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MEDBIOME: A Study Protocol to Evaluate the Effect of MEDiterranean Diet on the MicroBIOME of Individuals with Type 2 Diabetes*

*MEDBIOMA: Protocolo de um Estudo para Avaliar o Efeito da Dieta MEDiterrânea no MicroBIOMA de Indivíduos com Diabetes Tipo 2**

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Abstract

An imbalance in the homeostasis of gut microbiota, described as dysbiosis, is associated with the pathophysiology of type 2 diabetes. Mediterranean diet has proven to promote the integrity of gut barrier and has shown positive impact in the management of this disease. This study aims to describe a study protocol to evaluate if gut microbiota plays a role as a mediator of the impact of Mediterranean diet on metabolic control of subjects with type 2 diabetes.

In this 12-week, single-arm clinical trial, subjects with type 2 diabetes receive nutrition counselling sessions every 2 weeks where Mediterranean diet is constantly promoted. Stool and blood samples are collected, and body composition and blood pressure are accessed at baseline, at 4 weeks, and at 12 weeks. Every 2 weeks food intake is evaluated and PREDIMED questionnaire is applied to evaluate Mediterranean diet adherence.

It is expected that Mediterranean diet will significantly impact the gut microbiota profile, and that these changes will be observed prior to improvement in biochemical parameters. Additionally, this will be an opportunity to characterize the gut microbiota of Portuguese population with type 2 diabetes and to reinforce the importance of food and nutrition intervention in the treatment of this disease. This trial is registered at clinicaltrials.gov as NCT04403217.

Keywords: dysbiosis; gut microbiota; Mediterranean diet; metabolic control; type 2 diabetes

> INTRODUCTION

Type 2 diabetes mellitus (T2DM) is a complex metabolic disorder characterized by insulin deficiency, frequently on the background of insulin resistance, that leads to a progressive loss of islet β-cell. (1) In Portugal, around 1 million of the population has diabetes, 90% of those cases are T2DM. (2) The rapid rise of T2DM incidence in the last decades has been accredited to environmental factors and represents an important public health problem. (2) Gut microbiota has been suggested as one of the key

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Resumo

O desequilíbrio na homeostase do microbiota intestinal, descrito como disbiose, está associado à fisiopatologia da diabetes tipo 2. A dieta mediterrânica promove a integridade da barreira intestinal e tem demostrado um impacto positivo no controlo desta doença. Este trabalho tem como objetivo descrever o protocolo do estudo para avaliar se o microbiota intestinal é o mediador dos efeitos da dieta mediterrânica no controlo metabólico da diabetes tipo 2.

Neste estudo clínico de 12 semanas com um braço de intervenção, indivíduos com diabetes tipo 2 recebem consultas de nutrição, em que a adoção da dieta mediterrânica é promovida, a cada 2 semanas. No início do estudo, às 4 semanas e às 12 semanas são recolhidas amostras de fezes e de sangue, e avaliada a composição corporal e a pressão arterial. A cada 2 semanas a ingestão alimentar é avaliada e o questionário PREDIMED é aplicado, para avaliar a adesão à dieta mediterrânica.

Espera-se que a dieta mediterrânica tenha um impacto significativo no microbiota intestinal e que essas alterações observadas antecedam a melhoria clínica e analítica. Por outro lado, pretende-se caracterizar o microbiota intestinal de uma população portuguesa com diabetes tipo 2 e reforçar a importância da intervenção alimentar e nutricional no tratamento desta doença. Este estudo está registado na plataforma clinicaltrials.gov com o número NCT04403217.

Palavras-chave: controlo metabólico; diabetes tipo 2; dieta mediterrânica; disbiose; microbiota intestinal

metabolic targets of lifestyle factors that contributes to the pathophysiology of this metabolic disease. [3]

Gut microbiota is a complex ecosystem composed by trillions of microorganisms that inhabit the human intestine. [4] Components of gut microbiota are mostly bacteria, with a minority of viruses, fungi, and eukaryotic cells colonizing this ecosystem. The phylum *Firmicutes*, represented by the genera *Ruminiococcus*, *Clostridium* and *Lactobacillus*, is the most abundant (60-80%), followed by the phylum *Bacteroidetes* (20-30%), represented by the genera *Bacteroides* and *Prevotella*, and the phylum Actinobacteria (<10%), which represents a minority and is predominantly represented by the genus *Bifidobacterium*. Although less common, the phylum *Proteobacteria* (<1%) such as *Escherichia* and *Enterobacteriaceae* genera can also be found in gut microbiota. [5]

The gut microbiota has a symbiotic relationship with the host and contributes in several ways to the human metabolism, for instance, in the regulation of integrity and motility of gut barrier, with enzymes for the breakdown of polysaccharides, polyphenols and synthesis of vitamins, in the metabolism of bile acid and branched-chain aromatic amino acids, and in the modulation of immune and inflammatory response. [5-7]

However, an imbalance in gut microbiota homeostasis, characterized either by decreased bacterial diversity, decreased beneficial bacteria or increased pathobionts, known as dysbiosis, affect human health and is implicated in a wide range of diseases, including inflammatory bowel disease, [8] allergic disorders, [9] cancer, [10] obesity [11] and diabetes, specially T2DM. [12]

The process of dysbiosis can alter the permeability of intestinal barrier and increase the release of metabolic endotoxins. This might lead to chronic low-level inflammation, which is considered a possible trigger for impaired insulin resistance and development of T2DM. [13] Indeed, the gut microbiota profile of subjects with T2DM is different comparing with healthy individuals. Findings suggest that these subjects have an increase in the genera of *Ruminococcus*, *Fusobacterium* and *Blautia*, and a decrease in the genera of *Bifidobacterium*, *Bacteroides*, *Faecalibacterium*, *Akkermansia* and *Roseburia*. [14] This microbiota profile results in metabolic changes, such as an increase in lipopolysaccharides and inflammatory markers (e.g. tumour necrosis factor alpha and interleukin 6), and a decrease in interleukin 10, that promote metabolic endotoxemia and low-grade inflammation14. Additionally, there are also changes in the metabolites produced by the bacteria which may be implicated in disease development, including a reduction in short-chain fatty acids that are products of the fermentation of non-digestible carbohydrates and play an important role in the promotion of gut epithelial integrity, regulation of pancreatic β-cell proliferation and insulin biosynthesis. [15] An increase in trimethylamine, a bacterial-derived product of dietary choline, phosphatidylcholine or carnitine is also observed in disease development, later on trimethylamine is converted in the liver by flavin monooxygenase-3 in trimethylamine N-oxide, a metabolite that may enhance insulin resistance. [16]

Gut dysbiosis can be triggered by many lifestyle factors, such as diet, physical activity, age, antibiotics, and other medications. Out of these, diet is perhaps the single most important factor that has influence on gut microbiota by changing not only its composition but also its function which can modulate the susceptibility to disease. [3,17] In fact, evidence shows that typical western diet, rich in processed foods, rich in saturated fat, sugar and salt, and poor in fibre, enhance specific bacterial taxa characteristic of subjects with T2DM. [18] On the

other hand, a diet rich in fibre, phytochemicals, monounsaturated and polyunsaturated fatty acids promote intestinal barrier integrity and production of short-chain fatty acids with several beneficial effects. [18] This healthy pattern has similar characteristics to a dietary pattern that has an important role in the history of Portugal - the Mediterranean diet. [19] Mediterranean diet favours a high consumption of whole grains, fruits, vegetables, nuts, legumes, and fish, with olive oil as the main type of fat and water as a drink of choice. This pattern is also characterized by a moderate consumption of milk and dairy products, fermented foods, a low-to-moderate consumption of wine, a low consumption of meat and meat products and a low consumption of salt and sugary products. [20] The benefits of Mediterranean diet have been observed in the gut microbiota. In fact, several studies have shown an increase in microbiome diversity, an increase of specific bacteria taxa, such as *Bifidobacterium*, *Roseburia* and *Faecalibacterium* (that are decreased in T2DM), a decrease in *Firmicutes*, opportunistic pathobionts as well as inflammation, and an increase in short-chain fatty acids and insulin sensitivity. [21,22] Mediterranean diet has also been suggested to be one of the healthier eating pattern and recommended by different international dietary guidelines for prevention and management of T2DM, resulting in insulin sensitivity, glycaemic control and improvement in lipid profile. [23]

Thus, to date it well known that the Mediterranean diet has a positive impact in T2DM, but it is necessary to explore if gut microbiota plays a role as a mediator of the effect of this pattern in the management of T2DM. In top of this, until now, there is no scientific evidence derived from clinical trials that can support the existence of a diabetes-diet-microbiota triad.

> STUDY AIMS

This article intends to describe the protocol of MEDBIOME study, which aims to evaluate the role of gut microbiota in mediating the effect of the Mediterranean diet in T2DM, analysing if the adherence to this pattern results in changes on gut microbiota profile and if these changes are followed by an improvement on biochemical parameters related to glucose homeostasis (e.g. glycated haemoglobin and insulin resistance). Secondary aims of MEDBIOME study are a) to characterize the gut microbiota of Portuguese patients with T2DM, comparing it with other population groups and b) to identify which factors related to the Mediterranean diet impact the most in the management of T2DM.

> STUDY DESIGN

This 12-week study is a single-arm clinical trial conducted at *Faculdade de Ciências Médicas*/NOVA Medical School, *Universidade NOVA de Lisboa*. Participants have nutrition counselling sessions every 2 weeks where Mediterranean diet principles are constantly reinforced. Additionally, blood and stool samples are collected, body composition and blood pressure are analysed at baseline, after 4 weeks and at the end of the study. The PREDIMED questionnaire is used to evaluate Mediterranean diet adherence at baseline and 12 weeks after the intervention (Figure 1). The research protocol was approved by the Ethics Committee of *Administração Regional de Saúde de Lisboa e Vale do Tejo* (Ref. 016/CES/INV/2019) and *Faculdade de Ciências Médicas*/NOVA Medical School, *Universidade NOVA de Lisboa* (Ref. 55/2018/CEFCM), and is developed according to the Good Clinical Practice guidelines (Declaration of Helsinki) and applicable national law. Written informed consent was obtained from all participants before any study procedure was performed. This trial is registered at clinicaltrials.gov as NCT04403217.

> SAMPLE SIZE

The sample size was calculated in order to observe differences in glycated haemoglobin in subjects with T2DM after a Mediterranean diet intervention. A total of 30 patients will enter this study to observe a variation of 1.5% in glycated haemoglobin, considering a standard deviation of 1.4 [24] with a statistical power of 80% and a confidence level of 95%.

> ELIGIBILITY AND RECRUITMENT

Male and female subjects with diagnosed T2DM [according to the American Diabetes Association criteria (ADA)[1]] are recruited from different health centres of *ACES Lisboa Central*. Subjects aged between 40 and 80 years old and non-smokers willing and able to provide written informed consent are included in this study. Subjects are excluded if: diagnosed with diabetes before 40 years old, changes in oral glycaemic-control medications in the last 3 months, glycated haemoglobin levels under 6.4% or above 10%, under insulinotherapy and/or corticotherapy, triglycerides levels above 4.52 nmol/L (400 mg/dL), intake of antibiotics in the last 12 weeks, diagnosis of any digestive disease including functional bowel disorders such as irritable bowel syndrome.

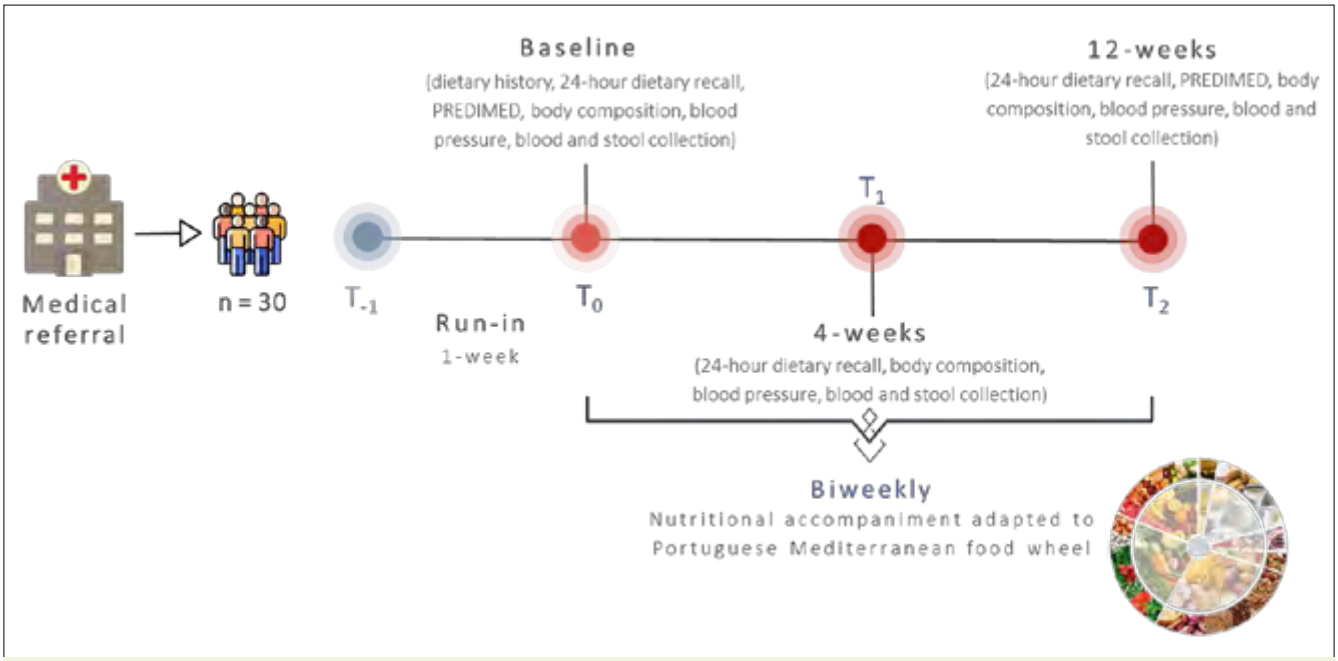

Figure 1 - Study design. Copyright of the Portuguese Mediterranean food wheel was authorized by the Directorate-General for Health.

> OUTCOMES

The main outcomes of this study are the changes in gut microbiota profile and glucose homeostasis (fasting glucose, insulin, glycated haemoglobin, insulin resistance and sensitivity, and β-cell function) in subjects with T2DM under a Mediterranean diet intervention from baseline to 4 weeks, and to 12 weeks.
Secondary outcomes are changes in diet quality, food intake, other biochemical parameters (e.g. lipid profile and hepatic enzymes), body composition, systolic and diastolic blood pressure from baseline to 4 weeks, and to 12 weeks.

> METHODS

Socio-demographic and Health Questionnaire

At baseline, information about participants education level, work situation, diagnosed diseases, food allergies and intolerances, and current medication is collected.

PREDIMED Questionnaire

Participants respond to a validated 14-item questionnaire composed by 12 questions on food consumption and frequency, and 2 questions on food intake habits considered principles of the Mediterranean diet. The final score ranges from 0 to 14, each question is scored 0 if the condition is not met or 1 if the condition is met. [25]

The PREDIMED score is used to evaluate the adherence to the Mediterranean diet. If the score is lower than 5 points the adherence is low, between 6 and 9 points is considered average adherence, and higher than 10 is high adherence. [26]

Body Composition and Blood Pressure Evaluation

Height is measured at baseline using a scale-mounted stadiometer to the nearest 0.5 cm, and waist circumference is measured using a measuring tape with a sensitivity of 0.1 cm at baseline, after 4 and 12 weeks, both measures are performed according to the protocol from *Directorate-General for Health*. [27] Body composition is determined while the participants are fasting for 10-12 hours using Inbody® model 770, according to manufacturer's procedures.
Systolic and diastolic blood pressure measurements are also performed according to the protocol from *Directorate-General for Health*. [28]

Nutrition Counselling Sessions

The participants have individualized nutrition counselling sessions provided by a registered nutritionist every 2 weeks.
In the first visit (at baseline), the nutritionist prescribes an individualized structured dietary plan according to the patient's dietary history. In order to do that, it is col-

lected the dietary history of the participant, asking about the usual intake of food (e.g. meat and alternatives, cereals, fruit and vegetables, dairy and 'extras'), schedules of the meals, favourite and deprecated foods, intake frequency of sweets, snacks, charcuterie products and alcoholic drinks, and used culinary methods. The 24-h dietary recall is collected in order to obtain detailed information about all foods and beverages consumed by the respondent in the past 24-hours, using a photographic manual for food quantification IAN-AF (*Inquérito Alimentar Nacional e de Atividade Física*). [29]
Subjects are asked about the use of sweeteners, in terms of type and quantity. This information is important because although some studies have shown an impact of sweeteners on gut microbiota and metabolic control, [30,31] the evidence is weak and clinical studies as MEDBIOME are needed to increase the knowledge in this area.
Participants are also asked about water consumption, and physical activity which is categorized according to type and frequency resulting in values of physical activity levels. [32]
To calculate the energy needs, the individual physical activity level is multiplied by the basal metabolism rate provided from the Inbody® results of the body composition. In the case of weight loss, it was decided to restrict energy requirements to 500-750 kcal/day, never lower than 1200 kcal/day for women and 1500 kcal/day for men. [33] The distribution of macronutrients followed the recommendations of ADA33 that recommend 26-45% of total energy from carbohydrates [34] and 20-30% of total energy from protein, [33] to increase satiety. For fats, there are no quantitative recommendations. All participants enrolled in this trial were instructed to follow a dietary plan with the same macronutrients' distribution: 40% carbohydrate, 25% protein and 35% fat. The importance of the quality of the fat, promoting monounsaturated and polyunsaturated fat, characteristic in Mediterranean diet is reinforced during nutrition counselling sessions. [33]
Fibre is also taken into account because of the different impact of each fibre type on health and gut microbiota, particularly soluble fibre that has a prebiotic effect. [18] Thus, a ratio 3 of insoluble fibre to 1 of soluble fibre is chosen for all the participants. [35]
The dietary plan is elaborated using the software Nutrium®. The foods chosen, the recommendations and culinary methods are based on the Mediterranean diet principles36 respecting the individual preferences. To reinforce Mediterranean diet, the participants also receive a poster with the Mediterranean food wheel36 and the principles to be followed.
In the subsequent nutrition counselling sessions, the nutritionist asks again about sweeteners and water consumption, and physical activity habits. It is also collected the 24-h dietary recall and promoted the Mediterranean diet. The participants are continuously revaluated, and the dietary plan is adapted accordingly.
The six 24-h dietary recall records collected during the study are inserted in the Food Processor Program® to analyse quantitatively and qualitatively the food intake of the participant.

Biochemical Parameters

Blood samples are collected while the participants are fasting for 10-12 hours in order to analyse glucose, insulin, glycated haemoglobin, total cholesterol, high-density lipoprotein cholesterol, low-density lipoprotein cholesterol, very low-density lipoprotein, aspartate aminotransferase, alanine aminotransferase, glutamine transferase, cholecystokinin, haemoglobin, iron, total protein, alkaline phosphatase, albumin, c-reactive protein, urea, creatinine, uric acid, sodium, potassium and chloride. Homeostatic Model Assessment for Insulin Resistance, Homeostatic Model Assessment for β-cell function and insulin sensitivity are calculated using fasting glucose and insulin. [37]

Gut Microbiota Analysis

Participants are asked to collect their own stool samples in sterile tubes with RNAlater provided by the research team. Subjects describe stool frequency and consistency of evacuation using the Bristol stool scale. [38] Stool samples are stored at −80°C until analysis. NZY Tissue gDNA Isolation Kit (NZY Tech) is used to extract and purify genomic DNA from stool samples, as previously described by Marques *et al.* [39] Libraries are prepared following the 16S Metagenomic Sequencing Library Preparation protocol from illumina (illumina; San Diego, CA, USA). A set of primers is used to capture the region V3–V4 of the bacterial 16S rRNA. [40] Samples are pooled and loaded into the illumina MiSeq System and, afterwards, sequenced using a 300PE combination according to manufacturer's specifications. Taxonomy of each sample is determined using the software Kraken and further improved using the software Bracken with our custom 16S database (GutHealth_DB) based on our own curation of 16S rRNA sequences and Greengenes 13_8 database (https://greengenes.secondgenome.com).

Statistical Analysis

Statistical analysis will be performed using SPSS V.25

software (IBM SPSS Statistics for Windows, IBM Corporation, Armonk, NY). Differences will be considered statistically significant when $p<0.05$.
The Kolmogorov-Smirnov test will be used to test normality of the distribution.
To analyse the differences for the continuous variables, from baseline, to 4 weeks, and to 12 weeks, different tests will be used depending on the data distribution. One-way ANOVA will be used for parametric variables and if there are significant interactions, the differences between pairs of means will be determined with a post-hoc test. For non-parametric variables, Friedman test will be used, and, if observed significant interactions, the differences between pairs of means will be determined by repeated Wilcoxon tests. Categorical variables will be analysed by X^2 test.
To analyse which factors are associated with a better management of T2DM different regression models will be used.

> EXPECTED RESULTS

It is expected to observe early changes (at 4 weeks) in gut microbiota profile compositions in the subjects with T2DM under Mediterranean diet intervention prior to significative modification in biochemical parameters. This will help to understand the mechanisms behind Mediterranean diet that have a positive effect in the health of these subjects, comprehending if weight management has a role or if gut microbiota is the main mediator. Additionally, it is expected with this study to obtain information on the gut microbiota profile of Portuguese subjects with T2DM and realize what features of the Mediterranean diet influence the most on this particular profile. It is likely that this study will show the importance of diet quality in the management of T2DM and will reinforce an approach focused on foods and total diet quality rather than on isolated nutrients quantity. Thus, it is also projected to validate a structured protocol of nutrition counselling sessions for subjects with T2DM to be implemented in the different health care units.
Some collected data from the participants who already completed the study have been analysed. Preliminary results indicate a reduction of glycated haemoglobin and a tendency in the reduction of Homeostatic Model Assessment for Insulin Resistance after 12 weeks of intervention. Results also indicate an increase in PREDIMED score and in the proportion of participants with high adherence to the Mediterranean diet after the intervention. These preliminary results are very promising and reinforce the positive impact that Mediterranean diet can have in the management of T2DM.

> DISCUSSION AND CONCLUSION

This clinical trial will be unprecedented due to investigating the effectiveness of a nutritional intervention, based on Mediterranean diet, in gut microbiota of subjects with T2DM. Promoting healthy dietary habits is important in the treatment of T2DM, individualized nutrition counselling sessions is an effective way to improve these habits and may result in the decrease of glycated haemoglobin up to 2.0% at 3-6 months. [34] Diet is also important in the modulation of gut microbiota, there is a strong evidence of how different nutrients can affect the human gut microbiota, especially those emphasized in Mediterranean diet, such as fibre, phytochemicals and unsaturated fatty acids that are associated with integrity of the gut barrier. [41] However, there are still lack of studies regarding the effect of total diet, in this case Mediterranean diet on intestinal microbiota. [42] In fact, evidence shows that considering isolated nutrients is a very reductionist approach and may not explain the effect on health because does not take in account the interaction between nutrients, [43] hence studying whole foods and diets is more important since it is a more realistic view. The few studies available intending to evaluate the adherence of Mediterranean diet as a whole and the influence of gut microbiota only analyse the dietary pattern in the context of specific diseases, for instance, chronic kidney disease, [44] non-alcoholic fatty liver disease [45] and psychiatric diseases. [46] Nevertheless, it is necessary to study other prevalent diseases as T2DM, that markedly affects the Portuguese population. There is also lack of information regarding the gut microbiota profile of Portuguese population, with or without the disease. The MEDBIOME study will fulfil these gaps of knowledge and identify what aspects within Mediterranean diet are more important in improving metabolic control of subjects with T2DM, describing the gut microbiota and how it modulates the effect of diet. This knowledge will allow to structure new therapeutic strategies for T2DM based on the gut microbiota. Additionally, gut microbiota could also be used to measure the metabolic control in T2DM, and because it may show modifications prior to any significant changes in the standard parameters (e.g. glycated haemoglobin), it may be used as an early predictor of metabolic state and of the impact of therapeutic interventions.
Nonetheless, this study has some limitations, including the absence of a control group, which makes it difficult

to establish a causal relationship. However, it is important to take in consideration that it would be unethical to have a group without any dietary intervention, since it is well established the importance of diet in the treatment of T2DM. Additionally, the study participants are referred from health centres without support from nutritionists, that is, without nutritional intervention, and therefore they can be considered their own "controls" at baseline. Another important limitation is that the dietary intake is self-reported, which can be inaccurate, though the nutritionist is trained to reduce this bias, using strategies such as the food quantification manual, food labels, and asking participants to take photos of the food whenever necessary. <

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Conflicts of interest/Conflitos de interesse:
The authors declare that they have no conflicts of interest/*Os autores declaram não ter conflitos de interesse*

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