated by comparing known spiked concentrations of the amino acids with expected concentrations. Linearity was tested in duplicate across the biological range from 0 to 600 μM by preparing dialyzed serum samples spiked with known amino acid concentrations.

Coefficients of variation for inter- and intra-assay precision ranged from 1.8-6.0, 1.7-5.4, 4.4-9.1, and 8.8-21.3%, for total BCAA, valine, leucine, and isoleucine, respectively. BCAA quantified from the same samples using NMR and LC-MS/MS were highly correlated (R2 = 0.97, 0.95 and 0.90 for valine, leucine and isoleucine) [1].

Reproducibility data for BP measurement

The between day coefficient of variation for blood pressure evaluated in a subset of patients from the control group of the Prevention of Renal and Vascular End-stage Disease Intervention Trial (PREVEND-IT) (n =417), was 9.7% for SBP and 10.4% for DBP over a three months time period.

Given the fact that all records of the SBP and DBP correspond to the measurements obtained by the automatic Dinamap XL Model 9300 series device, our data is not affected by zero (or any other) end-digit preference.

Supplemental References.

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Table S1. Prospective associations of plasma BCAA with risk of Hypertension, in sex-stratified quartiles, after exclusion of individuals with T2D at baseline. (N=4039)

BCAA Quartiles	Q1	Q2		Q3		Q4	
Incident Hypertension, n (%)	163/1010 (16.1)	183/1009 (18.1)		239/1010 (23.7)		295/1010 (29.2)	
		HR (95 % CI)	***P***	**HR (95 % CI)**	***P***	**HR (95 % CI)**	***P***
Crude Model	(ref)	1.09 [0.89;1.34]	0.41	1.31 [1.07;1.60]	0.008	1.84 [1.53;2.22]	< 0.001
Model 1	(ref)	1.02 [0.83;1.26]	0.84	1.19 [0.97;1.46]	0.08	1.68 [1.40;2.03]	<0.001
Model 2	(ref)	1.00 [0.80;1.24]	0.94	1.15 [0.93;1.42]	0.21	1.45 [1.19;1.79]	<0.001
Model 3	(ref)	1.00 [0.80;1.24]	0.97	1.13 [0.91;1.40]	0.28	1.35 [1.09;1.66]	0.005

Data are presented as hazard ratios (HRs) with 95% confidence intervals (CIs) and *P*-values.
Model 1: Model adjusted for age
Model 2: Model 1 + BMI + smoking + alcohol consumption + Family history of hypertension + eGFR + UAE
Model 3: Model 2 + TC/HDL-C ratio + TG + insulin
Abbreviations: BMI, body mass index; T2D, type 2 diabetes; eGFR, estimated glomerular filtration rate; UAE, urine albumin excretion; TG, triglycerides; TC, total cholesterol; HDL-C, high density lipoprotein cholesterol.

Table S2. Prospective associations of plasma BCAA with risk of Hypertension, in sex-stratified quartiles, after exclusion of individuals with T2D and use of lipid lowering medication at baseline. (N=3927)

BCAA Quartiles	Q1	Q2		Q3		Q4	
Incident Hypertension, n (%)	156/982 (15.9)	174/981 (17.7)		226/982 (23.0)		280/982 (28.5)	
		HR (95 % CI)	***P***	**HR (95 % CI)**	***P***	**HR (95 % CI)**	***P***
Crude Model	(ref)	1.06 [0.86;1.31]	0.57	1.32 [1.08;1.62]	0.008	1.82 [1.50;2.21]	< 0.001
Model 1	(ref)	1.00 [0.81;1.24]	0.98	1.21 [0.99;1.49]	0.06	1.67 [1.37;2.02]	<0.001
Model 2	(ref)	0.97 [0.78;1.22]	0.81	1.15 [0.93;1.44]	0.20	1.43 [1.16;1.77]	<0.001
Model 3	(ref)	0.98 [0.78;1.23]	0.85	1.14 [0.91;1.42]	0.25	1.33 [1.08;1.65]	0.008

Data are presented as hazard ratios (HRs) with 95% confidence intervals (CIs) and P-values.
Model 1: Model adjusted for age
Model 2: Model 1 + BMI + smoking + alcohol consumption + Family history of hypertension + eGFR + UAE
Model 3: Model 2 + TC/HDL-C ratio + TG + insulin
Abbreviations: BMI, body mass index; T2D, type 2 diabetes; eGFR, estimated glomerular filtration rate; UAE, urine albumin excretion; TG, triglycerides; TC, total cholesterol; HDL-C, high density lipoprotein cholesterol.

Table S3. Prospective associations of plasma BCAA with risk of Hypertension, in sex-stratified quartiles, after exclusion of individuals with decreased eGFR (<60 mL/min/1.73m^2). (N=4137)

BCAA Quartiles	Q1	Q2		Q3		Q4	
Incident Hypertension, n (%)	164/1032 (15.9)	187/1036 (18.0)		255/1034 (24.6)		305/1035 (29.5)	
		HR (95 % CI)	***P***	**HR (95 % CI)**	***P***	**HR (95 % CI)**	***P***
Crude Model	(ref)	1.10 [0.90;1.35]	0.35	1.35 [1.10;1.64]	0.003	1.84 [1.53;2.22]	< 0.001
Model 1	(ref)	1.03 [0.84;1.26]	0.78	1.21 [1.00;1.48]	0.05	1.65 [1.37;1.99]	<0.001
Model 2	(ref)	1.01 [0.82;1.26]	0.91	1.18 [0.95;1.46]	0.12	1.43 [1.17;1.76]	<0.001
Model 3	(ref)	1.01 [0.81;1.26]	0.91	1.16 [0.93;1.43]	0.18	1.33 [1.08;1.64]	0.008

Data are presented as hazard ratios (HRs) with 95% confidence intervals (CIs) and P-values.
Model 1: Model adjusted for age
Model 2: Model 1 + BMI + smoking + alcohol consumption + Family history of hypertension + eGFR + UAE
Model 3: Model 2 + TC/HDL-C ratio + TG + insulin

Abbreviations: BMI, body mass index; T2D, type 2 diabetes; eGFR, estimated glomerular filtration rate; UAE, urine albumin excretion; TG, triglycerides; TC, total cholesterol; HDL-C, high density lipoprotein cholesterol.

Table S5. Prospective associations of individual plasma BCAA with risk of Hypertension.

Amino Acid	Isoleucine Per 1 SD Increment		Valine Per 1 SD Increment		Leucine Per 1 SD Increment	
Incident Hypertension, n (%)	924/4169 (22.16)		924/4169 (22.16)		924/4169 (22.16)	
	HR (95 % CI)	***P***	**HR (95 % CI)**	***P***	**HR (95 % CI)**	***P***
Crude Model	1.22 [1.15;1.30]	<0.001	1.29 [1.20;1.38]	<0.001	1.29 [1.21;1.38]	<0.001
Model 1	1.15 [1.07;1.23]	<0.001	1.15 [1.07;1.24]	<0.001	1.18 [1.10;1.27]	<0.001
Model 2	1.11 [1.03;1.19]	0.004	1.10 [1.02;1.19]	0.01	1.13 [1.05;1.21]	0.001
Model 3	1.08 [1.01;1.16]	0.04	1.09 [1.01;1.18]	0.03	1.10 [1.02;1.18]	0.01

Data are presented as hazard ratios (HRs) with 95% confidence intervals (CIs) and P-values.

Model 1: Model adjusted for age + sex

Model 2: Model 1 + BMI + T2D + smoking + alcohol consumption + Family history of hypertension + eGFR + UAE

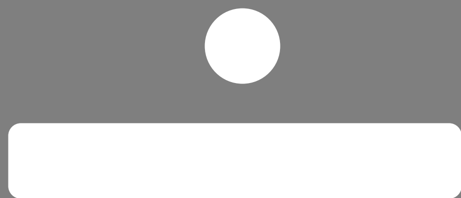
Model 3: Model 2 + TC/HDL-C ratio + TG + insulin

Abbreviations: BMI, body mass index; T2D, type 2 diabetes; eGFR, estimated glomerular filtration rate; UAE, urine albumin excretion; TG, triglycerides; TC, total cholesterol; HDL-C, high density lipoprotein cholesterol.

Table S4. Cross-sectional associations of individual BCAA.

Variables	Leucine	Isoleucine	Valine
	β (95% CI)	**β (95% CI)**	**β (95% CI)**
Sex, male, %	-0.29 [-0.33, -0.25]***	-0.37 [-0.40, -0.33]***	-0.32 [-0.36, -0.29]***
Age, years/10	-0.02 [-0.06, 0.02]	-0.09 [-0.13, -0.05]***	0.02 [-0.02, 0.06]
BMI, kg/m^2	0.12 [0.08, 0.16]***	0.06 [0.03, 0.10]***	0.15 [0.11, 0.19]***
SBP, mm Hg	-0.04 [-0.08, 0.01]	-0.02 [-0.06, 0.03]	-0.04 [-0.09, 0.00]
DBP, mm Hg	0.04 [-0.01, 0.08]	0.03 [-0.01, 0.07]	0.04 [-0.00, 0.08]
T2D, yes, %	0.02 [-0.01, 0.06]	0.02 [-0.01, 0.06]	0.03 [-0.00, 0.07]
Family history of HT, %	-0.01 [-0.04, 0.02]	-0.00 [-0.03, 0.03]	-0.00 [-0.03, 0.03]
Current smoking, %	-0.04 [-0.07, -0.01]**	-0.07 [-0.10, -0.04]***	-0.06 [-0.09, -0.03]***
Alcohol, ≥10 g/day, %	0.05 [0.02, 0.08]**	0.02 [-0.01, 0.05]	0.03 [-0.01, 0.06]
Lipid-lowering drugs, %	0.02 [-0.01, 0.05]	0.01 [-0.02, 0.04]	0.02 [-0.01, 0.05]
Glucose, mmol/L	0.09 [0.05, 0.13]***	0.08 [0.04, 0.11]***	0.08 [0.04, 0.12]***
Insulin, mU/l	0.06 [0.03, 0.10]***	0.12 [0.09, 0.15]***	0.14 [0.10, 0.17]***
TG, mmol/L	0.14 [0.10, 0.18]***	0.16 [0.13, 0.20]***	0.01 [-0.02, 0.05]
TC, mmol/L	0.05 [0.02, 0.09]**	-0.01 [-0.05, 0.02]	0.01 [-0.02, 0.05]
HDL-C, mmol/L	-0.05 [-0.09, -0.01]*	-0.05 [-0.09, -0.01]*	-0.04 [-0.08, -0.01]*
eGFR, mL/min/1.73m^2	-0.03 [-0.07, 0.01]	-0.07 [-0.10, -0.03]	-0.01 [-0.05, 0.02]
UAE, mg/24h	0.02 [-0.01, 0.05]	-0.00 [-0.03, 0.03]	0.01 [-0.03, 0.04]

Standardized regression coefficients of multivariable linear regression analyses with Leucine, Isoleucine and Valine as dependent variables are shown. Abbreviations: BCAA, branched-chain amino acids; BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; T2D, type 2 diabetes; HT, hypertension; TG, triglycerides; TC, total cholesterol; HDL-C, high-density lipoprotein cholesterol; eGFR, estimated glomerular filtration rate; UAE, urinary albumin excretion. * indicates $P < .05$; ** indicates $P < .01$; *** $P < .0001$



Chapter 6

A Metabolomic Index Based on Lipoprotein Subfractions and Branched Chain Amino Acids is Associated with Incident Hypertension.

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Abstract

Objective. The present study aims to evaluate the performance of the Diabetes Risk Index (DRI), a metabolomic index based on lipoprotein particles and branched chain amino acids, on the incidence of newly developed hypertension in a large community dwelling cohort.

Methods. The DRI was calculated by combining 6 lipoprotein parameters [sizes of very-low-density lipoprotein (VLDL), low-density lipoprotein (LDL) and high-density lipoprotein (HDL), concentrations of large VLDL, small LDL, and large HDL particles], and the concentrations of valine and leucine. DRI scores were estimated in 4169 participants from the PREVEND prospective cohort. Cox proportional hazards regression was used to evaluate the association of DRI scores with incident hypertension.

Results. During a median follow-up of 8.6 years, 924 new hypertension cases were ascertained. In analyses adjusted for age and sex, there was a significant association between DRI and incident hypertension with a hazard ratio (HR) per 1 SD increase of 1.45 (95% CI 1.36,1.54; $p < 0.001$). After additional adjustment for traditional risk factors, the HR remained significant (HRadj 1.21, 95% CI 1.10, 1.33, $p < 0.001$). Likewise, subjects in the top quartile of DRI presented with a higher risk of hypertension (HRadj 1.64, 95% CI 1.28, 2.10, $p < 0.001$). Furthermore, the net reclassification improvement assessment improved after the addition of DRI to a traditional risk model ($p < 0.001$), allowing proper reclassification of 34% of the participants.

Conclusion. Higher DRI scores were associated with an increased risk of incident hypertension. Such association was independent of traditional clinical risk factors for hypertension.

Introduction

Hypertension is the leading preventable risk factor for cardiovascular disease (CVD) and all-cause mortality worldwide, with a sustained increment in mortality over the last 40 years [1]. Over the same period of time, elevated blood pressure remains the risk factor with the highest disability-adjusted life-years burden [2]. Given the constant rise in hypertension incidence, improvement in the risk classification for hypertension development is needed.

Improvement in the performance of metabolite measurement platforms, such as nuclear magnetic resonance (NMR) spectroscopy, has allowed for the quantification of circulating biomarkers and exploration of their associations with incidence of hypertension [3]. Recently, the role of branched chain amino acids (BCAA) in the development of hypertension has gained attention. It has been shown that high concentrations of BCAA, quantified by means of NMR in normotensive subjects are associated with higher risk of hypertension incidence in a period of seven years [4].

Moreover, the association of insulin resistance with hypertension [5] has been further corroborated with the use of novel NMR biomarkers [6]. For instance, the lipoprotein insulin resistance index (LP-IR), a high-throughput multivariable marker of insulin resistance that combines the information from 6 lipoprotein particle parameters, has been shown to be strongly associated with insulin resistance [6, 7]. Notably high LP-IR scores have also been demonstrated to associate with elevated blood pressure in several studies, including participants from more than 27 countries [6, 8, 9].

Considering that circulating concentrations of BCAA have also been shown to be higher in insulin resistant conditions [10–12], a new multimarker called the Diabetes Risk Index (DRI) has been developed that integrates both BCAA and LP-IR into a score that predicts incident type 2 diabetes (T2D) [9].

Hypertension and T2D are two cardiometabolic entities that overlap in the general population. These conditions share common causes, such as sedentary lifestyle; but also, pathophysiological mechanisms that underlie their development with insulin resistance being most relevant [13]. Considering that the etiologic relationship between hypertension and T2D is bidirectional [14], we hypothesized that a T2D multimarker score which combines LP-IR and BCAA would provide an enhanced clinical ability to identify individuals at higher risk

of developing primary hypertension. The aim of the present study was to assess the performance of the DRI score to predict the development of primary hypertension in individuals from the PREVEND study, a large cohort of adults from the general population in The Netherlands.

Materials and Methods

Study Population

Briefly, the Prevention of Renal and Vascular End-stage Disease (PREVEND) Study is a Dutch cohort drawn from the general population of the city of Groningen in the northern part of the Netherlands. Briefly, from 1997 to 1998, all residents from Groningen aged 28–75 years were invited to participate. After exclusion of subjects with insulin-treated diabetes and pregnant women, subjects with a urinary albumin concentration ≥10 mg/L were invited to participate, resulting in cohort of 8592 subjects (aged 28–75 years) who completed the baseline survey. The second screening, which was the starting point of the current study, took place between 2001 and 2003 (n = 6892). For the current study, subjects with prevalent hypertension at baseline (defined as systolic blood pressure of ≥140 mm Hg or a diastolic blood pressure of ≥90 mm Hg), and those with missing data of the DRI at baseline and follow-up were excluded, leaving 4169 subjects for the present analyses. Cases of participants lost to follow-up were considered as censored cases. This report follows the Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) reporting guideline (Supplemental Table 1).

The PREVEND study was approved by the local medical ethics committee at the University Medical Center Groningen (approval number: MEC96/01/022). All participants provided written informed consent and all procedures were conducted according to the Declaration of Helsinki [15]. Details of the study design and recruitment have been described elsewhere [16].

Laboratory and Clinical Measurements

Plasma samples were obtained from participants after an overnight fast and 15 minutes of rest prior to sample collection. All blood samples were taken between 8:00 and 10:00 AM. EDTA plasma samples were prepared by centrifugation at 4 °C as per manufacturer's instructions and were stored at -80 ºC until analysis.

DRI Calculations

The DRI scores were determined in EDTA plasma samples using a Vantera® Clinical Analyzer (Labcorp, Morrisville, NC), a fully automated, high-throughput, 400 MHz proton (1H) NMR spectroscopy platform, as previously described [17, 18]. Briefly, the LP-IR scores were calculated using 6 NMR-measured lipoprotein parameters: the weighted average sizes of very-low- density lipoprotein, low-density lipoprotein and high-density lipoprotein (HDL), along with concentrations of large very-low-density lipoprotein, small low-density lipoprotein, and large HDL particles. LP-IR scores (0–100) reflect the magnitude of insulin resistance in individual patients and exhibit strong associations with homeostatic model assessment of insulin resistance (HOMA-IR) and the glucose disposal rate (GDR) assessed by hyperinsulinemic-euglycemic clamp, and have been shown to reflect both peripheral and hepatic insulin resistance on glucose metabolism [7]. BCAA concentrations were measured using the same fully automated, high-throughput, NMR platform; in a standalone assay that has been optimized to quantify only the BCAA concentrations. Plasma samples were prepared on board the instrument and automatically delivered to the flow probe in the NMR spectrometer's magnetic field. The validation of the use of NMR for quantification of BCAA has been previously described [19]. Coefficients of variation for inter- and intra-assay precision ranged from 1.8-6.0, 1.7-5.4, 4.4-9.1, and 8.8-21.3%, for total BCAA, valine, leucine, and isoleucine, respectively. BCAA quantified from the same samples using NMR and LC-MS/MS were highly correlated ($r2 = 0.97$, 0.95 and 0.90 for valine, leucine and isoleucine) [19].

DRI scores were determined using the following equation: $DRI = 0.0167 [LP\text{-}IR] + 1.907 [\ln (valine + 2 \times leucine)]$. DRI scores vary from 1–100 score, with the highest scores denoting the highest risk of progression to T2D. The coefficients of variation for intra- and inter-assay precision ranged from 3.9%–6.4% and 2.7%–7.9% for LP-IR and DRI, respectively.

Total cholesterol (TC), high density lipoprotein cholesterol (HDL-C), triglycerides (TG), insulin, serum creatinine, and serum cystatin C were measured using standard protocols, which have been previously described [20, 21]. Urinary albumin excretion (UAE) was measured as described in two 24-hour urine collections and the results were averaged for analysis [20, 21]. Fasting plasma glucose was measured by dry chemistry (Eastman Kodak, Rochester, New York). Estimated glomerular filtration rate (eGFR) was calculated using the

Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) combined creatinine-cystatin C equation [22].

Height and weight were measured with the participants standing without shoes and heavy outer garments. Body-mass index (BMI) was calculated by dividing weight in kilograms by height in meters squared.

Blood Pressure Measurement and Ascertainment of Hypertension

Participants were followed from the date of the baseline visit until end of follow-up. At both visits of each examination, blood pressure was assessed on the right arm in supine position, every minute for 10 and 8 minutes, respectively, with an automatic Dinamap XL Model 9300 series device. The mean of the last 2 recordings from each visit was used. The procedure has been previously described [23].

Use of antihypertensive medications was ascertained by a questionnaire at each examination and was complemented by information from a pharmacy-dispensing registry. For this study, incident hypertension was defined as hypertension that occurred after baseline, which included systolic blood pressure of ≥140 mm Hg, a diastolic blood pressure of ≥90 mm Hg, or the newly recorded use of antihypertensive drugs, in concordance with the recent Guidelines for the management of arterial hypertension from the European Society of Cardiology & European Society of Hypertension [24]. Antihypertensive medication use, for the definition of hypertension, included 5 second-level Anatomical Therapeutic Chemical codes: C02 (antihypertensives), C03 (diuretics), C07 (β-blockers), C08 (calcium channel blockers), and C09 (inhibitors of the renin-angiotensin system). Initiation of blood pressure lowering medication according to the central pharmacy registry follow-up data was complete as of 1 January 2011.

Statistical Analyses

Normally distributed data were presented as mean and standard deviation, whereas skewed data were expressed as median and interquartile range. Categorical data were presented as number and percentage. Linear trends across DRI quartiles were determined using ANOVA for normally distributed data, Kruskal-Wallis test for skewed distributed data, and $\chi 2$ test for categorical variables. Skewed data were log-transformed when appropriate. Baseline

associations between characteristics and DRI scores were analyzed through univariable and multivariable regression analysis.

For the prospective analysis, cumulative Kaplan-Meier curves for incidence of hypertension during follow-up according to quartiles of DRI were plotted. Time-to-event Cox proportional hazards models were used to compute hazard ratios (HRs) and 95% confidence interval (CI) of hypertension incidence among the 4169 participants with full information at baseline. HRs were calculated in models adjusted for age, sex, systolic blood pressure, history of T2D, tobacco and alcohol consumption, BMI, systolic blood pressure, glucose, insulin, total cholesterol, HDL-C, triglycerides, UAE and eGFR. The proportionality of hazards assumption was tested through the evaluation of independence between scaled Schoenfeld residuals with time for each variable and for every model as a whole [25]. Two-sided p-values < 0.05 were considered to be significant.

In view of the positive association between T2D and concentrations of BCAA [26], sensitivity analyses were performed to determine whether the association was present after the exclusion of participants with prevalent T2D at baseline. Moreover, considering the potential role of BCAA as regulator of lipid metabolism [27], a sensitivity analysis was conducted after exclusion of participants taking lipid-lowering medication. Finally, to further assess the association of DRI with incident hypertension in subjects with different BMI, particularly those with normal BMI, we analyzed two separated subgroups, those with a normal BMI (<25 kg/m2) and those with (BMI ≥25 kg/m2).

The Net Reclassification Index (NRI) and the Integrated Discrimination Improvement index (IDI) were calculated in order to evaluate whether DRI scores can improve the predictive ability of BCAA for incident hypertension. The base model included the variables: age, sex, parental history of hypertension, smoking behavior (current versus former or never smoker), according to the Framingham hypertension risk prediction model [28], as well as eGFR and albuminuria, which are predictors of incident hypertension in the general population [29, 30]. The second model included all the variables mentioned above as well as the DRI score. The NRI was calculated using predefined risk categories of hypertension development [31]: low (<10%), intermediate (10% to 20%), and high (≥20%). All statistical analyses were performed with R language for statistical computing software, v. 4.0.3 (2020), (Vienna, Austria) [32].

Results

Baseline characteristics

Of the PREVEND participants that completed the second round of screening, 4169 subjects who did not have hypertension and had complete data available on DRI and covariates at the time of screening were included in this study. Mean baseline DRI score was 37.71 (SD=19.53) in the subjects who developed new-onset hypertension vs. 29.70 (SD=18.01) in the subjects who did not ($p < 0.001$). Baseline characteristics, reported by quartiles of DRI scores, can be found in Table 1. Participants with higher DRI scores were more likely to be men and older. They were also more likely to have a history of T2D, be current smokers, consume more alcohol, and to be on lipid lowering medications. Those in the highest quartile of DRI scores had higher BMI, systolic and diastolic blood pressure, TC, TG, LDL-C, glucose, insulin, BCAA and lower HDL-C as well as lower eGFR and higher UAE.

Cross-sectional analysis

The associations of the concentration of DRI score with other variables of interest were evaluated with univariable and multivariable linear regression analyses. The univariable linear regression results were congruent with the trends reported in Table 1. DRI scores were positively associated with sex, age, history of T2D, smoking, alcohol consumption, lipid lowering medications, BMI, systolic and diastolic blood pressure, TC, TG, LDL-C, glucose, and BCAA. DRI was negatively associated with HDL-C (Table 2). In multivariable analyses, DRI scores were positively associated with the following variables: sex, BMI, waist circumference, diastolic blood pressure, alcohol consumption, glucose, insulin, TC, TG and inversely with HDL-C, ($p < 0.05$) (Table 3).

Longitudinal analysis

During a median follow-up period of 8.6 years (IQR, 8.0–8.2), 924 individuals were diagnosed with new-onset hypertension, of which 122 were prescribed diuretics; 194 were prescribed β-blockers; 153 were prescribed angiotensin-converting enzyme inhibitors (ACEi) and 53 were prescribed angiotensin receptor blockers (ARBs). 171 individuals received more than 1 prescription: 46 diuretics and β-blockers, 37 diuretics and ACEi, 16 diuretics and ARBs, 47 β-blockers and ACEi, 17 β-blockers and ARBs, 8 ACEi and ARBs. 24 individuals used more than 2 antihypertensive drugs.

Table 1. Baseline characteristics by quartiles of DRI scores in PREVEND participants (n=4169)

	Quartiles of DRI				p-value
	Q1	Q2	Q3	Q4	
Men, (%)	162 (15.3%)	382 (36.2%)	574 (55.9%)	785 (76.4%)	< 0.001
Age, years	47.82 (10.0)	49.09 (10.67)	50.13 (10.54)	50.17 (9.91)	< 0.001
BMI, kg/m^2	23.96 (3.46)	24.85 (3.26)	26.25 (3.86)	27.77 (3.98)	< 0.001
Waist circumference, cm	81.17 (9.67)	85.76 (9.50)	90.96 (10.12)	96.81 (10.19)	< 0.001
SBP, mmHg	112.72 (10.63)	115.02 (10.56)	118.72 (10.47)	122.33 (9.98)	< 0.001
DBP, mmHg	67.21 (7.05)	69.13 (6.99)	71.20 (6.90)	73.32 (6.76)	< 0.001
History of Cancer, (%)	43 (4.1%)	40 (3.8%)	39 (3.8%)	44 (4.3%)	0.49
History of CVD, (%)	10 (0.9%)	16 (1.5%)	12 (1.2%)	14 (1.4%)	0.67
History of T2D, (%)	9 (0.8%)	11 (1.0%)	32 (3.1%)	62 (6.0%)	< 0.001
Smoking status, (%)					< 0.001
never	363 (34.3%)	333 (31.5%)	323 (31.5%)	263 (25.6%)	
former	375 (35.4%)	408 (38.6%)	378 (36.8%)	404 (39.3%)	
<6 cig/day	65 (6.1%)	42 (4.0%)	53 (5.2%)	49 (4.8%)	
6-20 cig/day	208 (19.6%)	220 (20.8%)	213 (20.8%)	245 (23.8%)	
>20 cig/day	33 (3.1%)	43 (4.1%)	37 (3.6%)	59 (5.7%)	
Alcohol consumption, (%)					< 0.001
No, almost never	274 (25.9%)	221 (20.9%)	230 (22.4%)	213 (20.7%)	
1-4 drinks/month	190 (17.9%)	197 (18.7%)	160 (15.6%)	164 (16.0%)	
2-7 drinks/week	356 (33.6%)	394 (37.3%)	352 (34.3%)	342 (33.3%)	
1-3 drinks/day	221 (20.9%)	216 (20.5%)	239 (23.3%)	250 (24.3%)	
4 or more drinks/day	18 (1.7%)	28 (2.7%)	45 (4.4%)	59 (5.7%)	
Lipid-lowering medication, (%)	17 (1.6%)	19 (1.8%)	28 (2.7%)	55 (5.4%)	< 0.001
DRI score, median	9.00 (2.00, 13.00)	23.00 (20.00, 26.00)	36.00 (33.00, 40.00)	55.00 (49.00, 63.00)	< 0.001
Glucose, mmol/L	4.50 (4.30, 4.80)	4.60 (4.30, 5.10)	4.70 (4.40, 5.20)	5.00 (4.50, 5.40)	< 0.001
Insulin, mU/L	5.80 (4.50, 7.80)	6.50 (4.90, 8.80)	7.60 (5.60, 10.30)	10.70 (7.40, 15.30)	< 0.001
Isoleucine, µM/L	29.72 (23.67, 36.65)	38.14 (32.15, 44.36)	44.29 (36.65, 52.06)	53.77 (45.54, 63.38)	< 0.001
Leucine, µM/L	90.40 (28.25)	116.68 (13.33)	129.69 (17.09)	153.74 (23.37)	< 0.001
Valine, µM/L	155.87 (47.69)	193.82 (22.60)	212.90 (27.79)	238.46 (31.63)	< 0.001
Total Cholesterol, mmol/L	5.19 (1.01)	5.21 (0.99)	5.41 (1.05)	5.67 (1.04)	< 0.001
Triglycerides, mmol/L	0.73 (0.58, 0.93)	0.88 (0.68, 1.10)	1.12 (0.88, 1.40)	1.74 (1.36, 2.27)	< 0.001
HDL-C, mmol/L	1.48 (0.36)	1.36 (0.27)	1.22 (0.26)	1.06 (0.20)	< 0.001
LP-IR score	13.00 (6.00, 21.00)	24.00 (18.00, 35.00)	43.00 (34.00, 53.00)	70.50 (58.00, 82.00)	< 0.001
Large VLDL-P, nmol/L	1.30 (0.70, 2.15)	1.90 (1.20, 3.10)	3.60 (2.30, 5.60)	8.40 (5.50, 12.70)	< 0.001
VLDL size, nm	44.71 (10.49)	46.50 (7.47)	48.59 (7.66)	55.28 (8.60)	< 0.001
Small LDL-P, nmol/L	161.0 (0.0, 277.0)	245.5 (146.0, 364.2)	343.0 (221.2, 465.7)	574.5 (382.0, 789.2)	< 0.001
LDL size, nm	20.84 (3.35)	21.17 (1.80)	21.04 (1.42)	20.57 (0.90)	< 0.001
Large HDL Particles, µmol/L	7.49 (2.74)	6.06 (2.32)	4.50 (2.24)	2.80 (1.58)	< 0.001
HDL size, nm	9.58 (1.32)	9.39 (0.48)	9.02 (0.49)	8.65 (0.36)	< 0.001
eGFR, mL/min/1.73 m^2	98.05 (14.48)	97.35 (14.27)	96.25 (14.10)	95.92 (14.29)	0.002
UAE, mg/24 h	7.01 (5.62, 10.20)	7.41 (5.55, 11.37)	7.64 (5.78, 11.43)	8.84 (6.21, 15.06)	< 0.001

Continuous variables are reported as mean ± standard deviation, median (IQR, interquartile range) and categorical variables are reported as percentage. p values were determined using a one-way analysis of variance for normally distributed data, Kruskal–Wallis test for skewed distributed data, and chi-square test for categorical data and represent a significant difference across the quartiles of DRI score.

Table 2. Univariate associations of DRI scores with baseline characteristics.

Characteristic	Std. β (95% CI)	p value
Men	0.99 (0.90, 1.0)	**<0.001**
Age	0.09 (0.06, 0.12)	**<0.001**
BMI	0.39 (0.36, 0.42)	**<0.001**
Waist circumference	0.54 (0.52, 0.57)	**<0.001**
SBP	0.35 (0.32, 0.38)	**<0.001**
DBP	0.33 (0.30, 0.36)	**<0.001**
History of Cancer	0.02 (-0.14, 0.18)	0.82
History of CVD	0.11 (-0.16, 0.39)	0.42
History of T2D	0.93 (0.75, 1.1)	**<0.001**
Smoking status		
former	0.14 (0.06, 0.21)	**<0.001**
<6 cig/day	0.01 (-0.14, 0.16)	0.91
6-20 cig/day	0.18 (0.09, 0.26)	**<0.001**
>20 cig/day	0.35 (0.19, 0.51)	**<0.001**
Alcohol consumption		
1-4 drinks/month	0 (-0.10, 0.10)	0.97
2-7 drinks/ week	0.04 (-0.04, 0.13)	0.29
1-3 drinks/day	0.14 (0.05, 0.23)	**0.002**
4 or more drinks/day	0.53 (0.35, 0.70)	**<0.001**
Lipid-lowering medication	0.45 (0.27, 0.63)	**<0.001**
Glucose	0.27 (0.24, 0.30)	**<0.001**
Insulin	0.41 (0.38, 0.44)	**<0.001**
Isoleucine	0.71 (0.68, 0.73)	**<0.001**
Leucine	0.99 (0.94, 1.0)	**<0.001**
Valine	0.93 (0.90, 1.0)	**<0.001**
TC	0.21 (0.18, 0.24)	**<0.001**
Triglycerides	0.62 (0.59, 0.64)	**<0.001**
HDL-C	-0.58 (-0.61, -0.55)	**<0.001**
LP-IR score	0.88 (0.86, 0.89)	**<0.001**
Large VLDL-P	0.69 (0.67, 0.71)	**<0.001**
VLDL size	0.49 (0.46, 0.52)	**<0.001**
Small LDL-P	0.65 (0.63, 0.68)	**<0.001**
LDL size	-0.21 (-0.25, -0.17)	**<0.001**
Large HDL Particles	-0.66 (-0.68, -0.63)	**<0.001**
HDL size	-0.93 (-1.0, -0.90)	**<0.001**
eGFR	-0.07 (-0.10, -0.04)	**<0.001**
UAE	0.07 (0.04, 0.10)	**<0.001**

Standardized regression coefficients are shown. Abbreviations: DRI, Diabetes Risk Index; T2D, type 2 diabetes mellitus; CVD, cardiovascular disease; BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; TC, total cholesterol; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; TG, triglycerides; LP-IR, Lipoprotein Insulin Resistance Index; VLDL-P, very low-density lipoprotein particles; LDL-P, low-density lipoprotein particles; HDL-P, high-density lipoprotein particles.

Cox regression analyses revealed that high DRI scores were associated with increased risk of hypertension in the crude model, with a hazard ratio (HR) per 1 SD increase of 1.45 (95% CI: 1.36, 1.54; p <0.001) (Table 3). The adjusted HR for sex and age was (HR_{adj} per one 1 SD increase: 1.37, 95% CI: 1.28, 1.47; p <0.001). This association remained after adjustment for BMI, SBP, smoking, alcohol consumption, T2D, eGFR, and UAE (HR_{adj} per one 1 SD increase: 1.11, 95% CI: 1.03, 1.20; p =0.008), as well as TC, HDL-C, TG, glucose, insulin, eGFR, and UAE (HR_{adj} per 1 SD increase: 1.21, 95% CI: 1.10,1.33; p < 0.001) (Table 4).

Table 3. Multivariable associations of DRI with baseline characteristics.

Characteristic	Std. β (95% CI)	*p* value
Men	0.47 (0.41, 0.52)	**<0.001**
Age	-0.03 (-0.05, 0.00)	0.05
BMI	0.11 (0.07, 0.15)	**<0.001**
Waist circumference	0.05 (0.01, 0.09)	**0.017**
SBP	0.02 (-0.01, 0.04)	0.27
DBP	0.03 (0.00, 0.06)	**0.021**
History of Cancer	-0.06 (-0.16, 0.04)	0.22
History of CVD	-0.07 (-0.25, 0.11)	0.46
History of T2D	0.08 (-0.07, 0.23)	0.31
Smoking status		
former	0.02 (-0.03, 0.06)	0.54
<6 cig/day	-0.02 (-0.11, 0.08)	0.73
6-20 cig/day	-0.02 (-0.08, 0.04)	0.49
>20 cig/day	-0.03 (-0.14, 0.07)	0.54
Alcohol consumption		
1-4 drinks/month	0.1 (0.03, 0.16)	**0.002**
2-7 drinks/ week	0.15 (0.10, 0.21)	**<0.001**
1-3 drinks/day	0.28 (0.21, 0.34)	**<0.001**
4 or more drinks/day	0.38 (0.26, 0.49)	**<0.001**
Lipid-lowering medication	0.1 (-0.02, 0.22)	0.11
Glucose	0.06 (0.03, 0.09)	**<0.001**
Insulin	0.12 (0.10, 0.15)	**<0.001**
TC	0.06 (0.04, 0.09)	**<0.001**
Triglycerides	0.35 (0.32, 0.37)	**<0.001**
HDL-C	-0.27 (-0.30, -0.24)	**<0.001**
eGFR	-0.02 (-0.04, 0.01)	0.14
UAE	0.01 (-0.01, 0.03)	0.22

Standardized regression coefficients from multivariable linear regression are shown.

Similarly, Cox proportional hazard regression analysis was performed using quartiles of DRI scores. The crude model again revealed that DRI scores are associated with incident hypertension with a HR for the highest quartile of 2.74 (95% CI: 2.27, 3.32; $p < 0.001$). The association remained significant after adjustment for age, sex, BMI, systolic blood pressure, smoking, alcohol consumption, history of T2D, eGFR, and UAE (HRadj: 1.28, 95% CI: 1.03, 1.60; $p = 0.03$), as well as TC, HDL-C, TG, glucose, insulin, eGFR, and UAE (HRadj: 1.64, 95% CI: 1.28, 2.10; $p < 0.001$) (Table 5). Consistently, the Kaplan–Meier curves for hypertension according to quartiles of DRI score are presented in Figure 1. The graph depicts an increased risk of hypertension events in subjects in the top quartile of DRI multimarker (p log rank < 0.001).

Table 4. Association of DRI scores with incident hypertension in the PREVEND study (n= 4169).

	DRI Per 1 SD increment	
Participants	**4169**	
Events	**924**	
	HR (95 % CI)	**p value**
Crude model	1.45 [1.36;1.54]	< 0.001
Model 1	1.37 [1.28;1.47]	< 0.001
Model 2	1.11 [1.02;1.19]	0.008
Model 2b	1.14 [1.05;1.23]	<0.001
Model 3	1.21 [1.10;1.33]	< 0.001

Table 5. Association of DRI scores with incident hypertension by quartiles in the PREVEND study (n= 4169).

	Q1 DRI<17	**Q2 DRI 17-30**		**Q3 DRI 30-45**		**Q4 DRI >45**	
Participants	**1059**	**1056**		**1026**		**1028**	
Events	**155**	**197**		**228**		**344**	
		HR (95 % CI)	***p* value**	**HR (95 % CI)**	***p* value**	**HR (95 % CI)**	***p* value**
Crude model	(ref)	1.40 [1.13;1.73]	0.02	1.69 [1.37;2.07]	< 0.001	2.74 [2.27;3.32]	< 0.001
Model 1	(ref)	1.24 [1.00;1.54]	0.05	1.41 [1.14;1.75]	0.002	2.25 [1.82;2.77]	< 0.001
Model 2	(ref)	1.12 [0.90;1.40]	0.29	1.01 [0.79;1.23]	0.89	1.27 [1.01;1.59]	0.04
Model 2b	(ref)	1.07 [0.86;1.33]	0.55	1.05 [0.84;1.31]	0.67	1.37 [1.09;1.71]	0.007
Model 3	(ref)	1.18 [0.95;1.47]	0.13	1.22 [0.97;1.52]	0.08	1.64 [1.28;2.10]	< 0.001

Data are presented as hazard ratios (HRs) with 95% CIs and *p* values.
Model 1: Model adjusted for age and sex.
Model 2: Model 1 + BMI + SBP + heart rate + T2D + smoking + alcohol consumption + eGFR + UAE.
Model 2b: Model 1 + BMI + DBP + heart rate + T2D + smoking + alcohol consumption + eGFR + UAE.
Model 3: Model 1 + TC + HDL-C + TG + glucose + insulin.
Abbreviations. BMI, body mass index; DBP, diastolic blood pressure; eGFR, estimated glomerular filtration rate; HDL-C, high-density lipoprotein cholesterol; SBP, systolic blood pressure; T2D, type 2 diabetes; TC, total cholesterol; TG, triglycerides; and UAE, urinary albumin excretion.

Notably, the association of DRI was present both in participants with and without overweight. In the subgroup of participants with BMI < 25 kg/m2, DRI was associated with increased risk of hypertension in the crude model, with a HR per 1 SD increase of 1.34 (95% CI: 1.22, 1.48; p <0.001) as well as in the model adjusted for age and sex (HRadj per 1 SD increase: 1.25, 95% CI: 1.11,1.39; p < 0.001). Likewise, In the subgroup of participants with BMI ≥ 25 kg/m2, DRI was associated with increased risk of hypertension in the crude model, with a HR per 1 SD increase of 1.37 (95% CI: 1.26, 1.49; p <0.001) as well as in the model adjusted for age and sex (HRadj per 1 SD increase: 1.33, 95% CI: 1.21,1.45; p < 0.001).

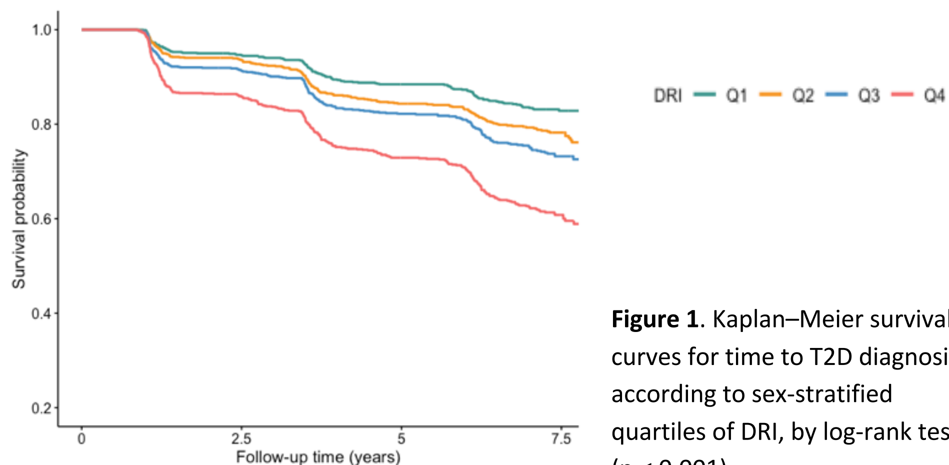

Figure 1. Kaplan–Meier survival curves for time to T2D diagnosis according to sex-stratified quartiles of DRI, by log-rank test ($p < 0.001$).

The Net Reclassification Index (NRI) was 0.13 (95% CI: 0.06, 0.20; $p < 0.001$), denoting that when DRI was added to the model, more subjects were correctly re-classified than with the Framingham Offspring Study risk score alone. The addition of DRI to the Framingham Offspring Study risk score allowed for the proper reclassification of 34% of subjects who developed hypertension during the follow-up. The IDI of the DRI enhanced model was 0.020 (95% CI: 0.015, 0.025), $p < 0.001$. Considering that DRI scores are higher in men, the cox regression analyses were performed using the sex-stratified quartiles of DRI scores. The results were similar to the non-sex-stratified quartiles analyses. The crude model again revealed that DRI scores were associated with incident hypertension with a HR for the highest quartile of 2.22 (95% CI: 1.85, 2.66; $p < 0.001$). This association remained after fully adjustment described above (HRadj: 1.55; 95% CI: 1.24, 1.93, $p < 0.001$) (Supplemental Table 3).

The sensitivity analysis conducted in participants free from T2D (n= 4056) revealed similar results to those found in the whole cohort. In the crude model, DRI scores were associated with incident hypertension with a HR for the highest quartile of 2.72 (95% CI: 2.24, 3.30; $p < 0.001$). Similarly, the association remained after fully adjustment described above (HRadj: 1.63; 95% CI: 1.27, 2.09, $p = 0.003$) (Supplemental Table 4).

Moreover, the sensitivity analysis conducted in subjects free from lipid lowering medication (n= 4050) revealed similar results to those found in the whole cohort. In the crude model, DRI scores were associated with incident hypertension with a HR for the highest quartile of 2.72 (95% CI: 2.24, 3.31; p <

0.001). Similarly, the association remained after fully adjustment described above (HRadj: 1.65; 95% CI: 1.28, 2.13, $p < 0.001$) (Supplemental Table 5).

Finally, DRI was compared to conventional metabolic indexes such as BMI and waist circumference. With incident hypertension as outcome, DRI showed a sensitivity and specificity of 0.68 and 0.74, respectively (Area under the curve (AUC): 0.78); meanwhile BMI showed a sensitivity and specificity of 0.69 and 0.46, respectively (AUC: 0.61). Consequently, the performance of the DRI was better than that of the BMI (p-value < 0.001). In addition, waist circumference showed a sensitivity and specificity of 0.63 and 0.57, respectively (AUC: 0.63), making that the performance of DRI was better than that of the waist circumference (p-value < 0.001).

Discussion

In this large prospective cohort, comprising 4169 participants, we report for the first time that higher scores of DRI, a newly developed risk algorithm based on BCAA and six lipoprotein particles parameters, are associated with incidence of hypertension. Multivariable adjusted time-to-event analyses showed that the positive association of DRI with hypertension was present after adjustment for age, sex, glucose, insulin and BMI. Additionally, we demonstrated that the Framingham Offspring Study model for hypertension risk enhanced with DRI scores improved reclassification of participants across clinical risk categories for hypertension compared to the model enriched with DRI scores, which comprises the information of six lipoprotein particles parameters: the weighted average sizes of VLDL, LDL and HDL, along with concentrations of large VLDL, small LDL, and large HDL particles, as well as the concentrations of two BCAA: leucine and valine.

The components of the DRI score are closely related to the development of hypertension. A recent study has revealed an association between BCAA and incident hypertension [4]. In addition, there is extensive evidence that high BCAA concentrations induces generation of reactive oxygen species and mitochondrial dysfunction [33]; which are known to be linked to the physio-pathogenesis of hypertension [34]. Moreover, it has been reported that circulating BCAA can induce pro-inflammatory responses through the transcription factor NF-κB, resulting in the release of intracellular adhesion molecule-1 (ICAM-1) and E-selectin; hence, contributing to the development of

hypertension [35]. Likewise, the HDL particles have been shown to be linked with different pathophysiology of hypertension, such as the regulation of fibrinolysis, particularly the transport of the plasmin regulator, alpha-2-antiplasmin [36], which recently had been identified as critical regulator of angiotensin II and vascular remodeling [37].

Moreover, several epidemiological studies have revealed the associations between the DRI components and hypertension. BCAA has been found to be consistently associated with prevalent hypertension in different studies [10, 38, 39]. Likewise, previous epidemiological studies have also reported the association between lipoprotein particle profiles and development of hypertension [40]. A study which involved 17,527 participants demonstrated that higher concentrations of small LDL, small HDL, and large VLDL particles were prospectively associated with incidence of hypertension in during 8 years of follow-up [41]. Furthermore, it has been reported that elevated concentrations of small dense LDL cholesterol associated with reduced blood flow and enhanced shear stress of the blood vessels, in early-stage hypertensive patients [42].

In the present study we found that high scores of DRI associated with increased risk of hypertension. Interestingly, the highest quartile of DRI was comprised predominantly of men. The increased proportion of men in the highest quartile of DRI, compared to LP-IR, could be explained by the fact that both plasma concentrations of BCAA and dietary intake of BCAA-rich foods are higher in men [43, 44]. Despite the fact that there is not a general consensus about what are the main determinants of circulating BCAA, some studies suggest that such differences may at least in part be attributed to differences in dietary patterns between men and women [45]. Importantly, the association of DRI with incident hypertension remained after adjustment for sex, as well as in in sex-stratified analyses.

It has been reported that the circulating concentrations of BCAA, which are an important component of the DRI score, found to be a useful biomarker of insulin sensitivity improvement in overweight participants of a lifestyle intervention [42]. Contemplating that the global hypertension practice guidelines from the International Society of Hypertension highlights that lifestyle modification is the first line of antihypertensive treatment and may also augment the effects of pharmacologic antihypertensive treatment [46], DRI

could also be an instrument to track the effects of lifestyle intervention in the context of hypertension. Further research is desirable to evaluate the usefulness of DRI on that concern.

We acknowledge several strengths of the present study. This study included a large population with a wide age range which allowed for the adjustment of the analysis with sufficient statistical power. Another strength of the present study is the implementation of a robust method of BCAA and lipoprotein particles quantification by means of NMR. The inclusion of lipoprotein particles in the multimarker may offer several advantages, given the fact that the distribution of HDL particles is closely associated with hypertensive status. Moreover, it has been recognized that subjects with different cardiovascular risk may have indistinguishable concentrations of traditional lipids (such as LDL), but have important differences in other lipid measurements such as lipoprotein size and particle concentration [47]. To the best of our knowledge this study explores for first time the performance of a test comprising the dual factors of lipoprotein particles and BCAA in the context of hypertension risk assessment.

We are also aware of the limitations of the study. First, the present study was conducted in the north of the Netherlands, and mainly comprises individuals of north European ancestry, which could limit the extrapolation of the current findings to other ethnicities. Secondly, the measurement of blood pressure was conducted in the outpatient clinic; twenty-four-hour ambulatory blood pressure monitoring, which remains the gold standard for diagnosing hypertension was not performed. Furthermore, this prospective cohort study did not record physical activity and therefore, our analyses could not be adjusted for such a variable. In addition, the observational nature of the study prevents the ability to draw causal conclusions. This fact restricts the capacity to describe the underlying biological mechanisms. Moreover, even after the adjustment for several variables, residual confounding remains a limitation in observational studies.

In conclusion, this prospective cohort study showed that high score of DRI, an NMR spectroscopy-measured multimarker of lipoprotein particles and BCAA, is associated with an increased risk of developing hypertension in both men and women in the general population during extended follow-up.

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Supplemental Table 1. STROBE Statement—Checklist of items that should be included in reports of cohort studies

	Item No	Recommendation	Page No
Title and abstract	1	(a) Indicate the study's design with a commonly used term in the title or the abstract (b) Provide in the abstract an informative and balanced summary of what was done and what was found	145 145
Introduction			
Background/rationale	2	Explain the scientific background and rationale for the investigation being reported	147
Objectives	3	State specific objectives, including any prespecified hypotheses	147
Methods			
Study design	4	Present key elements of study design early in the paper	148
Setting	5	Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection	148
Participants	6	(a) Give the eligibility criteria, and the sources and methods of selection of participants. Describe methods of follow-up (b) For matched studies, give matching criteria and number of exposed and unexposed	148
Variables	7	Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable	7,8
Data sources/ measurement	8*	For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group	6,7
Bias	9	Describe any efforts to address potential sources of bias	149-151
Study size	10	Explain how the study size was arrived at	148
Quantitative variables	11	Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen and why	149-151
Statistical methods	12	(a) Describe all statistical methods, including those used to control for confounding (b) Describe any methods used to examine subgroups and interactions (c) Explain how missing data were addressed (d) If applicable, explain how loss to follow-up was addressed (e) Describe any sensitivity analyses	151-153 b.NA 149 d.NA 153
Results			
Participants	13*	(a) Report numbers of individuals at each stage of study—eg numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analysed (b) Give reasons for non-participation at each stage (c) Consider use of a flow diagram	149 149
Descriptive data	14*	(a) Give characteristics of study participants (eg demographic, clinical, social) and information on exposures and potential confounders	154

		(b) Indicate number of participants with missing data for each variable of interest	149
		(c) Summarise follow-up time (eg, average and total amount)	153
Outcome data	15*	Report numbers of outcome events or summary measures over time	159
Main results	16	(a) Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (eg, 95% confidence interval). Make clear which confounders were adjusted for and why they were included	159
		(b) Report category boundaries when continuous variables were categorized	159
		(c) If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period	158
Other analyses	17	Report other analyses done—eg analyses of subgroups and interactions, and sensitivity analyses	160-162
Discussion			
Key results	18	Summarise key results with reference to study objectives	163
Limitations	19	Discuss limitations of the study, taking into account sources of potential bias or imprecision. Discuss both direction and magnitude of any potential bias	166
Interpretation	20	Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence	165
Generalisability	21	Discuss the generalisability (external validity) of the study results	165
Other information			
Funding	22	Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based	2

*Give information separately for exposed and unexposed groups.

Supplemental Table 2. Multivariable associations of covariables included in the cox regression analyses (Model 3 from Tables 4 and 5).

	DRI per 1 SD		DRI Q4 vs Q1	
	HR (95 % CI)	***p* value**	**HR (95 % CI)**	***p* value**
DRI	1.21 [1.10;1.33]	< 0.001	1.64 [1.28;2.10]	< 0.001
Age	1.04 [1.03;1.04]	<0.001	1.04 [1.03;1.04]	<0.001
Sex	1.13 [0.97;1.32]	0.08	1.14 [0.98;1.33]	0.08
TC	1.07 [1.00;1.15]	0.05	1.07 [1.00;1.15]	0.05
TG	1.04 [0.95;1.13]	0.21	1.05 [0.97;1.14]	0.21
HDL-C	0.74 [0.56;0.97]	0.02	0.73 [0.56;0.96]	0.02
Insulin	1.01 [1.00;1.02]	0.01	1.01 [1.00;1.02]	0.01
Glucose	1.05 [0.99;1.11]	0.07	1.05 [1.00;1.11]	0.07

Abbreviations: DRI, Diabetes Risk Index; TC, total cholesterol; HDL-C, high-density lipoprotein cholesterol; TG, triglycerides; HDL-P, high-density lipoprotein particles.

Supplemental Table 3. Association of DRI scores with incident hypertension by sex-stratified quartiles in the PREVEND study (n= 4169).

	Q1 DRI	Q2 DRI		Q3 DRI		Q4 DRI	
Women	<12	12-22		22-33		>33	
Men	<29	29-41		41-55		>55	
Participants	1116	1045		995		1013	
Events	182	198		215		329	
		HR (95 % CI)	***p* value**	**HR (95 % CI)**	***p* value**	**HR (95 % CI)**	***p* value**
Crude model	(ref)	1.17 [0.96;1.43]	0.13	1.39 [1.14;1.69]	0.001	2.22 [1.85;2.66]	< 0.001
Model 1	(ref)	1.13 [0.92;1.38]	0.23	1.32 [1.09;1.61]	0.005	2.05 [1.71;2.46]	< 0.001
Model 2	(ref)	0.97 [0.79;1.19]	0.77	1.02 [0.83;1.25]	0.87	1.24 [1.02;1.51]	0.03
Model 3	(ref)	1.11 [0.91;1.36]	0.30	1.21 [0.98;1.48]	0.07	1.55 [1.24;1.93]	< 0.001

Data are presented as hazard ratios (HRs) with 95% CIs and p values.
Model 1: Model adjusted for age and sex.
Model 2: Model 1 + BMI + SBP + T2D + smoking + alcohol consumption + eGFR + UAE.
Model 3: Model 1 + TC + HDL-C + TG + glucose + insulin.
Abbreviations. BMI, body mass index; eGFR, estimated glomerular filtration rate; HDL-C, high-density lipoprotein cholesterol; SBP, systolic blood pressure; T2D, type 2 diabetes; TC, total cholesterol; TG, triglycerides; and UAE, urinary albumin excretion.

Supplemental Table 4. Association of DRI scores with incident hypertension by quartiles in participants free from T2D at baseline (n= 4056).

	Q1 DRI<17	Q2 DRI 17-29		Q3 DRI 29-45		Q4 DRI >45	
Participants	**1051**	**984**		**1055**		**966**	
Events	**153**	**178**		**232**		**321**	
		HR (95 % CI)	***p* value**	**HR (95 % CI)**	***p* value**	**HR (95 % CI)**	***p* value**
Crude model	(ref)	1.36 [1.09;1.69]	0.005	1.69 [1.38;2.08]	< 0.001	2.72 [2.24;3.30]	< 0.001
Model 1	(ref)	1.22 [0.98;1.52]	0.07	1.42 [1.14;1.76]	0.001	2.26 [1.82;2.80]	< 0.001
Model 2	(ref)	1.10 [0.88;1.37]	0.41	1.03 [0.83;1.29]	0.78	1.28 [1.02;1.61	0.03
Model 2b	(ref)	1.15 [0.92;1.44]	0.20	1.22 [0.98;1.53]	0.07	1.63 [1.27;2.09]	< 0.001
Model 3	(ref)	1.36 [1.09;1.69]	0.005	1.69 [1.38;2.08]	< 0.001	2.72 [2.24;3.30]	< 0.001

Data are presented as hazard ratios (HRs) with 95% CIs and p values.

Model 1: Model adjusted for age and sex.

Model 2: Model 1 + BMI + SBP + smoking + alcohol consumption + eGFR + UAE.

Model 3: Model 1 + TC + HDL-C + TG + glucose + insulin.

Abbreviations. BMI, body mass index; eGFR, estimated glomerular filtration rate; HDL-C, high-density lipoprotein cholesterol; SBP, systolic blood pressure; T2D, type 2 diabetes; TC, total cholesterol; TG, triglycerides; and UAE, urinary albumin excretion.

Supplemental Table 5. Association of DRI scores with incident hypertension by quartiles in participants free from lipid-lowering medication (n= 4050).

	Q1 DRI<17	Q2 DRI 17-30		Q3 DRI 30-45		Q4 DRI >45	
Participants	**1042**	**1037**		**998**		**973**	
Events	**151**	**190**		**215**		**321**	
		HR (95 % CI)	***p* value**	**HR (95 % CI)**	***p* value**	**HR (95 % CI)**	***p* value**
Crude model	(ref)	1.38 [1.12;1.72]	0.003	1.64 [1.33;2.02]	<0.001	2.72 [2.24;3.31]	<0.001
Model 1	(ref)	1.24 [1.00;1.55]	0.05	1.38 [1.10;1.71]	0.004	2.25 [1.81;2.79]	<0.001
Model 2	(ref)	1.12 [0.90;1.39]	0.32	0.98 [0.78;1.22]	0.82	1.31 [1.04;1.65]	0.02
Model 2b	(ref)	1.18 [0.95;1.47]	0.13	1.19 [0.94;1.49]	0.14	1.65 [1.28;2.13]	<0.001
Model 3	(ref)	1.36 [1.09;1.69]	0.005	1.69 [1.38;2.08]	< 0.001	2.72 [2.24;3.30]	< 0.001

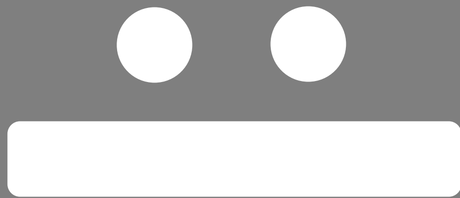
Data are presented as hazard ratios (HRs) with 95% CIs and p values.

Model 1: Model adjusted for age and sex.

Model 2: Model 1 + BMI + SBP + T2D + smoking + alcohol consumption + eGFR + UAE.

Model 3: Model 1 + TC + HDL-C + TG + glucose + insulin.

Abbreviations. BMI, body mass index; eGFR, estimated glomerular filtration rate; HDL-C, high-density lipoprotein cholesterol; SBP, systolic blood pressure; T2D, type 2 diabetes; TC, total cholesterol; TG, triglycerides; and UAE, urinary albumin excretion.



Chapter 7

Circulating Trimethylamine N-Oxide Is Associated With Increased Risk Of Cardiovascular Mortality In Type 2 Diabetes: Results From A Dutch Diabetes Cohort (ZODIAC-59).

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Abstract

Trimethylamine N-oxide (TMAO), a novel cardiovascular (CV) disease and mortality risk marker, is a gut microbiota-derived metabolite as well. Recently, plasma concentrations of branched chain amino acids (BCAA) have been reported to be affected by microbiota. The association of plasma TMAO with CV mortality in Type 2 Diabetes (T2D) and its determinants are still incompletely described. We evaluated the association between plasma BCAA and TMAO, and the association of TMAO with CV mortality in T2D individuals. We used data of 595 participants (mean age 69.5 years) from the Zwolle Outpatient Diabetes project Integrating Available Care (ZODIAC) cohort were analyzed. Plasma TMAO and BCAA were measured with nuclear magnetic resonance spectroscopy. CV mortality risk was estimated using multivariable-adjusted Cox regression models. Cross-sectionally, TMAO was independently associated with BCAA Std $\beta = 0.18$ (95% CI 0.09; 0.27), $p < 0.001$. During a median follow-up of 10 years, 113 CV deaths were recorded. In Cox regression analyses, adjusted for multiple clinical and laboratory variables including BCAA, TMAO was independently associated with CV mortality: $_{adj}HR$ 1.93 (95% CI 1.11; 3.34), $p = 0.02$ (for the highest vs. the lowest tertile of the TMAO distribution). The same was true for analyses with TMAO as continuous variable: $_{adj}HR$ 1.32 (95% CI 1.07; 1.63), $p = 0.01$ (per 1 SD increase). In contrast, BCAAs were not associated with increased CV mortality. In conclusion, higher plasma TMAO but not BCAA concentrations are associated with an increased risk of CV mortality in individuals with T2D, independent of clinical and biochemical risk markers.

Introduction

Trimethylamine-N-oxide (TMAO) is a microbiota derived metabolite [1,2] that recently has gained attention as a consequence of its potential role in the progress of ischemic heart disease [3,4] kidney disease [5–7], complications in the setting of type 2 diabetes (T2D) [8,9] and premature mortality in the general population [10]. Trimethylamine (TMA) is a by-product of a microbial fermentation, in which the gut microbiota metabolizes dietary components such as phosphatidylcholine, choline and L-carnitine in order to be used as carbon fuel supply by the gut microbiota. Subsequently, the conversion of TMA to TMAO occurs in the host liver by the flavin monooxygenase 3, after which it is cleared by the kidneys [11].

Despite the fact that some studies have identified an association of plasma concentrations of TMAO with adverse cardiovascular (CV) outcomes [10], studies in individuals with latent comorbidities are not always consistent [5]. Moreover, the association of TMAO with CV mortality in individuals with T2D has recently been identified to be present in high-risk individuals [12]

Recently, it has been shown that plasma concentrations of branched chain amino acids (BCAA) are also affected by the microbiota in both animal models and humans [13]. Moreover, it has been hypothesized that the contribution of the microbiota on BCAA concentrations may be more important on settings related to insulin resistance, such as T2D [14]. Therefore, the aim of the present study was to evaluate the association of circulating BCAA concentrations with concentrations of TMAO and the determine the potential association of TMAO with CV taking account concentrations of BCAA in a prospective cohort of outpatients with T2D.

Materials and Methods

Study Population

Briefly, the Zwolle Outpatient Diabetes project Integrating Available Care (ZO-DIAC) study is a Dutch cohort of patients with T2D from the Zwolle region in the northern part of the Netherlands. The design of the ZODIAC study has been described in detail elsewhere [15]. Briefly, the ZODIAC study was initiated in 1998, in this study, the effects of a shared-care project in a primary care population of patients with T2D were investigated. At the beginning of the

prospective cohort study 1,143 patients with T2D were enrolled; patients with a reduced life expectancy, i.e., with insufficient cognitive abilities or active cancer were excluded from participation. For the current study, subjects with missing data on outcome, and those with missing quantification of TMAO or BCAA by means of nuclear magnetic resonance (NMR) at baseline were excluded, leaving 594 subjects for the present analyses. This report follows the Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) reporting guideline (Supplementary Table S1).

The ZODIAC study and the informed consent procedure was approved by the local medical ethics committee of the Isala Clinics, Zwolle, the Netherlands (METC reference numbers 03.0316 and 07.0335). Informed consent was obtained for all patients by the participating diabetes specialist nurses and the consent was documented in the patients records. All procedures were conducted according to the Declaration of Helsinki [16].

During visits to the outpatient clinic, all baseline data were collected as previously described [15]. Blood pressure was measured twice with a Welch Allyn sphygmomanometer (Skaneateles Falls, USA) in the supine position after at least 5 minutes of rest. Pulse pressure was determined as the difference between systolic and diastolic blood pressure. Height and weight were measured with the participants standing without shoes and heavy outer garments. Body mass index (BMI) was calculated by dividing weight in kilograms by height in meters squared.

Baseline data included a medical history of CVD, tobacco consumption and use of medication, were collected during the annual check-up of the patient by the general practitioner or practice nurse. Patients were considered to have a history of macrovascular complications if they had a history of stroke, angina pectoris, myocardial infarction, transient ischemic attack, percutaneous transluminal coronary angioplasty, coronary artery bypass grafting, or peripheral vascular disease.

Microvascular complications were defined as the presence of one or more of the following entities: Neuropathy was defined as two or more errors out of three tests of foot sensibility, using a 5.07 Semmes-Weinstein monofilament, at least at one foot: diabetic retinopathy was investigated with a retinal camera, and the fundus photos were judged by an ophthalmologist: nephropathy was defined as eGFR <60 ml/min/1.73m2 and/or albuminuria,

which was defined as an albumin-to-creatinine ratio > 3.5 mg/mmol for women and > 2.5 mg/mmol for men.

Clinical Endpoint

The primary end point was CV mortality. In 2013, vital status and cause of death were retrieved from records maintained by the hospital and the general practitioners or from the Municipal Personal Records Database. Causes of death were coded according to the International Classification of Diseases, Ninth Revision. Cardiovascular death was defined as death in which the principal cause of death was cardiovascular in nature, using International Classification of Diseases, Ninth Revision codes 390 to 459 [17,18].

Laboratory measurements

Laboratory assessment included non-fasting lipid profile, glycated hemoglobin (HbA1c), serum creatinine, urinary albumin-to-creatinine ratio (ACR), and blood pressure. Serum creatinine was measured by a kinetic colorimetric Jaffe method (Modular P Analyzer; Roche, Almere, the Netherlands), The creatinine-based Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) equation was used to estimate glomerular filtration rate (eGFR) [19]. To calculate the eGFR, serum creatinine levels were reduced by 5%, because serum creatinine measurements in this study were not standardized to isotope dilution mass spectrometry[20]. Urinary albumin was measured using immunonephelometry (Behring Nephelometer, Mannheim, Germany).

TMAO and BCAA were measured in EDTA-anticoagulated plasma samples using a Vantera® Clinical Analyzer (LabCorp, Morrisville, NC), a fully automated, high-throughput, 400 MHz proton (1H) nuclear magnetic resonance (NMR) spectroscopy platform. TMAO were quantified from one-dimensional (1D) proton (1H) Carr-Purcell-Meiboom-Gill (CPMG) spectra by means of deconvolution assays as previously described[21,22]. The TMAO assay has intra- and inter-assay coefficients of variation (CV%) range from 4.3–10.3% and 9.8–14.5%, respectively, and a limit of quantitation of 3.3 µM.

The validation of the use of NMR for quantification of BCAA has been previously described by our group [23,24]. Briefly, coefficients of variation for inter- and intraassay precision ranged from 1.8% to 6.0%, 1.7% to 5.4%, 4.4% to 9.1%, and 8.8% to 21.3%, for total BCAA, valine, leucine, and isoleucine, respectively. BCAA quantified from the same samples using NMR and LC-MS/MS

were highly correlated, showing a r2 = 0.97, 0.95 and 0.90 for valine, leucine, and isoleucine, respectively [23,24].

Statistical Analysis

Normally distributed data were presented as mean and standard deviation, whereas skewed data were expressed as median and interquartile range. Categorical data were presented as number and percentage. Skewed data were log-transformed when appropriate. Linear trends across TMAO tertiles were determined using ANOVA for normally distributed data, Kruskal-Wallis test for skewed distributed data, and $\chi 2$ test for categorical variables. For the cross-sectional analysis, a multivariable linear regression analysis was performed using the plasma concentration of TMAO as dependent variable. Given the fact that eGFR is calculated using creatinine and age information, eGFR was not included in the cross-sectional analysis. Therefore, we were able to better evaluate the association of age and creatinine with plasma concentrations of TMAO. To further investigate the potential association of TMAO with BCAA, as suggested by previous research [13,14], we included total BCAA.

For the prospective analysis, we plotted cumulative Kaplan-Meier curves for risk of CV mortality during follow-up according to tertiles of TMAO. Time-to-event Cox proportional hazards models were used to compute hazard ratios (HRs) and 95% CI of CV mortality risk among the 594 participants. HRs were calculated in models adjusted for age, sex, T2D duration, smoking behavior, prevalent macrovascular complications, systolic blood pressure, HbA1c, total cholesterol, high-density lipoprotein cholesterol (HDL-cholesterol), triglycerides, albuminuria and reduced eGFR at baseline (<60 mL/min/1.73 m2). The Cox proportional hazard assumption was tested through the evaluation of independence between scaled Schoenfeld residuals with time for each variable and for every model as a whole; this assumption was met, with no indication for a violation [25]. To further evaluate the robustness of the association and the risk of bias, a sensitivity analysis was conducted to calculate the Robustness of Inference to Re-placement [26].

The net reclassification improvement (NRI) [27] was calculated to evaluate whether the inclusion of TMAO into a model can improve the risk reclassification of participants. As a base model, a conventional model for cardiovascular mortality [28] already used to evaluate the NRI for mortality in people with T2D was used. Such model includes age, sex, smoking behavior,

systolic blood pressure, total cholesterol, HDL-cholesterol, and antihypertensive medications. For the computation of NRI three predefined risk categories of cardiovascular mortality previously described in the literature were used, i.e.: low (<7%), medium (7% to 20%), and high (>20%) [28]. All statistical analyses were per-formed with R language for statistical computing software [29], v. 4.0.2.

Results

Baseline characteristics

Out of the 594 subjects with available measurements of TMAO that were included in the current study, the mean age of the population was 69.5 ± 11.2 years and 42.1% (n= 250) were men. The median (IQR) plasma total TMAO concentration was 3.9 (2.4–6.5) µmol/L. Participant characteristics at baseline are shown in Table 1. Subjects with higher TMAO concentrations were more likely to be older, have higher systolic blood pressure and lower diastolic blood pressure, have a pronounced increased prevalence of macrovascular complications, have a slightly increased prevalence of microvascular complications and albuminuria as well as a lower eGFR (Table 1).

Cross-sectional analysis

The association of the concentration of TMAO with baseline characteristics was evaluated with multivariable linear regression analysis (Table 2). In a multivariable model, including the variables presented in Table 1, TMAO remained positively associated with systolic blood pressure (Std. β = 0.27 (95% CI 0.17; 0.38), $p < 0.001$), and negatively associated with diastolic blood pressure (Std. β = -0.19 (95% CI -0.29; -0.09), $p<0.001$) ((Supplementary Figure S1). TMAO was also associated with microvascular complications (Std. β = 0.23 (95% CI 0.06; 0.41), $p = 0.009$), total cholesterol, (Std. β= 0.12(95% CI 0.02;0.22), $p = 0.03$), BCAAs (Std. β = 0.18 (95% CI 0.09; 0.27), $p < 0.001$) and serum creatinine (Std. β = 0.17 (95% CI 0.09; 0.26), $p < 0.001$). Those results were comparable to the coefficients calculated in univariable regression analyses. (Supplementary Table S2). Moreover, higher plasma concentrations of TMAO were positively associated with greater pulse pressure (Std. β = 0.10 (95% CI 0.06; 0.14), $p <0.001$) (Supplementary Figure S2).

Table 1. Baseline participant characteristics according to tertiles of plasma TMAO concentration.

	All (*n*=594)	Tertile 1 (*n*=194)	Tertile 2 (*n*=193)	Tertile 3 (*n*=207)	p-value
Men, *n* (%)	250 (42.1%)	94 (48.5%)	74 (38.3%)	82 (39.6%)	0.08
Age, years	69.45 (11.19)	66.93 (11.30)	68.64 (11.60)	72.55 (9.93)	< 0.001
BMI, kg/m^2	28.84 (4.53)	28.73 (4.29)	29.12 (4.51)	28.69 (4.77)	0.58
SBP, mmHg	154.40 (24.02)	151.06 (24.68)	156.39 (22.48)	155.68 (24.57)	0.05
DBP, mmHg	83.38 (10.88)	83.55 (11.24)	85.17 (10.32)	81.54 (10.82)	0.004
Never smoked, *n* (%)	123 (20.7%)	37 (19.1%)	47 (24.4%)	39 (18.8%)	0.31
Diabetes duration, years	4.62 (2.00, 9.11)	4.00 (2.0, 8.0)	5.00 (2.0, 10.0)	5.00 (2.0, 11.0)	0.58
Macrovascular comp., *n* (%)	220 (37.0%)	69 (35.6%)	60 (31.1%)	91 (44.0%)	0.02
Microvascular comp., *n* (%)	136 (48.4%)	44 (45.8%)	44 (44.9%)	48 (55.2%)	0.31
Glucose, mg/dL	155.62 (63.15)	157.99 (60.02)	157.96 (64.88)	151.22 (64.44)	0.24
HbA1c, %	7.32 (1.26)	7.28 (1.35)	7.35 (1.26)	7.33 (1.19)	0.88
TMAO, µmol/L	3.90 (2.40, 6.50)	1.90 (1.30, 2.40)	3.90 (3.30, 4.50)	8.20 (6.30, 11.7)	< 0.001
TC, mmol/L	5.58 (1.12)	5.53 (1.13)	5.59 (0.98)	5.61 (1.23)	0.76
HDL-C, mmol/L	1.11 (0.93, 1.36)	1.10 (0.92, 1.37)	1.11 (0.93, 1.37)	1.14 (0.95, 1.35)	0.69
Triglycerides, mmol/L	2.55 (1.50)	2.58 (1.58)	2.64 (1.59)	2.44 (1.34)	0.38
BCAA, µmol/L	512.94 (126.69)	510.97 (120.61)	508.90 (125.24)	518.55 (133.80)	0.72
Valine, µmol/L	273.47 (54.40)	274.56 (52.37)	273.26 (53.50)	272.65 (57.28)	0.93
Leucine, µmol/L	171.21 (53.46)	169.80 (49.66)	166.44 (51.17)	176.98 (58.47)	0.13
Isoleucine, µmol/L	68.27 (30.03)	66.63 (28.17)	69.22 (30.34)	68.93 (31.48)	0.64
Serum creatinine, mmol/L	94.16 (18.94)	90.09 (15.09)	92.18 (16.33)	99.81 (22.79)	< 0.001
eGFR, mL/1.73 m^2/min) [2]	68.44 (18.80)	72.37 (17.66)	71.22 (18.29)	62.16 (18.76)	< 0.001
Albuminuria, *n* (%)	194 (33.9%)	49 (25.9%)	70 (37.6%)	75 (37.9%)	0.02

Abbreviations: BCAA, Branched chain amino acids; BMI, body mass index; DBP, diastolic blood pressure; eGFR, estimated glomerular filtration rate; HbA1c glycated hemoglobin; HDL-C, high-density lipoprotein cholesterol; SBP, systolic blood pressure; TC, total cholesterol; TMAO, Trimethylamine N-Oxide. Values are shown as mean (standard deviation) or median (interquartile range), accordingly. *p* values were determined using ANOVA for normally distributed data, Kruskal-Wallis test for skewed distributed data, and $\chi 2$ test for categorical variables.

Longitudinal Analyses on TMAO and CV mortality

After a median (IQR) follow-up of 10.4 (5.7 – 11.8) years, 113 deaths attributed to cardiovascular disease were recorded. Kaplan-Meier curves for cardiovascular mortality according to tertiles of TMAO plasma concentration are presented in Figure 1. There was an increased risk of cardiovascular mortality associated with the top tertile of TMAO concentrations (*p* for log-rank test <0.001).

Table 2. Multivariable associations of baseline characteristics with plasma concentrations of TMAO.

Characteristic	Std. β (95% CI)	p value
Men, (yes)	-0.17 (-0.36; 0.01)	0.06
Age, years	-0.04 (-0.13; 0.06)	0.45
BMI, kg/m^2	0.00 (-0.08; 0.08)	0.98
SBP, mmHg	0.27 (0.17; 0.38)	**<0.001**
DBP, mmHg	-0.19 (-0.29; -0.09)	**<0.001**
Never smoked, (yes)	-0.07 (-0.28; 0.13)	0.47
Diabetes duration, years	-0.05 (-0.13; 0.03)	0.24
Macrovascular comp., (yes)	0.11 (-0.06; 0.28)	0.22
Microvascular comp., (yes)	0.23 (0.06; 0.41)	**0.009**
Glucose, mg/dL	-0.04 (-0.13; 0.05)	0.35
HbA1c, %	-0.01 (-0.11; 0.08)	0.75
TC, mmol/L	0.12 (0.02; 0.22)	**0.02**
HDL-C, mmol/L	-0.02 (-0.11; 0.07)	0.69
Triglycerides, mmol/L	-0.10 (-0.19; 0.00)	0.05
BCAA, µmol/L	0.18 (0.09; 0.27)	**<0.001**
Serum creatinine, mmol/L	0.17 (0.09; 0.26)	**<0.001**
Albuminuria, (yes)	-0.10 (-0.28; 0.08)	0.28

Standardized regression coefficients are shown. Abbreviations: BCAA, Branched chain amino acids; BMI, body mass index; DBP, diastolic blood pressure; HbA1c, glycated hemoglobin; HDL-C, high-density lipoprotein cholesterol; SBP, systolic blood pressure; TC, total cholesterol.

Table 3. Multivariable associations of DRI with baseline characteristics.

Characteristic	Std. β (95% CI)	*p* value
Men	0.47 (0.41, 0.52)	**<0.001**
Age	-0.03 (-0.05, 0.00)	0.05
BMI	0.11 (0.07, 0.15)	**<0.001**
Waist circumference	0.05 (0.01, 0.09)	**0.017**
SBP	0.02 (-0.01, 0.04)	0.27
DBP	0.03 (0.00, 0.06)	**0.021**
History of Cancer	-0.06 (-0.16, 0.04)	0.22
History of CVD	-0.07 (-0.25, 0.11)	0.46
History of T2D	0.08 (-0.07, 0.23)	0.31
Smoking status		
former	0.02 (-0.03, 0.06)	0.54
<6 cig/day	-0.02 (-0.11, 0.08)	0.73
6-20 cig/day	-0.02 (-0.08, 0.04)	0.49

>20 cig/day	-0.03 (-0.14, 0.07)	0.54
Alcohol consumption		
1-4 drinks/month	0.1 (0.03, 0.16)	**0.002**
2-7 drinks/ week	0.15 (0.10, 0.21)	**<0.001**
1-3 drinks/day	0.28 (0.21, 0.34)	**<0.001**
4 or more drinks/day	0.38 (0.26, 0.49)	**<0.001**
Lipid-lowering medication	0.1 (-0.02, 0.22)	0.11
Glucose	0.06 (0.03, 0.09)	**<0.001**
Insulin	0.12 (0.10, 0.15)	**<0.001**
TC	0.06 (0.04, 0.09)	**<0.001**
Triglycerides	0.35 (0.32, 0.37)	**<0.001**
HDL-C	-0.27 (-0.30, -0.24)	**<0.001**
eGFR	-0.02 (-0.04, 0.01)	0.14
UAE	0.01 (-0.01, 0.03)	0.22

Standardized regression coefficients from multivariable linear regression are shown.

In Cox proportional hazard regression analyses that examined the TMAO as HR per 1 Ln SD, increased plasma concentrations of TMAO were associated with increased risk of cardiovascular mortality independent of age and sex (adjusted HR, 1.39 (95% CI 1.16;1.67), $p < 0.001$, model 1, Table 3); T2D duration, smoking and prevalent macrovascular complications ($_{adj}$HR, 1.29 (95% CI 1.07; 1.56), p = 0.007, model 2, Table 3); systolic blood pressure, HbA1c, total cholesterol, HDL-cholesterol, triglycerides and total BCAA ($_{adj}$HR, 1.26 (95% CI 1.04; 1.54), p = 0.02, model 3, Table 3); albuminuria ($_{adj}$HR, 1.27 (95% CI 1.03; 1.56), p = 0.02, model 4, Table 3) and reduced eGFR (<60 mL/min/1.73 m2) ($_{adj}$HR, 1.32 (95% CI 1.07; 1,63) p = 0.01, model 5, Table 3). There was a marginally significant interaction between TMAO and BCAA (p = 0.05), which is graphically depicted in Figure 2. The proportional hazards assumptions were not violated for any of the variables in the cox regression models. The analyses of plasma concentration of TMAO as a categorical variable, using the first tertile as the reference group, showed that the third tertile of TMAO plasma concentration was also associated with higher risk of cardiovascular mortality in all the cox regression models described, resulting in a fully $_{adj}$HR 1.93 (95% CI 1.11; 3.34), p = 0.02 (Table 3). According to the sensitivity analyses, to invalidate the inference about the association of TMAO with CVD mortality, 57.4 % of the estimated effect would have to be due to bias. Likewise, to invalidate the inference, in 341 out of the 594 participants the effect of TMAO on cardiovascular mortality should be 0.

Table 3. Association of TMAO with cardiovascular mortality, assessed with Cox Proportional Hazard ratios.

	TMAO per 1 Ln SD Increment		T1	T2		T3	
Participants, *n*	595		194	193		208	
Events, *n*	113		22	32		59	
	HR (95 % CI)	***p* value**		**HR (95 % CI)**	***p* value***	**HR (95 % CI)**	***p* value****
Crude Model	1.58 (1.33; 1.87)	<0.001	(ref)	1.61 (0.94; 2.77)	0.08	3.26 (1.99; 5.34)	<0.001
Model 1	1.39 (1.16; 1.67)	<0.001	(ref)	1.49 (0.86; 2.56)	0.15	2.18 (1.33; 3.58)	0.002
Model 2	1.29 (1.07; 1.56)	0.007	(ref)	1.58 (0.91; 2.72)	0.1	2.06 (1.25; 3.41)	0.004
Model 3	1.26 (1.04; 1.54)	0.02	(ref)	1.42 (0.82; 2.46)	0.21	1.87 (1.12; 3.12)	0.02
Model 4	1.27 (1.03; 1.56)	0.02	(ref)	1.39 (0.78; 2.48)	0.26	1.92 (1.12; 3.30)	0.02
Model 5	1.32 (1.07; 1.63)	0.01	(ref)	1.30 (0.73; 2.32)	0.38	1.93 (1.12; 3.35)	0.02

Data are presented as hazard ratios (HRs) with 95% confidence intervals (CIs) and *p* values. *p* values correspond to the comparison between T1 vs T2 (*) and T1 vs T3 (**). Model 1. Model adjusted for age +sex. Model 2. Model 1 + T2D duration + smoking + macrovascular complications. Model 3. Model 2 + SBP + HbA1c + TC + HDL-C+ TG. Model 4. Model 3 + albuminuria. Model 5. Model 4 + reduced eGFR (<60 mL/min/1.73 m^2) + total BCAA. Abbreviations: BCAA, Branched chain amino acids; eGFR, estimated glomerular filtration rate; HbA1c, glycated hemoglobin; HDL-C, high-density lipoprotein cholesterol; SBP, systolic blood pressure; T2D, type 2 diabetes; TC, total cholesterol; TG, triglycerides.

The addition of TMAO to a model for cardiovascular mortality [28], which included age, sex, smoking behaviour, systolic blood pressure, total cholesterol, HDL-cholesterol, and antihypertensive medications, allowed the reclassification of 21% of the sample. Seven percent of the participants in the low-risk category were correctly reclassified to medium-risk and 14% of the participants in the middle risk category were correctly reclassified to the high-risk category. The improvement in the classification of participants into predicted risk categories was statistically significant with a NRI of 0.24 (95% CI 0.03; 0.44; p = 0.02).

Longitudinal analysis on BCAA and CV mortality

Plasma concentrations of BCAAs were not independently associated with increased risk of mortality, neither when analyzed per 1 Ln SD ($_{adj}$HR 0.90 (95% CI 0.72;1.12), p = 0.19), nor when analyzed as tertiles, using the first tertile as the reference group ($_{adj}$HR 0.88 (95% CI 0.52;1.49), p = 0.64) (Supplementary Table S3).

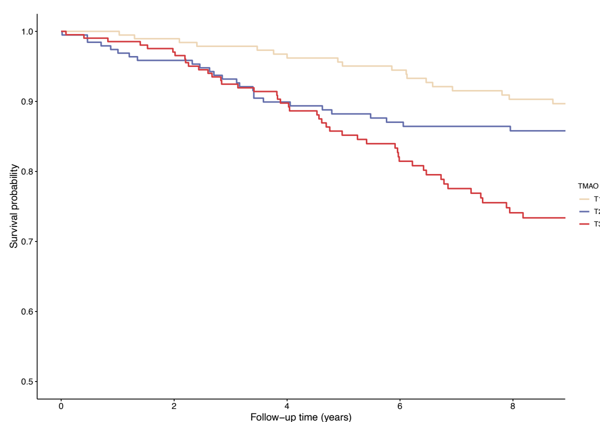

Fig. 1. Kaplan-Meier plot for cardiovascular mortality comparing tertiles of TMAO (log-rank test, p<0.001).

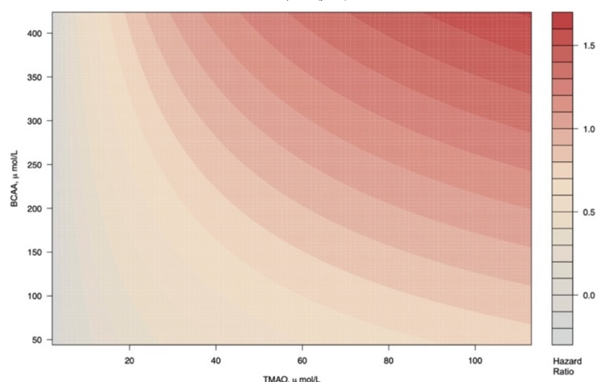

Fig. 2. Cardiovascular mortality as a function of TMAO and BCAA in T2D. Subjects with highest concentrations of both BCAA and TMAO present higher risk of cardiovascular mortality.

Discussion

In this prospective study, we have shown that higher circulating TMAO concentrations were associated with an increased risk of cardiovascular mortality in individuals with T2D. TMAO remained significantly associated, after the adjustment for several CVD risk markers, history of macrovascular complications and circulating concentrations of BCAAs. Moreover, addition of circulating concentrations of TMAO to a base model of cardiovascular mortality risk, improved the patient reclassification from a lower to a higher risk category. In cross-sectional analyses, plasma concentrations of TMAO at baseline were associated with renal function, blood pressure and plasma BCAA.

As expected [7], plasma TMAO was associated with creatinine, reflecting accumulation of TMAO in the context of impaired renal function [10]. Moreover, systolic and diastolic blood pressure respectively displayed strong positive and negative associations with plasma concentrations of TMAO, resembling the increase of systolic and the decrease of diastolic blood over adulthood [30]. The association of systolic and diastolic blood pressure with plasma concentrations of TMAO were independent of age, and the decline of the diastolic blood pressure is more evident in the group with high concentration of TMAO (Supplementary Figure S1). Further analysis demonstrated that higher plasma concentrations of TMAO were associated with elevated pulse pressure (Supplementary Figure 2), probably reflecting an association of TMAO with arterial stiffness. Although this association cannot provide a causal link, it has been recently reported that TMAO supplementation in rats induced an aging-like artery dysfunction, via dysregulation of endothelium-dependent dilation [31].

Different mechanisms are involved in the deleterious effect of TMAO: oxidative stress characterized by excess of nitrotyrosine, endothelial nitric oxide synthase and impaired nitric oxide-mediated dilation. Importantly, such mechanisms were confirmed in human endothelial cells [31]. Those findings were in line with the results from this cohort study. Of further note, experimental studies have provided evidence about the importance and causal role of TMAO in cardiovascular disease [11]. It has been proposed that the role of TMAO in the risk of cardiovascular disease could be mediated by several pathways, such as the acceleration of atherosclerosis by enhancing the

formation of foam cells and atherosclerotic plaques [1], the inhibition of reverse cholesterol process whereby cholesterol is transported form the arterial wall back to the liver where it is metabolized and excreted in the bile [2], as well as the platelet hyper-activity, nevertheless the entire mechanisms are not fully understood [32]. Such biological background provides a rationality to the growing body of epidemiological evidence about the association of TMAO with cardiovascular mortality, summarized in a meta-analysis that included 19, 256 subjects. In such meta-analysis subjects with high concentrations of TMAO had a higher relative risk of with major adverse cardiovascular events [33].

The role of gut microbiota derived metabolites in the development of cardiovascular disease has gained recent attention [11], particularly in individuals with T2D [34]. Nevertheless, the association of TMAO with risk of cardiovascular mortality in subjects with T2D has not being sufficiently studied. To the best of our knowledge, there is only one study that reported on such an association in a time-to-event analysis. Croyal and colleagues reported that T2D patients with high concentrations of TMAO presented a higher risk for mortality during a seven year follow up [8]. The reported association remained after adjustment for several confounding factors. However, the concentrations of TMAO were only analyzed as a categorical variable [8], while in our study, TMAO was analyzed both as a categorical and as a continuous variable, which improves the robustness of the analysis [35].

Strengths and Limitations

This study has some strengths. Firstly, this study comprises a long-term follow-up, and includes the record of several important confounders. The study population consisted of T2D patients exclusively treated in primary care setting, and therefore those results could be extrapolated to patients in real practice. Several limitations of the present study deserve mention. First, the present study was conducted in the north of the Netherlands, and mainly comprises individuals of Caucasian ancestry, which could limit the extrapolation of our findings to other ethnicities. Likewise, there was no available data on dietary patters, which is major contributor of TMAO plasma concentration, therefore it was not possible to further evaluate if the association was independent of any particular dietary pattern, particularly those which are closely related to cardiovascular disease and high concentrations of TMAO production, such as meat-rich diets [2]. In addition, patients whose markers were not measured,

where excluded from the analyses, which could lead to selection bias; nevertheless, those missing values were missing completely at random.

Importantly, various other uremic toxins have been described to be involved in the development of cardiovascular disease, i.e. p-cresyl sulfate and indoxyl sulfate [36], it remains to be explored how important is role of TMAO in comparison with other gut-derived uremic toxins in the prediction of CV related outcomes in T2D subjects. Finally, the association of TMAO with BCAAs, was apparently non-linear (Table 1); probably due to the fact that circulating concentrations of TMAO does not reflects the whole array of microbiota metabolism. A more comprehensive study with a wider range of microbiota-derived biomarkers is desirable to better depict the association of BCAAs with the microbiota. Likewise, considering that the BCAA are closely related with T2D and insulin resistance [23], which is a major risk factor for CVD; therefore, it may be possible the association between BCAA and risk of cardiovascular mortality may be neglected in an analysis restricted to patients with a history of T2D.

In conclusion, this prospective T2D cohort study indicates that that high concentrations of circulating TMAO were associated with a higher risk of cardiovascular mortality, independent of traditional risk factors. In contrast, BCAA, were not associated with cardiovascular mortality, although the highest mortality was found in patients with high TMAO and high BCAA. Further investigation is needed to determine whether TMAO is a potential treatment target in individuals with T2D to possibly reduce the risk of cardiovascular mortality.

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Supplemental Table S1. STROBE Statement—Checklist of items that should be included in reports of cohort studies

	Item No	Recommendation	Page No
Title and abstract	1	(a) Indicate the study's design with a commonly used term in the title or the abstract (b) Provide in the abstract an informative and balanced summary of what was done and what was found	177 177
Introduction			
Background/rationale	2	Explain the scientific background and rationale for the investigation being reported	179
Objectives	3	State specific objectives, including any prespecified hypotheses	179
Methods			
Study design	4	Present key elements of study design early in the paper	180
Setting	5	Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection	180
Participants	6	(a) Give the eligibility criteria, and the sources and methods of selection of participants. Describe methods of follow-up (b) For matched studies, give matching criteria and number of exposed and unexposed	180
Variables	7	Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable	181, 182
Data sources/ measurement	8*	For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group	180-182
Bias	9	Describe any efforts to address potential sources of bias	184
Study size	10	Explain how the study size was arrived at	180
Quantitative variables	11	Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen and why	181
Statistical methods	12	(a) Describe all statistical methods, including those used to control for confounding (b) Describe any methods used to examine subgroups and interactions (c) Explain how missing data were addressed (d) If applicable, explain how loss to follow-up was addressed (e) Describe any sensitivity analyses	183-184 b.NA 180 d.NA 184
Results			
Participants	13*	(a) Report numbers of individuals at each stage of study—eg numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analysed (b) Give reasons for non-participation at each stage (c) Consider use of a flow diagram	180 180

*Give information separately for exposed and unexposed groups.

Supplemental Table S2. Univariable associations of baseline characteristics with plasma concentrations of TMAO.

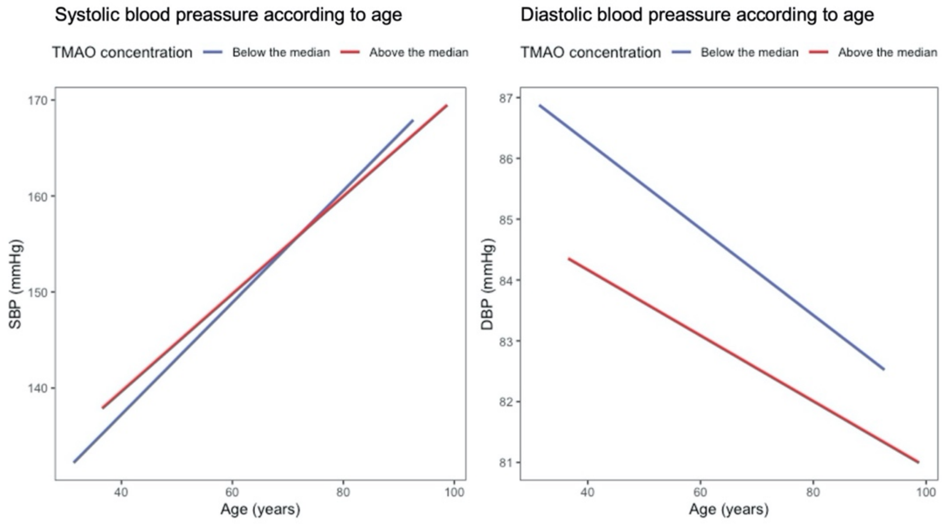
	HR (95 % CI)	***p* value**
Men, *n* (%)	-0.06 (-0.22; 0.11)	0.50
Age, years	0.11 (0.03; 0.19)	**0.007**
BMI, kg/m^2	-0.04 (-0.12; 0.05)	0.39
SBP, mmHg	0.15 (0.07; 0.23)	**<0.001**
DBP, mmHg	-0.04 (-0.12; 0.04)	0.33
Never smoked, *n* (%)	-0.10 (-0.30; 0.10)	0.31
Diabetes duration, years	0.02 (-0.06; 0.10)	0.60
Macrovascular comp., *n* (%)	0.17 (0.00; 0.33)	0.05
Microvascular comp., *n* (%)	0.19 (0.03; 0.35)	**0.02**
Glucose, mg/dL	-0.01 (-0.09; 0.07)	0.81
HbA1c, %	-0.02 (-0.10; 0.06)	0.58
TC, mg/dL	0.08 (0.00; 0.16)	0.06
HDL-C, mg/dL	0.01 (-0.07; 0.09)	0.83
Triglycerides, mg/dL	-0.03 (-0.11; 0.05)	0.50
BCAA, µmol/L	0.10 (0.02; 0.18)	**0.01**
Serum creatinine, mmol/L	0.16 (0.09; 0.24)	**<0.001**
Albuminuria, *n* (%)	0.05 (-0.12; 0.22)	0.53

Standardized regression coefficients are shown. Abbreviations: BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; TC, total cholesterol; HDL-C, high-density lipoprotein cholesterol; BCAA, Branched chain amino acids.

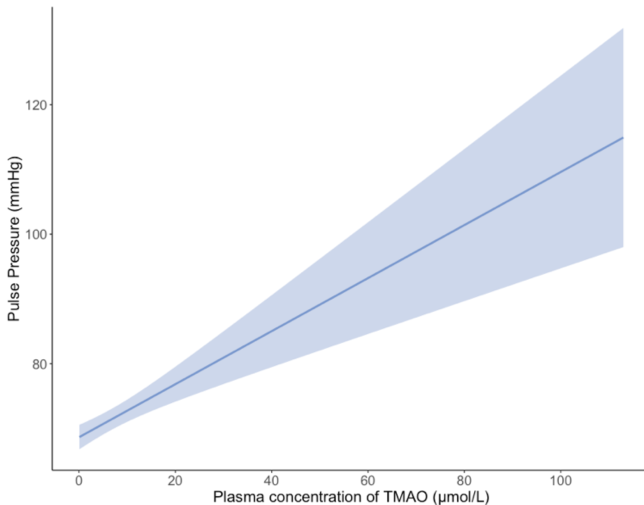
Supplementary Table S3. Association of branched chain amino acids with cardiovascular mortality, assessed with Cox Proportional Hazard ratios.

	BCAA per 1 Ln SD Increment		T1	T2		T3	
Participants, *n*	595		190	203		202	
Events, *n*	113		35	45		33	
	HR (95 % CI)	***p* value**		**HR (95 % CI)**	***p* value***	**HR (95 % CI)**	***p* value****
Crude Model	0.84 (0.70;1.01)	0.06	(ref)	0.69 (0.44;1.08)	0.11	0.67 (0.43;1.05)	0.07
Model 1	0.99 (0.82;1.21)	0.95	(ref)	0.91 (0.58;1.44)	0.7	1.06 (0.66;1.69)	0.8
Model 2	0.99 (0.81;1.20)	0.89	(ref)	0.84 (0.54;1.33)	0.46	0.95 (0.60;1.52)	0.83
Model 3	0.93 (0.76;1.15)	0.51	(ref)	0.74 (0.46;1.17)	0.2	0.89 (0.54;1.45)	0.63
Model 4	0.97 (0.78;1.20)	0.74	(ref)	0.73 (0.47;1.24)	0.27	1.04 (0.63;1.73)	0.86
Model 5	0.90 (0.72;1.12)	0.34	(ref)	0.72 (0.44;1.18)	0.19	0.88 (0.52;1.49)	0.64

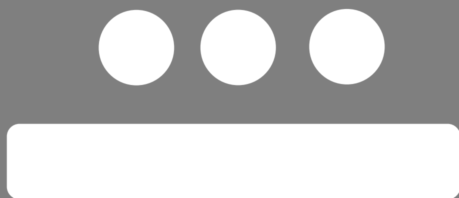
Data are presented as hazard ratios (HRs) with 95% confidence intervals (CIs) and *p* values. *p* values correspond to the comparison between T1 vs T2 (*) and T1 vs T3 (**). Model 1. Model adjusted for age +sex. Model 2. Model 1 + T2D duration + smoking + macrovascular complications. Model 3. Model 2 + SBP + HbA1c + TC + HDL-C+ TG. Model 4. Model 3 + albuminuria. Model 5. Model 4 + reduced eGFR (<60 mL/min/1.73 m^2) + total BCAA. Abbreviations: BCAA, Branched chain amino acids; eGFR, estimated glomerular filtration rate; HbA1c, glycated hemoglobin; HDL-C, high-density lipoprotein cholesterol; SBP, systolic blood pressure; T2D, type 2 diabetes; TC, total cholesterol; TG, triglycerides.



Supplementary Figure S1. Association of age with blood pressure by plasma concentrations of Trimethylamine N-oxide. The left panel depicts the association of age with systolic blood pressure in individuals above and below the median. The right panel depicts the association of age with diastolic blood pressure in individuals above and below the median.



Supplementary Figure S2. Association of pulse pressure with plasma concentrations of Trimethylamine N-oxide. Higher concentrations of TMAO in plasma are associated with elevated pulse pressure. Std. β= 0.10 (95% CI 0.06; 0.14), $p<0.001$.



Chapter 8

Circulating TMAO is Associated with All-Cause Mortality in Subjects with Non-Alcoholic Fatty Liver Disease

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Abstract

Background and Aims: Trimethylamine-N-oxide (TMAO), a gut microbiota-liver metabolite, has been associated with cardiometabolic disease. However, whether TMAO is associated with NAFLD and NAFLD related health outcomes remains unclear. We aimed to investigate the association of TMAO with NAFLD and to assess the extent to which the association of TMAO with all-cause mortality is dependent on the presence of NAFLD in the general population.

Methods: We included 5292 participants enrolled in the PREVEND (Prevention of Renal and Vascular End-stage Disease) cohort study. Cox proportional-hazards regression analyses were performed to study the association of TMAO with all-cause mortality in subjects with and without a Fatty Liver Index (FLI) ≥ 60, which was used as a proxy of NAFLD.

Results: During a median follow-up of 8.2 years, 307 subjects died, of whom 133 were classified with NAFLD. TMAO was positively and independently associated with baseline FLI (Std β 0.08, 95% CI 0.05; 0.11, $p < 0.001$). Higher TMAO was associated with increased risk of all-cause mortality in subjects with NAFLD, in crude analysis (Hazard Ratio (HR) per 1 SD, 2.55, 95% CI 1.60, 4.05, $p < 0.001$) and after full adjustment ($_{adj}$HR 1.90, 95% CI 1.18, 3.04, $p = 0.008$). Such an association was not present in subjects without NAFLD, (crude HR 1.14, 95% CI 0.81,1.71, $p = 0.39$; $_{adj}$HR 0.95, 95% CI 0.65, 1.39, $p = 0.78$).

Conclusion: This prospective study revealed that plasma concentrations of TMAO were associated with all-cause mortality in subjects with NAFLD, independently of traditional risk factors.

Introduction

According to the latest reports, Non-Alcoholic Fatty Liver Disease (NAFLD) has a worldwide prevalence of 25%, being even more prevalent in countries with concomitant obesity, i.e. in the United States, where NAFLD affects 30% of the population (1). Although the deleterious effect of lipid accumulation in the liver was proposed back in 1849 (2), the impact of NAFLD as a global health issue and its association with increased risk of mortality was only recognized 150 years later (3). The understanding of the etiology and risk factors of this global health challenge has evolved over the last few years. Recent evidence from in vivo models of NAFLD had pointed to the role of the gut microbiome in the development and progression of NAFLD (4, 5).

It has been proposed that because the liver is the "first pass" organ of gut microbiota-derived metabolites, it is exposed to the highest concentrations of such metabolites and therefore more vulnerable to their deleterious effects. Similarly, it may be likely that hepatic tissues already affected by inadvertent lipid accumulation are more susceptible to such effects, possibly worsening the clinical prognosis of patients with NAFLD (6). Trimethylamine-N-oxide (TMAO) is a microbiota derived metabolite (7, 8) that has recently gained attention as a consequence of its potential role in the development and progression of type 2 diabetes (T2D) (9), cardiovascular disease (CVD) (10) and its association with increased mortality risk in the general population (11, 12). Trimethylamine (TMA) is a by-product of a microbial fermentation of dietary components such as choline, phosphatidylcholine and L-carnitine. Subsequently, TMA is oxidized to TMAO by the liver enzyme flavin monooxygenase 3, whereas circulating TMAO is cleared by the kidneys (7).

Clinical studies have revealed an association between higher circulating TMAO and NAFLD as well as with non-alcoholic steatohepatitis (NASH) (13, 14, 15). Besides the deleterious effects of TMAO in the development of CVD in the context of metabolic disease, TMAO may underlie different mechanisms that affect the clinical course of NAFLD. For instance, it has been reported that TMAO inhibits cholesterol conversion into bile acids, promoting steatosis and worsening the progression of NAFLD (15).

Given the fact that most patients diagnosed with NAFLD remain asymptomatic, and the progression of the disease is extremely variable (16), it

is relevant to further investigate whether novel risk factors, i.e. microbiota-derived biomarkers, are associated with and increased risk of mortality in subjects with NAFLD. Therefore, the aim of this study was to interrogate the association of circulating concentrations of TMAO with NAFLD and to assess the extent to which the association of TMAO with all-cause mortality is dependent on the presence of NAFLD in the general population. Furthermore, considering that the most common cause of death in patients with NAFLD is CVD, patients being twice as likely to die of CVD than of liver disease (17), we further explored the association of plasma concentrations of TMAO with cardiovascular mortality in participants with NAFLD.

Materials and Methods

Study Cohort

The Prevention of Renal and Vascular END-stage Disease (PREVEND) Study is a prospective population-based cohort study in the city of Groningen, The Netherlands. The design of the PREVEND Study has been described in detail elsewhere (18). Briefly, from 1997 to 1998, all residents from Groningen, excluding pregnant women and people with type 1 diabetes or T2D using insulin, aged 28–75 years were invited to participate. A total of 40 856 subjects (47.8%) responded to the invitation to participate. From this group, 30890 subjects had a urinary albumin concentration of < 10 mg/L and 9966 subjects had a urinary albumin concentration of ≥ 10 mg/L in their morning urine sample. After exclusion of subjects with type 1 diabetes and pregnant women, all subjects with a urinary albumin concentration of ≥ 10 mg/L (n = 7768) and a randomly selected control group with a urinary albumin concentration of < 10 mg/L (n = 3395) were invited for further investigations in an outpatient clinic. A total of 8592 individuals completed an extensive examination.

We used data of participants who completed the second screening and were free from liver disease assessed by questionnaire (n = 6894), excluding those with missing samples for assessment of TMAO concentrations (n = 1425) or missing values for assessment of FLI (n = 177), leaving a cohort of 5292 participants with complete information for the analysis. Cases of participants lost to follow-up were considered as censored cases. This report follows the Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) reporting guideline (Supplemental Table 1). The protocol for the PREVEND study

was approved by the local ethics committee of the University Medical Center Groningen (approval number: MEC96/01/022). All participants provided written informed consent and all procedures were conducted according to the Declaration of Helsinki (19).

Laboratory measurements

Laboratory measurements were performed at the Central Laboratory of the University Medical Center Groningen, The Netherlands. Venous blood samples were drawn after an overnight fast while participants rested for 15 min. Ethylenediaminetetraacetic acid (EDTA) - anticoagulated plasma samples and sera were stored at -80 ° C until analysis.

TMAO concentrations were measured in EDTA-anticoagulated plasma samples using a Vantera® Clinical Analyzer (Labcorp, Morrisville, NC), a fully automated, high-throughput, 400 MHz proton (1H) nuclear magnetic resonance (NMR) spectroscopy platform. TMAO was quantified from one-dimensional (1D) proton (1H) Carr-Purcell-Meiboom-Gill (CPMG) spectra by spectral deconvolution algorithm as previously described (20, 21). The TMAO assay has intra- and inter-assay coefficients of variation (CV%) range from 4.3–10.3% and 9.8–14.5%, respectively (21). Total cholesterol (TC), triglycerides, and serum creatinine were measured using standard protocols, as described previously (22). Serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were measured using the standardized kinetic method with pyridoxal phosphate activation (Roche Modular P, Roche Diagnostics, Mannheim, Germany). Serum gamma-glutamyl transferase (GGT) was assayed by an enzymatic colorimetric method (Roche Modular P, Roche Diagnostics, Mannheim, Germany). Standardization of ALT, AST and GGT was performed according to the International Federation of Clinical Chemistry guidelines (23, 24, 25).

Urinary albumin excretion (UAE) was measured by nephelometry (Dade Behring Diagnostic, Marburg, Germany) as described in two 24-hour urine collections and the results were averaged for analysis. Serum creatinine was measured by an enzymatic method on a Roche Modular analyzer (Roche Diagnostics, Mannheim, Germany). Serum cystatin C was measured using Gentian Cystatin C Immunoassay (Gentian AS, Moss, Norway) reagents on a modular analyzer (Roche Diagnostics). The estimated glomerular filtration rate

(eGFR) was calculated using the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) combined creatinine-cystatin C equation (26).

Clinical Measurements

During two outpatient visits, baseline data were collected on demographics, lifestyle factors, anthropometric measurements, medical history, parental history of T2D and medication use. Information on medication use was combined with information from a pharmacy-dispensing registry, which had complete information on the drug usage of > 95% of subjects in the PREVEND study. Height and weight were measured in standing position without shoes and heavy outer garments. Body mass index (BMI) was calculated as weight (kg) divided by height (meter) squared. Waist circumference was measured as the smallest girth between the rib cage and iliac crest. Systolic and diastolic blood pressure values were measured with an automatic Dinamap XL Model 9300 series device and recorded as the means of the last two recordings of the second visit.

The Fatty Liver Index (FLI) was used as proxy for the diagnosis of NAFLD (27, 28). The FLI was calculated from BMI, GGT, triglycerides and waist circumference data according to the following formula: [e (0.953 × loge (triglycerides + 0.139 × BMI + 0.718 × loge (GGT) + 0.053 × waist circumference − 15.745)] / [1 + e (0.953 × loge (triglycerides) + 0.139 × BMI + 0.718 × loge (GGT) + 0.053 × waist circumference − 15.745)] × 100. The optimum cut-off value for the FLI is accepted to be 60 with an accuracy of 84%, a sensitivity of 61% and a specificity of 86% for detecting NAFLD as determined by ultrasonography (27). FLI ≥ 60 was therefore used for this study. The FLI is currently considered as one of the best-validated steatosis scores for larger scale screening studies (29). In alternative analyses, we used the hepatic steatosis index (HSI). The HSI is defined as follows: HSI = 8 × ALT / AST ratio + BMI (+2, if diabetes; +2, if female). A cut-off of HSI ≥ 36 was used as a second proxy of NAFLD) (30).

Ascertainment of End Point

Participants were followed from the date of the baseline visit until end of follow-up (January first 2011). Data on mortality were obtained from the municipal register, and the cause of death was obtained by linking the number of the death certificate to the primary cause of death as coded by a physician from the Central Bureau of Statistics.

Statistical Analysis

Data are presented as the mean (SD) or median (interquartile range, IQR) for continuous variables and percentages for categorical variables. Cross-sectional group differences among FLI groups at baseline were assessed by unpaired t-tests for normally distributed and loge transformed variables, by Mann–Whitney U tests for non-normally distributed variables or by chi-squared tests for categorical variables where appropriate. Multivariable linear regression analyses were carried out to disclose the associations of TMAO concentrations with clinical covariates and laboratory parameters, after adjustment for age and sex. To further evaluate whether TMAO was associated with FLI and HSI, two models were built including those variables associated with TMAO and mutually excluding FLI and HSI as well as its determinants. In order to identify the risk of multicollinearity in the multivariable regression analyses, the Variance-Inflation Factors (VIF) were calculated. A high risk of multicollinearity was considered present if the calculated VIF was > 5 (31).

For the prospective analysis, the data of the two groups of participants: with NAFLD (n = 1598) and without NAFLD (n =3694) were analyzed separately, given the significant interaction between TMAO and NAFLD ($p_{int} < 0.05$). We plotted cumulative Kaplan-Meier curves for risk of all-cause mortality during follow-up according to tertiles of TMAO. Cox proportional hazards models were used to compute hazard ratios (HRs) and 95% CI of all-cause mortality risk. HRs were adjusted for age, sex, T2D medication, smoking behavior, alcohol consumption, history of cancer, systolic blood pressure, antidiabetic medication, lipid lowering medication, glucose, total cholesterol, high-density lipoprotein cholesterol (HDL-cholesterol), albuminuria and eGFR at baseline.

The Cox proportional hazard assumption was tested through the evaluation of independence between scaled Schoenfeld residuals with time for each variable and for every model as a whole; this assumption was met, with no indication for a violation (32). In the two groups of participants (with and without NAFLD) the interactions of TMAO with age and eGFR were also evaluated. To further evaluate the robustness of the association and the risk of bias, a sensitivity analysis was conducted to calculate the Robustness of Inference to Replacement (33). All statistical analyses were performed with R language for statistical computing software, v. 4.0.3 (2020), (Vienna, Austria) (34).

Results

Baseline characteristics

Out of the 5292 participants with available measurements of TMAO that were included in the current study, 1671 (31.6 %) participants had a FLI ≥ 60. Table 1 shows clinical characteristics and laboratory data of the study population according to FLI categorization. Participants with an FLI ≥60 were older, more likely to be men and use antihypertensive- and lipid-lowering drugs. Alcohol consumption and cigarette smoking were also more common among participants with elevated FLI. BMI, waist circumference, systolic and diastolic blood pressure, blood glucose, transaminases, GGT, TC, triglycerides and UAE were higher in participants with FLI ≥ 60, whereas eGFR and HDL-cholesterol were lower in participants with an elevated FLI. The median (IQR) plasma total TMAO concentration was 3.58 (2.02, 6.25) µmol/L and 2.99 (1.60, 5.46) in participants with FLI ≥ 60 and < 60, respectively ($p < 0.001$) (Table 1).

Cross-sectional analysis

The association of the plasma concentrations of TMAO with baseline characteristics was evaluated with both univariable and multivariable linear regression analyses. In the univariable analyses sex, systolic blood pressure, diastolic blood pressure, history of CVD, former smoking, antihypertensive medication, GGT, and plasma albumin were significantly associated with plasma concentrations of TMAO (Table 2). In a multivariable model, TMAO remained independently associated with age, BMI, waist circumference, alcohol consumption, medication for T2D, glucose, HOMA-IR, HDL-cholesterol, triglycerides, ALT, FLI, HSI, eGFR and UAE (Table 2). To further evaluate the strength of the association of TMAO with NAFLD, FLI and HSI were evaluated in two separate multivariable models without risk of multicollinearity (VIF<5) that included the variables above mentioned. In such models, circulating concentrations of TMAO remained significantly associated with an elevated FLI (Std. β = 0.10 (95% CI 0.03, 0.17), p = 0.003) and an elevated HSI (Std. β = 0.14 (95% CI 0.08, 0.20), p < 0.001) (Supplemental Table 2 and 3).

Although the circulating TMAO concentrations were higher in participants with NAFLD, in comparison with participants without NAFLD; the concentrations of TMAO remained negatively associated with eGFR in participants with and

Table 1. Clinical and laboratory characteristics in 3694 subjects with a fatty liver index (FLI) < 60 and 1598 subjects with an FLI ≥60.

	FLI < 60 (N=3694)	FLI ≥ 60 (N=1598)	*p* value
Male, n (%)	1523 (41.2%)	1048 (65.6%)	< 0.001
Age, mean (SD), years	51.7 (11.9)	56.9 (11.2)	< 0.001
BMI, mean (SD), kg/m^2	24.8 (2.8)	30.9 (4.2)	< 0.001
Waist circumference, mean (SD), cm	85.9 (9.2)	105.3 (9.4)	< 0.001
SBP, mean (SD), mmHg	121.6 (17.4)	135.1 (17.9)	< 0.001
DBP, mean (SD), mmHg	71.4 (8.6)	77.3 (8.7)	< 0.001
History of Cancer, n (%)	179 (4.8%)	67 (4.2%)	0.55
History of CVD, n (%)	106 (2.9%)	97 (6.1%)	< 0.001
Smoking status, n (%)			< 0.001
never	1147 (31.1%)	379 (23.7%)	
former	1440 (39.0%)	784 (49.1%)	
current <6 cigarettes per day	175 (4.7%)	61 (3.8%)	
current 6-20 cigarettes per day	760 (20.6%)	275 (17.2%)	
current >20 cigarettes per day	124 (3.4%)	78 (4.9%)	
Alcohol consumption, n (%)			< 0.001
No, almost never	879 (23.8%)	461 (28.8%)	
1-4 drinks per month	653 (17.7%)	250 (15.6%)	
2-7 drinks per week	1233 (33.4%)	442 (27.7%)	
1-3 drinks per day	810 (21.9%)	347 (21.7%)	
>3 drinks per day	119 (3.2%)	98 (6.1%)	
Glucose lowering medication, n (%)	68 (1.8%)	120 (7.5%)	< 0.001
Antihypertensive medication, n (%)	525 (14.2%)	573 (35.9%)	< 0.001
Lipid lowering medication, n (%)	219 (5.9%)	221 (13.8%)	< 0.001
TMAO, µmol/L	2.99 (1.60, 5.46)	3.58 (2.02, 6.25)	< 0.001
Glucose, mmol/L	4.70 (4.40, 5.10)	5.20 (4.70, 5.90)	< 0.001
HOMA-IR, median (IQR), mUmmol/L^2/22.5	1.44 (1.05, 2.03)	3.06 (2.13, 4.67)	< 0.001
TC, mean (SD), mmol/L	5.28 (0.99)	5.72 (1.08)	< 0.001
HDL-C, mean (SD), mmol/L	1.32 (0.31)	1.09 (0.24)	< 0.001
Triglycerides, median (IQR), mmol/L	0.95 (0.72, 1.27)	1.74 (1.31, 2.38)	< 0.001
ALT, median (IQR), U/L	15.0 (12.0, 20.0)	23.0 (17.0, 32.0)	< 0.001
AST, median (IQR), U/L	21.0 (19.0, 25.0)	25.0 (21.0, 30.0)	< 0.001
GGT, median (IQR), U/L	19.0 (14.0, 28.0)	41.0 (29.0, 63.0)	< 0.001
FLI, median (IQR), A.U.	22.56 (10.18, 38.99)	80.33 (70.03, 90.21)	< 0.001
HSI, median (IQR), A.U.	31.82 (29.34, 34.58)	39.25 (35.86, 43.02)	< 0.001
Plasma albumin, g/L	44.0 (42.0, 45.0)	44.0 (42.0, 45.0)	0.10
eGFR, mean (SD), mL/min/1.73 m^2	94.50 (16.39)	87.79 (17.62)	< 0.001
UAE, median (IQR), mg/24 h	7.81 (5.79, 12.82)	11.42 (7.08, 26.65)	< 0.001

Abbreviations. ALT, alanine aminotransferase; AST, aspartate aminotransferase; A.U., arbitrary units; BMI, body mass index; DBP, diastolic blood pressure; eGFR, estimated glomerular filtration rate; FLI, fatty liver index; GGT, gamma-glutamyltransferase; HDL-C, high-density lipoprotein cholesterol; HSI, hepatic steatosis index; HOMA, Homeostasis Model Assessment; SBP, systolic blood pressure; TC, total cholesterol; TMAO, Trimethylamine N-Oxide; UAE, urinary albumin excretion.

without NAFLD: (Std. β = -0.17 (95% CI -0.22, -0.11), p < 0.001) and (Std. β = -0.14 (95% CI -0.18, -0.09), p < 0.001, respectively. (Supplemental Fig. 1).

Longitudinal Analyses

In the all-cause mortality analysis conducted in the whole population, there was a significant interaction between TMAO and NAFLD (p_{int} <0.05); therefore, we conducted the analyses separately in the groups with and without NAFLD.

All-cause mortality in subjects with NAFLD

After a median (IQR) follow-up of 8.2 (5.7 – 11.8) years, 133 deaths were recorded. Kaplan-Meier curves for all-cause mortality according to tertiles of TMAO plasma concentration are presented in Figure 1. There was an increased risk of all-cause mortality associated with the top tertile of TMAO concentrations (*p* for log-rank test < 0.001).

Table 2. Univariable and multivariable associations of baseline characteristics with plasma concentrations of TMAO in 5292 PREVEND participants.

	Univariable regression analysis		**Multivariable regression analysis**	
	Std. β (95% CI)	***p* value**	**Std. β (95% CI)**	***p* value**
Men, n	0.06 (0.00, 0.11)	0.04	0.04 (-0.02, 0.09)	0.20
Age, years	0.12 (0.10, 0.15)	<0.001	0.12 (0.09, 0.14)	**<0.001**
BMI, kg/m^2	0.09 (0.07, 0.12)	<0.001	0.07 (0.04, 0.10)	**<0.001**
Waist circumference, cm	0.10 (0.08, 0.13)	<0.001	0.08 (0.05, 0.11)	**<0.001**
SBP, mmHg	0.06 (0.03, 0.08)	<0.001	0.00 (-0.03, 0.03)	0.91
DBP, mmHg	0.04 (0.01, 0.07)	0.004	0.00 (-0.03, 0.03)	0.88
History of Cancer, n	-0.01 (-0.14, 0.12)	0.89	-0.03 (-0.16, 0.10)	0.65
History of CVD, n	0.19 (0.05, 0.33)	0.008	0.07 (-0.07, 0.22)	0.32
Smoking status, n				
former	0.08 (0.02, 0.15)	0.01	0.04 (-0.03, 0.11)	0.23
current <6 cig/d	0.06 (-0.08, 0.20)	0.4	0.06 (-0.07, 0.20)	0.37
current 6-20 cig/d	-0.05 (-0.13, 0.03)	0.19	-0.06 (-0.13, 0.02)	0.16
current >20 cig/d	0.04 (-0.11, 0.18)	0.61	0.05 (-0.09, 0.20)	0.49
Alcohol consumption, n				
1-4 drinks per month	0.01 (-0.07, 0.10)	0.78	0.03 (-0.05, 0.12)	0.47
2-7 drinks per week	0.06 (-0.01, 0.13)	0.10	0.10 (0.03, 0.17)	**0.01**
1-3 drinks per day	0.05 (-0.03, 0.13)	0.19	0.06 (-0.02, 0.14)	0.13
>3 drinks per day	0.17 (0.03, 0.31)	0.02	0.17 (0.03, 0.32)	**0.02**
Glucose lowering medication, n	0.37 (0.23, 0.52)	<0.001	0.27 (0.13, 0.42)	**<0.001**
Antihypertensive medication, n	0.12 (0.05, 0.19)	<0.001	0.01 (-0.06, 0.08)	0.76
Lipid lowering medication, n	0.14 (0.04, 0.24)	0.005	0.05 (-0.05, 0.15)	0.29
Glucose, mmol/L	0.08 (0.05, 0.11)	<0.001	0.06 (0.03, 0.08)	**<0.001**
HOMA-IR, mU mmol/L^2/22.5	0.08 (0.05, 0.11)	<0.001	0.06 (0.03, 0.09)	**<0.001**
TC, mmol/L	0.01 (-0.01, 0.04)	0.31	-0.01 (-0.04, 0.02)	0.56
HDL-C, mmol/L	-0.05 (-0.07, -0.02)	<0.001	-0.04 (-0.07, -0.01)	**0.004**
Triglycerides, mmol/L	0.05 (0.02, 0.07)	0.001	0.03 (0.00, 0.06)	**0.02**
ALT, U/L	0.04 (0.01, 0.06)	0.008	0.03 (0.01, 0.06)	**0.01**
AST, U/L	0.02 (-0.01, 0.04)	0.26	0.00 (-0.03, 0.03)	0.90
GGT, U/L	0.04 (0.02, 0.07)	0.002	0.03 (-0.00, 0.05)	0.06
FLI, ≥ 60 A. U.	0.19 (0.13, 0.25)	<0.001	0.14 (0.08, 0.21)	**<0.001**
HSI, ≥ 36 A. U.	0.19 (0.13, 0.25)	<0.001	0.16 (0.11, 0.22)	**<0.001**

Plasma albumin, g/L	-0.04 (-0.06, -0.01)	0.009	-0.02 (-0.05, 0.01)	0.13
eGFR, mL/min/1.73 m^2	-0.16 (-0.19, -0.13)	<0.001	-0.15 (-0.19, -0.12)	**<0.001**
UAE, mg/24 h	0.07 (0.04, 0.09)	<0.001	0.06 (0.03, 0.08)	**<0.001**

Standardized beta regression coefficients (95% Confidence Intervals) are shown. Multivariable regression coefficients are adjusted for age and sex.

Abbreviations. β, standardized beta regression coefficient; ALT, alanine aminotransferase; AST, aspartate aminotransferase; A.U., arbitrary units; BMI, body mass index; DBP, diastolic blood pressure; eGFR, estimated glomerular filtration rate; FLI, fatty liver index; GGT, gamma-glutamyl transferase; HDL-C, high-density lipoprotein cholesterol; HSI, hepatic steatosis index; HOMA, Homeostasis Model Assessment; SBP, systolic blood pressure; TC, total cholesterol; TMAO, Trimethylamine N-Oxide; UAE, urinary albumin excretion.

In Cox proportional hazard regression analyses that examined the TMAO concentrations as HR per 1 Ln SD, increased plasma concentrations of TMAO were associated with increased risk of all-cause mortality independent of age and sex ($_{adj}$HR, 1.25 (95% CI 1.04,1.49), p = 0.01, model 1, Table 3); systolic blood pressure, smoking status, alcohol consumption, history of cancer, glucose lowering medication and lipid lowering medication ($_{adj}$HR, 1.24 (95% CI 1.04, 1.48), p = 0.02, model 2, Table 3); total cholesterol, HDL-cholesterol and glucose ($_{adj}$HR, 1.23 (95% CI 1.03, 1.47), p = 0.03, model 3, Table 3); albuminuria and eGFR < 90 mL/min/1.73 m^2 ($_{adj}$HR, 1.21 (95% CI 1.01, 1.46), p = 0.04, model 4, Table 3). The proportional hazards assumptions were not violated for any of the variables in the additive models. Analyses of plasma concentration of TMAO as a categorical variable, using the first tertile as the reference group, showed that the third tertile of TMAO plasma concentration was also associated with higher risk of all-cause mortality in all the cumulative models described, resulting in a fully $_{adj}$HR 1.90 (95% CI 1.18, 3.04), p = 0.008 (model 4, Table 3). In the longitudinal analysis, there was a significant interaction of TMAO with eGFR (p_{int} < 0.01), but there was no significant interaction with age (p_{int} > 0.10). Similar results were obtained in the analysis using HSI as proxy of NAFLD (Supplemental Table 4). According to the sensitivity analyses, to invalidate the inference about the association of TMAO with all-cause mortality, 48.6 % of the estimated effect would have to be due to bias. Likewise, in order to invalidate the inference, the effect of TMAO on all-cause mortality should be equal to 0 in 777 out of the 1598 participants, which highlights the robustness of the association.

Table 3. Association of TMAO with all-cause mortality, assessed with Cox Proportional Hazard ratios in subjects with NAFLD (FLI ≥ 60).

	TMAO per 1 Ln SD Increment		**T1**	**T2**		**T3**	
Participants, *n*	1598		533	532		533	
Events, *n*	133		25	45		63	
	HR (95 % CI)	***p* value**		**HR (95 % CI)**	***p* value**	**HR (95 % CI)**	***p* value**
Crude Model	1.41 (1.18,1.69)	<0.001	(ref)	1.80 (1.10,2.93)	0.01	2.55 (1.60,4.05)	<0.001
Model 1	1.25 (1.04,1.49)	0.01	(ref)	1.66 (1.02,2.70)	0.04	2.16 (1.36,3.44)	0.01
Model 2	1.24 (1.04,1.48)	0.02	(ref)	1.66 (1.02,2.71)	0.04	2.01 (1.26,3.21)	0.003
Model 3	1.23 (1.03,1.47)	0.03	(ref)	1.71 (1.04,2.79)	0.03	1.96 (1.23,3.12)	0.005
Model 4	1.21 (1.01,1.46)	0.04	(ref)	1.69 (1.03,2.77)	0.04	1.90 (1.18,3.04)	0.008

Data are presented as hazard ratios (HRs) with 95% confidence intervals (CIs) and p values.
Model 1. Model adjusted for age +sex
Model 2. Model 1 + SBP + Smoking status + alcohol consumption + cancer history + glucose lowering medication + lipid lowering medication
Model 3. Model 2 + TC + HDL-C + Glucose
Model 4. Model 3 + albuminuria + reduced eGFR (< 90 mL/min/1.73 m^2)

Table 4. Association of TMAO with all-cause mortality, assessed with Cox Proportional Hazard ratios in subjects without NAFLD (FLI < 60).

	TMAO per 1 Ln SD Increment		T1	T2		T3	
Participants, *n*	3694		1232	1231		1231	
Events, *n*	174		51	62		61	
	HR (95 % CI)	***p* value**		**HR (95 % CI)**	***p* value**	**HR (95 % CI)**	***p* value**
Crude Model	1.14 (0.98,1.33)	0.09	(ref)	1.22 (0.84,1.76)	0.3	1.18 (0.81,1.71)	0.39
Model 1	1.02 (0.87,1.20)	0.81	(ref)	0.86 (0.59,1.24)	0.41	0.90 (0.62,1.31)	0.59
Model 2	1.07 (0.91,1.26)	0.41	(ref)	0.90 (0.62,1.30)	0.56	0.94 (0.65,1.37)	0.75
Model 3	1.07 (0.91,1.25)	0.42	(ref)	0.90 (0.62,1.31)	0.56	0.94 (0.65,1.37)	0.75
Model 4	1.07 (0.91,1.26)	0.42	(ref)	0.91 (0.62,1.32)	0.61	0.95 (0.65,1.39)	0.78

Data are presented as hazard ratios (HRs) with 95% confidence intervals (CIs) and p values.
Model 1. Model adjusted for age +sex
Model 2. Model 1 + SBP + Smoking status + alcohol consumption + cancer history + glucose lowering medication + lipid lowering medication
Model 3. Model 2 + TC + HDL-C + Glucose
Model 4. Model 3 + albuminuria + reduced eGFR (< 90 mL/min/1.73 m^2)

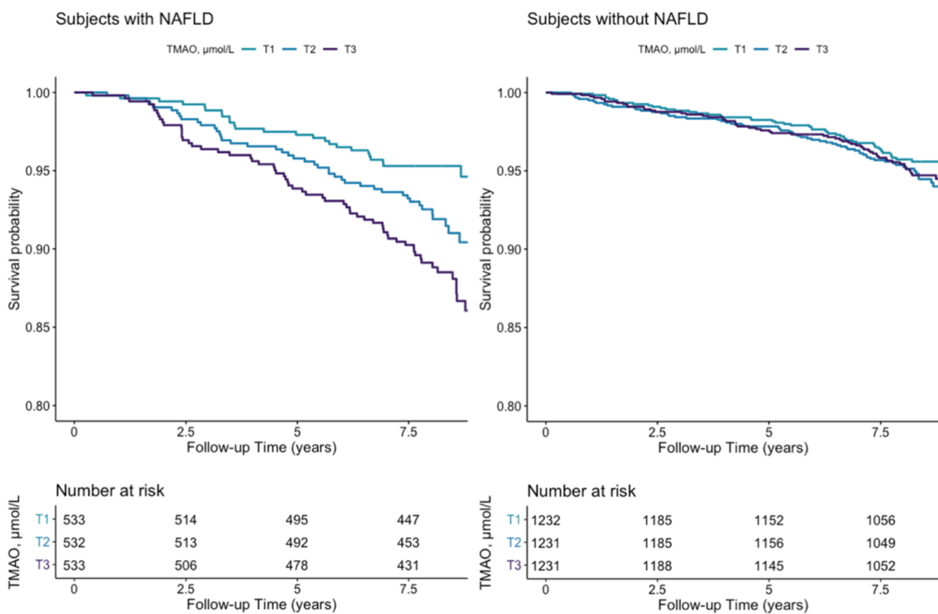

Figure 1. Kaplan-Meier plots for all-cause mortality comparing tertiles of TMAO.

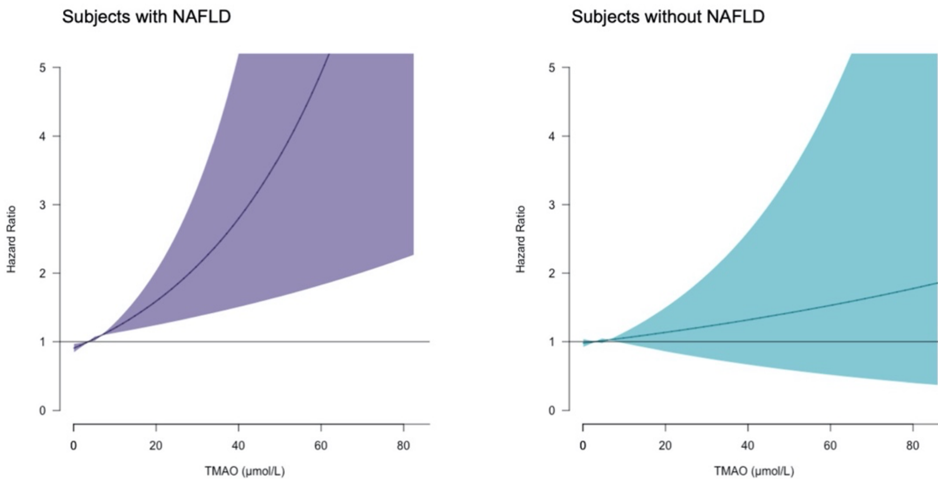

Figure 2. Association between circulating concentrations of TMAO and all-cause mortality.

The association of TMAO with all-cause mortality in NAFLD was also evaluated on sensitivity analysis after excluding subjects with high alcohol consumption (>3 drinks per day). Analyses of plasma concentration of TMAO as a categorical variable, using the first tertile as the reference group, showed that the third tertile of TMAO plasma concentration was associated with increased risk of all-cause mortality (HR, 2.48 (95% CI 1.55, 3.99), $p < 0.001$) (Supplemental Table 5); after adjustment for the above-described variables, the TMAO plasma concentration remained associated with higher risk of all-cause mortality, resulting in a adjusted $_{adj}$HR 1.75 (95% CI 1.08, 2.84), $p = 0.02$ (Supplemental Table 5).

Additionally, the association of TMAO with all-cause mortality was also evaluated on sex-specific groups. The association did not reach formal significance in women, having a crude HR 2.14 (95% CI 0.81, 5.64), $p = 0.12$. Men presented a crude HR 3.56 (95% CI 2.04, 6.21), $p < 0.001$ (Supplemental Table 6). After adjustment for the above-described variables, the associations of TMAO plasma concentration with risk of cardiovascular mortality remained consistent in both groups; having a fully $_{adj}$HR 2.46 (95% CI 0.91, 6.13), $p = 0.08$ in women, and a $_{adj}$HR of 2.17 (95% CI 1.23, 3.84), $p = 0.007$ in men (Supplemental Table 6).

All-cause mortality in subjects without NAFLD

In subjects without NAFLD, plasma concentrations of TMAO were not associated with an increased risk of all-cause mortality (*p* for log-rank test >0.05) (Figure 1). In Cox proportional hazard regression analyses that examined the circulating TMAO concentrations as HR per 1 Ln SD, increased plasma concentrations of TMAO were not associated with increased risk of all-cause mortality, neither in the crude model (HR, 1.14 (95% CI 0.98, 1.33), $p = 0.09$, Table 4), nor in the full model adjusted for age, sex, systolic blood pressure, smoking status, alcohol consumption, history of cancer, antidiabetic medication and lipid lowering medication, total cholesterol, HDL-cholesterol, glucose, albuminuria and reduced eGFR ($_{adj}$HR, 1.07 (95% CI 0.91, 1.26), $p = 0.42$, model 4, Table 4). Similarly, the analyses of plasma concentration of TMAO as a categorical variable, using the first tertile as the reference group, showed that the third tertile of TMAO plasma concentration was not associated with higher risk of all-cause mortality in all the cumulative model described above, resulting in a fully

$_{adj}$HR 0.95 (95% CI 0.65, 1.39), p = 0.78 (model 4, Table 4). Similar results were obtained in the analysis using HSI as proxy of NAFLD (Supplemental Table 7).

Cardiovascular mortality in subjects with NAFLD

In subjects with NAFLD, the Cox proportional hazard regression analyses that examined the circulating TMAO concentrations as HR per 1 Ln SD, increased plasma concentrations of TMAO were associated with increased risk cardiovascular mortality, only in the crude model (HR, 1.49 (95% CI 1.04, 2.12), p = 0.02, Supplemental Table 8), but not in the full adjusted model ($_{adj}$HR, 1.14 (95% CI 0.80, 1.63), p = 0.48, Supplemental Table 8). Similarly, the analyses of plasma concentration of TMAO as a categorical variable, using the first tertile as the reference group, showed that the third tertile of TMAO plasma concentration was associated with higher risk of cardiovascular mortality only in the crude model (HR, 4.20 (95% CI 1.58, 11.15), p = 0.003, Supplemental Table 8), but not in the fully adjusted model ($_{adj}$HR, 2.50 (95% CI 0.91, 6.81), p = 0.07, Supplemental Table 8).

The association of TMAO with cardiovascular mortality in NAFLD was also evaluated on sensitivity analysis after excluding subjects with high alcohol consumption (>3 drinks per day). Analyses of plasma concentration of TMAO as a categorical variable, using the first tertile as the reference group, showed that the third tertile of TMAO plasma concentration was associated with increased risk of cardiovascular mortality (HR, 2.48 (95% CI 1.55, 3.99), $p < 0.001$) (Supplemental Table 9); after adjustment for the above-described variables, the TMAO plasma concentration remained associated with higher risk of cardiovascular mortality, resulting in a adjusted $_{adj}$HR 1.75 (95% CI 1.08, 2.84), p = 0.02 (Supplemental Table 9).

Cardiovascular mortality in subjects without NAFLD

In subjects without NAFLD, the Cox proportional hazard regression analyses that examined the circulating TMAO concentrations as HR per 1 Ln SD, increased plasma concentrations of TMAO were not associated with increased risk cardiovascular mortality, neither in the crude model (HR, 1.35 (95% CI 0.97, 1.86), p = 0.07, Supplemental Table 10), nor in the full adjusted model ($_{adj}$HR, 1.26 (95% CI 0.89, 1.79), p = 0.20, Supplemental Table 10). Similarly, the analyses of plasma concentration of TMAO as a categorical variable, using the first tertile as the reference group, showed that the third tertile of TMAO plasma concentration was not associated with higher risk of cardiovascular mortality

neither in the crude model (HR, 1.69 (95% CI 0.77. 3.69), p = 0.19, Supplemental Table 10), nor in the fully adjusted model ($_{adj}$HR, 1.30 (95% CI 0.58, 2.91), p = 0.52, Supplemental Table 10).

Discussion

In this prospective cohort, we have shown that higher plasma TMAO concentrations were significantly associated with an increased risk of all-cause mortality in individuals with NAFLD, as judged by a FLI score ≥ 60. Importantly, such association was not present in subjects without NAFLD. Plasma concentrations of TMAO at baseline were higher in subjects with NAFLD, compared with those without NAFLD. Cross-sectionally, TMAO was associated with several metabolic risk factors including adiposity, reduced eGFR, older age and plasma glucose, as well as with the use of glucose lowering medication. The most relevant clinical and biochemical variables associated with TMAO reported in the present study and the literature are summarized in Table 5 (35, 36). Notably, the association of TMAO with increased risk of all-cause mortality was independent of these variables. These results are in line with previous studies that have shown that altered gut microbiota composition may control the rate of progression of multiple metabolic syndrome-associated pathologies such as NAFLD (6).

In this study, NAFLD was assessed with two validated scores: FLI and HSI. Both scores were positively associated with plasma concentrations of TMAO. Our results are in accordance with a prior pilot study conducted in 137 subjects with metabolic syndrome (57% women), aged 21–56 years. In that study, circulating TMAO, measured by means of High-Performance Liquid Chromatography-Mass Spectrometry, was linearly associated with FLI score values (β = 0.82, p < 0.001) (37). Similarly, in a small case-control study comprising 34 subjects with biopsy-proven NAFLD, it was demonstrated that circulating TMAO concentrations were associated with a more advanced disease status (15). A more recent and larger study (n=357, 76% women) also provided consistent evidence about the association of biopsy-proven NAFLD with plasma concentrations of TMAO. In this report, higher concentrations of TMAO were also associated with worse clinical and histological characteristics in patients with NAFLD. Importantly, the authors found that circulating TMAO concentrations were not associated with hepatic FMO3 expression, suggesting that the concentrations of TMAO are not mainly dependent on the human liver

metabolism of TMAO, but rather on its clearance rate by the kidneys (13). Accordingly, we found that TMAO concentrations were strongly associated with eGFR at baseline (Supplemental Figure 1) as previously reported (12). Likewise, in the prospective analysis of all-cause mortality, we found that there was a significant interaction of TMAO with eGFR.

Table 5. Clinical and biochemical variables associated with TMAO.

Clinical and Biochemical variables associated with TMAO
Use of glucose lowering medication
Age
Fatty Liver Index
Estimated glomerular filtration rate
Alcohol consumption
Waist circumference
Body mass index
Vitamin D (reference 35) *
Glucose
Homeostasis Model Assessment of Insulin Resistance
High-density lipoprotein cholesterol
Triglycerides
Reactive oxygen species (reference 36) *
Interleukin 18 (reference 36) *

* denotes variables did not include in the present analysis.

TMAO has been shown to play a role in the development of atherosclerosis, by promoting platelet hyperreactivity (38). Zhu and colleagues demonstrated that circulating TMAO promotes a hyper response of platelets aggregation in response to thrombin, in an in vitro setting. They also demonstrated that TMAO promotes an increased platelet adhesion to collagen surfaces. Furthermore, in in vivo thrombosis assays, they have shown that the formation of thrombus after an arterial injury is enhanced in animals fed with TMAO-enriched diets (38). Accordingly, we found that higher TMAO concentrations were associated cross-sectionally with history of CVD. Nevertheless, the prospective analysis showed that elevated concentrations of TMAO were not particularly associated with an increased risk of cardiovascular mortality (Supplemental Tables 6 and 7).

The mechanisms underlying the association between TMAO and all-cause mortality in subjects with NAFLD remain to be investigated. Of note, previous studies conducted in subjects with NAFLD, had shown that subject with high concentrations of circulating TMAO present a more advanced disease

stage, characterized by more steatosis, hepatocellular ballooning and lobular inflammation (14).

The worsening of NAFLD associated to high TMAO might be caused by its effect on decreasing the bile acids pool (14). Some mechanism had been proposed: decreasing synthesis of bile acids due to the inhibition of the key enzymes CYP7A1 and CYP27A116 (8); and constraining the enterohepatic circulation of bile acids between the liver and the gut due to the repression of organic anion transporter family protein expression (8). Furthermore, there is evidence from experimental models of NAFLD on which it had been shown that TMAO increases the hepatic triglyceride accumulation by inhibition the farnesoid X receptor signaling (15).

Future interventions to ameliorate the excess of mortality in patients with NAFLD, with special focus in improving the microbiota deserve attention. Previous studies have reported that adherence to the Mediterranean diet, characterized by reduced consumption of animal-derived protein, is associated with lower concentrations of TMAO (39). Furthermore, it has been reported that the micronutrient, vitamin D, was strongly associated to both NAFLD and TMAO concentrations (35), therefore, the study of nutritional interventions to explore the effect of micronutrient supplementation, also merit further research.

NMR represents a methodology capable of offering high-throughput metabolite quantifications in a cost-effective manner, in comparison with other metabolomic technologies (40). Therefore, it is plausible that TMAO quantification by means of NMR could represent a useful tool for the evaluation of clinical interventions to ameliorate adverse cardiometabolic consequences of NAFLD.

Strengths and Limitations

This study has strengths. Firstly, this study comprises a long-term follow-up, and includes the record of several important confounders for the analysis of both all-cause and cardiovascular mortality. In addition, the large population enrolled in the study enabled us to carry out sufficiently powered multivariable-adjusted analyses and test the robustness of the findings using sensitivity analyses to provide solid evidence. In addition, the sample size also facilitates the generalization of our findings to similar populations, in fact, our cross-sectional results were in agreement with a larger Dutch study that included a total of 37,496 participants (41). Furthermore, patients enrolled in

the PREVEND cohort were followed-up for a period of time that is long enough to allow us the study of metabolites with potentially subtle and cumulative effects, which seems to be the case of microbiota-derived metabolites such as TMAO (42). Finally, to the best of our knowledge, this is the first study to report the association between elevated circulating TMAO concentrations and increased risk of all-cause mortality in subjects with NAFLD. Several limitations of the present study deserve mentioning. First, the present study was conducted in the north of the Netherlands, and mainly comprises individuals of Caucasian ancestry, which could limit the extrapolation of our findings to other ethnicities.

In our report, categorization of NAFLD rely on the FLI and the HSI, which are proxies of NAFLD for which nuclear magnetic resonance imaging or liver biopsy are preferred diagnostic procedures in small scale studies. Similarly, alcohol consumption was self-reported and therefore we cannot disregard the possibility of residual confounding due to imprecise reports. In addition, the observational nature of the study prevents us to draw causal conclusions. Nevertheless, the robust body of external experimental evidence would suggest a causal role of TMAO in the increased risk of mortality within NAFLD populations. Finally, it is worth mentioning that residual confounding is an important limitation of all observational studies.

In conclusion, we presented the first evidence about the increased risk of mortality associated to elevated concentrations of TMAO, in subjects with NAFLD. Such prospective association was independent of traditional risk factors and comorbidities. Further investigation is needed to determine if TMAO-lowering interventions could improve the prognosis of patients with NAFLD.

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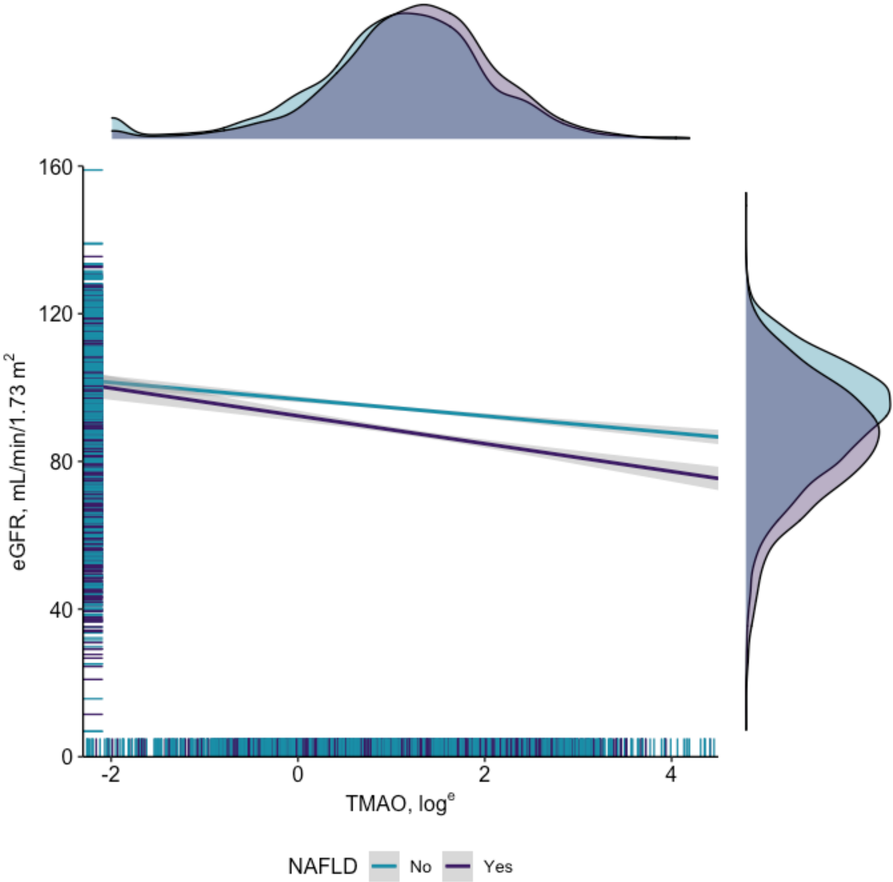
Supplemental Table S1. STROBE Statement—Checklist of items that should be included in reports of cohort studies

	Item No	Recommendation	Page No
Title and abstract	1	(a) Indicate the study's design with a commonly used term in the title or the abstract (b) Provide in the abstract an informative and balanced summary of what was done and what was found	207 208
Introduction			
Background/rationale	2	Explain the scientific background and rationale for the investigation being reported	209
Objectives	3	State specific objectives, including any prespecified hypotheses	210
Methods			
Study design	4	Present key elements of study design early in the paper	210
Setting	5	Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection	211
Participants	6	(a) Give the eligibility criteria, and the sources and methods of selection of participants. Describe methods of follow-up (b) For matched studies, give matching criteria and number of exposed and unexposed	211
Variables	7	Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable	212-214
Data sources/ measurement	8*	For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group	212
Bias	9	Describe any efforts to address potential sources of bias	213
Study size	10	Explain how the study size was arrived at	211
Quantitative variables	11	Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen and why	213
Statistical methods	12	(a) Describe all statistical methods, including those used to control for confounding (b) Describe any methods used to examine subgroups and interactions (c) Explain how missing data were addressed (d) If applicable, explain how loss to follow-up was addressed (e) Describe any sensitivity analyses	214-215 b.NA 211 d.NA 215
Results			
Participants	13*	(a) Report numbers of individuals at each stage of study—eg numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analysed (b) Give reasons for non-participation at each stage (c) Consider use of a flow diagram	216 211
Descriptive data	14*	(a) Give characteristics of study participants (eg demographic, clinical, social) and information on exposures and potential confounders	216

		(b) Indicate number of participants with missing data for each variable of interest	211
		(c) Summarise follow-up time (eg, average and total amount)	10
Outcome data	15*	Report numbers of outcome events or summary measures over time	220
Main results	16	(a) Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (eg, 95% confidence interval). Make clear which confounders were adjusted for and why they were included	221
		(b) Report category boundaries when continuous variables were categorized	221, 222
		(c) If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period	225
Other analyses	17	Report other analyses done—eg analyses of subgroups and interactions, and sensitivity analyses	224-226
Discussion			
Key results	18	Summarise key results with reference to study objectives	227
Limitations	19	Discuss limitations of the study, taking into account sources of potential bias or imprecision. Discuss both direction and magnitude of any potential bias	230
Interpretation	20	Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence	228-230
Generalisability	21	Discuss the generalisability (external validity) of the study results	230
Other information			
Funding	22	Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based	2

*Give information separately for exposed and unexposed groups.

Supplemental Fig 1. Association of Trimethylamine N-oxide with eGFR in participants with and without NAFLD.



Supplemental Table 2. Multivariable associations of baseline characteristics, excluding HSI and its determinants, with plasma concentrations of TMAO in 5292 PREVEND participants

Characteristic	**Std β**	**95% CI**	***p* value**
Men, n	0.02	-0.04, 0.08	0.46
Age, years	0.01	-0.03, 0.05	0.64
SBP, mmHg	-0.04	-0.08, 0.01	0.11
DBP, mmHg	0.01	-0.03, 0.05	0.53
History of CVD, n	0.02	-0.13, 0.17	0.78
Glucose lowering medication, n	0.15	-0.01, 0.31	0.07
Antihypertensive medication, n	-0.04	-0.11, 0.04	0.33
Lipid lowering medication, n	-0.03	-0.13, 0.08	0.60
Glucose, mmol/L	0.04	0.01, 0.07	**0.004**
HDL-C, mmol/L	-0.01	-0.04, 0.02	0.66
FLI, ≥ 60 A. U.	0.10	0.03, 0.17	**0.003**
Plasma albumin, g/L	-0.02	-0.05, 0.01	0.19
eGFR, mL/min/1.73 m^2	-0.15	-0.18, -0.11	**<0.001**
UAE, mg/24 h	0.03	0.01, 0.06	**0.02**

Standardized beta regression coefficients (95% Confidence Intervals) are shown.
Abbreviations. β, standardized beta regression coefficient, A.U., arbitrary units, DBP, diastolic blood pressure, eGFR, estimated glomerular filtration rate, FLI, fatty liver index, HDL-C, high-density lipoprotein cholesterol, HSI, hepatic steatosis index, SBP, systolic blood pressure, TMAO, Trimethylamine N-Oxide, UAE, urinary albumin excretion.

Supplemental Table 3. Multivariable associations of baseline characteristics, excluding FLI and its determinants, with plasma concentrations of TMAO in 5292 PREVEND participants

Characteristic	**Std β**	**95% CI**	***p* value**
Men, n	0.05	-0.01, 0.11	0.12
Age, years	0.01	-0.03, 0.05	0.57
SBP, mmHg	-0.04	-0.08, 0.00	0.07
DBP, mmHg	0.01	-0.03, 0.05	0.48
History of CVD, n	0.03	-0.12, 0.17	0.74
Glucose lowering medication, n	0.14	-0.02, 0.30	0.10
Antihypertensive medication, n	-0.04	-0.11, 0.04	0.32
Lipid lowering medication, n	-0.03	-0.13, 0.08	0.59
Glucose, mmol/L	0.04	0.01, 0.07	**0.010**
HDL-C, mmol/L	0.00	-0.04, 0.03	0.79
HSI, ≥ 36 A. U.	0.14	0.08, 0.20	**<0.001**
Plasma albumin, g/L	-0.02	-0.04, 0.01	0.21
eGFR, mL/min/1.73 m^2	-0.15	-0.19, -0.11	**<0.001**
UAE, mg/24 h	0.04	0.01, 0.06	**0.01**

Standardized beta regression coefficients (95% Confidence Intervals) are shown.
Abbreviations. β, standardized beta regression coefficient, A.U., arbitrary units, DBP, diastolic blood pressure, eGFR, estimated glomerular filtration rate, FLI, fatty liver index, HDL-C, high-density lipoprotein cholesterol, HSI, hepatic steatosis index, SBP, systolic blood pressure, TMAO, Trimethylamine N-Oxide, UAE, urinary albumin excretion.

Supplemental Table 4. Association of TMAO with all-cause mortality, assessed with Cox Proportional Hazard ratios in subjects with NAFLD (HSI ≥ 36).

	T1	T2		T3	
Participants, *n*	586	585		585	
Events, *n*	21	30		58	
		HR (95 % CI)	***p* value**	**HR (95 % CI)**	***p* value**
Crude Model	(ref)	1.41 (0.81,2.47)	0.22	2.81 (1.71,4.63)	
Model 1	(ref)	1.16 (0.66,2.02)	0.60	2.20 (1.33,3.63)	0.002
Model 2	(ref)	1.19 (0.68,2.09)	0.54	2.17 (1.31,3.58)	0.003
Model 3	(ref)	1.20 (0.68,2.10)	0.53	2.03 (1.23,3.36)	0.006
Model 4	(ref)	1.14 (0.65,1.99)	0.66	1.83 (1.10,3.07)	0.02

Data are presented as hazard ratios (HRs) with 95% confidence intervals (CIs) and *p* values.
Model 1. Model adjusted for age +sex
Model 2. Model 1 + SBP + Smoking status + alcohol consumption + cancer history + glucose lowering medication + lipid lowering medication
Model 3. Model 2 + TC + HDL-C + Glucose
Model 4. Model 3 + albuminuria + reduced eGFR (< 90 mL/min/1.73 m^2)

Supplemental Table 5. Association of TMAO with all-cause mortality, assessed with Cox Proportional Hazard ratios in subjects with NAFLD (FLI > 60), after exclusion of subjects with high alcohol consumption (>3 drinks per day).

	T1	T2		T3	
Participants, *n*	499	499		499	
Events, *n*	24	44		59	
		HR (95 % CI)	***p* value**	**HR (95 % CI)**	***p* value**
Crude Model	(ref)	1.84 (1.12,3.03)	0.01	2.48 (1.55,3.99)	<0.001
Model 1	(ref)	1.54 (0.94,2.53)	0.09	1.98 (1.23,3.19)	0.004
Model 2	(ref)	1.60 (0.97,2.63)	0.06	1.86 (1.15,3.00)	0.01
Model 3	(ref)	1.63 (0.99,2.69)	0.05	1.79 (1.11,2.90)	0.02
Model 4	(ref)	1.61 (0.98,2.66)	0.06	1.75 (1.08,2.84)	0.02

Data are presented as hazard ratios (HRs) with 95% confidence intervals (CIs) and *p* values.
Model 1. Model adjusted for age
Model 2. Model 1 + SBP + Smoking status + alcohol consumption + cancer history + glucose lowering medication + lipid lowering medication
Model 3. Model 2 + TC + HDL-C + Glucose
Model 4. Model 3 + albuminuria + reduced eGFR (< 90 mL/min/1.73 m^2)

Supplemental Table 6. Association of TMAO with all-cause mortality, assessed with Cox Proportional Hazard ratios in men and women with NAFLD (FLI > 60) separately.

MEN	T1	T2		T3	
Participants, *n*	184	183		183	
Events, *n*	6	8		13	
		HR (95 % CI)	***p* value**	**HR (95 % CI)**	***p* value**
Crude Model	(ref)	1.35 (0.47,3.89)	0.57	2.14 (0.81,5.64)	0.12
Model 1	(ref)	1.22 (0.42,3.52)	0.71	2.25 (0.85,5.91)	0.10
Model 2	(ref)	1.24 (0.43,3.58)	0.69	2.30 (0.86,6.14)	0.10
Model 3	(ref)	1.19 (0.41,3.50)	0.74	2.37 (0.88,6.37)	0.09
Model 4	(ref)	1.25 (0.42,3.71)	0.69	2.46 (0.91,6.63)	0.08
WOMEN	**T1**	**T2**		**T3**	
Participants, *n*	350	350		349	
Events, *n*	16	35		55	
		HR (95 % CI)	***p* value**	**HR (95 % CI)**	***p* value**
Crude Model	(ref)	2.17 (1.20,3.92)	0.01	3.56 (2.04,6.21)	<0.001
Model 1	(ref)	1.83 (1.01,3.30)	0.05	2.55 (1.46,4.45)	0.001
Model 2	(ref)	1.91 (1.06,3.47)	0.03	2.40 (1.37,4.21)	0.002
Model 3	(ref)	1.95 (1.07,3.55)	0.03	2.26 (1.29,3.98)	0.004
Model 4	(ref)	1.93 (1.06,3.52)	0.03	2.17 (1.23,3.84)	0.007

Data are presented as hazard ratios (HRs) with 95% confidence intervals (CIs) and *p* values.
Model 1. Model adjusted for age
Model 2. Model 1 + SBP + Smoking status + alcohol consumption + cancer history + glucose lowering medication + lipid lowering medication
Model 3. Model 2 + TC + HDL-C + Glucose
Model 4. Model 3 + albuminuria + reduced eGFR (< 90 mL/min/1.73 m^2)

Supplemental Table 7. Association of TMAO with all-cause mortality, assessed with Cox Proportional Hazard ratios in subjects without NAFLD (FLI < 60).

	T1	T2		T3	
Participants, *n*	1179	1178		1179	
Events, *n*	54	71		73	
		HR (95 % CI)	***p* value**	**HR (95 % CI)**	***p* value**
Crude Model	(ref)	1.32 (0.93,1.88)	0.12	1.33 (0.93,1.88)	0.12
Model 1	(ref)	0.98 (0.68,1.39)	0.89	1.00 (0.70,1.43)	0.99
Model 2	(ref)	0.98 (0.69,1.41)	0.93	0.98 (0.68,1.39)	0.89
Model 3	(ref)	1.00 (0.70,1.42)	0.98	0.98 (0.69,1.40)	0.90
Model 4	(ref)	1.01 (0.71,1.45)	0.95	1.01 (0.71,1.45)	0.96

Data are presented as hazard ratios (HRs) with 95% confidence intervals (CIs) and *p* values.
Model 1. Model adjusted for age +sex
Model 2. Model 1 + SBP + Smoking status + alcohol consumption + cancer history + glucose lowering medication + lipid lowering medication
Model 3. Model 2 + TC + HDL-C + Glucose
Model 4. Model 3 + albuminuria + reduced eGFR (< 90 mL/min/1.73 m^2)

Supplemental Table 8. Association of TMAO with cardiovascular mortality, assessed with Cox Proportional Hazard ratios in subjects with NAFLD (FLI ≥ 60).

	T1	T2		T3	
Participants, *n*	533	532		533	
Events, *n*	5	10		21	
		HR (95 % CI)	***p* value**	**HR (95 % CI)**	***p* value**
Crude Model	(ref)	1.99 (0.68,5.82)	0.20	4.20 (1.58,11.15)	0.003
Model 1	(ref)	1.78 (0.61,5.21)	0.29	3.32 (1.25,8.81)	0.01
Model 2	(ref)	1.96 (0.67,5.74)	0.22	2.90 (1.08,7.79)	0.03
Model 3	(ref)	1.94 (0.66,5.75)	0.23	2.56 (0.94,6.93)	0.07
Model 4	(ref)	1.97 (0.66,5.86)	0.22	2.50 (0.91,6.81)	0.07

Data are presented as hazard ratios (HRs) with 95% confidence intervals (CIs) and *p* values.
Model 1. Model adjusted for age +sex
Model 2. Model 1 + SBP + Smoking status + alcohol consumption + cancer history + glucose lowering medication + lipid lowering medication
Model 3. Model 2 + TC + HDL-C + Glucose
Model 4. Model 3 + albuminuria + reduced eGFR (< 90 mL/min/1.73 m^2)

Supplemental Table 9. Association of TMAO with cardiovascular mortality, assessed with Cox Proportional Hazard ratios in subjects with NAFLD (FLI > 60), after exclusion of subjects with high alcohol consumption (>3 drinks per day).

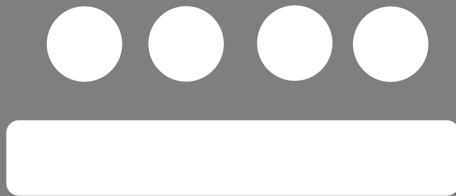
	T1	T2		T3	
Participants, *n*	499	499		499	
Events, *n*	5	10		20	
		HR (95 % CI)	***p* value**	**HR (95 % CI)**	***p* value**
Crude Model	(ref)	2.01 (0.69,5.87)	0.20	3.99 (1.50,10.64)	0.005
Model 1	(ref)	1.66 (0.57,4.85)	0.35	2.94 (1.10,7.85)	0.03
Model 2	(ref)	1.80 (0.61,5.28)	0.29	2.58 (0.95,6.98)	0.06
Model 3	(ref)	1.84 (0.62,5.44)	0.27	2.28 (0.83,6.23)	0.11
Model 4	(ref)	1.87 (0.63,5.58)	0.26	2.24 (0.81,6.17)	0.12

Data are presented as hazard ratios (HRs) with 95% confidence intervals (CIs) and *p* values.
Model 1. Model adjusted for age
Model 2. Model 1 + SBP + Smoking status + alcohol consumption + cancer history + glucose lowering medication + lipid lowering medication
Model 3. Model 2 + TC + HDL-C + Glucose
Model 4. Model 3 + albuminuria + reduced eGFR (< 90 mL/min/1.73 m^2)

Supplemental Table 10. Association of TMAO with cardiovascular mortality, assessed with Cox Proportional Hazard ratios in subjects without NAFLD (FLI < 60).

	T1	T2		T3	
Participants, *n*	1232	1231		1231	
Events, *n*	10	16		17	
		HR (95 % CI)	***p* value**	**HR (95 % CI)**	***p* value**
Crude Model	(ref)	1.60 (0.73,3.53)	0.24	1.69 (0.77,3.69)	0.19
Model 1	(ref)	0.98 (0.44,2.16)	0.95	1.15 (0.52,2.52)	0.72
Model 2	(ref)	1.08 (0.48,2.41)	0.85	1.31 (0.59,2.91)	0.50
Model 3	(ref)	1.05 (0.47,2.35)	0.91	1.33 (0.60,2.94)	0.49
Model 4	(ref)	1.03 (0.46,2.32)	0.93	1.30 (0.58,2.91)	0.52

Data are presented as hazard ratios (HRs) with 95% confidence intervals (CIs) and *p* values.
Model 1. Model adjusted for age +sex
Model 2. Model 1 + SBP + Smoking status + alcohol consumption + cancer history + glucose lowering medication + lipid lowering medication
Model 3. Model 2 + TC + HDL-C + Glucose
Model 4. Model 3 + albuminuria + reduced eGFR (< 90 mL/min/1.73 m^2)



Chapter 9

Association of Circulating Trimethylamine N-oxide, and its dietary determinants with risk of kidney graft failure: Results of the TransplantLines Cohort Study.

Jose L. Flores-Guerrero, Maryse C.J. Osté, Paula B. Baraldi, Margery A. Connelly, Erwin Garcia, Gerjan Navis, Stephan J. L. Bakker and Robin P. F. Dullaart

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Abstract

Due to the critical shortage of kidneys for transplantation, the identification of modifiable factors related to graft failure is highly desirable. The role of trimethylamine-N-oxide (TMAO) in graft failure remains undetermined. Here we investigated the clinical utility of TMAO and its dietary determinants for graft failure prediction in renal transplant recipients (RTRs). We included 448 RTRs who participated in the TransplantLines Cohort Study. Cox proportional-hazards regression analyses were performed to study the association of plasma TMAO with graft failure. Net Benefit, a decision analysis method, was performed to evaluate the clinical utility of TMAO and dietary information in the prediction of graft failure. Among RTRs (age 52.7 ± 13.1 years; 53% males), baseline median TMAO was 5.6 (3.0–10.2) µmol/L. In multivariable regression analysis, the most important dietary determinants of TMAO were egg intake (Std. β = 0.09 [95%CI, 0.01;0.18]; P=0.03), fiber intake (Std. β = -0.14 [95%CI, -0.22, -0.05]; P =0.002), and fish and seafood intake (Std. β = 0.12 [95%CI, 0.03,0.21]; P=0.01). After a median follow-up of 5.3 (4.5–6.0) years, graft failure was observed in 58 subjects. TMAO was associated with increased risk of graft failure, independent of age, sex, BMI, blood pressure, lipids, albuminuria and eGFR (Hazard Ratio per 1-SD increase of TMAO, 1.62 (95% confidence interval (CI): 1.22; 2.14, P<0.001). A TMAO and dietary enhanced prediction model offered approximately double Net Benefit compared to a previously reported, validated prediction model for future graft failure, allowing the detection of 21 RTRs per 100 RTRs tested with no false positives versus 10 RTRs respectively. In conclusion, a predictive model for graft failure, enriched with TMAO and its dietary determinants yielded a higher Net Benefit compared with an already validated model. This study suggests that TMAO and its dietary determinants are associated with an increased risk of graft failure and that it is clinically meaningful.

Introduction

Renal transplantation is the treatment of choice for patients with end-stage kidney disease, being superior to dialysis in terms of quality of life, long-term survival, and healthcare cost [1]. Unfortunately, worldwide there is a massive shortage of organs. E.g., in the United States of America, approximately 84% of the patients with end-stage kidney disease will not receive a transplant and their prognosis is reduced to an average of 5 years survival on dialysis before premature death [2].

Due to the scarcity of organs, approaches to lengthen graft and patient survival are highly desirable [3]. Over the last decades, there has been a remarkable improvement in 1-year graft survival, nonetheless, long-term survival still needs to be improved [3,4]. The investigation of biomarkers that reflect pathophysiological changes in the interplay of the kidney with other organs, such as the crosstalk between the gut microbiome and the kidney, could be of utility in the development of future interventions to preserve the graft function. The evaluation of the above-mentioned biomarkers, should also take into account the clinical utility of the biomarkers, in addition to its predictive accuracy. The application of decision theory analytic methods; i.e. the calculation of Net Benefit, has been proposed as an alternative to evaluate the clinical value of biomarkers and predictive models [5,6].

Trimethylamine-N-oxide (TMAO) is a methylamine osmolyte [7] that has gained attention due its potential role in the development of heart [8,9] and kidney disease [10], and mortality [11]. TMAO is a product of the gut microbiome–host metabolism of dietary components such as choline and L-carnitine [12,13]. Its metabolism includes the production of trimethylamine (TMA) by the gut microbiota, subsequent conversion to TMAO by host liver flavin monooxygenase 3, and clearance of circulating TMAO by the kidneys [14].

Despite the fact that some studies have identified an association between plasma concentrations of TMAO with adverse cardiovascular outcomes in patients with chronic kidney disease [15,16], and in hemodialysis patients [17], the potential role of these osmolytes in graft survival in renal transplant recipients is still unknown. Considering that modification of gut microbiome by dietary intervention may enhance survival of the allograft, as observed in a rodent kidney transplant model [18], and the fact that TMAO may

contribute to renal fibrosis [19], the aim of the present study was to evaluate the potential association of TMAO and its dietary determinants with risk of graft failure in renal transplant recipients (RTRs) and its clinical value.

Materials and Methods

Study Population

In this study stable adult RTRs (≥18 years old) with a functioning graft for at least one year after transplantation (i.e., on maintenance immunosuppression and with a stable renal function) were included. Briefly, between November 2008 and May 2011, patients who visited the outpatient clinic of the University Medical Center Groningen were invited to participate. Subjects with malignancies, opportunistic infections or addictions were excluded. Furthermore, participants with missing data on TMAO were excluded, resulting in 448 renal transplant recipients. The protocol for the present study was approved by the local ethics committee of the University Medical Center Groningen (METc 2008/186), and all procedures were conducted according to the Declaration of Helsinki [20]. This report follows the Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) reporting guideline (Supplemental Table 1).

During a morning visit to the outpatient clinic, all baseline data were collected as previously described [21]. Height and weight were measured with the participants standing without shoes and heavy outer garments. Body mass index (BMI) was calculated by dividing weight in kilograms by height in meters squared. Systolic and diastolic blood pressure and heart rate were measured every minute for 15 minutes in a half-sitting position using a semi-automatic device (Dinamap®1846; Critikon, Tampa, FL, USA). The average of the last three measurements was taken as blood pressure value. Information on medication was derived from patient records. Information on smoking behavior was obtained by questionnaire.

Dietary intake assessment

Dietary intake was assessed with a validated semi-quantitative food frequency questionnaire (FFQ) developed and updated at the Wageningen University (Wageningen, Netherlands) [22]. The intake of 177 food items during the previous month was inquired, taking seasonal variations into account. For each

item, the frequency was registered daily, weekly and monthly. In this study, only measurements of daily intake were considered.

The FFQ was self-administered and its completeness was checked by a trained researcher on the day of the visit to the outpatient clinic. Inconsistent answers were verified with the patients. The results of the FFQ were converted into total energy (in kcal) per day by using the Dutch Food Composition Table (NEVO 2006). The FFQ was validated by comparing the protein intake of the FFQ with the protein intake calculated by the Maroni Equation, using urinary urea excretion values [23].

Laboratory measurements

Blood samples were taken after an 8–12 h overnight fasting period in the morning after completion of 24 h urine collection. Serum creatinine was determined using a modified version of the Jaffé method (MEGA AU 510, Merck Diagnostica, Darmstadt, Germany). TMAO concentrations were measured in EDTA anticoagulated plasma samples using a Vantera® Clinical Analyzer (LabCorp, Morrisville, NC), a fully automated, high-throughput, 400 MHz proton (1H) nuclear magnetic resonance (NMR) spectroscopy platform. TMAO concentrations were quantified from one-dimensional (1D) proton (1H) Carr-Purcell-Meiboom-Gill (CPMG) spectra by means of deconvolution assays as previously described [24]. The TMAO assay has intra- and inter-assay coefficients of variation (CV%) range from 4.3-10.3% and 9.8-14.5%, respectively, and a limit of quantitation of 3.3 µM. Estimated Glomerular Filtration Rate (eGFR) was calculated using the serum creatinine-based Chronic Kidney Disease Epidemiology Collaboration equation [25]. Chronic Kidney Disease (CKD) stage was determined according to the latest nomenclature from Kidney Disease: Improving Global Outcomes (KDIGO) Consensus Conference [26] (Normal eGFR (G1): ≥ 90 ml/min/1.73 m^2, Mildly decreased (G2): 60-89, Mildly to moderately decreased (G3a): 45-59, Moderately to severely decreased (G3b): 30-44, Severely decreased (G4): 15-29, Kidney failure (G5): <15).

Clinical Endpoint

The primary outcome of this study, graft failure, was defined as re-transplantation or return to dialysis and was censored for death. The endpoint was recorded until September 2015 by a qualified physician, with no loss to follow-up.

Statistical Analysis

Normally distributed data were presented as mean and standard deviation, whereas skewed data were expressed as median and interquartile range. Categorical data were presented as number and percentage. Linear trends across TMAO tertiles were determined using ANOVA for normally distributed data, Kruskal-Wallis test for skewed distributed data, and χ^2 test for categorical variables. Skewed data were log-transformed when appropriate. Baseline associations between characteristics and TMAO concentrations were analyzed through univariable regression analysis and through backward stepwise regression analysis, which involves a sequence of automated steps on which the least statistically significant explanatory variables are removed from a full model, until a the most parsimonious model is achieved. In order to identify the risk of multicollinearity in the multivariable regression analysis, Variance-Inflation Factors were calculated. A high risk of multicollinearity was considered present if the calculated Variance-Inflation Factor (VIF) was >5 [27].

For the prospective analysis, we plotted cumulative Kaplan-Meier curves for risk of graft failure during follow-up according to tertiles of TMAO. Time-to-event Cox proportional hazards models were used to compute hazard ratios (HRs) and 95% CI of graft failure risk among the 448 participants. HRs were calculated in models adjusted for age, sex, pre-emptive transplantation, donor type, immunosuppressive medication, HLA-A,-B and HLA-DR broad antigen mismatch, history of T2D, tobacco and alcohol consumption, BMI, systolic blood pressure, glucose, total cholesterol, high-density lipoprotein cholesterol (HDL-cholesterol), triglycerides, insulin, urinary albumin excretion (UAE), CKD stage [26] (Normal eGFR (G1): ≥ 90 ml/min/1.73 m^2, Mildly decreased (G2): 60-89, Mildly to moderately decreased (G3a): 45-59, Moderately to severely decreased (G3b): 30-44, Severely decreased (G4): 15-29, Kidney failure (G5): <15), vegetable intake, meat intake and fish and seafood intake. The Cox proportional hazard assumption was tested through the evaluation of independence between scaled Schoenfeld residuals with time for each variable and for every model as a whole; this assumption was met, with no indication for a violation [28].

To determine whether the model with higher HR can improve the predictive ability of an independent multicenter validated predictive model for kidney graft failure that includes age, sex, serum albumin, urine albumin-

creatinine ratio and eGFR [29], the net reclassification improvement (NRI) [30] was calculated for an enhanced model that included TMAO and its dietary determinants according to the cross-sectional relationships and previous reports in the literature [13,31–34], i.e. fish and seafood, red meat and eggs. In order to avoid bias, for the computation of NRI four predefined risk categories for kidney graft failure previously described in the literature were used, i.e.: low (<5%), medium low (5% to 10%), medium high (11% to 20) and high (>20%) [29].

Brier scores were calculated in order to assess the difference between the predicted probability of graft failure development and the cases of actual outcome development [35]. The Brier scores of two predictive models for kidney graft failure were compared: a model already validated [29] and our model enhanced with TMAO and dietary information.

Since Brier scores could not properly assess the clinical utility of some prediction models [36], and considering that traditional measures such as sensitivity, specificity, or area under the curve, correspond to statistical abstractions which could not provide direct information about the clinical value of biomarkers or prediction models, we decided to calculate the Net Benefit, which is a decision-analytic measure that can overcome such limitations [5]. Therefore, the clinical utility of enhancing the previously validated model with TMAO and dietary information, was further assessed by calculating the Standardized Net Benefit and plotting decision curves for both models after 1000 bootstraps.

Briefly, Net Benefit, is a type of decision analysis method in which an exchange rate is calculated between the benefit and harms of a prognostic model. In the clinical research of prediction biomarkers and models, the above-mentioned exchange rate is related to the probabilities of patient to be classified as a developer of the disease or not. Net Benefit informs about the proportion of “net” true positives. The “net” can be understood as the observed number of true positives corrected for the observed proportion of false positives and weighted by the odds of the risk threshold [37]. Further details about the methods and rationally of decision theory in clinical research can be found somewhere else [6,38].

All statistical analyses were performed with R language for statistical computing software [39], v. 4.0.2.

Results

Baseline characteristics

Of the 632 participants that were enrolled in the prospective cohort, 448 subjects with available measurements of TMAO were included in the current study. Among the study participants, 238 (53.1%) were men and the mean age of the population was 54.9 ± 13.1 years. The median (IQR) plasma total TMAO concentration was 5.65 (3.1–11.2) µM. Participant characteristics at baseline are shown in Table 1.

Participants within the highest tertile of TMAO plasma concentrations (>8.07 µmol/L) were more likely to be older, have lower concentrations of total and HDL-cholesterol, decreased eGFR, higher urinary albumin excretion, and belong to an advanced CKD stage. Those patients reported a higher consumption of fish and seafood, less frequently received renal allografts from living donors and more frequently were taking cyclosporine, azathioprine, mycophenolic acid and diuretic medication.

Cross-sectional analysis

The association of the concentration of TMAO with baseline characteristics was evaluated with both, univariable and multivariable linear regression analyses. In the univariable analyses, HDL-C (Std. β = 0.-0.12 [95%CI, -0.21, -0.02]; P = 0.01), eGFR (Std. β = -0.29 [95%CI, -0.38, -0.20]; P <0.001), stage 4 of CKD (Std. β = 0.88 [95%CI, 0.29, 1.15]; P = 0.003), stage 5 of CKD (Std. β = 1.00 [95%CI, 0.02, 1.90]; P = 0.003),), fish and seafood intake (Std. β = 0.15 [95%CI, 0.06, 0.24]; P <0.001), living donor (Std. β = -0.24 [95%CI, -0.43, -0.05]; P = 0.02 and tacrolimus medication (Std. β = 0.31 [95%CI, 0.08, 0.54]; P = 0.009), were significantly associated with plasma concentrations of TMAO (Table 2).

Table 1. Participant Characteristics According to Tertiles of Plasma Concentration of TMAO

Characteristic	All (N=448)	Tertile 1 (N=147) (<3.93 µmol/L)	Tertile 2 (N=149) (3.93, 8.07 µmol/L)	Tertile 3 (N=152) (>8.07 µmol/L)	P value
Men, n (%)	238 (53.1%)	70 (47.6%)	79 (53.0%)	89 (58.6%)	0.17
Age, y	52.71 (13.09)	50.32 (13.14)	53.43 (12.64)	54.32 (13.23)	0.02
BMI, kg/m2	26.41 (4.79)	26.47 (5.01)	26.14 (4.37)	26.63 (4.97)	0.67
SBP, mmHg	135.85 (17.56)	135.25 (15.58)	134.47 (17.55)	137.77 (19.24)	0.24
DBP, mmHg	81.86 (10.95)	82.35 (10.49)	81.50 (10.85)	81.73 (11.54)	0.79
Heart Rate, bpm	67.97 (11.67)	68.20 (11.58)	68.03 (11.63)	67.69 (11.86)	0.93
Glucose, mmol/L	5.20 (4.70, 5.90)	5.30 (4.80, 5.70)	5.15 (4.70, 5.90)	5.30 (4.70, 6.00)	0.69

TG, mmol/L	1.88 (0.94)	1.80 (0.91)	1.89 (1.01)	1.95 (0.89)	0.21
TC, mmol/L	5.12 (1.14)	5.18 (1.15)	5.11 (1.19)	5.07 (1.08)	0.72
HDL-C, mmol/L	1.37 (0.46)	1.47 (0.48)	1.35 (0.44)	1.31 (0.46)	0.01
HbA1c, %	5.80 (5.50, 6.10)	5.80 (5.45, 6.25)	5.70 (5.40, 6.00)	5.80 (5.50, 6.10)	0.32
TMAO, µmol/L	5.66 (3.08, 11.20)	2.41 (1.44, 3.06)	5.62 (4.82, 6.69)	15.09 (11.08, 22.11)	< 0.001
eGFR, mL/min/1.73 m²	49.93 (19.38)	62.12 (16.70)	49.35 (15.58)	38.71 (18.23)	< 0.001
CKD stage, n (%)					< 0.001
G1	12 (2.7%)	8 (5.4%)	2 (1.3%)	2 (1.3%)	
G2	125 (27.9%)	72 (49.0%)	38 (25.5%)	15 (9.9%)	
G3a	115 (25.7%)	41 (27.9%)	47 (31.5%)	27 (17.8%)	
G3b	128 (28.6%)	25 (17.0%)	47 (31.5%)	56 (36.8%)	
G4	62 (13.8%)	1 (0.7%)	15 (10.1%)	46 (30.3%)	
G5	6 (1.3%)	0 (0.0%)	0 (0.0%)	6 (3.9%)	
UAE, mg/24h	43.97 (11.75, 207.12)	37.70 (9.62, 177.82)	30.55 (8.48, 108.65)	90.03 (16.54, 384.67)	< 0.001
Current smokers, n (%)	56 (13.2%)	15 (11.1%)	16 (11.3%)	25 (17.0%)	0.24
Alcohol consumption, g/d	2.20 (0.04, 10.47)	2.01 (0.03, 11.33)	2.49 (0.06, 9.65)	2.62 (0.03, 10.33)	0.94
Total energy intake, kcal/d	2138.61 (613.46)	2204.56 (558.62)	2137.56 (650.91)	2072.21 (626.80)	0.21
Egg intake, g/day	8.93 (4.46, 14.29)	8.93 (4.46, 14.29)	7.14 (4.46, 14.29)	8.93 (7.14, 14.29)	0.25
Vegetable intake, g/day	107.00 (71.50, 147.90)	110.71 (81.69, 144.21)	112.67 (72.81, 156.02)	95.75 (65.83, 140.15)	0.10
Fruit intake, g/day	105.43 (49.71, 191.53)	90.11 (39.63, 178.02)	123.79 (53.07, 212.64)	99.43 (52.71, 191.41)	0.25
Fiber intake, g/day	20.86 (16.32, 26.39)	21.07 (15.88, 27.15)	21.47 (17.93, 28.23)	19.95 (15.28, 25.29)	0.08
Fish and sea food intake, g/day	10.81 (4.42, 18.71)	9.94 (3.89, 17.07)	10.69 (4.68, 16.93)	14.87 (4.68, 22.96)	0.02
Meat intake, g/day	83.82 (60.95, 100.30)	84.06 (59.58, 102.90)	80.51 (64.76, 98.25)	84.60 (59.92, 99.49)	0.86
Transplant vintage, y	4.95 (1.59, 11.54)	4.88 (1.79, 10.26)	4.08 (1.31, 10.58)	6.14 (1.76, 14.43)	0.09
Preemptive, n (%)	69 (17.5%)	28 (20.9%)	26 (20.3%)	15 (11.3%)	0.07
Living donor, n (%)	158 (35.3%)	62 (42.2%)	57 (38.3%)	39 (25.7%)	0.007
HLA-A, -B broad antigen mismatch, n (%)					0.99
0	94 (22.8%)	29 (21.6%)	32 (22.7%)	33 (23.9%)	
1	97 (23.5%)	35 (26.1%)	33 (23.4%)	29 (21.0%)	
2	151 (36.6%)	48 (35.8%)	51 (36.2%)	52 (37.7%)	
3	46 (11.1%)	14 (10.4%)	16 (11.3%)	16 (11.6%)	
4	25 (6.1%)	8 (6.0%)	9 (6.4%)	8 (5.8%)	
HLA-DR broad antigen mismatch, n (%)					0.49
0	175 (42.6%)	60 (45.1%)	55 (39.0%)	60 (43.8%)	
1	192 (46.7%)	60 (45.1%)	66 (46.8%)	66 (48.2%)	
2	44 (10.7%)	13 (9.8%)	20 (14.2%)	11 (8.0%)	
Tacrolimus, n (%)	68 (17.2%)	17 (12.7%)	22 (17.2%)	29 (21.8%)	0.14
Cyclosporine, n (%)	173 (43.8%)	48 (35.8%)	60 (46.9%)	65 (48.9%)	0.07
Azathioprine, n (%)	72 (18.2%)	21 (15.7%)	17 (13.3%)	34 (25.6%)	0.02
Mycophenolic acid, n (%)	262 (66.3%)	98 (73.1%)	90 (70.3%)	74 (55.6%)	0.005
Prednisolone dose, mg/day	10.00 (7.50, 10.00)	10.00 (7.50, 10.00)	10.00 (7.50, 10.00)	10.00 (7.50, 10.00)	0.42
Statins, n (%)	233 (52.0%)	70 (47.6%)	73 (49.0%)	90 (59.2%)	0.09
Diuretics, n (%)	177 (39.5%)	47 (32.0%)	55 (36.9%)	75 (49.3%)	0.007

Abbreviations. BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; BPM, beats per minute; TG, triglycerides; TC, total cholesterol; HDL-C, high-density lipoprotein cholesterol; eGFR, estimated glomerular filtration rate; UAE, urinary albumin excretion; HLA, human leukocyte antigen.

In a multivariable model, without risk of multicollinearity (VIF<5), TMAO remained independently associated with SBP (Std. β = 0.15 [95%CI, 0.03, 0.27]; P = 0.01), DBP (Std. β = -0.13 [95%CI, -0.25,-0.02]; P = 0.03), TC (Std. β = -0.15 [95%CI, -0.24,-0.06]; P = 0.01), eGFR (Std. β = -0.28 [95%CI, -0.36, -0.19]; P <0.001), egg intake (Std. β = 0.09 [95%CI, 0.01;0.18]; P= 0.03), fiber intake (Std. β = -0.14 [95%CI, -0.22, -0.05]; P = 0.002), and fish and seafood intake (Std. β = 0.12 [95%CI, 0.03,0.21]; P = 0.01) (Table 2). Notably, the distribution of the concentrations of TMAO in RTRs in the highest tertiles of fish and sea food intake differed among the participants with normal and declined renal function. The median plasma concentration of TMAO in RTRs with eGFR>60 ml/min * 1.73m^2 was 3.04 (1.84 – 5.16), whereas the median for RTRs with eGFR <60 ml/min * 1.73m^2 was 12.82 (6.69 – 19.58), P <0.001 (Supplemental Figure 1A and Supplemental Figure 1B). A similar effect modification was observed in RTRs in the highest tertiles of meat intake, (Supplemental Figure 2A and Supplemental Figure 2B).

Longitudinal Analyses

After a median (IQR) follow-up of 5.3 (4.6-6.0) years, 58 (12.9%) RTRs developed graft failure. Kaplan-Meier curves for graft failure according to tertiles of TMAO plasma concentration are presented in Figure 1. There was an increased risk of graft failure associated with the top tertile of TMAO concentrations (P for log-rank test <0.001).

In Cox regression analyses that examined the TMAO plasma concentration as a categorical variable with first tertile as the reference group, the third tertile of TMAO plasma concentration [8.07 – 154.3 μmol/L] was associated with increased risk of graft failure independent of age and sex (adjusted HR, 8.17 [95%CI, 3.44; 19.43]; P <0.001, model 1, Table 3); pre-emptive transplantation, donor type (living, post mortem), T2D, smoking, alcohol consumption and immunosuppressive medication (tacrolimus, cyclosporine and prednisolone), HLA-A,-B and HLA-DR broad antigen mismatch (adjusted HR, 5.37 [95%CI, 2.15; 13.42]; P <0.001, model 2, Table 3); BMI, systolic blood pressure, HDL-cholesterol, triglycerides, total cholesterol, UAE and CKD stage (adjusted HR, 3.01 [1.10; 8.26]; P =0.03, model 3, Table 3); vegetable intake, meat intake, fish and seafood intake and egg intake (adjusted HR, 9.15 [95%CI, 3.79; 22.04]; P <0.001, model 4, Table3).

Table 2. Univariable and multivariable associations of baseline characteristics with plasma concentrations of TMAO in Renal Transplant Recipients.

	Univariable regression analysis		Multivariable regression analysis	
	Std. β (95% CI)	P value	Std. β (95% CI)	P value
Sex, male, yes	0.02 (-0.17, 0.21)	0.83	—	—
Age, y	0.03 (-0.06, 0.12)	0.54	—	—
BMI, kg/m^2	0.06 (-0.04, 0.15)	0.24	—	—
SBP, mm Hg	0.06 (-0.03, 0.15)	0.21	0.15 (0.03,0.27)	0.01
DBP, mm Hg	-0.05 (-0.14, 0.05)	0.32	-0.13 (-0.25,-0.02)	0.03
Heart rate, bpm	0.03 (-0.07, 0.12)	0.56	—	—
Glucose, mmol/L	0.02 (-0.08, 0.11)	0.72	—	—
TG, mmol/L	0.03 (-0.06, 0.12)	0.53	—	—
TC, mmol/L	-0.09 (-0.19, 0.00)	0.05	-0.15 (-0.24,-0.06)	0.001
HDL-C, mmol/L	-0.12 (-0.21, -0.02)	0.01	—	—
HbA1C, %	0.02 (-0.08, 0.11)	0.71	—	—
eGFR, mL/min/1.73 m^2	-0.29 (-0.38, -0.20)	<0.001	-0.28 (-0.36,-0.19)	<0.001
CKD stage, yes				
G2	0.01 (-0.56, 0.57)	0.99	—	—
G3a	0.10 (-0.47, 0.67)	0.74	—	—
G3b	0.46 (-0.10, 1.00)	0.11	—	—
G4	0.88 (0.29, 1.50)	0.003	—	—
G5	1.00 (0.02, 1.90)	0.04	—	—
UAE, mg/24h	0.08 (-0.01, 0.17)	0.08	—	—
Current smoking, yes	0.10(-0.19, 0.39)	0.49	—	—
Alcohol consumption, g/d	-0.04 (-0.13, 0.05)	0.39	—	—
Total energy intake, kcal/d	-0.07 (-0.16, 0.02)	0.12	—	—
Egg intake, g/day	0.03 (-0.06, 0.11)	0.57	0.09 (0.01,0.18)	0.03
Vegetable intake, g/day	-0.07 (-0.16, 0.01)	0.10	—	—
Fruit intake, g/day	-0.04 (-0.13, 0.04)	0.33	—	—
Vegetable and Fruit intake, g/day	-0.07 (-0.16, 0.02)	0.13	—	—
Fiber intake, g/day	-0.08 (-0.17, 0.01)	0.06	-0.14 (-0.22,-0.05)	0.002
Fish and Seafood intake, g/day	0.15 (0.06, 0.24)	<0.001	0.12 (0.03,0.21)	0.01
Meat intake, g/day	-0.01 (-0.09, 0.08)	0.89	—	—
Transplant vintage, y	0.01 (-0.08, 0.10)	0.84	—	—
Preemptive, yes	-0.23 (-0.47, 0.00)	0.05	—	—
Living donor, yes	-0.24 (-0.43, -0.05)	0.02	-0.08 (-0.17,0.00)	0.06
HLA -A, -B broad antigen mismatch, yes				
1	-0.12 (-0.41, 0.16)	0.39	—	—
2	0.04 (-0.22, 0.30)	0.75	—	—
3	0.05 (-0.31, 0.40)	0.80	—	—
4	-0.16 (-0.60, 0.29)	0.48	—	—
HLA-DR broad antigen mismatch, yes				
1	0.08 (-0.12, 0.29)	0.43	—	—
2	0.07 (-0.26, 0.41)	0.66	—	—
Tacrolimus, yes	0.31 (0.08, 0.54)	0.009	—	—
Cyclosporine, yes	-0.01 (-0.19, 0.17)	0.92	—	—
Azathioprine, yes	0.15 (-0.08, 0.38)	0.19	—	—
Mycophenolic acid, yes	-0.16 (-0.35, 0.03)	0.09	—	—
Prednisolone dose, mg/day	-0.01 (-0.10, 0.09)	0.89	—	—
Statins, yes	0.02 (-0.17, 0.21)	0.83	—	—
Diuretics, yes	0.23 (0.04, 0.42)	0.02	—	—

Standardized beta regression coefficients (95% Confidence Intervals) are shown. Abbreviations. BPM, beats per minute; TG, triglycerides; TC, total cholesterol; HDL-C, high-density lipoprotein cholesterol; eGFR, estimated glomerular filtration rate; UAE, urinary albumin excretion; HLA, human leukocyte antigen.

Table 3. Prospective associations of plasma TMAO with risk of Graft Failure in Renal Transplant Recipients.

	TMAO per 1 Ln SD Increment		T1	T2		T3	
Participants, *n*	448		147	149		152	
Events, *n*	58		6	14		38	
	HR [95 % CI]	**P-value**		**HR [95 % CI]**	**P-value**	**HR [95 % CI]**	**P-value**
Crude Model	2.22 [1.73; 2.84]	<0.001	(ref)	2.41 [0.93; 6.27]	0.07	7.62 [3.22; 18.03]	<0.001
Model 1	2.20 [1.73; 2.80]	<0.001	(ref)	2.54 [0.97; 6.62]	0.06	8.17 [3.44; 19.43]	<0.001
Model 2	2.27 [1.64; 3.14]	< 0.001	(ref)	1.73 [0.60; 4.95]	0.31	5.41 [2.17; 13.48]	<0.001
Model 3	1.39 [1.01; 1.91]	0.04	(ref)	2.06 [0.75; 5.70]	0.16	3.01 [1.10; 8.26]	0.03
Model 4	2.86 [2.13; 3.83]	< 0.001	(ref)	2.02 [0.73; 5.57]	0.17	9.15 [3.79; 22.04]	<0.001

Data are presented as hazard ratios (HRs) with 95% confidence intervals (CIs) and P-values.
Model 1: Model adjusted for age + sex
Model 2: Model 1 + pre-emptive transplantation + donor type + T2D + smoking + alcohol consumption + immunosuppressive medication (tacrolimus, cyclosporine and prednisolone) + HLA-A, -B + HLA-DR
Model 3: Model 1 + BMI + Systolic Blood Pressure + HDL-C + TG + TC + UAE + CKD stage
Model 4: Model 1 + vegetable intake + meat intake + fish and seafood intake + egg intake
Abbreviations. T2D, type 2 diabetes; BMI, body mass index; HDL-C, high density lipoprotein cholesterol; TG, triglycerides; TC, total cholesterol; UAE, urine albumin excretion; eGFR, estimated glomerular filtration rate.

The proportional hazards assumptions were not violated for any of the variables in the additive models. The interaction terms of TMAO plasma concentration with age, sex, and eGFR with graft failure were not significant (P >0.10 for each) when included in either the crude or the sex- and age-adjusted models.

Plasma concentrations of TMAO, when analyzed as HR per 1 log SD increase, were also associated with risk of graft failure. In Cox regression analyses that examined TMAO plasma concentration as a continuous variable, the crude model displayed a HR of 2.22 [95%CI, 1.73; 2.84]; P <0.001 (Table 3). The association was independent of adjustment for age and sex (adjusted HR, 2.20 [95%CI, 1.73; 2.80]; P <0.001, model 1, Table 3); pre-emptive transplantation, donor type (living, post mortem), T2D, smoking, alcohol consumption and immunosuppressive medication (tacrolimus, cyclosporine and prednisolone), HLA-A,-B and HLA-DR broad antigen mismatch (adjusted HR, 2.27 [95%CI, 1.64; 3.14]; P <0.001, model 2, Table 3); BMI, systolic blood pressure, HDL-cholesterol, triglycerides, total cholesterol, urinary albumin excretion and CKD stage (adjusted HR, 1.39 [95%CI, 1.01; 1.91]; P = 0.04, model 3, Table3); vegetable intake, meat intake, fish and seafood intake and egg intake (adjusted HR, 2.86 [95%CI, 2.13; 3.83]; P <0.001 , model 4, Table 3).

Clinical Utility of TMAO and Diet Assessment

The NRI of a previously validated model [29] that included age, sex, serum albumin, urine albumin-creatinine ratio and eGFR, was compared with a model that included of TMAO and its dietary determinants. Ten percent of the participants in the low-risk category were correctly reclassified, 48% of the participants in the medium risk category were correctly reclassified and 17% of the participants in the high-risk category were correctly reclassified with the addition of TMAO and dietary information to the risk model. The improvement in the classification of participants into predicted risk categories was statistically significant with an NRI of 0.35 [95% CI, 0.08–0.62]; P =0.01. The Brier Score of the updated model containing TMAO and dietary information, showed a discrete, albeit insignificant, improvement from 0.0879 to 0.0857 (P>0.5) (Supplemental Table 3).

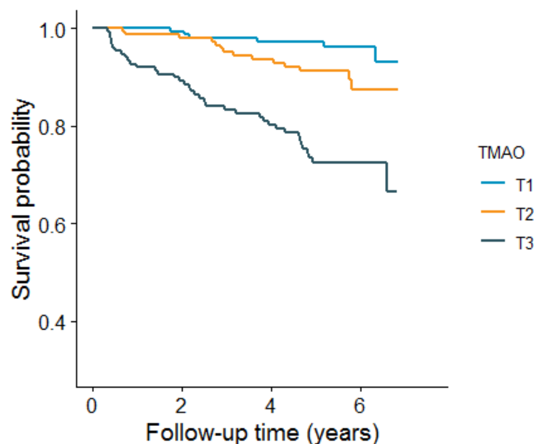

Figure 1. Kaplan-Meier plot for graft failure comparing tertiles of TMAO. (log-rank test, P value <0.001).

The Net Benefit analysis demonstrated a higher clinical utility of the model containing TMAO and dietary information over the traditional model for graft failure risk prediction (Figure 2). The TMAO and dietary enhanced model offered a greater Net Benefit, compared with the traditional model, along all the risk thresholds. The already validated model showed a Net Benefit of 0.466 at the 20% graft failure risk threshold, which is equivalent to detecting 46.6 RTR with future graft failure and suggesting no false positive results per 100 RTRs with future graft failure tested. At the same risk threshold, the TMAO and dietary enhanced model offered the greatest Net Benefit of 0.509, meaning that 51 RTRs with future graft failure could be detected per 100 RTRs tested with no false positives. The TMAO and dietary enhanced model offered a double Net Benefit, compared with the traditional model at the 60% graft failure risk threshold, allowing the detection of 21 RTRs (per 100 RTRs tested with no false positives) with future graft failure, in contrast, the previously published model allowed the detection of with 10 RTRs (per 100 RTRs tested with no false positives) (Supplemental Table 4).

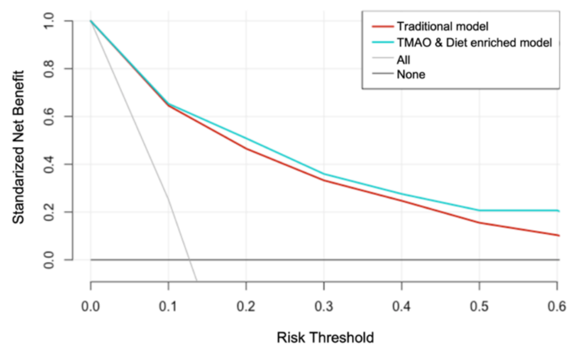

Figure 2. Decision curve for graft failure prediction. The model enriched with TMAO and diet information show superior clinical utility that the traditional risk model for graft failure.

Discussion

In this RTR cohort, the associations of plasma concentrations of TMAO with risk of graft failure were investigated. Plasma concentrations of TMAO at baseline were associated with reduced eGFR, and fish and seafood intake. The association of TMAO with increased risk of graft failure remained significant when taking into account important risk factors, including BMI, lipid profile, smoking, donor characteristics, diet and renal function at baseline.

Furthermore, addition of circulating TMAO and diet information to an already validated predictive model of graft failures enhanced its predictive ability in terms of NRI as well as Net Benefit. To the best of our knowledge, the present study is the first to extensively evaluate the clinical utility of circulating TMAO and diet information in the context of graft failure prediction in RTRs.

In this cohort study, baseline renal function evaluated by means of eGFR was the variable with the strongest TMAO association (standardized regression coefficient (-0.28 [-0.37, -0.19], P<0.001). This result is in line with a previous study conducted in patients with end-stage renal disease where pre- and post-hemodialysis TMAO concentrations were measured, and showed a marked decline in TMAO concentrations, from 99.9 ± 31.9 µM to 41.3 ± 18.8 µM after hemodialysis [40]. In relation to the dietary components, after multiple adjustment, fish and seafood intake combined was associated with TMAO plasma concentrations (standardized regression coefficient 0.12 [95%CI, 0.03,0.21], P=0.01). These findings are explained by the role of fish and seafood as sources of TMAO, given the fact that such foods are the only source of TMAO that do not require intermediate metabolism [41]. Egg consumption displayed a similar regression coefficient with TMAO plasma concentrations (standardized regression coefficient 0.09 [95%CI, 0.01,0.18], P=0.03). Such association is in line with previous studies which have reported that increased egg intake was associated with elevated concentrations of TMAO in both plasma and urine [42]. Moreover, the consumption of fiber was inversely associated to the plasma concentrations of TMAO, (standardized regression coefficient -0.14 [-0.22, -0.05], P=0.002). Notably, subjects in the highest tertile of TMAO consumed less vegetables and fruits and more eggs and fish and seafood, than the average Dutch population (Supplemental Table 2).

Post-hemodialysis concentrations were closer to the values in healthy control group (37.8 ± 20.4 µM). Notably, the prospective association of TMAO

concentrations with graft failure was independent of CKD stage. The role of the gut microbiome in the context of chronic kidney disease has gained recent attention [43]. However, little is known about its role and clinical utility on graft survival after kidney transplantation. In a pilot study comprising 26 RTRs, it was found that posttransplant diarrhea, which associates with allograft failure [44] and affects 1 in 5 RTRs in the first year after kidney transplantation, was associated with gut microbiome dysbiosis, characterized by a reduction in the relative abundance of commensal bacterial taxa [45]. In this context, the positive association of TMAO with graft failure risk, suggests a potential link between the gut microbiome and the patency of the graft in RTRs.

It is necessary to have reliable information about the clinical benefit of gut microbiome related biomarkers and its modifiable determinants (i.e., diet) for prediction of graft survival in RTRs. Due to the fact that traditional measures such as sensitivity, specificity, or area under the curve, correspond to statistical abstractions which could not provide direct information about the clinical value of biomarkers or prediction models, the use of decision-analytic measures, such as Net Benefit, have been proposed [5].

Our results from Net Benefit analysis showed that the addition of TMAO measurements and dietary information increases the Net Benefit of an already validated graft failure predicted model [29]. The previous prediction model of 5-year graft failure was developed in Birmingham, United Kingdom (n= 651) alive, and it was externally validated in independent cohorts from Tours, France (n= 736); Leeds, United Kingdom (n= 787); and Halifax, Canada (n= 475). In our study, the clinical utility of the model was improved by the addition of TMAO measurements and dietary information, identifying more RTRs with future graft failure, without increasing the false positives cases. Such improvement is more marked in a threshold risk of graft failure <40%. Interestingly, RTRs under such threshold risk are those who obtain greater benefit of more sensitive predictive biomarkers, given the fact that they have better renal function at baseline and presumably are those in which a dietary intervention may have a greater impact.

According to the authors who first introduced the decision curve analysis as a method to evaluate prediction models and diagnostic tests, in classical decision theory, the strategy with the highest expected utility should be chosen, irrespective of the size or statistical significance of the benefit [6]. Therefore, even a marginal improvement may have a clinical significance.

Strengths and Limitations

This single-center study has several strengths. In our analysis, TMAO improved the reclassification of patients from a lower to a higher graft failure risk category. It is interesting to note that, in a relatively small study (n= 53) urinary TMAO adequately discriminated patients with limited graft function from those with prolonged graft function [46]. The results from Net Benefit analysis of the present study are important due to the fact that the variables that enriched our model, TMAO concentrations and dietary information, do not involve any invasive, dangerous, or costly procedures to obtain. To the best of our knowledge this is the first report to include such approach in the context of graft survival analyses. Moreover, those variables, contrarily to other biomarkers (i.e., genetic risk scores), represent modifiable factors, that can be translated to nutritional recommendations in order to improve graft survival in RTRs. However, randomized clinical trials are required to prove that implementation of TMAO lowering diets can actually prolong graft function in RTRs.

Several limitations of the present study deserve mention. First, the present study mainly comprises individuals of Caucasian ancestry, which could limit extrapolation of our findings. The previously validated model used to predict graft failure included ethnicity [29], which was not included in the present study. Moreover, another model used for graft loss prediction revealed the utility of assessing the type of medical insurance [47], in our model it was not possible to evaluate this, because of the Dutch universal healthcare system. In addition, the diet in this cohort was assessed only at baseline, and therefore we could not provide any inference about the changes or permanence of the dietary habits over the follow-up. Finally, due the nature of the observational studies, the associations reported on the present study could not immediately be translated in causal relationships, and therefore, further research is needed.

In conclusion, this study of stable RTRs revealed that high concentrations of circulating TMAO are associated with a higher risk of graft failure, independent of other risk factors such as impaired renal function. Furthermore, this study provides for first time an extensive evaluation of the clinical utility of TMAO and dietary information for risk classification and prediction of graft failure in RTRs. Further investigation is needed to unravel the complexity behind the relationship between circulating gut microbiome derived metabolites such as TMAO and CKD.

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Supplemental Table S1. STROBE Statement—Checklist of items that should be included in reports of cohort studies

	Item No	Recommendation	Page No
Title and abstract	1	(a) Indicate the study's design with a commonly used term in the title or the abstract	249
		(b) Provide in the abstract an informative and balanced summary of what was done and what was found	250
Introduction			
Background/rationale	2	Explain the scientific background and rationale for the investigation being reported	451
Objectives	3	State specific objectives, including any prespecified hypotheses	452
Methods			
Study design	4	Present key elements of study design early in the paper	252
Setting	5	Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection	252-253
Participants	6	(a) Give the eligibility criteria, and the sources and methods of selection of participants. Describe methods of follow-up	253
		(b) For matched studies, give matching criteria and number of exposed and unexposed	
Variables	7	Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable	254-255
Data sources/ measurement	8*	For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group	254-255
Bias	9	Describe any efforts to address potential sources of bias	256
Study size	10	Explain how the study size was arrived at	5
Quantitative variables	11	Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen and why	255
Statistical methods	12	(a) Describe all statistical methods, including those used to control for confounding	255-257
		(b) Describe any methods used to examine subgroups and interactions	b.NA
		(c) Explain how missing data were addressed	253
		(d) If applicable, explain how loss to follow-up was addressed	d.NA
		(e) Describe any sensitivity analyses	257
Results			
Participants	13*	(a) Report numbers of individuals at each stage of study—eg numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analysed	258
		(b) Give reasons for non-participation at each stage	253
		(c) Consider use of a flow diagram	
Descriptive data	14*	(a) Give characteristics of study participants (eg demographic, clinical, social) and information on exposures and potential confounders	258

		(b) Indicate number of participants with missing data for each variable of interest	253
		(c) Summarise follow-up time (eg, average and total amount)	10
Outcome data	15*	Report numbers of outcome events or summary measures over time	261
Main results	16	(a) Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (eg, 95% confidence interval). Make clear which confounders were adjusted for and why they were included	264
		(b) Report category boundaries when continuous variables were categorized	264
		(c) If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period	263
Other analyses	17	Report other analyses done—eg analyses of subgroups and interactions, and sensitivity analyses	265
Discussion			
Key results	18	Summarise key results with reference to study objectives	267
Limitations	19	Discuss limitations of the study, taking into account sources of potential bias or imprecision. Discuss both direction and magnitude of any potential bias	270
Interpretation	20	Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence	269
Generalisability	21	Discuss the generalisability (external validity) of the study results	270
Other information			
Funding	22	Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based	2

*Give information separately for exposed and unexposed groups.

Supplemental Table 2. Diet components in subjects at the third TMAO tertile.

	Median (IQ range)	Mean (SD)	Population mean consumption[1]
Egg intake, g/day	8.92(7.14,14.28)	12.68 (9.9)	12
Vegetable Intake, g/day	95.75(65.83,140.15)	106.81 (70.1)	127
Fruit Intake, g/day	99.43(52.71,191.41)	121.25 (84.8)	122
Fish and Seafood Intake, g/day	14.87(4.679,22.95)	17.242 (16.0)	15

1. The diet of the Dutch: Results of the first two years of the Dutch National Food Consumption Survey 2012-2016 | RIVM. https://www.rivm.nl/publicaties/diet-of-dutch-results-of-first-two-years-of-dutch-national-food-consumption-survey-2012. Accessed 22 Dec 2020

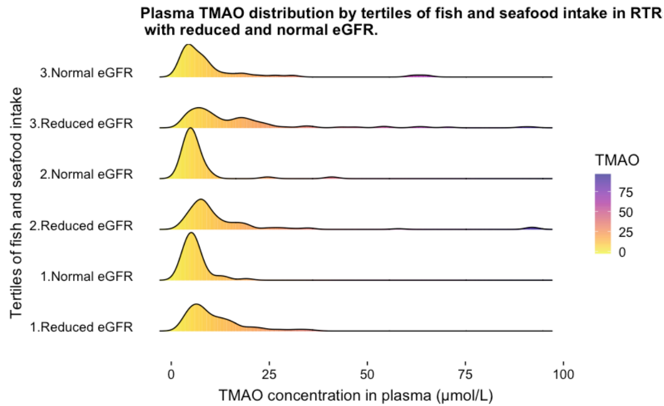
Supplemental Table 3. Reclassification table of RTRs.

		Updated Model				
		RTRs in Risk categories (*n*)				
		Low	Medium low	Medium high	High	RTR reclassified (%)
Initial Model	Low	188	19	2	0	10
	Medium low	22	34	10	0	48
	Medium high	7	15	38	13	48
	High	0	2	15	83	17

Supplemental Table 4. Comparison of Standardized Net Benefits of two predictive models.

Risk Threshold	Traditional Model	TMAO & Diet enriched Model
0.1	0.646	0.653
0.2	0.466	0.509
0.3	0.333	0.360
0.4	0.247	0.276
0.5	0.155	0.207
0.6	0.103	0.207
0.7	0.017	0.080

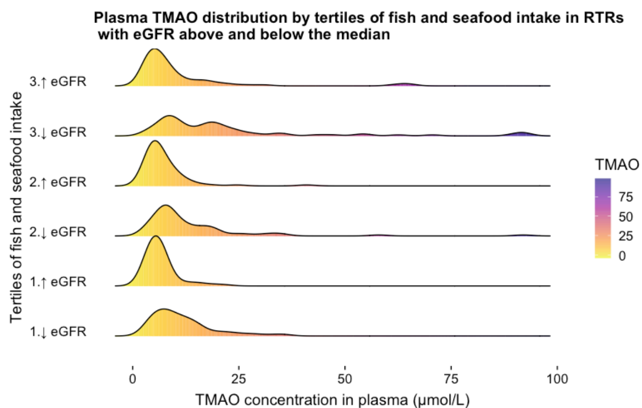
Supplemental Figure 1. A



Tertiles of fish and seafood intake in RTRs with reduced eGFR (<60 ml/min * 1.73m^2)
1.Reduced eGFR = 0 – 7.55 g/day
2.Reduced eGFR = 7.55 – 17.1 g/day
3.Reduced eGFR = 17.1 – 106 g/day

Tertiles of fish and seafood intake in RTRs with normal eGFR (>=60 ml/min * 1.73m^2)
1.Normal eGFR = 0 – 4.69 g/day
2.Normal eGFR = 4.69 – 15.7 g/day
3.Normal eGFR = 15.7 – 80.8 g/day

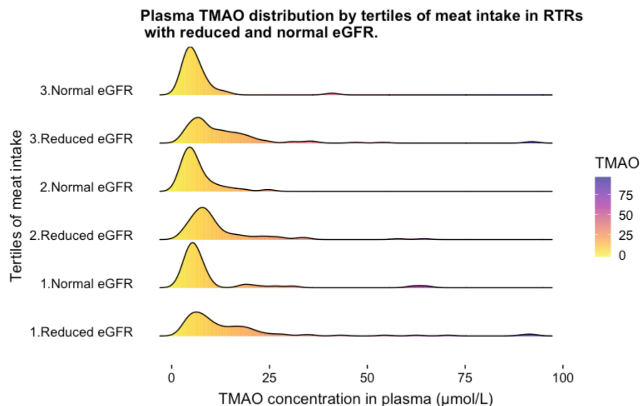
Supplemental Figure 1. B



Tertiles of fish and seafood intake in RTRs with eGFR below the median (< 47.96 ml/min * 1.73m^2)
1. ↓ eGFR = 0 – 6.45 g/day
2. ↓ eGFR = 6.45 – 16.7 g/day
3. ↓ eGFR = 16.7 – 106 g/day

Tertiles of fish and seafood intake in RTRs with eGFR above the median (>=47.96 ml/min * 1.73m^2)
1. ↑ eGFR = 0 – 6.97 g/day
2. ↑ eGFR = 6.97 – 16.8 g/day
3. ↑ eGFR = 16.8 – 80.8 g/day

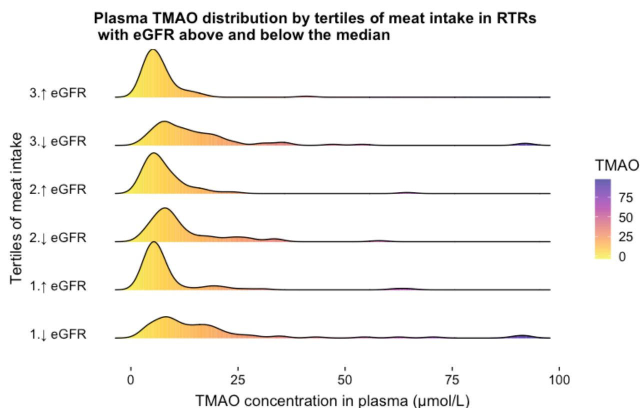
Supplemental Figure 2.A



Tertiles of meat intake in RTRs with reduced eGFR (< 60 ml/min * 1.73m^2)
1. Reduced eGFR = 0.1 – 71.4 g/day
2. Reduced eGFR = 71.4 – 93.5 g/day
3. Reduced eGFR = 93.5 – 270 g/day

Tertiles of meat intake in RTRs with normal eGFR (>= 60 ml/min * 1.73m^2)
1. Normal eGFR = 0.1 – 70 g/day
2. Normal eGFR = 70 – 97.4 g/day
3. Normal eGFR = 97.4 – 158 g/day

Supplemental Figure 2.B



Tertiles of meat intake in RTRs with eGFR below the median (<47.96 ml/min * 1.73m^2)
1. ↓ eGFR = 0.1 – 70 g/day
2. ↓ eGFR = 70 – 93.4 g/day
3. ↓ eGFR = 93.4 – 205 g/day

Tertiles of meat intake in RTRs with eGFR below the median (>=47.96 ml/min * 1.73m^2)
1. ↑ eGFR = 0.1 – 71.3 g/day
2. ↑ eGFR = 71.3 – 96.1 g/day
3. ↑ eGFR = 96.1 – 270 g/day

Chapter 10

Mahalanobis Distance, a Novel Statistical Proxy of Homeostasis Loss is Longitudinally Associated with Risk of Type 2 Diabetes.

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Abstract

Background. The potential role of individual plasma biomarkers in the pathogenesis of type 2 diabetes (T2D) has been broadly studied, but the impact of biomarkers interaction remains underexplored. Recently, the Mahalanobis distance (MD) of plasma biomarkers has been proposed as a proxy of physiological dysregulation. Here we aimed to investigate whether the MD calculated from circulating biomarkers is prospectively associated with development of T2D.

Methods. We calculated the MD of the Principal Components (PCs) integrating the information of 32 circulating biomarkers (comprehending inflammation, glycemic, lipid, microbiome and one-carbon metabolism) measured in 6247 participants of the PREVEND study without T2D at baseline. Cox proportional-hazards regression analyses were performed to study the association of MD with T2D development.

Findings. After a median follow-up of 7.3 years, 312 subjects developed T2D. The overall MD (mean (SD)) was higher in subjects who developed T2D compared to those who did not: 35.65 (26.67) and 30.75 (27.57), respectively (P =0.002). The highest hazard ratio (HR) was obtained using the MD calculated from the first 31 PCs (per 1 log-unit increment) (1·72 (95% CI 1·42,2·07), $P <$ 0·001). Such associations remained after the adjustment for age, sex, plasma glucose, parental history of T2D, lipids, blood pressure medication, and BMI (HR_{adj} 1·37 (95% CI 1·11,1·70), P = 0·004).

Interpretation. Our results are in line with the premise that MD represents an estimate of homeostasis loss. This study suggests that MD is able to provide information about physiological dysregulation also in the pathogenesis of T2D.

Introduction

The idea of an internal body regulation mechanism as a cornerstone in health and disease has been around in medicine since the pre-Socratic era [1]. Bernard and Cannon, helped to integrate such ideas under the concept of homeostasis, defined as a self-regulating process by which an organism can maintain internal stability while adjusting to changing external conditions [2].

The role of homeostasis in the study of chronic diseases had gained attention over time as they became more prevalent [3]. Amongst the most prevalent chronic diseases, type 2 diabetes (T2D) has become one of the leading causes of morbidity and disability worldwide [4]; consequently, several studies had been performed to understand the underlying mechanism of the homeostasis loss in T2D [3, 5–7].

The impact of homeostasis loss in the development of T2D has included the role of the pancreas [8], skeletal muscle [9], adipose tissue [10], and more recently the gut microbiota [11]. Although the interconnection of such systems in health and disease has been recognized, analyses of their related biomarkers has largely been confined to individual biomarkers to date. For instance, in a study comprising 11896 subjects from four well characterized prospective cohorts, 113 out of 229 metabolites were associated with risk of T2D development [12]. Several other observational studies have confirmed these findings [13–15]. Remarkably, the assessment of how the simultaneous variation of such biomarkers among themselves could reflect the loss of homeostasis remains underexplored in the context of T2D development.

Recently, it has been suggested that the analysis of the statistical distances of multivariate probability distributions, using circulating biomarkers, could identify abnormalities in the overall biomarker profile of subjects in relation to the studied population [16]. Particularly, the Mahalanobis distance (MD) calculated from circulating biomarkers has been suggested as a proxy of homeostasis loss, with a higher MD being associated with ageing related outcomes [16]. Therefore, the aim of this study was to investigate whether the MD calculated from circulating biomarkers is longitudinally associated with development of T2D among participants from a large general population-based cohort study.

Materials and Methods

Study design and subjects

The Prevention of Renal and Vascular END-stage Disease (PREVEND) Study is a population-based cohort study in the city of Groningen, The Netherlands. The PREVEND study was designed to prospectively investigate the natural course of increased levels of urinary albumin excretion and its relation to renal and cardiovascular disease in a large cohort drawn from the general population [17]. The design of the PREVEND Study has been described in detail elsewhere [18]. Briefly, from 1997 to 1998, all residents from Groningen, excluding pregnant women and people with type 1 diabetes or T2D using insulin, aged 28–75 years were invited to participate. A total of 40856 subjects (47.8%) responded the invitation to participate. From this group, 30890 subjects had a urinary albumin concentration of < 10 mg/L and 9966 subjects had a urinary albumin concentration of ≥ 10 mg/L in their morning urine sample. After exclusion of subjects with type 1 diabetes and pregnant women, all subjects with a urinary albumin concentration of ≥ 10 mg/L (n = 7768) and a randomly selected control group with a urinary albumin concentration of < 10 mg/L (n = 3395) were invited for further investigations in an outpatient clinic. A total of 8592 individuals completed an extensive examination.

We used data of participants who completed the second screening (n = 6894), and were free from T2D at baseline (n = 6447) excluding those with insufficient samples for quantification of biomarkers by means of Nuclear Magnetic Resonance (NMR), leaving a cohort of 6247 participants with complete information for the analyses. Cases of participants lost to follow-up were considered as censored cases. This report follows the Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) reporting guideline (Supplemental Table 1). The study conforms to the ethical guidelines of the 1975 Declaration of Helsinki [19] and was approved by the local ethics committee of the University Medical Center Groningen (approval number: MEC96/01/022). All participants provided written informed consent.

Clinical measurements

During two outpatient visits, baseline data were collected on demographics, lifestyle factors, anthropometric measurements, medical history, parental history of T2D and medication use. Information on medication use was

combined with information from a pharmacy-dispensing registry, which had complete information on the drug usage of > 95% of subjects in the PREVEND study. Height and weight were measured in standing position without shoes and heavy outer garments.

Body mass index (BMI) was calculated as weight (kg) divided by height squared (meter). Waist circumference was measured as the smallest girth between the rib cage and iliac crest. Systolic and diastolic blood pressure values were measured with an automatic Dinamap XL Model 9300 series device and recorded as the means of the last two recordings of the second visit.

End Point of the Study

Participants were followed from the date of the baseline center visit until end of follow-up. Incident T2D was established if one or more of the four criteria were met during follow-up: (1) blood glucose ≥ 7·0 mmol/L (126 mg/dL); (2) random sample plasma glucose ≥ 11·1 mmol/L (200 mg/dL); (3) self-report of a physician diagnosis; (4) initiation of glucose lowering medication according to the central pharmacy registry follow-up data, which was completed as of 1 January 2011.

Laboratory measurements

Laboratory measurements were performed at the Central Laboratory of the University Medical Center Groningen. The Netherlands. Venous blood samples were drawn after an overnight fast of at least 8 h, while participants rested for 15 min. Ethylene diamine tetra acetic acid (EDTA) - anticoagulated plasma samples and sera were stored at -80° C until analysis.

Fasting plasma glucose (FPG) was measured by dry chemistry (Eastman Kodak, Rochester, NY, USA). Insulin was measured with an immunoturbidometric assay (Diazyme Laboratories, Poway, CA, USA). Total cholesterol (TC), triglycerides, and serum creatinine were measured using standard protocols, as described previously [20]. Serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were measured using the standardized kinetic method with pyridoxal phosphate activation (Roche Modular P, Roche Diagnostics, Mannheim, Germany). Serum gamma-glutamyl transferase (GGT) was assayed using an enzymatic colorimetric method (Roche Modular P, Roche Diagnostics, Mannheim, Germany). Standardization of ALT, AST and GGT was performed according to the

International Federation of Clinical Chemistry guidelines [21–23]. High-sensitivity C-reactive protein (hs-CRP) albumin and urea concentrations were measured with Roche routine chemistry analyzers (Modular P/Cobas C, Roche Diagnostics, Mannheim, Germany). Urinary albumin was measured by nephelometry (Dade Behring Diagnostic, Marburg, Germany) and urinary albumin excretion (UAE) was determined in two 24-hour urine collections with the results being averaged for analysis. Serum creatinine was measured by an enzymatic method on a Roche Modular analyzer (Roche Diagnostics, Mannheim, Germany). Serum ferritin was measured using immunoassay (Roche Diagnostics, Mannheim, Germany). Hemoglobin, hematocrit, and mean corpuscular volume, were measured on a Coulter Counter STKS sample testing system (Coulter Corp) in fresh venous blood according to standard procedures.

Trimethylamine N-oxide (TMAO), betaine, branched chain amino acids (BCAA), GlycA (a pro-inflammatory glycoprotein biomarker), high density lipoprotein (HDL) and ketone body concentrations were measured in EDTA-anticoagulated plasma samples using a Vantera® Clinical Analyzer (LabCorp, Morrisville, NC), a fully automated, high-throughput, 400 MHz NMR spectroscopy platform using spectral deconvolution algorithm as previously described [24–30]. A detailed description of the NMR biomarker measurements is found in the Supplement.

Statistical Analysis

Normality distribution was assessed with the visualization of density plots and Q-Q plots. Variables with a skewed distribution were natural log transformed. Baseline data were presented as the mean (standard deviation, SD) or median (interquartile range, IQR) for continuous variables and percentages for categorical variables. All statistical analyses were performed with R language for statistical computing software, v. 4·0·3 (2020), (Vienna, Austria) [31].

Mahalanobis Distance

We used a set of thirty-two circulating metabolites to calculate the MD. Twenty-three out of the thirty-two biomarkers have already been reported to be useful in the calculation of MD as proxy of homeostasis loss (Supplemental Table 2) [16]. We replaced HDL cholesterol, used in a previous report [16] by seven HDL subspecies that have lately been reported to be differently associated with the risk of T2D [32]. Additionally, BCAA (valine, leucine, isoleucine) were included in the analysis because of their association with T2D [15], which has been shown

to be causal [33]. Finally, TMAO and betaine, were included as gut-microbiota derived metabolites, given the recently recognized relationship with the gut microbiome in the context of T2D [34].

To better depict the intervariability of the circulating biomarkers, a Principal Component Analysis (PCA) was performed using the biomarker data, in order to obtain a new set of variables which integrates the information of the 32 biomarkers. Considering that the circulating biomarkers are measured in different units, the input data was standardized in order to have mean equals to zero and variance equals to one before doing PCA. PCA is a dimensionality reduction technique, which comprehends a series of orthogonal linear transformations of the original variables, generating a new set of transformed variables (denominated Principal Components (PCs)). Each PC is a linear combination of all p variables, and it is intended that new set of transformed variables preserves as much as possible of the information contained in the original variables [35].

Starting with the addition of PC1 and PC2, the 32 PCs were added one by one into cumulative sets of PCs that were used to calculate 31 different MDs.

The MD is a multivariate distance measure related to the familiar Euclidean Distance; yet, it provides two further benefits. Firstly, it is scale invariant, meaning that the differences in the unit measurements of the diverse biomarkers do not bias the analysis. Secondly, it includes the correlations between the covariates, allowing to capture the information not only for the difference in one variable, but rather, the differences among a set of variables [36]. The MD is defined as: $MD\ (x_i, x_j) = [(x_i - x_j)^T \boldsymbol{S}^{-1}(x_i - x_j)]^{1/2}$ where x_i is the ith row of the (n×p) covariate matrix $\boldsymbol{X}$, with n subjects in the rows and p covariates in the columns, and $\boldsymbol{S}$ is the (p×p) covariance matrix of $\boldsymbol{X}$ [36].

Survival Analysis

Time-to-event Cox proportional hazards models were used to compute hazard ratios (HRs) and 95% CI of T2D development risk, using the MDs calculated from subsets of the PCs. In order to evaluate the potential overfitting of the different models, the Bayesian Information Criterion (BIC) and the Akaike Information Criterion (AIC) were computed for the MDs that contained different subsets of the PCs. Given the fact that mean and median of the MD increases with a larger number the variables included in its calculation, we further calculated the Relative Risk difference over 95% of the observed MD distribution; this was

calculated by subtracting the risk of being in the 97·5th percentile of MD relative to the 2·5th percentile, this method was previously reported to be appropriate to compare different MDs [37]. HRs were calculated per 1-unit increase in the log scale. HRs were adjusted for age and sex, BMI (or waist circumference), plasma glucose, lipid lowering medication and anti-hypertensive medication. The Cox proportional hazard assumption was tested through the evaluation of independence between scaled Schoenfeld residuals with time for each variable and for every model as a whole; this assumption was met, with no indication for a violation [38].

To further evaluate the performance of the MD to improve the T2D risk reclassification, two risk prediction models were fitted: The first model included the clinical variables used in the FINDRISC T2D risk score, which has been reported as a reasonably good predictor of incident T2D in the Netherlands [39] (age, family history of T2D, BMI, waist circumference, hypertension and FPG). The second model included the MD in addition to the variables above mentioned. Using predefined risk categories of T2D development (<10%), intermediate (10% to 20%), and high (≥20%) [40], reclassification was assessed using the categorical net reclassification improvement (NRI) approach; additionally, a category-free NRI was also computed [41].

Role of funding source

The funders did not have any role in study design, data collection, data analyses, interpretation, or writing of report.

Results

Clinical characteristics at baseline

A total of 6247 participants of the PREVEND cohort were included in this study. Among the participants, 3089 (49·4%) were men, and the mean age of the population was 53·2 (11·0) years. Participant characteristics at baseline are shown in Table 1. During a median follow-up of 7·3 (IQR 6·1–7·3) years, a total of 312 participants developed T2D. Participants who developed T2D during the follow-up, were more likely to be men and to be older, and were more likely to have a family history of diabetes when compared to people who did not develop T2D. Likewise, those who developed T2D, presented a higher BMI, waist circumference and blood pressure. Among T2D developers it was more common to have a history of CVD and parental history of T2D; those participants also

used antihypertensive medications and lipid-lowering drugs more frequently. There was no difference in terms of a history of cancer, smoking or alcohol consumption.

Biochemical characteristics at baseline

Between the groups of participants who developed T2D and those who did not there were marked differences in almost all the biochemical biomarkers, except for plasma albumin, HDL particle 5 (H5P), and mean corpuscular volume (Fig. 1). The following circulating biomarkers were higher in T2D developers: ketone bodies (β-hydroxybutyrate, acetoacetate and acetone), ALP, ALT, AST, creatinine, hsCRP, FPG, ferritin, GGT, GlycA, H2P, hemoglobin, hematocrit, insulin, BCAAs (isoleucine, leucine, valine), total cholesterol, triglycerides, TMAO, transferrin, and urea. Some circulating biomarkers were lower in T2D developers: betaine, H1P, H3P, H4P, H6P and H7P. In participants who developed T2D, UAE was higher, and eGFR was lower (Table 1).

Table 1. Baseline characteristics of 6247 participants of the PREVEND prospective cohort.

Variable	Total (N=6247)	Incident T2D (N=312)	No Incident T2D (N=5935)	*P* value
Men, (%)	3089 (49·4%)	196 (62·8%)	2893 (48·7%)	< 0·001
Age, years	53·18 (11·94)	57·47 (9·97)	52·96 (12·00)	< 0·001
BMI, kg/m^2	26·50 (4·22)	29·96 (4·67)	26·32 (4·12)	< 0·001
Waist circumference, cm	91·55 (12·56)	102·27 (12·38)	90·99 (12·32)	< 0·001
SBP, mmHg	125·64 (18·60)	136·84 (21·05)	125·05 (18·27)	< 0·001
DBP, mmHg	73·24 (9·08)	77·60 (9·39)	73·01 (9·01)	< 0·001
History of Cancer, (%)	279 (4·5%)	12 (3·9%)	267 (4·5%)	0·68
History of CVD, (%)	231 (3·7%)	22 (7·1%)	209 (3·5%)	0·001
Parental history of T2D, (%)	898 (14·9%)	87 (29·3%)	811 (14·1%)	< 0·001
Smoking status, (%)				0·47
never	1788 (28·6%)	81 (26·0%)	1707 (28·8%)	
former	2626 (42·0%)	140 (44·9%)	2486 (41·9%)	
current <6 cig/day	284 (4·5%)	14 (4·5%)	270 (4·5%)	
current 6-20 cig/day	1231 (19·7%)	58 (18·6%)	1173 (19·8%)	
current >20 cig/day	243 (3·9%)	17 (5·4%)	226 (3·8%)	
Alcohol consumption, (%)				
No, almost never	1512 (24·4%)	89 (28·6%)	1423 (24·2%)	0·14
1-4 drinks/ month	1058 (17·1%)	49 (15·8%)	1009 (17·2%)	

2-7 drinks/ week	1976 (31·9%)	88 (28·3%)	1888 (32·1%)	
1-3 drinks/ day	1380 (22·3%)	66 (21·2%)	1314 (22·4%)	
4 or more drinks/ day	264 (4·3%)	19 (6·1%)	245 (4·2%)	
Lipid-lowering drugs, (%)	446 (7·1%)	50 (16·0%)	396 (6·7%)	< 0·001
Antihypertensive drugs, (%)	1130 (18·1%)	115 (36·9%)	1015 (17·1%)	< 0·001
Glucose, mmol/L	4·70 (4·40, 5·20)	5·80 (5·20, 6·20)	4·70 (4·40, 5·20)	< 0·001
eGFR, mL/min/1·73 m^2	92·45 (16·97)	88·28 (16·67)	92·67 (16·96)	< 0·001
UAE, mg/24 h	8·55 (6·02, 15·12)	12·81 (7·92, 30·91)	8·42 (5·99, 14·73)	< 0·001
Biomarkers				
AcAc, µmol/L	37·83 (25·58, 56·85)	41·06 (27·91, 61·37)	37·72 (25·44, 56·58)	0·01
Acetone, µmol/L	19·54 (12·53, 28·88)	23·12 (15·66, 33·20)	19·33 (12·41, 28·53)	< 0·001
Albumin, g/L	44·00 (42·00, 45·00)	44·00 (42·00, 45·75)	44·00 (42·00, 45·00)	0·44
ALP, U/L	66·00 (55·00, 78·00)	72·00 (62·00, 86·00)	65·00 (54·00, 78·00)	< 0·001
ALT, U/L	17·00 (13·00, 24·00)	21·50 (16·00, 32·75)	17·00 (12·00, 23·00)	< 0·001
AST, U/L	22·00 (19·00, 26·00)	24·00 (20·00, 29·00)	22·00 (19·00, 26·00)	< 0·001
Betaine, µmol/L	36·90 (31·00, 43·90)	34·90 (30·20, 42·10)	37·00 (31·10, 44·00)	0·03
BHB, µmol/L	120·18 (91·99, 166·30)	140·55 (111·74, 187·54)	119·12 (91·17, 164·90)	< 0·001
Creatinine, mmol/L	71·00 (62·00, 80·00)	73·00 (63·00, 82·00)	71·00 (62·00, 80·00)	0·05
CRP, mg/L	1·29 (0·60, 2·89)	2·15 (1·13, 3·86)	1·26 (0·59, 2·84)	< 0·001
Ferritine, µg/L	94·00 (46·00, 169·00)	144·0 (77·75, 258·25)	92·00 (45·00, 165·75)	< 0·001
GGT, U/L	23·00 (16·00, 37·00)	37·00 (26·00, 57·00)	23·00 (15·00, 36·00)	< 0·001
GlycA, mmol/L	369.46 (333.22, 413.16)	397.27 (351.68, 437.28)	368.40 (332.21, 411.19)	< 0·001
H1P, µmol/L	3·45 (1·82)	3·13 (1·76)	3·46 (1·83)	0·002
H2P, µmol/L	10·43 (2·81)	11·51 (2·99)	10·37 (2·78)	< 0·001
H3P, µmol/L	3·15 (1·91, 4·42)	2·78 (1·63, 4·05)	3·17 (1·93, 4·44)	< 0·001
H4P, µmol/L	1·70 (1·09, 2·44)	1·32 (0·68, 2·04)	1·71 (1·12, 2·46)	< 0·001
H5P, µmol/L	0·29 (0·03, 0·61)	0·29 (0·06, 0·57)	0·29 (0·03, 0·61)	0·83
H6P, µmol/L	0·62 (0·24, 1·37)	0·35 (0·14, 0·69)	0·64 (0·25, 1·40)	< 0·001
H7P, µmol/L	0·32 (0·12, 0·62)	0·17 (0·05, 0·37)	0·33 (0·13, 0·64)	< 0·001
Hemoglobin, mmol/L	8·51 (0·76)	8·76 (0·79)	8·50 (0·76)	< 0·001
Hematocrit, %	0·41 (0·38, 0·43)	0·42 (0·40, 0·44)	0·41 (0·38, 0·43)	< 0·001
Insulin, mU/L	8·00 (5·70, 11·80)	13·40 (9·00, 20·25)	7·80 (5·60, 11·50)	< 0·001
Isoleucine, µM/L	41·98 (32·57, 52·05)	50·10 (41·09, 62·51)	41·52 (32·29, 51·52)	< 0·001
Leucine, µM/L	124·79 (32·50)	142·49 (35·98)	123·86 (32·05)	< 0·001
MCV, µm3	90·48 (4·64)	90·01 (5·27)	90·50 (4·60)	0·06
TC, mmol/L	5·44 (1·04)	5·63 (1·14)	5·43 (1·03)	0·001

Triglycerides, mmol/L	1·10 (0·80, 1·58)	1·57 (1·07, 2·28)	1·08 (0·79, 1·55)	< 0·001
TMAO, µmol/L	3·20 (1·80, 5·70)	3·50 (1·90, 5·70)	3·20 (1·80, 5·70)	0·43
Transferrin, g/L	2·58 (0·41)	2·65 (0·39)	2·58 (0·41)	0·007
Urea, mmol/L	5·00 (4·30, 6·00)	5·25 (4·50, 6·00)	5·00 (4·20, 6·00)	0·01
Valine, µM/L	203·13 (46·50)	226·56 (51·44)	201·90 (45·90)	< 0·001

Abbreviations: AcAc, Acetoacetate; ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BMI, body mass index (calculated as weight in kilograms divided by height in meters squared); BHB, beta-hydroxybutyrate; CRP, C-reactive protein; CVD, cardiovascular disease; DBP, diastolic blood pressure; eGFR, estimated glomerular filtration rate; GGT, γ-glutamyltransferase; H1P – H7P: High-density lipoprotein 1-7 particles; IQR, interquartile range; MCV, mean corpuscular volume; SBP, systolic blood pressure; TC, total cholesterol; TMAO, Trimethylamine N-Oxide; UAE, urinary albumin excretion.

Values are shown as mean (SD) or median (25th and the 75th percentile). P-values represent the significance of between developers and non-developers of T2D. P-values were determined using a 1-way analysis of variance for normally distributed data, Kruskal-Wallis test for skewed distributed data, and χ2 test for categorical data.

PCA of circulating biomarkers

Data of 32 circulating biomarkers: acetoacetate, acetone, albumin, ALP, ALT, AST, betaine, β-hydroxybutyrate (BHB), creatinine, CRP, ferritin, GGT, FPG, H1P, H2P, H3P, H4P, H5P, H6P, H7P, hemoglobin, hematocrit, insulin, isoleucine, leucine, MCV, total cholesterol, triglycerides, TMAO, transferrin, urea, valine were used in the PCA (Supplemental Table 2). The potential biological significance of the PCs is depicted in a heatmap based on pairwise correlations (Fig. 2). The five biomarkers with the highest positive correlation coefficients were: GGT (PC24, ρ= 0·49), total cholesterol (PC11, ρ= 0·47), TMAO (PC12, ρ= 0·46), urea (PC12, ρ= 0·46), betaine (PC4, ρ= 0·45), (*P*-value for all < 0·0001). The five biomarkers with the highest negative correlation coefficients were: GlycA (PC2, ρ= -0·83), BHB (PC3, ρ= -0·75), leucine (PC1, ρ= -0·71), isoleucine (PC1, ρ= -0·69), valine (PC1, ρ= -0·69) (*P*-value for all < 0·0001).

Biomarker loadings (the contribution of each biomarker to the PCs) from the first two PCs were congruent with the above reported correlations. The biomarker loadings for the first two PCs are depicted across participants subsets (females, males, participants younger and older than the median age of the cohort, 52 years). Together, the first two PCs captured 26·4% of the total data variation. PC1 captured 16·7% of the variation and PC2 9·7%. In the PC1, the loadings corresponding to BCAA, displayed the biggest differences, being those loadings higher in the group of participants older than 52 years (Δ= 3%), the same was true in the comparison between men and women, being those loadings higher in the group of men (Δ= 2.2%). In the PC2, the loadings

corresponding to ketone bodies (acetoacetate, beta-hydroxybutyrate and acetone), displayed the biggest differences, being those loadings higher in the group of participants older than 52 years (Δ= 24%, 19% and 13%, respectively).

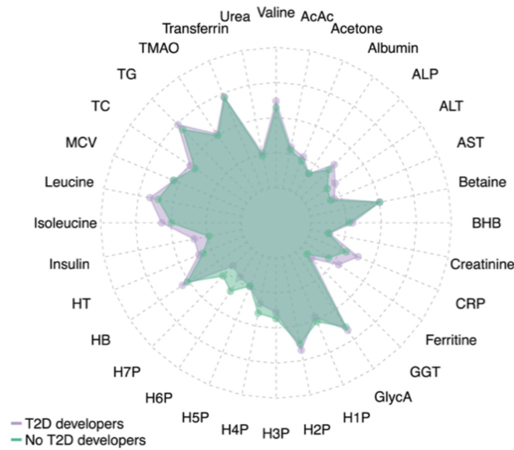

Figure 1. Radarplot showing levels of 32 circulating biomarkers used to calculate the MD in the groups of those developed and did not develop T2D during the follow-up. Biomarkers concentrations are displayed in a scale from 0 to 1, were 0 represents the minimum value and 1 represents the maximum value. Albumin, betaine, creatinine, CRP, ferritin, GlycA, hematocrit, hepatic enzymes, insulin, ketone bodies, triglycerides, TMAO and urea were log transformed prior scalation. (n= 6247).

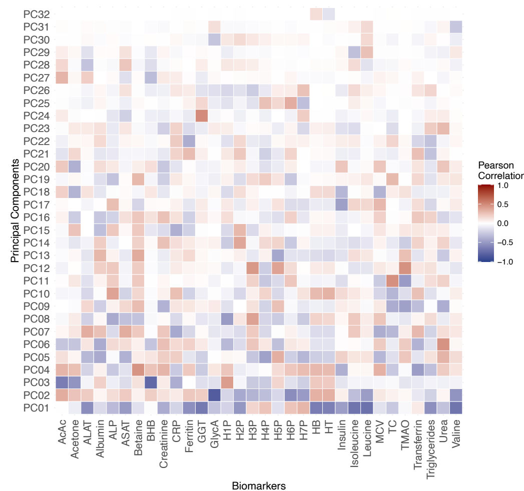

Figure 2. Heatmap showing the correlations between the circulating biomarkers and the PCs (n= 6247).

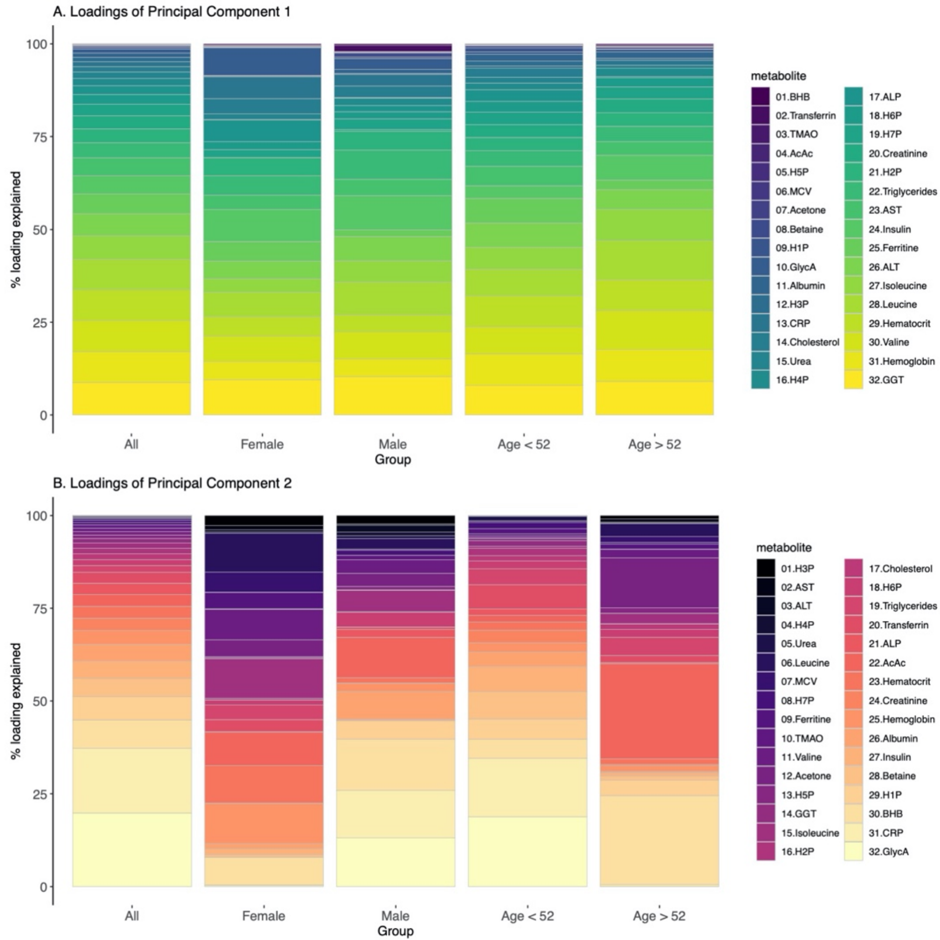

Figure 3. Barplots showing circulating biomarker loadings for the 1st and 2nd PCs across participants subsets (Fig. 3A and Fig. 3B, respectively). Each panel contains the loadings (from left to right) for the whole population (n= 6247), females (n= 3158) , males (n= 3089), participants younger than 52 years (n= 3146) and older than 52 years (n=3101) (the median age was selected as cutoff point).

The PC2 loadings in men and women displayed more differences, being the contribution of hemoglobin and hematocrit smaller in men, compared to women (Δ= 8·6% for hemoglobin and 8·5%, respectively for hematocrit), and the contribution of c-reactive protein and GlycA higher in men compared to women (Δ= 12·8% for c-reactive protein and 12·7% for GlycA) (Fig. 3).

Mahalanobis Distance and risk of T2D

During a median follow-up of 7·3 (IQR 6·1–7·3) years, a total of 312 participants developed T2D. Cox proportional hazard regression analyses were first

performed using the 31 MDs calculated based on the subsets of cumulative PCs. All of the MDs were positively associated with an increased risk of T2D (Supplemental Table 3). The association of the MDs with the risk of T2D differed depending on the subset of PCs used to calculate the MDs (Supplemental Table 3). The MD of the subset holding the first 31 PCs was the one with the strongest association, with a HR of 1·72 (95% CI 1·42,2·07), *P* < 0·001, per 1 log-unit increase. The association remained significant after adjustment for age and sex (HR_{adj} 1·70 (95% CI 1·41,2·06), *P* < 0·001), parental history of T2D, plasma glucose, lipid lowering medication and antihypertensive medication (HR_{adj} 1·46 (95% CI 1·19,1·78), *P* < 0·001), BMI (HR_{adj} 1·37 (95% CI 1·11,1·70), *P*= 0·004), and waist circumference (HR_{adj} 1·33 (95% CI 1·07,1·64), *P* = 0·01) (Table 2).

Similarly, the analyses of MD as a categorical variable, using the first tertile as the reference group, showed that the third tertile of MD was associated with a higher risk of T2D in all of the models described above, resulting in a HR of 1·99 (95% CI 1·51,2·63), *P* < 0·001 and a fully adjusted HR_{adj} of 1·42 (95% CI 1·10,1·82), *P* = 0·01 (Table 2). The potential overfitting of the models was assessed with their BIC and their AIC, showing no major difference when using the MD of the subset holding the first 31 PCs in comparison with a smaller number of PCs, i.e., 3 PCs. (BIC: 5166 and 5173, respectively and AIC: 5160 and 5169), respectively), showing that in fact, the model that contains the MD of the subset holding the first 31 PCs performs better. Moreover, the association between risk of T2D and the separated PCs was evaluated. We identified that only the first 6 PCs were associated with the risk of T2D; these associations were less robust than those obtained when using the MD. (Supplemental Table 4)

The association of the MDs with the risk of T2D was also evaluated in men and women, separately. Notably, the association of the MDs with the risk of T2D was greater in women than men for all the PCs subsets (Supplemental Fig. 1.). The MD displaying the strongest association with T2D risk did not correspond to the same PCs subset in men and women. In women, the MD from the first 20 PCs displayed a HR of 2·27 (95% CI 1·74, 2·96), *P* <0.001; meanwhile, in men, the MD from the 32 PCs displayed a HR of 1·47 (95% CI 1·14, 1·89), *P* <0·001. To further asses the benefit of calculating the MD from the PCs instead of using the biomarker raw information, we further evaluate the association of the MD of the 32 circulating biomarkers with the risk of T2D. The MD of the 32 circulating biomarkers was associated with the risk of T2D, showing an unadjusted HR of 1·68 (95% CI 1·46,1·94), *P* < 0·001, per 1 log-unit increase.

Table 2. Prospective associations of MD as continuous and as categorical variable with risk of T2D.

	MD per 1 Ln SD Increment		T1	T2		T3	
Participants, *n*	6247		2083	2082		2082	
Events, *n*	312		78	92		142	
	HR (95 % CI)	***P* value**		**HR (95 % CI)**	***P* value**	**HR (95 % CI)**	***P* value**
Crude Model	1·72 (1·42,2·07)	<0·001	(ref)	1·15 (0·85,1·56)	0·36	1·99 (1·51,2·63)	<0·001
Model 1	1·70 (1·41,2·06)	<0·001	(ref)	1·18 (0·87,1·60)	0·28	2·00 (1·52,2·64)	<0·001
Model 2	1·46 (1·19,1·78)	<0·001	(ref)	1·10 (0·80,1·50)	0·55	1·49 (1·12,1·98)	0·006
Model 2b	1·42 (1·16,1·75)	<0·001	(ref)	1·03 (0·75,1·41)	0·86	1·44 (1·08,1·92)	0·01
Model 3	1·37 (1·11,1·70)	0·004	(ref)	1·02 (0·75,1·40)	0·88	1·42 (1·10,1·82)	0·01
Model 4	1·33 (1·07,1·64)	0·01	(ref)	1·01 (0·74,1·39)	0·96	1·29 (1·07,1·77)	0·02

Data are presented as hazard ratios (HRs) with 95% confidence intervals (CIs) and P values of MD as continuous variable (per 1 log unit increment) and as categorical variable (with the first tertile of MD as reference). MD was calculated using the subset of the first 31 PCs.
Model 1. Model adjusted for age and sex
Model 2. Model 1 plus adjusted for plasma glucose, parental history of T2D, lipid lowering medication and antihypertensive medication.
Model 2b. Model 1 plus adjusted for plasma glucose, parental history of T2D, lipid lowering medication, systolic blood pressure and personal history of CVD.
Model 3. Model 2 plus adjusted for BMI.
Model 4. Model 2 plus adjusted for waist circumference.

The association remained after the adjustment for age and sex, (HR_{adj} 1·60 (95% CI 1·38,1·85), P < 0·001. Nonetheless, the association was not hold after a full adjustment (HR_{adj} 1·18 (95% CI 0·98,1·40), P = 0·07 (Supplemental Table 5). Similarly, the analyses of MD as a categorical variable, using the first tertile as the reference group, showed that the third tertile of MD was associated with a higher risk of T2D in the crude model, resulting in a HR of 1·61 (95% CI 1·20,2·15), P < 0·001; and the association did not hold in the fully adjusted model: HR_{adj} 1·30 (95% CI 0·96,1·76), P = 0·09 (Supplemental Table 5).

The comparison of the traditional T2D risk model against the enriched model that included the MD revealed that inclusion of the MD led to a significant improvement in the classification of participants into predicted T2D risk categories, with a NRI of 0·24 (95% CI: 0·18, 0·31) (P= 0·001). A category-free version of the NRI (often denoted as NRI > 0) was also computed, resulting in a NRI of 0·74 (95% CI: 0·64, 0·85) (P= 0·001).

Discussion

In this prospective population-based cohort study, we demonstrated the association of MD, a proxy of homeostasis loss, with incidence of T2D. MD is a novel approach for studying changes in collections of biomarkers based on the concept of multivariate statistical distance. In this study, MD measured the abnormality of the whole biomarker profile at baseline in relation to the population mean. The association of MD with increased risk of T2D was independent of age and sex, as well as of anthropometric variables such as BMI and waist circumference.

Along with the development of more efficient high throughput techniques, it is recognized that most of the of circulating metabolites are stable over time in healthy subjects, and variations in biomarker profiles could offer a wide-ranging indicator of changes in an individual's health status [42]. The MD of circulating biomarkers has become an alternative means to analyze such variations in high throughput biomarkers and provide a quantifiable proxy of homeostasis loss. Recently, the calculation of MD has being upgraded by replacing the raw biomarker information with PCs [43]. The rationality of replacing the raw biomarkers with PCs is based on the fact that PC analysis could detect underlying processes that might simultaneously regulate the levels of the variables used in the analysis, but may not be directly measurable [44].

Importantly, plasma glucose was not included as part of the PC analysis, in order to prevent the prevailing influence of glucose in assessing degree of homeostasis loss.

In this study, the PC1 loadings corresponding to BCAA were higher in older participants compared to younger participants. This could reflect the already described altered BCAA metabolism in ageing, due to the impaired activity of the mammalian target of rapamycin (mTOR) and mitochondrial dysfunction in ageing [45], characterized by the downregulation of the branched chain aminotransferase 2 [46]. The PC2 loading depicted important differences among the subgroups. The this could further correspond to the aging-induced impairment of ketone body oxidation, regulated by the succinyl-CoA-acetoacetate transferase [47]. Remarkably differences were identified between men and women, in relation to the contribution of inflammatory markers to the PC2 loading, C-reactive protein and GlycA were among the more important contributors to PC2 in men, but its contribution to PC2 in women was remarkably low. Such findings are in line with the reports of a reduced activation and recruitment of leucocytes in women, due to an enhanced response of proresolving mediators, including the D-resolvins. Those sex-differences in inflammatory response have been suggested to underly lower incidence of cardiovascular disease in women [48].

The use of PCs accounts for the assessment of the intervariability of biomarkers; previous studies have shown that the PCs could provide an insight of homeostasis dysregulation across multiple physiological systems in patients with chronic diseases, such as T2D and chronic kidney disease [44, 49]. The current analyses revealed that, when MD was calculated based on different cumulative sets of PCs, the association of MD with the risk of T2D was stronger (Supplemental Fig. 1.). This finding reflects the fact that the interactions among biomarkers, better depicts the metabolic changes in the subjects at risk of T2D, rather than the independent effects of individual circulating biomarkers. These results are in line with previous findings reporting similar performance of MD, but in the context of ageing-related outcomes [43]. In our study, the calculation of the MD based on the cumulative set of PCs, instead of the raw biomarker information, helped to depict the association of the MD with the incidence of T2D. Of note, this approach has been previously employed for the investigation of the association of the MD with mortality. Leung et al. computed the MD using the cumulative PCs of 36 circulating biomarkers, and reported a positive

association between the MD and the risk of mortality [50]. They found the highest association using the MD based on the first half of PCs, and they reported a critical decline in the association when the last PCs were included in the calculation of the MD. They have argued that the last PCs may represent measurement error or other types of noise [50]. In our analysis, the inclusion of the first 31 PCs represented the strongest association with T2D risk, and the inclusion of the last PC did not further improve the association. Moreover, based on the Akaike Information Criterion and the Bayesian Information Criterion, we found no evidence of overfitting.

The MD was originally developed as a tool to classify subjects based on the joint distribution of different variables and since its application has been restricted to such purposes [51]. It is worth noting that the results of the MD calculation do not merely represent a combination of the biomarkers concentrations, (such as through a PCA), and its association with physiological dysregulation in humans and animals, could remain even when the MD is uncorrelated with its component biomarkers or if such biomarkers are not individually associated with a higher risk of developing a specific clinical outcome [37].

Milot et al, had previously reported the non-significant association between the MD calculated from two different sets of biomarkers [37]. The first set included the concentrations of alanine amino transferase, albumin, albumin/globulin ratio, aspartate amino transferase, calcium, C-reactive protein, Hemoglobin, hematocrit, interleukin 6, iron, and red blood cell count, the MD calculated from this set resulted in a Relative Risk of 1·23 (95% CI 0·77, 2·00), P >0·05, The second set of biomarkers included the plasma concentrations of albumin, basophil count, urea/creatinine ratio, calcium, cholesterol, chloride, creatinine, bilirubin, hematocrit, hemoglobin, osteocalcin, potassium, red blood cell count and sodium, the MD calculated from this set resulted in a Relative Risk of 1·07 (95% CI 0·76, 1·50), P >0·05 [37].

Bearing in mind that T2D has different prevalence and consequences between men and women, with women having T2D being at higher risk of complications [52], we considered it of interest to further explore the association of the MDs with the risk of T2D separately in men and women separately. The association of the MDs with the risk of T2D was greater in women than men (Supplemental Fig. 1.). These results are in line with the

findings previously reported by Li et al about the association between T2D with the MD calculated from 37 biomarkers. In their study, the association was evaluated in two cohorts: Aging in Chianti, (InCHIANTI) and the Women's Health and Aging Study (WHAS), in such study, the association of MD with T2D was stronger in the WHAS cohort (OR: 1·22, 95% C.I. 1·08, 1·39), compared to the CHIANTI cohort that included men and women (OR: 1·12, 95% CI 1·00, 1·25) [53]. These results highlight sex differences in the context of T2D pathogenesis. Considering that a higher MD represents a higher degree of homeostasis lost, this finding could signify that once homeostasis regulation is lost, clinical and biochemical risk factors for T2D with sexual dimorphism, such as the higher body fat percentage, fetuin-A, a protein secreted primarily by the liver that regulates insulin signaling [54], neurotensin, a neuro peptide associated with satiety and gut motility [55], sex hormone–binding globulin [56], among others, may exert a major effect. The fact that the MD could better assess the risk of T2D in women could be of pathophysiological relevance, given that typical risk factors are insufficiently able to distinguish the shift from a healthy to an unhealthy phenotype. For instance, in a recent study including more than 90,000 women, 84% of the participants progressed from a from metabolic healthy phenotype to a metabolic unhealthy phenotype, irrespectively of BMI category [57]. Here we reported that the inclusion of MD to a risk model improved the NRI, using predefined T2D risk categories, and also using a category-free version of the NRI (often denoted as NRI > 0). Whereas some authors argue that a category-free NRI may represent a more objective measurement of the improvement in risk prediction because it does not lose information due categorization [58], other authors have argued that it may overestimates the risk prediction improvement and may not reflect its clinical utility [59].

We acknowledge several strengths of the present study. This study included a large number of participants which allowed us to conduct our analysis with sufficient statistical power. Another strength of the present study is the implementation of robust and validated methods of quantification of novel biomarkers such as betaine, BCAAs, HDL subspecies, and TMAO by means of NMR spectroscopy. To the best of our knowledge, this study is the first to assess the loss of homeostasis with the use of MD of the PCs that contain the information of traditional and novel biomarkers associated with the risk of T2D development.

We are also aware of the limitations of the study. The PREVEND population is mainly comprised of individuals with European ancestry, which limits the generalizability of our findings to persons with different ethnicities. We did not have measurements of biomarkers beyond baseline assessment, which impedes us from evaluating the evolution of the biomarker profiles and therefore the MDs and its association with T2D risk. This fact limits our ability to describe the underlying biological mechanisms. For the same reason, the absence of repeated biomarker measurements prevents us from correcting our analysis for regression dilution. Moreover, the sample size of our study, prevents us from performing a cross-validation analysis. Finally, considering that the MD is a metric that has been proposed to identify outliers in multidimensional datasets [60], it is important to further investigate how the presence of outliers could potentially affect the performance of the MD in the assessment of T2D risk. In this study, a sensitivity analysis conducted in a dataset after outliers removal, the association of the MD with T2D risk remained similar to the association found in the original dataset, being the HRs (1·85 (95% CI 1·47,2·33), $P < 0·001$) and (1·72 (95% CI 1·42,2·07), $P < 0·001$), in the dataset after removal of outliers and in the original dataset, respectively. Further research in this regard is needed.

In conclusion, this large-scale cohort study demonstrated that higher MD, a novel method for measuring homeostasis loss, is positively associated with incident T2D in both men and women in the general population during extended follow-up. The performance of MD increased by including a larger set of PCs in its calculation, supporting the notion that diminished homeostasis regulation is a result of the interactions among biomarkers, not just their independent effects.

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Supplemental Table S1. STROBE Statement—Checklist of items that should be included in reports of cohort studies

	Item No	Recommendation	Page No
Title and abstract	1	(a) Indicate the study's design with a commonly used term in the title or the abstract	285
		(b) Provide in the abstract an informative and balanced summary of what was done and what was found	286
Introduction			
Background/rationale	2	Explain the scientific background and rationale for the investigation being reported	287
Objectives	3	State specific objectives, including any prespecified hypotheses	288
Methods			
Study design	4	Present key elements of study design early in the paper	288
Setting	5	Describe the setting, locations, and relevant dates, including periods of recruitment, exposure, follow-up, and data collection	288
Participants	6	(a) Give the eligibility criteria, and the sources and methods of selection of participants. Describe methods of follow-up	289
		(b) For matched studies, give matching criteria and number of exposed and unexposed	
Variables	7	Clearly define all outcomes, exposures, predictors, potential confounders, and effect modifiers. Give diagnostic criteria, if applicable	290-291
Data sources/ measurement	8*	For each variable of interest, give sources of data and details of methods of assessment (measurement). Describe comparability of assessment methods if there is more than one group	290
Bias	9	Describe any efforts to address potential sources of bias	293
Study size	10	Explain how the study size was arrived at	291
Quantitative variables	11	Explain how quantitative variables were handled in the analyses. If applicable, describe which groupings were chosen and why	292
Statistical methods	12	(a) Describe all statistical methods, including those used to control for confounding	291-294
		(b) Describe any methods used to examine subgroups and interactions	b.NA
		(c) Explain how missing data were addressed	290
		(d) If applicable, explain how loss to follow-up was addressed	d.NA
		(e) Describe any sensitivity analyses	294
Results			
Participants	13*	(a) Report numbers of individuals at each stage of study—eg numbers potentially eligible, examined for eligibility, confirmed eligible, included in the study, completing follow-up, and analysed	295
		(b) Give reasons for non-participation at each stage	290
		(c) Consider use of a flow diagram	
Descriptive data	14*	(a) Give characteristics of study participants (eg demographic, clinical, social) and information on exposures and potential confounders	295-298

		(b) Indicate number of participants with missing data for each variable of interest	NA
		(c) Summarise follow-up time (eg, average and total amount)	301
Outcome data	15*	Report numbers of outcome events or summary measures over time	302
Main results	16	(a) Give unadjusted estimates and, if applicable, confounder-adjusted estimates and their precision (eg, 95% confidence interval). Make clear which confounders were adjusted for and why they were included	300-302
		(b) Report category boundaries when continuous variables were categorized	302
		(c) If relevant, consider translating estimates of relative risk into absolute risk for a meaningful time period	301, 318
Other analyses	17	Report other analyses done—eg analyses of subgroups and interactions, and sensitivity analyses	301-303
Discussion			
Key results	18	Summarise key results with reference to study objectives	304
Limitations	19	Discuss limitations of the study, taking into account sources of potential bias or imprecision. Discuss both direction and magnitude of any potential bias	308
Interpretation	20	Give a cautious overall interpretation of results considering objectives, limitations, multiplicity of analyses, results from similar studies, and other relevant evidence	305-307
Generalisability	21	Discuss the generalisability (external validity) of the study results	307
Other information			
Funding	22	Give the source of funding and the role of the funders for the present study and, if applicable, for the original study on which the present article is based	2

*Give information separately for exposed and unexposed groups.

Supplemental Table 2. Biomarkers included in the calculation of Mahalanobis distance.

Biomarker	T2D association	Most associated PC
AcAc	Positive	PC3
Acetone	Positive	PC3
Albumin ˜	Negative	PC5
ALP ˜	Positive	PC8
ALT ˜	Positive	PC1
AST ˜	Positive	PC1
Betaine	Negative	PC4
BHB	Positive	PC3
Creatinine ˜	Positive	PC1
CRP ˜	Positive	PC7
Ferritin ˜	Positive	PC1
GGT ˜	Positive	PC1
GlycA	Positive	PC2
H1P ˜ ˜	Negative	PC8
H2P ˜ ˜	Positive	PC1
H3P ˜ ˜	Negative	PC12
H4P ˜ ˜	Negative	PC5
H5P ˜ ˜	Positive	PC13
H6P ˜ ˜	Negative	PC2
H7P ˜ ˜	Negative	PC2
Hemoglobin ˜	Positive	PC1
Hematocrit ˜	Positive	PC1
Insulin	Positive	PC1
Isoleucine	Positive	PC1
Leucine	Positive	PC1
MCV ˜	Negative	PC7
TC ˜	Positive	PC11
Triglycerides ˜	Positive	PC1
TMAO	Positive	PC11
Transferrin ˜	Positive	PC4
Urea ˜	Positive	PC6
Valine	Positive	PC1

˜ denotes biomarkers used in original report of Mahalanobis distance as proxy of Homeostasis loss (Cohen et al, 2015, DOI 10.1371/journal.pone.0116489). ˜ ˜ Original report included HDL. Abbreviations: AcAc, Acetoacetate; ALP, alkaline phosphatase; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BHB, beta-hydroxybutyrate; CRP, C-reactive protein; GGT, γ-glutamyltransferase; H1P – H7P: High-density lipoprotein 1-7 particles; MCV, mean corpuscular volume; TC, total cholesterol; TMAO, Trimethylamine N-Oxide.

Supplemental Table 3. Prospective associations of MD calculated from different subsets of PCs with risk of T2D.

	HR (95 % CI)	*P* value	$RR_{95\%}$
PC1-PC2	1·21 (1·12,1·32)	<0·001	1.61 (1.33, 2.01)
PC1-PC3	1·22 (1·10,1·35)	<0·001	1.93 (1.37,2.71)
PC1-PC4	1·28 (1·14,1·44)	<0·001	2.45 (1.61,3.77)
PC1-PC5	1·32 (1·16,1·50)	<0·001	2.97 (1.79,4.92)
PC1-PC6	1·29 (1·13,1·48)	<0·001	2.98 (1.68,5.35)
PC1-PC7	1·25 (1·08,1·45)	<0·001	2.82 (1.43,5.64)
PC1-PC8	1·29 (1·11,1·51)	<0·001	3.52 (1.67,7.69)
PC1-PC9	1·31 (1·11,1·54)	<0·001	3.73 (1.66,8.22)
PC1-PC10	1·39 (1·18,1·64)	<0·001	4.87 (2.21,10.79)
PC1-PC11	1·44 (1·21,1·71)	<0·001	5.75 (2.49,13.13)
PC1-PC12	1·43 (1·20,1·71)	<0·001	5.50 (2.38,12.92)
PC1-PC13	1·44 (1·20,1·72)	<0·001	5.69 (2.38,13.28)
PC1-PC14	1·47 (1·22,1·77)	<0·001	6.09 (2.54,14.54)
PC1-PC15	1·48 (1·23,1·78)	<0·001	6.36 (2.65,15.20)
PC1-PC16	1·48 (1·23,1·79)	<0·001	6.61 (2.71,16.54)
PC1-PC17	1·56 (1·30,1·88)	<0·001	8.64 (3.56,21.36)
PC1-PC18	1·60 (1·33,1·92)	<0·001	10.24 (4.10,25.25)
PC1-PC19	1·59 (1·32,1·92)	<0·001	9.83 (3.93,24.92)
PC1-PC20	1·61 (1·34,1·93)	<0·001	10.92 (4.34,27.13)
PC1-PC21	1·60 (1·34,1·93)	<0·001	11.96 (4.68,32.19)
PC1-PC22	1·58 (1·32,1·90)	<0·001	10.89 (4.25,28.51)
PC1-PC23	1·61 (1·35,1·93)	<0·001	12.47 (4.90,35.39)
PC1-PC24	1·64 (1·36,1·96)	<0·001	13.82 (5.11,35.63)
PC1-PC25	1·67 (1·39,2·00)	<0·001	15.38 (5.78,40.22)
PC1-PC26	1·68 (1·40,2·02)	<0·001	17.34 (6.36,47.80)
PC1-PC27	1·65 (1·37,1·98)	<0·001	16.02 (5.72,44.00)
PC1-PC28	1·64 (1·37,1·98)	<0·001	16.36 (5.92,47.44)
PC1-PC29	1·68 (1·39,2·02)	<0·001	18.94 (6.46,53.86)
PC1-PC30	1·67 (1·39,2·02)	<0·001	20.50 (6.95,62.88)
PC1-PC31	1·72 (1·42,2·07)	<0·001	24.65 (7.94,73.68)
PC1-PC32	1·70 (1·42,2·05)	<0·001	23.88 (8.14,73.16)

Data are presented as unadjusted hazard ratios (HRs) with 95% confidence intervals (CIs) and *P* values. HRs were calculated per 1-unit increase in the log scale. The fourth column presents the Relative Risk difference over 95% ($RR_{95\%}$) of the observed MD distribution; this was calculated by subtracting the risk of being in the 97.5th percentile of MD relative to the 2.5th percentile.

Supplemental Table 4. Prospective associations of different subsets of PCs with risk of T2D.

	HR (95 % CI)	*P* value
PC1	0·74 (0·70,0·77)	<0·001
PC2	1·13 (1·06,1·21)	<0·001
PC3	0·78 (0·72,0·84)	<0·001
PC4	0·81 (0·75,0·87)	<0·001
PC5	1·21 (1·11,1·32)	<0·001
PC6	0·89 (0·80,0·99)	0·03
PC7	1·01 (0·91,1·11)	0·87
PC8	1·07 (0·97,1·17)	0·18
PC9	1·12 (1·01,1·24)	0·04
PC10	1·08 (0·97,1·20)	0·17
PC11	0·99 (0·89,1·11)	0·91
PC12	1·00 (0·89,1·13)	0·94
PC13	0·93 (0·81,1·05)	0·25
PC14	0·96 (0·84,1·09)	0·51
PC15	1·11 (0·96,1·29)	0·15
PC16	0·89 (0·77,1·03)	0·11
PC17	0·88 (0·78,0·99)	0·04
PC18	0·93 (0·80,1·08)	0·33
PC19	1·05 (0·91,1·21)	0·48
PC20	0·98 (0·83,1·16)	0·83
PC21	1·28 (1·07,1·52)	0·01
PC22	1·04 (0·86,1·24)	0·71
PC23	0·86 (0·72,1·02)	0·08
PC24	1·06 (0·90,1·25)	0·46
PC25	1·12 (0·95,1·32)	0·18
PC26	1·07 (0·91,1·26)	0·43
PC27	1·02 (0·82,1·26)	0·86
PC28	0·84 (0·68,1·04)	0·11
PC29	1·15 (0·94,1·42)	0·17
PC30	1·13 (0·88,1·46)	0·35
PC31	0·81 (0·61,1·06)	0·13
PC32	0·75 (0·49,1·15)	0·19

Supplemental Table 5. Prospective associations of MD calculated from 32 circulating biomarkers concentrations with risk of T2D.

	MD per 1 Ln SD Increment		T1	T2		T3	
Participants, *n*	6247		2083	2082		2082	
Events, *n*	312		70	92		150	
	HR (95 % CI)	***P* value**		**HR (95 % CI)**	***P* value**	**HR (95 % CI)**	***P* value**
Crude Model	1·68 (1·46,1·94)	<0·001	(ref)	1·26 (0·91,1·73)	0·15	1·61 (1·20,2·15)	0·001
Model 1	1·60 (1·38,1·85)	<0·001	(ref)	1·21 (0·88,1·67)	0·23	1·51 (1·13,2·04)	0·005
Model 2	1·18 (1·00,1·40)	0·05	(ref)	1·21 (0·88,1·67)	0·24	1·45 (1·07,1·96)	0·01
Model 3	1·18 (0·99,1·40)	0·06	(ref)	1·12 (0·81,1·55)	0·50	1·31 (1·97,1·77)	0·08
Model 4	1·18 (0·98,1·40)	0·07	(ref)	1·11 (0·80,1·54)	0·51	1·30 (0·96,1·76)	0·09

Data are presented as hazard ratios (HRs) with 95% confidence intervals (CIs) and P values of MD as continuous variable (per 1 log unit increment) and as categorical variable (with the first tertile of MD as reference). MD was calculated using the circulating concentrations of 32 biomarkers.
Model 1. Model adjusted for age and sex
Model 2. Model 1 adjusted for plasma glucose, parental history of T2D, lipid lowering medication and antihypertensive medication.
Model 3. Model 2 adjusted for BMI.
Model 4. Model 2 adjusted for waist circumference.

Supplementary methods.

TMAO, betaine, BCAA and ketone body concentrations were measured in EDTA-anticoagulated plasma samples using a Vantera® Clinical Analyzer (LabCorp, Morrisville, NC), a fully automated, high-throughput, 400 MHz proton (1H) nuclear magnetic resonance (NMR) spectroscopy platform. TMAO was quantified from one-dimensional (1D) proton (1H) Carr-Purcell-Meiboom-Gill (CPMG) spectra by spectral deconvolution algorithm as previously described (1,2). The TMAO assay has intra- and inter-assay coefficients of variation (CV%) range from 4.3–10.3% and 9.8–14.5%, respectively, and a limit of quantitation of 3.3 µM (2).

The validation of the use of NMR for quantification of BCAAs has been previously described by our group (3). In brief, coefficients of variation for inter- and intraassay precision ranged from 1.8% to 6.0%, 1.7% to 5.4%, 4.4% to 9.1%, and 8.8% to 21.3%, for total BCAAs, valine, leucine, and isoleucine, respectively. BCAAs quantified from the same samples using NMR and LC-MS/MS were highly correlated (r = 0.97, 0.95 and 0.90 for valine, leucine, and isoleucine) (3).

For determining ketone body concentrations, a method comparison study was performed comparing quantification by NMR to platforms commonly used, i.e LC/MS/MS for β-hydroxybutyrate and acetoacetate and GC/MS for acetone. A comparison of plasma concentrations using the comparator platforms correlated well by Deming regression with R2 values of 0.996, 0.994 and 0.994 for β-hydroxybutyrate, acetoacetate and acetone, respectively. The limits of quantification were calculated to be 65.0, 45.0, 26.3 and 19.7 µM for total ketone bodies, β-hydroxybutyrate, acetoacetate and acetone, respectively. For β-hydroxybutyrate, acetoacetate and acetone coefficients of variation for intra-assay and inter-assay precision were 1.3%–9.3%, 3.1%–7.7%, and 3.8%–9.1%, respectively. A more detailed description of the data acquisition and method validation has been previously reported (4).

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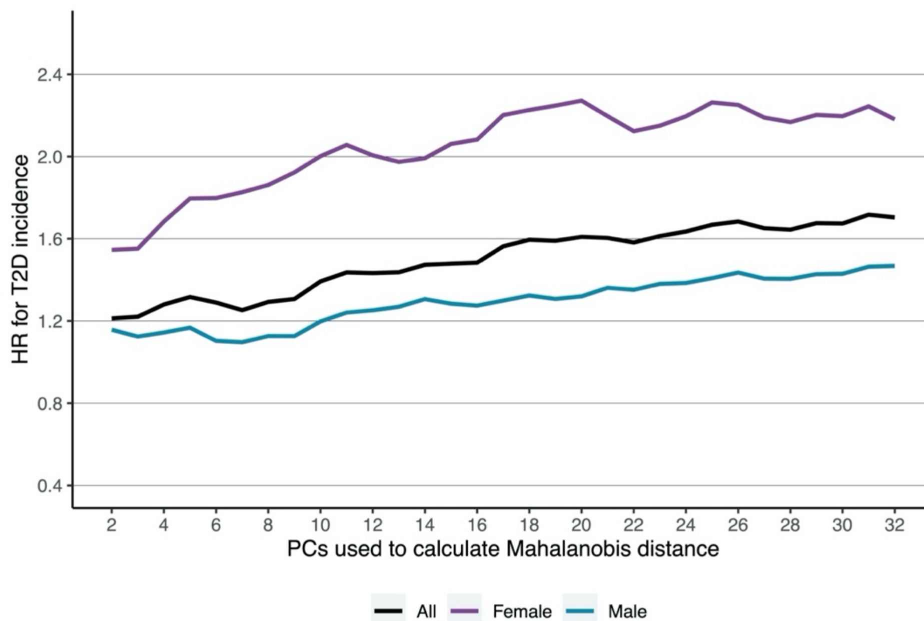
3. Connelly MA, Wolak-Dinsmore J, Dullaart RPF. Branched Chain Amino Acids Are Associated with Insulin Resistance Independent of Leptin and Adiponectin in Subjects with Varying Degrees of Glucose Tolerance. Metabolic Syndrome and Related Disorders 2017;15(4):183–186.

4. Garcia E, Shalaurova I, Matyus SP, Oskardmay DN, Otvos JD, Dullaart RPF, Connelly MA. Ketone Bodies Are Mildly Elevated in Subjects with Type 2 Diabetes Mellitus and Are Inversely Associated with Insulin Resistance as Measured by the Lipoprotein Insulin Resistance Index. Journal of Clinical Medicine 2020;9(2):321.

Supplementary Figure 1.



Supplementary Fig.1. Plot showing the association of MD and risk of T2D. HRs for incidence of T2D (*y*-axis), were obtained for 32 different MDs, calculated from cumulative subsets of PCs (*x*-axis). HRs were computed for the whole population (black line), men (blue) and women (purple).

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Chapter 11

Summary, discussion, and future perspectives

BRANCHED-CHAIN AMINO ACIDS AND TRIMETHYLAMINE N-OXIDE AS BIOMARKERS OF CARDIOMETABOLIC OUTCOMES

The identification of biomarkers that could improve the individual risk prediction of developing cardiometabolic disease is one of the salient goals of the 21th century paradigm in medicine captured as precision medicine [1–3].

Branched-chain amino acids (BCAA) and trimethylamine N-oxide (TMAO) are two novel biomarkers of which the metabolism is linked to key pathophysiologic pathways of cardiometabolic diseases, such as insulin resistance, microbiome response to diet, kidney function, and atherogenesis [4–18].

Given the steady rise of incidence and prevalence of the whole cardiometabolic disease spectrum (e.g. Type 2 Diabetes (T2D), non-alcoholic fatty liver disease (NAFLD) and hypertension) [19–22], the robust characterization of promising biomarkers to assess its role and clinical utility in the development of cardiometabolic disease entities and its complications is highly relevant. Despite the prominent development of cutting-edge instruments that allow the simultaneous measurement of several metabolites [23,24], most of the studies report the associations of individual biomarkers with clinical outcomes but do not take into account the interrelationships among biomarkers. Therefore, it is necessary to investigate new approaches to integrate the information of novel and traditional cardiometabolic disease biomarkers.

The first aim of this thesis was to evaluate the role of circulating BCAA as biomarker of T2D and hypertension incidence in the general population. Secondly, we aimed to study whether TMAO was prospectively associated with higher cardiometabolic risk in patients with comorbidities such as T2D, NAFLD and CKD. Finally, we aimed to investigate whether a statistical proxy of homeostasis loss, that integrates the information of several biomarkers could also provide information about the risk of T2D.

Summary

In **Chapter 2,** we investigated whether concentrations of circulating BCAA were prospectively associated with a higher risk of incidence of T2D in a large population-based cohort. In this study, we found that higher plasma concentrations of BCAA are strongly associated with an elevated risk of incident T2D, independently of traditional risk factors such as sex, age, BMI, family history of T2D and fasting plasma glucose. Given these findings, in **Chapter 3**, we explored the extent to which BCAAs influence T2D development in participants with NAFLD. After conducting a mediation analysis, we found that the association of NAFLD with incident T2D was in part mediated by elevated BCAAs (proportion mediated 19.6%). Driven by the results obtained in chapters 2 and 3, we aimed to investigate whether a multimarker that contains the information of BCAA and lipoproteins could also be prospectively associated with the development of T2D. Consequently, in **Chapter 4** we assessed the association of the diabetes risk index (DRI), a multimarker that comprehends the concentrations of valine and leucine, as well as 6 lipoprotein subfractions with the incidence of T2D. We found that higher DRI scores were associated with an increased risk of T2D incidence. This association was independent of clinical risk factors for T2D, including insulin resistance (HOMA-IR), BMI and conventional lipids.

Considering that T2D and hypertension are two highly prevalent non-communicable diseases, that commonly overlap in the population [25], which share common risk factors [26–30] as well as pathophysiologic mechanisms [31–34], including a potential role of BCAA [35–37], we were interested to know whether plasma concentrations of BCAA were associated with incident hypertension. In **Chapter 5**, we provided the first evidence from a well-characterized prospective study about the association of BCAA with an increased risk of developing hypertension among individuals from the general population. Consistent with our previous findings, such an association was independent of traditional risk factors, such as age, sex, BMI, family history of hypertension and kidney function. Moreover, in **Chapter 6**, we investigated the association of the multimarker called Diabetes Risk Index (DRI) with the risk of incident hypertension. Here we found that higher DRI scores were associated with an increased risk of hypertension. Furthermore, we found that the addition

of DRI to a traditional risk model allowed a proper reclassification of 34% of the participants from lower to higher risk categories.

Considering that TMAO, a microbiota-derived biomarker [15,38] has recently gained attention due to its potential role in the development and progression of T2D complications [39–42], CVD [13,18,43–45], and kidney disease [46–49], and also because its association with increased mortality risk in the general population [50,51], subsequent studies were performed to evaluate its relationship with cardiometabolic disease.

In **Chapter 7**, we used data from a cohort of T2D patients to evaluate the association of TMAO concentrations in plasma with the risk of cardiovascular mortality. We found that higher plasma TMAO concentrations are associated with an increased risk of CV mortality in individuals with T2D, even after the adjustment for multiple clinical and laboratory variables, including duration of T2D, glycemic and lipid profile and kidney function.

Thereafter, the association of plasma concentrations of TMAO with all-cause mortality in patients with NAFLD was described in **Chapter 8**. In both men and women with NAFLD, circulating concentrations of TMAO were prospectively associated with the risk of all-cause mortality, with this association being independent of traditional risk factors such as blood pressure, BMI, smoking and alcohol consumption, kidney function as well as glycemic and lipid profile.

Given the fact that a reduced renal function results in increased circulating concentrations of TMAO [15,52], while at the same time, high concentrations of TMAO could also exert a deleterious effect on kidney function [53,54], we investigated in **Chapter 9**, the longitudinal association of plasma concentrations of TMAO, with the risk of kidney graft failure in a cohort of RTRs.

Here we found that higher concentrations of circulating TMAO are associated with an increased risk of kidney graft failure, independently of age, sex, BMI, blood pressure, lipids, albuminuria and, importantly, CKD stage. We furthermore identified that egg and fish consumption are the main dietary determinants of TMAO concentrations in this population. We reported that dietary information along with the concentrations of TMAO improve the risk assessment for graft failure.

In order to gain a deeper insight into the interplay between the different biomarkers of glucose and lipid metabolism, as well the novel biomarkers such

as BCAA and TMAO, in **Chapter 10** we evaluate the performance of the Mahalanobis distance of circulating biomarkers for the risk assessment of T2D. In this study, we calculate the Mahalanobis distance using a set of 32 circulating biomarkers, that had been previously postulated as a proxy of homeostasis loss [55]. Here we found that a higher Mahalanobis distance of circulating biomarkers, including lipids and lipoproteins, as well as inflammation markers and microbiome-derived metabolites, suggest a more atypical biomarker profile and it is prospectively associated with a higher risk of incident T2D. Of note, this association was independent of age, sex, plasma glucose, parental history of T2D, lipid, blood pressure medication and BMI. These results suggested that the Mahalanobis distance provides information about the physiological dysregulation not only in the ageing process, as previously reported, but also in the development of T2D.

Discussion and future perspectives

BCAA, T2D and hypertension

T2D and hypertension are two of the most prevalent non-communicable diseases, sharing common clinical and lifestyle risk factors [26–30] as well as some underlying molecular mechanisms [31–34]. Former studies have reported the cross-sectional association of high concentrations of BCAA with prevalent T2D [56,57] and prevalent hypertension [35,58,59].

It has been described at least two common pathways on which the BCAA could potentially play a role in the development of these two pathologies. On the one hand, the BCAA boost the activation of the mammalian target of rapamycin (mTOR1), which activates the S6KI, responsible for the phosphorylation of insulin receptor substrate 1 (IRS-1), thereby impairing insulin signaling [60,61]. On the other hand, it has been reported that chronic activation of mTOR1 also increases the production of the reactive oxide species, via stimulation of NADPH oxidase [8,62]. Since both, insulin resistance and the oxidative stress well-known components of the pathophysiology of both T2D and hypertension [63–67], it is plausible that the strong association that we reported between the high concentrations of BCAA and the augmented risk of T2D and hypertension development, could at least in part be explained by these novel underlying mechanisms.

In this thesis, we have presented that BCAA longitudinally associates with the incidence of T2D and hypertension. This association was independent of traditional clinical and laboratory risk factors. Furthermore, we demonstrated that these biomarkers improve the risk classification for T2D and hypertension, in adults from the general population.

We found that the association of BCAA with both T2D and hypertension was present in men and women. Notably, the plasma concentrations of BCAA and the multimarker DRI were higher in men, compared to women. At the moment, one of the plausible explanations for the elevated concentrations of BCAA in men could be that the dietary intake of BCAA-rich foods is higher in men [68,69]. There is evidence suggesting that such differences may at least in part be attributed to differences in dietary patterns between men and women [70].

In our studies, we used data mainly from a community-dwelling population, nevertheless, previous studies have also reported the existence of the association of BCAA with obesity and progression to insulin resistance in children and adolescents [71]. This is relevant, given the rise of incidence and prevalence of obesity among young people, and its well-known association with a higher risk of premature death in adulthood [72–75].

The design of our studies prevents us to draw causal conclusions about the association of BCAA with T2D and hypertension. Nonetheless, Luca A. Lotta and colleagues have conducted a large-scale mendelian randomization study (which is one of the most important methods for causal inference in epidemiology [76]) and found evidence of the causal role of BCAA in the development of T2D [77]. Similar studies in the context of hypertension incidence need to be conducted to further confirm whether or not the association of BCAA with incident hypertension is causal.

TMAO and cardiometabolic disease

T2D has been considered a cardiovascular disease of metabolic origin [78]. Given the fact that not only T2D, but most of metabolic disorders represent an increased risk of cardiovascular disease [79,80], the study of biomarkers that could provide an early signal of the development of such complications has become a common endeavor in medical research.

In this thesis, we reported the association of TMAO, a microbiota-derived metabolite [15,38], with increased risk of mortality, as well as kidney graft

failure. We have shown that higher plasma TMAO concentrations were significantly associated with an increased risk of mortality in individuals with T2D and with NAFLD, in individuals from two different Dutch populations. Interestingly, the increased risk of mortality in subjects with T2D was due to cardiovascular disease, meanwhile, in subjects with NAFLD, it was due to all-cause mortality. In both populations, TMAO was cross-sectionally associated with several metabolic risk factors including adiposity, reduced renal function, and older age. Such findings are in line with the literature [81,82]. Notably, the association of TMAO with increased risk mortality was independent of these variables.

Our longitudinal analyses in the T2D population are in line with basic research reports that reveal that the role of TMAO in the risk of cardiovascular disease could be mediated by several pathways, such as the acceleration of atherosclerosis by enhancing the formation of foam cells and atherogenic plaque [15], the inhibition of reverse cholesterol process whereby cholesterol is transported from the arterial wall back to the liver [38] as well as the increased platelet adhesion to collagen surfaces [13].

The increased mortality risk in subjects with NAFLD could also be explained by other deleterious actions of TMAO on liver physiology. Evidence from in vivo models of NAFLD had shown that TMAO increases hepatic triglyceride accumulation by inhibiting the farnesoid X receptor signaling [83]. Moreover, it has been proposed that TMAO could reduce the enterohepatic circulation of bile acids between the liver and the gut due to the repression of organic anion transporter family protein expression [38].

Although the association of TMAO with kidney function is already described [51,84], in this thesis, we reported for the first time the association between higher concentrations of circulating TMAO and kidney graft failure. Our study finding is in line with previous reports showing that in patients with end-stage renal disease, TMAO concentrations remain remarkedly high until undergoing hemodialysis [52]. In our study, we further evaluated the association of TMAO with diet components, confirming the association of TMAO with animal-derived products, such as seafood and eggs [17,85].

These results highlight the role of the gut microbiome biomarkers in the prolonged functionality of the graft in renal transplant recipients, and the importance of the continuous investigation of diet interventions in frail

populations, beyond reductionistic analyses of the mere nutrient concentrations, but taking into account the role of the microbiota-derived products.

Cardiometabolic risk assessment

Over the last century, medicine has shifted from a merely responsive (curative) approach to a preventive one [86]. In order to make such a shift, it is necessary to have a framework to interpret the different health hazards. The German Scientific Advisory Council for Global Environmental Change had proposed a novel approach to risk classification assessment composed of six risk clusters, illustrated with characters from Greek mythology, based on the similarities between the risk clusters and the features of those characters [87].

Clusters differ in terms of their probability of occurrence and the degree of associated damage (Figure 1). For instance, the well-known character Medusa represents a cluster of risks that could be perceived as a major threat, but in fact, its potential damage could be easily controlled and therefore its extent of damage and probability of occurrence is low, which could be the case of non-ionizing radiation. Another example is the Damocles cluster risk, (named after Damocles who took the place of king Dionysius for one day, just to discover that a sword was hanging over the king's seat); this cluster includes those threats that have a high disaster potential, but an extremely low probability, i.e. nuclear energy facilities disasters. Along with Damocles, there are other 2 risk clusters in the same area of risk management. Cyclops, despite being strong, having a single eye affected his dimensionality perception. This cluster includes risks with an extent of damage well know, but an uncertain probability of occurrence (i.e. natural disasters such as earthquakes). The cluster Pythia is named after the Oracle of Delphi, famous for her ambiguous prophecies; in this cluster, the risks are characterized for a high incertitude in both the probability of occurrence and the extent of damage (i.e. the instability of the West Antarctic ice sheet). The Pandora cluster covers the three areas of risk management and includes those interventions that could potentially cause a wide-range collection of damage, for instance pesticides that affect both the environment and human health [87].

The Cassandra cluster (named after Cassandra, a seeress cursed to see the future, but never to be believed) is of particular interest. Risks of this cluster have a high probability of occurrence and the extent of damage is also high.

Nevertheless, those risks are commonly downplayed, mainly due to their slow development.

Considering the above-described clusters, T2D itself could be classified as a Cassandra-type risk, being responsible of a remarkable health burden worldwide, with a high degree of certainty, but still underestimated due to its slow (silent) development.

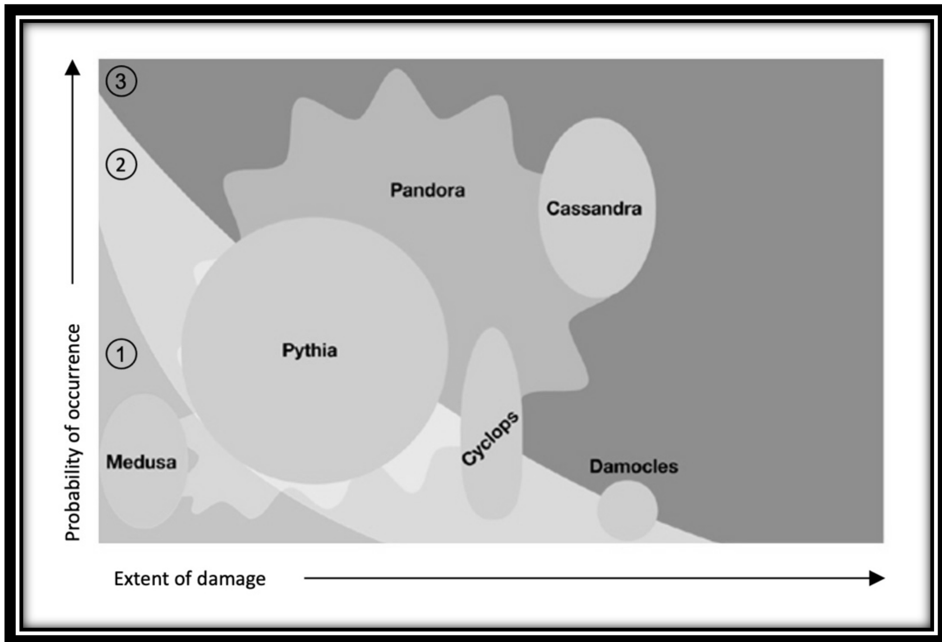

Figure 1. Schematic representation of the six risk clusters proposed by The German Scientific Advisory Council for Global Environmental Change. 1: Normal area of risk management. 2: Intermediate area of risk management. 3: Intolerable area of risk management. Figure reprinted from "Systemic risks: a new challenge for risk management" by Ortwin Renn & Andreas Klinke, *EMBO reports*, 2004, 5 (s41-s46).

Overall, this thesis provided further epidemiological evidence which supports the notion that BCAA and TMAO are longitudinally associated with a high risk of cardiometabolic disease. Here, we studied biomarkers that are involved in slow, but certainly deleterious mechanisms, which extend of damage and probability are high; fulfilling the characteristics of the Cassandra risk cluster. These observational findings should be taken with caution, but not be downplayed (as the Cassandra cluster), instead, should be interpreted under the light of recent experimental findings that suggest a pivotal role of the BCAA in the development of diseases characterized by insulin resistance, such as T2D and

hypertension [88,89], as well a critical role of TMAO in the development of cardiovascular complications, due to its atherogenic mechanisms CVD [13,15,16].

An important aspect of the risk assessment is the optimal utilization of the available information. Currently, the amount of information biomarkers overpasses the traditional analyses methods and therefore it is necessary to explore novel statistical approaches. In this thesis, we found that the use calculation of the Mahalanobis distance (MD) after a principal component analysis could help to integrate the information from novel and traditional biomarkers in the context of T2D incidence.

The MD, which is a metric that reflects the variation of a set of elements among themselves, applied to circulating biomarkers data, has been proposed as a quantifiable proxy of homeostasis loss outcomes [90]. Recently, the calculation of MD has been upgraded by replacing the raw biomarker information with PCs [90]. The substitution of the raw biomarkers data with its principal components is founded on the fact that principal component analysis could detect underlying processes that might simultaneously regulate the levels of the variables used in the analysis, but may not be directly measurable [91]. Notably, in our study, we intentionally excluded fasting plasma glucose from the principal component analysis and therefore from the MD calculation with the intention to avoid the prevailing influence of glucose in assessing the degree of homeostasis loss in the context of T2D development.

Due to the nature of BCAA and TMAO, its concentrations are importantly influenced by changes in dietary patterns [70,92]; therefore, future interventional studies are needed to explore the extent to which diet modifications (i.e. reduction of foods rich in BCAA and TMAO precursors) could reduce its associated risk associated. Furthermore, it would be of interest to investigate if gut microbiome-target interventions (i.e. fecal transplant [93,94]) could also have a clinical impact in the progression of the cardiometabolic disease outcomes reported in this thesis and whether such effects are mediated, at least in part, by the BCAA and TMAO.

Finally, it is worth mentioning that among the multiplicity of novel biomarkers that have been recently discovered, BCAA and TMAO have the potential to become biomarkers that could be used in the context of One Health. Briefly, One Health is defined as a collaborative, multisectoral, and transdisciplinary

approach to accomplish optimal health outcomes, which is aware of the interconnection between human health, animals and plants wellbeing and the environment [95,96] (Figure 2).

It has recently been proposed that the quest for biomarkers that can provide information not only about human health outcomes but about its close relationship with the environment, could help to improve the application of the One Health approach in addressing the global health challenges [97]. The general scientific consensus points towards the agreement that the reduction of animal-based food is a benefit for both the planet and the population [98–100].

Because of the link between BCAA and TMAO with certain foods, particularly those of animal origin, it seems plausible that these metabolites could also provide information on the degree of consumption of such foods, and in combination with other biomarkers could depict a more accurate picture of dietary patterns within populations, and its associated health impact; this information could certainly be integrated into robust and multimodal analyses under the perspective of One Health in order to produce the evidence needed for public health decisions.

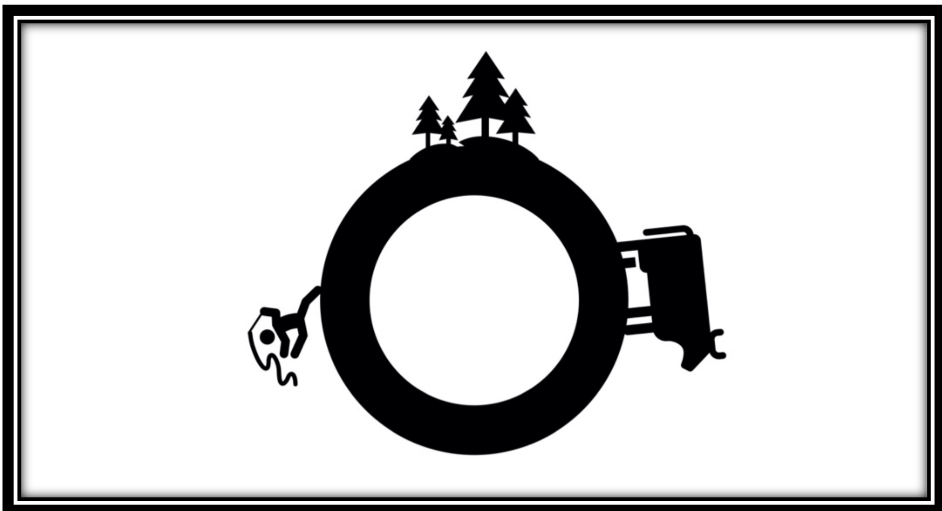

Figure 2. One Health is a collaborative, multisectoral, and transdisciplinary approach to accomplish optimal health outcomes, recognizing the deep interconnection between human health, animals and plants wellbeing and the environment.

Conclusion

In this thesis, we studied the potential role of two novel biomarkers, BCAA and TMAO in the context of cardiometabolic disease. We characterized the association of BCAA with the development of T2D, hypertension, and progression of NAFLD; moreover, we depict the clinical impact of TMAO in the development of cardiovascular and all-cause mortality in patients with T2D and NAFLD, respectively, as well as the development of kidney graft failure in post-transplant recipients. Overall, the results of this thesis support the conception that BCAA and TMAO provide valuable information that can be used to perform a better risk assessment of the early development of cardiometabolic disease, such as hypertension, TD2 and its complications. Given the nature of BCAA and TMAO, these results also pave the way for future studies of pharmacological and non-pharmacological interventions as strategies to mitigate the deleterious consequences of the still-growing pandemic of obesity and T2D. Finally, this thesis also reinforces the idea that it is possible and desirable to improve the application of novel statistical methods that cope with the advanced methods of metabolites quantification, in order to better understand the underlying complexity of T2D and cardiometabolic disease.

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Appendices

Dutch Summary

De identificatie van biomarkers die de individuele risicovoorspelling van het ontwikkelen van cardiometabole ziekten zouden kunnen verbeteren, is een van de belangrijkste doelen van het 21e-eeuwse paradigma in de geneeskunde, vastgelegd als precisiegeneeskunde.

Aminozuren met vertakte keten (BCAA) en trimethylamine N-oxide (TMAO) zijn twee nieuwe biomarkers waarvan het metabolisme is gekoppeld aan belangrijke pathofysiologische routes van cardiometabole ziekten, zoals insulineresistentie, microbioomreactie op dieet, nierfunctie en atherogenese. In dit proefschrift hebben we de rol van plasma BCAA concentratie als biomarker van T2D en hypertensie onderzocht. Evenzo hebben we onderzocht of TMAO prospectief geassocieerd was met een hoger cardiometabool risico bij patiënten met comorbiditeiten zoals T2D, NAFLD en CKD. Ten slotte wilden we onderzoeken of een statistische proxy van homeostaseverlies, die de informatie van verschillende biomarkers integreert, ook informatie zou kunnen geven over het risico op T2D.

In **Hoofdstuk 2** hebben we onderzocht of concentraties van circulerend BCAA prospectief geassocieerd waren met een hoger risico op incidentie van T2D in een groot populatiegebaseerd cohort. In deze studie vonden we dat hogere plasmaconcentraties van BCAA sterk geassocieerd zijn met een verhoogd risico op incidente T2D, onafhankelijk van traditionele risicofactoren zoals geslacht, leeftijd, BMI, familiegeschiedenis van T2D en nuchtere plasmaglucose. Gezien deze bevindingen hebben we in **Hoofdstuk 3** onderzocht in hoeverre BCAA's de T2D-ontwikkeling beïnvloeden bij deelnemers met NAFLD. Na het uitvoeren van een mediatieanalyse, ontdekten we dat de associatie van NAFLD met incident T2D gedeeltelijk werd gemedieerd door verhoogde BCAA's (aandeel gemedieerd 19,6%). Gedreven door de resultaten verkregen in hoofdstuk 2 en 3, wilden we onderzoeken of een multimarker die de informatie van BCAA en lipoproteïnen bevat ook prospectief geassocieerd zou kunnen worden met de ontwikkeling van T2D. Daarom hebben we in **Hoofdstuk 4** de associatie van de Diabetes Risk Index (DRI) onderzocht, een multimarker die de concentraties van valine en leucine omvat, evenals 6 lipoproteïne-subfracties met de incidentie van T2D. We vonden dat hogere DRI-scores geassocieerd waren met een verhoogd risico op T2D-incidentie. Deze associatie was onafhankelijk van klinische risicofactoren voor T2D, waaronder insulineresistentie (HOMA-IR), BMI en conventionele lipiden.

In **Hoofdstuk 5** hebben we het eerste bewijs geleverd van een goed gekarakteriseerde prospectieve studie over de associatie van BCAA met een verhoogd risico op het ontwikkelen van hypertensie bij individuen uit de algemene bevolking. In overeenstemming met onze eerdere bevindingen was een dergelijke associatie

onafhankelijk van belangrijke risicofactoren, zoals BMI en nierfunctie. Bovendien vonden we in **Hoofdstuk 6** dat de multimarker genaamd DRI geassocieerd was met een verhoogd risico op hypertensie. Verder vonden we dat de toevoeging van DRI aan een traditioneel risicomodel een goede herclassificatie van 34% van de deelnemers van lagere naar hogere risicocategorieën mogelijk maakte.

In **Hoofdstuk 7** hebben we gegevens van een cohort T2D-patiënten gebruikt om de associatie van TMAO-concentraties in plasma met het risico op cardiovasculaire mortaliteit te evalueren. We vonden dat hogere plasma-TMAO-concentraties geassocieerd zijn met een verhoogd risico op CV mortaliteit bij personen met T2D, zelfs na correctie voor belangrijke klinische variabelen, waaronder duur van T2D, glykemie- en lipidenprofiel en nierfunctie. Daarna werd de associatie van plasmaconcentraties van TMAO met mortaliteit door alle oorzaken bij patiënten met NAFLD beschreven in **hoofdstuk 8**. Bij zowel mannen als vrouwen met NAFLD waren de plasma TMAO concentraties prospectief geassocieerd met het risico op mortaliteit door alle oorzaken. De associatie is onafhankelijk van traditionele risicofactoren zoals bloeddruk, BMI, roken en alcoholgebruik, nierfunctie evenals glycemisch en lipidenprofiel.

Gezien het feit dat een verminderde nierfunctie resulteert in verhoogde plasma TMAO concentraties, terwijl tegelijkertijd hoge concentraties van TMAO ook een schadelijk effect kunnen hebben op de nierfunctie, hebben we in **Hoofdstuk 9** de longitudinale associatie van plasmaconcentraties van TMAO onderzocht, met het risico op niertransplantaatfalen in een cohort van niertransplantatiepatiënten. Hier vonden we dat hogere concentraties van circulerend TMAO geassocieerd zijn met een verhoogd risico op niertransplantaatfalen, onafhankelijk van BMI, bloeddruk, lipiden, albuminurie en, CKD-stadium. We hebben verder vastgesteld dat de consumptie van eieren en vis de belangrijkste voedingsdeterminanten zijn van TMAO-concentraties in deze populatie.

Om een dieper inzicht te krijgen in het samenspel tussen de verschillende biomarkers van glucose- en lipidemetabolisme, evenals de nieuwe biomarkers zoals BCAA en TMAO, evalueerden we in **Hoofdstuk 10** de prestatie van de Mahalanobis-afstand van plasma biomarkers voor de risicobeoordeling van T2D. In deze studie berekenen we de Mahalanobis-afstand met behulp van een set van 32 plasma biomarkers, die eerder waren gepostuleerd als een proxy voor homeostaseverlies. Hier ontdekten we dat een hogere Mahalanobis-afstand van plasma biomarkers, waaronder lipiden en lipoproteïnen, evenals ontstekingsmarkers en microbioom-afgeleide metabolieten, een meer atypisch biomarkerprofiel suggereert en prospectief geassocieerd is met een hoger risico op incident T2D. Merk op dat deze associatie onafhankelijk was van leeftijd, geslacht, plasmaglucose, ouderlijke voorgeschiedenis van T2D, lipiden, bloeddrukmedicatie en BMI. Deze resultaten suggereerden dat de Mahalanobis-afstand informatie geeft over de fysiologische ontregeling, niet alleen in het verouderingsproces, zoals eerder gemeld, maar ook in de ontwikkeling van T2D.

List of Publications

- Sokooti S, **Flores-Guerrero JL**, Heerspink HJL, Garcia E, Connelly MA, Bakker SJL, Dullaart RPF. Lipoprotein Particle Sizes and Incident Type 2 Diabetes: the PREVEND Study. *Diabetologia.* 2021; in press.
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Awards

- 1st Place. Falling Walls Lab. German Academic Exchange Service-EURAXESS (Mexico-Germany, 2021)
- PhD Image contest prize. Groningen Organisation for PhD Education and Recreation (The Netherlands, 2021)
- Highest Impact Paper in *Hypertension* (Population Sciences category). American Heart Association (U.S.A., 2020)
- 2nd Best oral Presentation. International Forum for Donation and Transplantation Research. Health Service of the State (Mexico, 2020)
- 2nd Best oral Presentation. VII European Young Endocrine Scientists Meeting. European Society of Endocrinology (Greece, 2019)
- International Writing Prize. Multidisciplinary Digital Publishing Institute (Switzerland, 2019)
- Finalist. 3M Thesis Competition. University of Groningen (The Netherlands, 2019)

About the author

José Luis Flores Guerrero was born in Puebla, Mexico, on March 29th in 1988. In 2006, after finishing high school at the Emiliano Zapata High School, he studied Medicine at the Meritorious University Autonomous of Puebla, in Puebla, Mexico. As undergrad, he collaborated in several Diabetes related research projects at the Physiology Institute (BUAP) as well as the Center for Research and Advanced Studies of the National Polytechnic Institute. From 2011 to 2012 he did his medical internship at the National Institute of Medical Sciences and Nutrition. His MD thesis received some prizes, including the Expo-sciences prize, granting him the right to attend the Nobel Prize ceremony representing Latin-American students. After receiving his MD degree in 2013 he joined an NGO in order to teach Natural Sciences in public schools, aiming to close the achievement gap. In 2016, José Luis obtained the MSc degree in Medical Sciences Diabetes from the University of Glasgow. Afterwards, he received a grant from the Finnish government to do a research stay at The University of Helsinki.

From 2017 to 2021, José Luis was supported by the National Council of Science and Technology of Mexico (CONACYT) to do his PhD under the supervision of Prof. S.J.L. Bakker, Prof. G.J. Navis and Dr. R. P. Dullaart at the Department of Internal Medicine, Division of Nephrology, the University Medical Center Groningen, Groningen, The Netherlands. The work of this thesis led to several oral and poster presentations at international conferences in the field of Diabetes.

After finishing his PhD, José Luis will continue his academic work in the field of cardiometabolic disease.

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José Luis

Γίνε αστέρι, γίνε το κύμα, γίνε ο ήλιος, που γεννιέται πρωί. Γίνε για λίγο, μαύρο σκοτάδι, για να μπορεί, της ψυχής μας, το φως να φανεί.

Γίνε το χάδι, σε κάθε τραύμα, γίνε στον πόνο μας, κρεβάτι ζεστό. Φώτισε δρόμους, που έχουμε χάσει, Γίνε βροχή, να πλυθεί, η καμένη μας γη.

Έρημος τόπος, τα άδεια όνειρα μας, Γίνε γεφύρι, στης χαράς το κενό.

Άνοιξε δρόμους, δώσε το βήμα, ρίξε το θάνατο, στης φωτιάς το χορό.