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**Dietary metabolome profiles of a Healthy Australian Diet and a Typical Australian Diet: protocol for a randomised cross-over feeding study in Australian adults**

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**ABSTRACT**

**Introduction** Traditional dietary assessment methods such as 24-hour recalls and food frequency questionnaires rely on self-reported data and are prone to error, bias and inaccuracy. Identification of dietary metabolites associated with different dietary patterns can provide objective markers of whole diet patterns that account for metabolism and individual responses to dietary interventions. Additionally, few studies have investigated country-specific healthy and unhealthy dietary patterns using metabolomics. Therefore, the current study aims to identify urinary and plasma metabolites that characterise a ‘healthy’ (aligned with current national dietary guidelines) and an ‘unhealthy’ dietary pattern (Typical Australian Diet) in Australian adults.

**Methods and analysis** The Diet Quality Feeding Study (DQFS) is an 8-week cross-over feeding study that will recruit 40 healthy adults from the Hunter region (NSW, Australia). Data collected includes biospecimens (whole blood, urine, stool) for quantification of dietary metabolite biomarkers; questionnaires (medical history/demographic, physical activity, quality of life); physical measures (anthropometry, body composition, waist circumference, blood pressure, arterial pressure); skin carotenoids and dietary intake (24-hour recalls, food frequency questionnaire). Participants will attend the research facility every 2 weeks (end of the run-in, diet intervention and washout period) for collection of physical measures. All food will be provided to participants for each dietary intervention and participants will return to their usual diet during the run-in and washout periods. Targeted and untargeted metabolomics using liquid chromatography-mass spectrometry and/or proton nuclear magnetic resonance (1H-NMR) spectroscopy will be used to identify metabolites in biospecimens associated with dietary intake.

**Ethics and dissemination** This study is approved by the Hunter New England Human Research Ethics Committee (HNEHREC; 2022/ETH01649) and the University of Newcastle’s Human Research Ethics Committee (HREC; H-2022-0330). Findings will be disseminated to study participants, funding bodies supporting the DQFS, peer-review publications and presented at scientific conferences within the field of research.

**STRENGTHS AND LIMITATIONS OF THIS STUDY**

⇒ Usage of objective methods (ie, the metabolome) to compare dietary metabolites between healthy and unhealthy dietary patterns among Australian adults.

⇒ All food is provided for each diet intervention feeding periods thus optimising adherence to study protocol and standardisation between participants.

⇒ The study integrates multimics (metabolites, microbiome and human genetic risk scores) and the cross-over study design enables participants to serve as their own controls and accounts for potential inter-individual variation in the dietary metabolome. However, a potential limitation is residual carry-over effects if the washout period is insufficient.

⇒ The use of specific indicator foods and measurement of their respective metabolites to objectively monitor adherence to study protocol for each diet intervention.

⇒ Typical Australian Diet is based on apparent consumption data and therefore represents typical eating patterns of Australians which are suboptimal in comparison to current national dietary guidelines.

**Trial registration number** Australian New Zealand Clinical Trials Registry (ACTRN12622001321730).

**INTRODUCTION**

Dietary intake can be assessed using a range of methods such as food frequency questionnaires, 24-hour recalls and food records.1 These methods provide detailed information about an individual’s food and nutrient intakes, but typically rely on self-report, which is associated with risk of error and bias2–3 that varies by method and mode.4 Objective biomarkers of dietary intake provide an understanding of what people eat and how this may impact health outcomes. Current validated objective dietary intake biomarkers...
are limited to specific foods and nutrients, rather than whole dietary patterns. Current studies for predicting change in metabolites detected in biological fluids such as plasma and urine in response to dietary interventions, are not extensive. However, advances in technology have resulted in the identification of smaller metabolites that have potential to be used to characterise aspects of dietary intake and/or overall dietary patterns.8

The emergence of the research field of personalised nutrition has generated interest in identifying individual metabolic responses to consumed foods and/or specific dietary patterns. Dietary metabolomics is a field of research that identifies the presence of a range of metabolites in biological samples, including blood and urine, as potential biomarkers of foods or whole diet patterns, accounting for metabolic responses. Development of this field in humans requires understanding of the variation in individual responses to specific dietary patterns with an appreciation of the interplay between metabolism, the microbiome and genetics. Identification of biomarkers of dietary patterns will enhance understanding of the complexities of personalising diets for optimal health. Together with identification of genetic risk for chronic conditions, precision nutrition strategies based on this knowledge may lead to further personalisation of dietary interventions.7 A systematic review of 11 randomised controlled trials (RCTs) concluded that improvements in dietary intake following personalised nutrition advice compared with generic advice, and these improvements were evident at least one dietary outcome (eg, reduced sodium intake) for at least one time point.3

There is limited evidence that genetic information alone is effective in improving dietary intake and health behaviours.9 10 however, phenotypic characteristics such as drivers of taste preferences and metabolomics have been shown to be beneficial when included in the design of personalised nutrition advice.8 11 12 Eight of the 11 studies reported improvements in dietary intake following personalised nutrition advice compared with generic advice, and these improvements were evident in at least one dietary outcome (eg, reduced sodium intake) for at least one time point.3

Metabolic flux exhibits a dynamic pattern with rapid turnover of metabolites occurring within seconds to minutes, thus making the metabolome a more sensitive and accurate indicator of overall metabolism.13 Short-term variation in specific metabolite concentrations can result from a single meal, highlighting the relevance of biomarkers that reflect short-term dietary intakes and not only longer-term dietary patterns.6 Due to the variability in the half-life of metabolites, with some as short as 2 hours in the case of proline betaine following citrus consumption,12 it may be desirable to combine short-lived metabolites with self-reported dietary intake measurements, or with metabolites that have a longer half-life, to minimise intraindividual variation.13 14 To address this, clinical trials administering controlled feeding interventions where participants consume target foods or meals repeatedly over a specific duration (ranging from a few days to several months), can be employed. Clinical trials are invaluable for accurately evaluating dietary exposures and identifying objective markers that capture responses to diet interventions and compliance, particularly clinical trials that are short-term to medium-term in duration. A cross-over study design is favoured as it leverages each participant as their own control, effectively accounting for inter-individual variation. Furthermore, well-conducted clinical trials address confounding factors and measurement errors, while enabling closely monitored and controlled conditions according to a standardised protocol, rendering them more reliable with a higher degree of consistency than observational studies.

Our recent scoping review summarised the methodological components of human feeding studies designed to identify the diet-related metabolome in biospecimens in response to feeding interventions.15 We identified that study methods used to date were highly variable, as were the dietary patterns explored, sample collections and analytic techniques.15 Moreover, few dietary metabolomic studies have examined country specific recommended or ‘healthier’ dietary patterns for example compared with population usual or ‘unhealthy’ dietary patterns.16–18 These studies have identified plasma and urinary metabolites with potential to distinguish between ‘healthier’ and ‘unhealthier’ dietary patterns, however, require further evaluation. However, no dietary metabolomics interventions have been conducted in Australia that compare the current relatively unhealthy Typical Australian Diet to dietary patterns consistent with recommendations in Australian Dietary Guidelines.

Therefore, the aim of the current study research is to conduct a randomised cross-over feeding study to identify plasma and urinary metabolites that characterise ‘healthy’ and ‘unhealthy’ dietary patterns in healthy adults. The ‘healthy’ dietary pattern will align with the current Australian Guide to Healthy Eating19 recommendations, while the ‘unhealthy’ dietary pattern will align with a current Typical Australian Diet, modelled on the most recent apparent consumption data.20 It is hypothesised that distinct dietary metabolome patterns will be characterised for both a ‘healthy’ and ‘unhealthy’ dietary pattern.

METHODS

This protocol follows the 2013 Standard Protocol Items: Recommendations for Interventional Trials guidance on clinical trials (online supplemental file 1).

Patient and public involvement

Patients and the public were not involved in any way in the design of this protocol.

Open access

Study setting
Participants will be recruited from the Hunter region (NSW, Australia) via the following: flyers on public notice-boards; local pharmacies, pathologies, shopping centres (with permission); media advertising such as radio announcements and newspaper articles; Hunter Medical Research Institute’s (HMRI’s) media releases, social networks and volunteer register; and social media platforms. All data collection and measures for the study will be conducted by the research team at the Nutrition and Dietetics Clinical Research laboratory at the University of Newcastle, Callaghan, NSW, 2308 Australia. This study is expected to run from September 2022 until January 2024.

Eligibility criteria
Eligibility screening will use a two-phased approach. Interested volunteers will submit their minimum eligibility requirements via REDCap (https://www.project-redcap.org/). Volunteers who are eligible after the first phase will then be screened and interviewed by phone by a study investigator according to the screening checklist. Eligible volunteers include healthy males and females (≥18 years); currently taking no medications or who have been on stable medication therapy not known to influence study outcomes for the previous 3 months (eg, lipid-lowering medication); willing to adhere strictly to the dietary protocols for each 2-week intervention period; have access to a smart phone or tablet with access to the internet; in the Newcastle region for at least the entirety of the two feeding intervention periods; and received at least two vaccinations against COVID-19. Volunteers are ineligible if they have any food allergies or intolerances; uncontrolled hypertension as defined by the National Heart Foundation of Australia (systolic blood pressure ≥180 and/or diastolic blood pressure ≥110)21; type 1 diabetes or insulin-dependent type 2 diabetes; cancer receiving treatment; following strict diet regimen, for example, vegan, intermittent fasting; non-compliant during run-in phase; individuals who have lost or gained ≥5% body weight in the past 2 months; history of gastric ulcers, gastrointestinal disorders including severe digestive disorders such as inflammatory or irritable bowel syndrome; on medication known to influence study outcomes, for example, antibiotics, probiotics or prebiotics, hormone therapy, anti-inflammatory medications, corticosteroids, medications affecting metabolism; routinely taking any supplements known to influence the study outcomes for example, fish oils; currently participating in another research intervention trial involving dietary intervention and/or physical activity intervention; alcohol consumption greater than National Health and Medical Research Council (NHMRC) Guidelines to Reduce Health Risks from Drinking Alcohol (>10 standard drinks per week); pregnant or breast feeding.

Study design
This study is an 8-week, randomised, cross-over feeding study consisting of a 2-week run-in period (habitual/usual diet) followed by a 2-week dietary intervention period, then a 2-week washout period (habitual/usual diet), followed by a final 2-week dietary intervention period. Allocation to dietary intervention will be randomly allocated using a computer-generated list. Participants will be randomly assigned to one of two dietary interventions, a ‘Healthy Australian Diet’ or a ‘Typical Australian Diet’, which they will consume daily for 2 weeks. After a 2-week washout period, they will switch to the other dietary intervention. Blinding of participants and investigators for outcome assessments after the first baseline visit is not feasible due to the nature of a feeding intervention. A biostatistician independent to the research team who is blinded to the allocation, will be consulted, and involved in the statistical analysis of biospecimens. Sample size determination was informed by our recent scoping review,15 whereby majority of the cross-over studies had between 20 and 40 participants. Therefore, the target sample for the current study will be to achieve a minimum of 20 participants who complete all study arms and study procedures for biological sample collection after dropout or withdrawals, with an equal balance of males and females, for inclusion in the final analyses.

Dietary interventions
The Healthy Australian Diet provides foods to adequately meet the recommended servings of the five core food groups according to the current Australian Dietary Guidelines for adults.19 The diet will also aim to meet the acceptable macronutrient distribution ranges and some emphasis will be given to specific nutrient targets such as fibre, added sugars and sodium. For fruits and vegetables, those rich in beta-carotene (eg, carrots, pumpkins, tomatoes, red capsicum and sweet potato) will be emphasised during this dietary pattern. The inclusion of beta-carotene-rich foods in this dietary pattern serves to create a clear distinction from the comparator dietary pattern (Typical Australian Diet) in terms of carotenoid intake. This distinction is particularly relevant for analysing plasma samples and, more importantly, for detecting differences in measured skin carotenoid levels. Characteristics of the food choices will reflect a high diet quality consistent with recommendations for the five core food groups in the Australian Dietary Guidelines.

The typical Australian Diet is based on the most recent data on the nutritional profile of Australians,20 from the Apparent Consumption of Australians report, which is the amount of food and non-alcoholic beverages purchased from food and retail sectors, for example, major supermarkets, smaller outlets, delis, fresh food markets, butchers, etc from July 2020 to June 2021.20 Fruits and vegetables that are low in beta-carotene (eg, white potato, onion, cauliflower, pears) will be emphasised during this dietary pattern. Characteristics of the food choices will reflect a poor diet quality consistent with the Apparent Consumption of Foods Among Australians in 2020–202120 (table 1).
A repeated 7-day menu cycle including all meals and snacks will be prepared and provided for each dietary intervention to ensure participants maintain habitual caloric intake determined during the run-in period to ensure weight stability. All meals and snacks will be sourced from a supermarket chain and assembled by the research team specifically for the study and participants will collect the food from the nominated supermarket chain closest to their residence. A ‘study meal box’, reminder meal cards and takeaway/eating out resources respective for each dietary intervention will be provided to participants to assist with consumption of study foods away from the home. The entirety of the intervention diets (ie, three main meals and snacks per day) are provided to participants. Participants will provide their own tea, coffee, fluids and will be instructed to record their intake of all beverages and any non-study foods during each dietary intervention phase using the Easy Diet Diary app (Xyris Pty Ltd., Brisbane, Queensland, Australia). Written records will be used for participants who do not wish to use the smartphone application. Participants will supply their own meals and foods during the run-in and washout periods (usual diet).

Adherence to the protocol will be examined during the 2nd week run-in period prior to the feeding interventions and will be reassessed throughout the feeding intervention periods. Participants who do not adhere to the study procedures and data collection (outlined in table 2) during the run-in period will be withdrawn from the study prior to randomisation. Dietary adherence will be assessed throughout the entire study using: three 24-hour recalls per dietary intervention phase, and record keeping of habitual beverage (including alcohol) consumption and food consumed out of the home for example, takeaway, restaurant or café using the Easy Diet Diary app. Presence of plasma metabolites for respective indicator foods for each diet intervention will also be monitored to assess dietary adherence retrospectively. Participants will receive 150 mL orange juice to
consume daily at lunchtime in both dietary phases. This food was chosen to monitor compliance and dietary adherence as its metabolite proline betaine is well documented in previous feeding studies.\(^ {22-25} \) 2,5-dihydroxybenzoic acid, 3,5 dihydroxyphenylpropionic acid,\(^ {26,27} \) alkylresorcinols\(^ {28,29} \) and picpeolic acid betaine\(^ {30} \) will signify daily wholegrain bread/cereal intake during the Healthy Australian Diet, and theobromine will signify daily chocolate intake in the Typical Australian Diet.\(^ {22,31} \)

### Study procedures

Eligible participants will attend four 60–90 min clinic appointments after an overnight (12 hours) fast, refraining from vigorous physical activity or alcohol consumption for at least 24 hours prior to the appointment. Study participation involves completing various questionnaires, collection of biospecimen samples (fasted blood and urine), faecal sample (collected within 3 days of the study appointment), blood pressure, arterial pressure, skin carotenoids, anthropometry and body composition measurements at the nominated timepoints (table 2). Participants will attend the research facility at the end of the run-in period, end of each dietary intervention phase, and end of the washout period and will be provided with light refreshments at the conclusion of each appointment. Participants will also undergo two short (10–15 min) virtual check-in appointments with study researchers at weeks three and seven via telephone/video call to monitor compliance and adherence to

<table>
<thead>
<tr>
<th>Table 2</th>
<th>Summary of data collection at each time-point</th>
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<tr>
<td></td>
<td>Run-in period</td>
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<td>Week 1</td>
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<tr>
<td>Eligibility screening</td>
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<tr>
<td>Informed consent and randomisation</td>
<td>X</td>
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<tr>
<td>Medical and demographic questionnaire</td>
<td>X</td>
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<td>AES FFQ</td>
<td>X</td>
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<tr>
<td>Active Australia Survey</td>
<td>X</td>
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<tr>
<td>Social desirability, approval and quality of life</td>
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<tr>
<td>24-hour recall</td>
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<tr>
<td>Visual Analogue Scale appetite/hunger</td>
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<tr>
<td>Urine sample</td>
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<td>Blood sample</td>
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<td>Faecal sample</td>
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<td>Height</td>
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<td>Body composition</td>
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<td>Blood pressure</td>
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<td>Skin carotenoids</td>
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<td>Virtual check-in with study staff</td>
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<tr>
<td>Compliance monitoring: Easy Diet Diary—recording of uneaten food items and additional non-study foods eaten</td>
<td>X (Daily)</td>
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<tr>
<td>Compliance monitoring: biospecimen food indicator measures</td>
<td>X</td>
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<tr>
<td>Adverse event reporting</td>
<td>X</td>
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X denotes that the data were collected at the respective timepoint.

AES FFQ, Australian Eating Survey Food Frequency Questionnaire.
study protocol, monitor adverse events, and have an opportunity to ask any questions.

**Clinical assessments and laboratory investigations**

**Medical history, demographics, physical activity, social desirability/approval and quality of life**

Demographic data will be recorded at baseline for medical history, current medication/supplement usage, smoking status, alcohol consumption, recent changes to diet or physical activity, employment, education and ethnicity. At weeks 4, 6 and 8 participants will be asked if there have been any changes in the previous 2 weeks in health, medications and supplement usage. The validated Active Australia Survey questionnaire will be completed at all study phases. The Marlowe-Crowne Social Desirability Scale, a 33-item measure of individual-level social desirability bias will be completed at baseline. This questionnaire explores individual-level social desirability bias, such as the need to respond in a socially and culturally acceptable way as well as perceived social approval. The EuroQol-5D (EQ-5D) will be used at baseline during the run-in period to assess participants’ quality of life.

**Dietary intake assessment**

The Automated Self-Administered 24-hour—Australia (ASA24 AUS) Dietary Assessment Tool will be used to assess dietary intake three times during each dietary feeding period to quantify intakes during each feeding period and to inform adherence. The first recall falls within the first week of each phase, the second and the third recalls fall within the second of the phase aligned with biospecimen collection. The ASA24 is an online, multiple pass, self-administered recall tool reporting information on all food and beverages consumed in the preceding 24 hours. The ASA24 uses the AUSNUT 2011–2013 food composition database and quantified intakes of up to 65 nutrients for each ASA24 conducted.

The Australian Eating Survey (AES) is a 135-item self-administered validated semi-quantitative FFQ that measures usual food and nutrient intakes over the past 3 months. The AES will be used to measure participant’s habitual dietary intake and diet quality at baseline (the previous 3 months), as well as examine changes in dietary intake and diet quality after each study period (the previous 2 weeks) that is, end of each diet intervention phase and washout period. The AES uses the AUSNUT 2011–2013 food composition database and quantified intakes of up to 65 nutrients for each ASA24 conducted. Diet quality will be assessed using the Australian Recommended Food Score (ARFS), which uses a subset of 70 questions from the AES. ARFS range from 0 to 73, with higher scores reflective of greater diet quality. Both the AES and ARFS have been validated in Australian populations from the age of 2 years and is based on 15 years of research.

A 10-point Visual Analogue Scale will be used to measure hunger and satiety throughout each study period (run-in, both feeding phases and washout). Participants will log hunger/appetite alongside each 24-hour recall, and this will be provided via a link with the ASA24 recall.

**Anthropometry and body composition**

Height, waist circumference, body mass index (BMI), body composition, arterial stiffness, systolic and diastolic blood pressure will be collected by the research team. A tensile tape measure positioned across the abdomen at its narrowest point between the lower costal (10th rib) border and the top of the iliac crest, perpendicular to the long axis of the trunk. If there is no obvious narrowing, the measurement is taken at the mid-point between the lower costal border and iliac crest. Measurements will be recorded to the nearest 0.1 cm. If values differ by more than 1% of waist circumference total, repeat measure. An average of two waist measures will be taken as the final value for analysis. Body composition will be measured via bioimpedance using InBody (Inbody 270; Biospace Co, Seoul, Korea) to quantify fat, fat mass, lean muscle mass, fat free mass and water weight. Participants will be instructed to wear light clothing, removing socks, shoes and all metal items including jewellery or belts.

**Skin carotenoids**

Skin colour will be measured using a CM700D spectrophotometer (Konica Minolta) with an 8 mm diameter aperture, 2° observer angle and illuminant D65. The spectrophotometer will be white point calibrated for each participant at each measurement session. Skin colour (CIE L*a*b* values, where positive L*, a* and b* values represent lightness, redness, and yellowness, respectively) will be recorded for each participant. The measurements will be repeated three times at each site (palm, inner and outer arm) and the average recorded. Body locations will be selected according to anatomical landmarks using the International Society for the Advancement of Kinanthropometry standards for anthropometric assessment. Data will be analysed using spectrophotometer software (SpectraMagic NX).

**Blood pressure and arterial stiffness**

Participants will have sat relaxed and comfortably for 5 min prior to blood pressure and arterial stiffness collection. Participants will be seated with legs uncrossed and feet are placed evenly flat on floor and asked to maintain even breathing throughout the tests. Blood pressure will be measured in the seated position and three serial measurements with 1 min rest in between will be collected from a supported left arm. The first measurement will be discarded and an average of the remaining two will be considered as the final measurement. Arterial stiffness and blood pressure will be measured using the Uscom BP+ supra-systolic oscillometric central blood pressure device with the cuff positioned around the upper arm at the strongest pulse signal location. An initial test measurement will be performed to ensure a good signal is obtained, after which the participant will sit quietly for 5 min to ensure blood pressure is not falsely high. A second
reading will then be collected with a 2 min rest before taking any other measures (if required).

Biological measures
A fasted blood sample will be collected by a qualified phlebotomist. Whole blood collected into EDTA tubes will be divided into plasma, red blood cells (RBCs) and buffy coat aliquots according to standard operating procedures approved by the University of Newcastle Institutional Biosafety Committee and stored at −80°C until ready for analysis. RBC aliquots will be used to assess RBC fatty acid composition and concentrations by employing published methods we have used previously. Polygenic risk scores for relevant disease outcomes will be generated for genotyped samples using the respective genome-wide association studies summary statistics, in collaboration with genetic research colleagues from the University of Newcastle. In each case, DNA will be extracted from buffy coat layer of blood prior to genome-wide single nucleotide polymorphism analysis using microarray or low-pass whole genome sequencing. This established prediction method integrates known DNA variants into a statistical model, enabling the assessment of an individual participants’ genetic susceptibility to a given disorder across the genome.

Spot urine samples will be collected mid-stream in sterile containers and aliquoted for storage at −80°C according to standard operating procedures in order.

Faecal samples will be collected within 3 days of individual clinic appointments using at-home collection kits, which includes Zymo Research FecesCatcher catchment paper and DNA/RNA Shield™ faecal collection tubes. Clear instructions, both verbal and written, will be provided to participants regarding how to collect the samples correctly. Participants will be instructed to store the samples at ambient temperature and return them either at their next clinic appointment or via prepaid reply post bags, whichever is more convenient and time-lower. According to the manufacturer’s specifications, faecal samples can be viably stored at ambient temperatures for at least 30 days. On receipt, faecal samples will be stored at −80°C until ready for analysis. The microbial DNA extracted from faecal samples will undergo whole genome metagenomics sequencing and will be conducted by Microba Life Sciences Pty Limited, Brisbane, QLD, Australia. Taxonomic assignment will be conducted using MetaPhlAn, and functional reconstruction will be performed using HUMAnN pathways with default settings carried out by trained bioinformaticians. Gene annotation will be accomplished by employing KEGG and Metacyc pathways.

Metabolite identification
Untargeted and targeted metabolomics using liquid chromatography-mass spectrometry and/or proton nuclear magnetic resonance (1H-NMR) spectroscopy will be used to identify metabolites in plasma and urine associated with the Healthy Australian Diet and Typical Australian Diet pattern interventions. Metabolite identification will be guided by available standards, currently available analysis packages, previous research and reviews and data captured in online databases such as FooDB (www.fooddb.ca).

Statistical plan
Descriptive analyses of the study cohort’s measured parameters will be presented as mean/SD for normally distributed variables and median/IQR for non-normally distributed variables. Data will be evaluated by mixed model analysis of variance (ANOVA) with sequence and interaction between diet and start/end of treatment periods as fixed effects, and participants within sequence and visit as random effects. Least square means will be estimated for the start (Healthy Australian Diet-week 0, Typical Australian Diet-week 0) and end values (Healthy Australian Diet-week 2, Typical Australian Diet-week 2) for each diet period for outcomes of interest (eg, dietary intake, metabolites, microbial function, and composition outcomes, genetic, cardiometabolic risk factors, etc). Least square means and confidence intervals will be calculated for the differences between Healthy Australian Diet/week 0, Typical Australian Diet/week 0, Healthy Australian Diet/week 2 and Typical Australian Diet/week 2, as well as for the net difference between the diets, that is (Healthy Australian Diet/week 2−Healthy Australian Diet/week 0)−(Typical Australian Diet/week 2−Typical Australian Diet/week 0) for study outcomes.

Various visual techniques, including principal components analysis, partial least squares discriminant analysis plots, VIP/S plots and random-forest plots may be undertaken to detect a panel of metabolites that represent the two dietary interventions. Feature selection based on permunational multivariate ANOVA will estimate the contributions of diet, phenotypic, genetic and microbial data to inter-individual variations of the whole plasma and/or urinary metabolome. Spearman correlation coefficients (rs) will be used to examine the relationship between changes in the cardiometabolic-related biomarkers (and other health markers/outcomes) and the significantly differentiated metabolites as well as the relationship between Healthy Australian Diet and Typical Australian Diet interventions and biological markers/metabolites. Linear regression will be employed to assess the relationship between individual metabolites and dietary intake, with crude regression models as well as multivariable regression models adjusted for potential covariates such as age, BMI and sex will be conducted. As this is an exploratory intervention, adjustment will be conservative, however, additional confounders identified may be included in these models where necessary. This study will also employ advanced biologically driven machine learning techniques to conduct multilevel computational analysis of dietary, clinical, microbial and genetic data to explore potential non-linear relationships between the input and output variables of the model. Several other approaches will be used to examine
associations between measured parameters and the faecal microbiome, to identify all phyla, genera, species or pathways with study outcomes including cluster-based analysis, correlations and regression analysis. P values will be adjusted for multiple comparisons using the Benjamini-Hochberg procedure where relevant.

Data availability statement
Data from the study may be made available in the future for collaborative research questions. Such requests must be authorised by the principal investigators and the appropriate Human Research Ethics Committee.

Ethics and dissemination
This study has been approved by the Hunter New England Human Research Ethics Committee (HNEHREC; 2022/ETH01649) and registered with the University of Newcastle Human Research Ethics Committee (HREC; H-2022-0330). Subjects will voluntarily confirm their willingness to participate after being informed in writing and verbally, of all aspects of the study that are relevant to the subject’s decision to participate. Written informed consent is mandatory for study enrolment.

Participant safety
All aspects of data collection and recruitment will be undertaken and managed by appropriately trained and experienced research investigators with necessary certification to undertake clinical measures including a trained and certified phlebotomist to conduct venepuncture blood sample collections. Study staff will conduct these activities according to standard operating procedures for collection of blood from the vein, urine and faecal sampling which have been approved by the University of Newcastle Institutional Biosafety Committee. Data will be collected through REDcap and stored securely via the HMRI server. Once data collection closes, the REDCap survey will be closed and the data will be downloaded and stored in the University of Newcastle online cloud network (OneDrive) in an encrypted file that is password protected.

Unexpected findings during examinations
The study governance committee (CC, EC, JJAF, JS) will meet weekly and discuss any unexpected clinical findings and agree on whether a clinical result requires immediate action via forwarding of results to the consenting participant’s general practitioner (GP) for routine clinical follow-up and care. Where necessary, participants may be contacted directly by the study investigators and their participation terminated if indicated by necessary GP follow-up/intervention.

Dissemination
Study findings will be disseminated as published manuscripts in peer-reviewed journals, scientific communication via conference abstracts, presentations, posters and potential undergraduate/postgraduate research theses. Reports developed to the funding body(s) will also be conducted as part of dissemination. Members of the research team will have publishing and authorship rights in accordance with the NHMRC Australian Code for the Responsible Conduct of Research and as described in existing research agreements.

DISCUSSION
This is the first human feeding study to investigate and identify plasma and urinary metabolites that characterise both the current Typical Australian Diet and a dietary pattern consistent with national dietary guidelines for Australians. The authors anticipate that study results will identify a metabolomic profile that characterises both ‘healthy’ and ‘unhealthy’ dietary patterns in a sample of healthy Australian adult males and females. The primary strength of this study is the utilisation of indicator foods (including orange juice, wholegrains and dark chocolate), with known metabolites (such as proline betaine; 2,5-dihydroxybenzoic acid, 3,5 dihydroxyphenylpropionic acid, alkylresorcinols; and theobromine, respectively), which will be used to objectively monitor adherence to the dietary prescriptions. Other strengths include usage of objective data collection methods such as biospecimen collection, blood pressure and arterial pressure and body composition, as well as provision of all foods to participants during the two feeding phases in order to reduce heterogeneity for the comparison of the dietary metabolome between the two different dietary patterns. This will serve as a basis for enhancing understanding of metabolic signatures for two distinct, nationally relevant, dietary patterns. The cross-over study design allows for participants to serve as their own control and account for sources of inter-individual variation. Moreover, how these metabolic signatures differ between individuals, as well as relationships with specific polygenic genetic risks for chronic disease pathways may potentially be identified. This will provide greater insight into the relationship between dietary patterns to inform personalised nutrition strategies in relation to chronic disease risk in the future. This study is not without limitations. These include the potential for residual carry-over effects if the washout period is not long enough. Second, the Typical Australian Diet is based on apparent consumption data from food purchasing habits. The most recent National Nutrition Survey is approximately 10 years old and therefore was not deemed recent enough to inform the current ‘Typical Australian Diet’. Overall, findings from the current study and learnings from the methodological implementation of a human feeding intervention designed to identify the diet-related metabolome, will inform future studies examining personalised dietary interventions for the treatment and/or management of chronic conditions, including type 2 diabetes and cardiovascular disease.

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