Title	**Randomised controlled trial to investigate *N*-nitrosamine formation after meat intake: protocol for Study 1 of the Nitrate INFORMER Studies** (Nitrate INFORMER study: **I**s **N**itrosamine **FORM**ation d**E**penent on sou**R**ce)
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Hypothesis	Endogenous *N*-nitrosamine levels will be higher after ingestion of meat with added nitrate compared to meat without added nitrate.
Aims	***Primary aim***: To investigate nitrate to *N*-nitrosamine formation after ingestion of meat with and without added nitrate.
Background and significance	Nitrate is a controversial component of vegetables, meat, and drinking water. The now well-established benefits of nitrate, through the enterosalivary nitrate-nitrite-nitric oxide (NO) pathway[1], on cardiovascular risk factors and long term cardiovascular disease risk [2, 3] are tarnished by a continuing concern about a link between nitrate ingestion and cancer. This can result in misguided advice to avoid consumption of high-nitrate leafy green vegetables by both the media and the scientific literature. A recent media headline stated, "Cancer alert over rocket: trendy salad leaves exceed safe levels of carcinogenic nitrates in one in every ten samples". One scientific review stated, "the presence of nitrate in vegetables, as in water and generally in other foods, is a serious threat to man's health" [4]. Controversy in the literature, and gaps in our knowledge are leading to confusing messages around vegetables that may play a critical role in cardiovascular health. Cancer in relation to nitrate consumption has been a concern among researchers since the 1970's when it was demonstrated that dietary nitrate had the potential to form carcinogenic *N*-nitrosoamines[5, 6]. *N*-nitroso compounds (NOCs) are highly carcinogenic in laboratory animals[7] and endogenously formed NOCs are associated with cancers in humans, specifically gastric cancer, oesophageal cancer, colorectal cancer and bladder cancer[8]. The International Agency for Research on Cancer/World Health Organization (WHO) has summarized that: "Ingested nitrate or nitrite under conditions that result in endogenous nitrosation is probably carcinogenic to humans (group 2A)" [9]. However, convincing epidemiological evidence for an increased risk of human cancer is still lacking. A review of all studies in 2003 by the Joint Food and Agriculture Organisation (FAO) and WHO Expert Committee on Food Additives (JECFA) found no evidence of an increased risk of cancer with dietary nitrate consumption[10]. The major dietary sources of nitrate are vegetables, meat, and drinking water. Nitrate from vegetables accounts for approximately 80% of dietary nitrate intake[11]. Nitrate, being an important part of the nitrogen cycle, plays an important role in the growth and development of plants. Vegetables such as lettuce, rocket, spinach and beetroot have high concentrations of nitrate while other vegetables, such as peas, tomato and potato have low concentrations of nitrate[12]. Meat is a dietary source of nitrate due to

	its use as a food additive, primarily to improve the microbiological safety of cured meat and extend its shelf life[13]. Nitrate in drinking water is a contaminant, originating primarily from the agricultural use of fertilizers and manure, as well as wastewater treatment and septic tanks[14]. Source of nitrate could be a crucial factor determining whether the consumption of nitrate is linked with beneficial (such as improving cardiovascular health) versus harmful (potentially carcinogenic *N*-nitrosamine formation) effects. One reason for which effects could differ between source is that, unlike meat and water-derived nitrate, vegetables contain high levels of vitamin C and/or polyphenols that may inhibit the production of *N*-nitrosamines[15]. To the best of our knowledge, no study has investigated the formation of *N*-nitrosamines after consumption of the different sources of nitrate in humans. Our overarching objective is to measure *N*-nitrosamine formation after intake of the major sources of nitrate (meat, vegetables, water, or a combination). Currently, guidelines on nitrate intake do not differentiate between dietary sources of nitrate. The results of these randomised, controlled, cross-over clinical trials will inform international policymakers on safe source-dependent nitrate levels in the environment and diet, particularly the Joint FAO/WHO Expert Committee on Food Additives (JECFA) and European Food Safety Authority (EFSA) guidelines. Currently, EFSA has set the Acceptable Daily Intake (ADI) for nitrate at 3.7 mg/kg (approximately 260 mg for a 70 kg adult), regardless of source[16]. ***The first of these studies will investigate N-nitrosamine formation after intake of meat with and without added nitrate***.
Study setting	All Nitrate INFORMER study visits will take place at the Royal Perth Hospital Research Foundation, Nutrition and Health Innovation Research Institute, School of Medical and Health Sciences, Edith Cowan University, Perth, Western Australia.
Participants	We will recruit 25 healthy, ambulant, community-dwelling men and women aged between 18 to 70 years old and with no history of major chronic disease, from the Perth general population. Exclusion criteria. Individuals volunteering to participate in the study will be excluded according to the following criteria: current or recent (<12 months) smoking; body mass index (BMI) <18 or > 35 kg/m^2; systolic blood pressure > 160 mmHg ; diastolic blood pressure > 100 mmHg;; any major illness such as cancer, psychiatric illness, diagnosed diabetes; use of any of the following medications: statins, antihypertensives, nitric oxide donors, antithrombotic medication, anti-coagulant medication, anti-arrhythmic drugs, beta-blockers, regular aspirin use, regular proton pump inhibitor use; alcohol consumption > 30g/day; women who are pregnant, lactating, or wishing to become pregnant during the study; use of antibiotics within the previous 12 weeks of the study; regular use of mouthwash and not willing to cease mouthwash use for the duration of the study; participation on other research studies; major GIT condition e.g. Crohn's disease and inflammatory bowel disease; and inability or unwillingness to follow the study protocol.
Recruitment	Eligibility to participate in the study will be assessed via a two-step process: **1. Telephone screening.** A telephone questionnaire will be used to screen volunteers according to the study's inclusion / exclusion criteria. Volunteers who are eligible to participate will be sent an information pack explaining the study in detail and a clinic assessment visit will be scheduled for further physical screening. 2. **Clinic assessment visit.** Prior to commencement of physical screening, volunteers will be required to sign an informed consent form. Physical screening will comprise of anthropometric measurements (height and weight), blood pressure, an electrocardiogram, and a fasting blood test for measurement of lipids (cholesterol, triglycerides, LDL cholesterol and HDL cholesterol) and glucose. Blood pressure will be assessed using a CARES-CAPE^{TM} Dinamap v100 Vital Signs Monitor (GE Health-care, Buckinghamshire, UK). Participants will rest in a supine position for 10 minutes prior to having 5 blood pressure and heart rate measurements performed at 2-minute intervals. The first measurement will be discarded and the mean of the remaining 4 measurements will be used to calculate resting blood pressure.

Study design A crossover study design will be used with a 1-week washout period between interventions. Participants diet for the day before and the day of the intervention will be standardised (food choices on visit 1 will be matched for visit 2) as follows:

For the day before the study visit:

- A choice of 3 breakfasts: cornflakes with milk; rice krispies with milk; white bread toast with eggs.
- Lunch will be provided comprising pumpkin soup and white bread/or rice
- Dinner: a low nitrate vegetarian meal
- All water will be provided
- Participants will be asked to refrain from drinking coffee [17] and any alcoholic beverage [18] and do any exercise 24 hours prior to their study visit.

For the day of the study visit:

- Participants will arrive at the study unit fasting
- Breakfast and lunch will comprise the intervention
- Participants will be provided with a standardised dinner: a low nitrate vegetarian meal
- All water will be provided
- Participants will be asked to refrain from drinking coffee [17] and any alcoholic beverage [18] and do any exercise for 24 hours from the time of the first intervention.

Dietary interventions

1. *Meat with added nitrate:* 50 g salami and 35 g ham on white bread sandwich at breakfast and lunch.

   This intervention will allow us to determine both endogenous formation of *N*-nitrosamines as well as *N*-nitrosamines present in the commercially prepared meat.

2. *Meat without added nitrate*: 65 g Pork mince on white bread sandwich at breakfast and lunch Nitrate is not an allowed additive in pork mince[13].

   This intervention will allow us to determine if there is endogenous formation of *N*-nitrosamines as well as *N*-nitrosamines present in the prepared meat due to the natural content of nitrate in meat.

3. *Control*: low nitrate vegetable protein burger on white bread. Protein content matched to interventions 1 and 2.

Allocation

Sequence generation. The sequence of intervention allocation will be generated via block randomisation using computer-generated random numbers. Random block sizes of 2 and 4 will be used.

Concealment mechanism. Fifty randomly generated sequences of the interventions for each of the study participants will be printed on separate pieces of paper and sealed in opaque envelopes, numbered 1-50, by a study investigator not involved in performing the intervention, the data collection or the data analysis.

Implementation. Once a participant is deemed eligible and enrolled in the study, the study coordinator will contact the study investigator responsible for randomisation and intervention allocation to obtain the next available envelope and randomly generated intervention sequence.

Blinding

Given the nature of the interventions, participants, and the investigators responsible for delivering the interventions will be unblinded throughout the trial. However, all researchers performing the laboratory analyses and data analyses will be blinded to the interventions that the participants received until after the data analysis has been performed.

Participant timeline

Prior to the first clinic visit, each participant will complete a food frequency questionnaire (FFQ) to assess background habitual diet.

At each clinic visit (**Figure 1**), a baseline urine and faecal stool sample will be collected for measurement of *N*-nitrosamines. Prior to the intervention participants will be instructed to void their bladder into baseline urine collection container. After the first intervention all urine for the subsequent 24-hour period will be collected for measurement of *N*-nitrosamines, nitrate, and nitrite. A faecal stool will be collected for measurement of *N*-nitrosamines, nitrate, and nitrite.

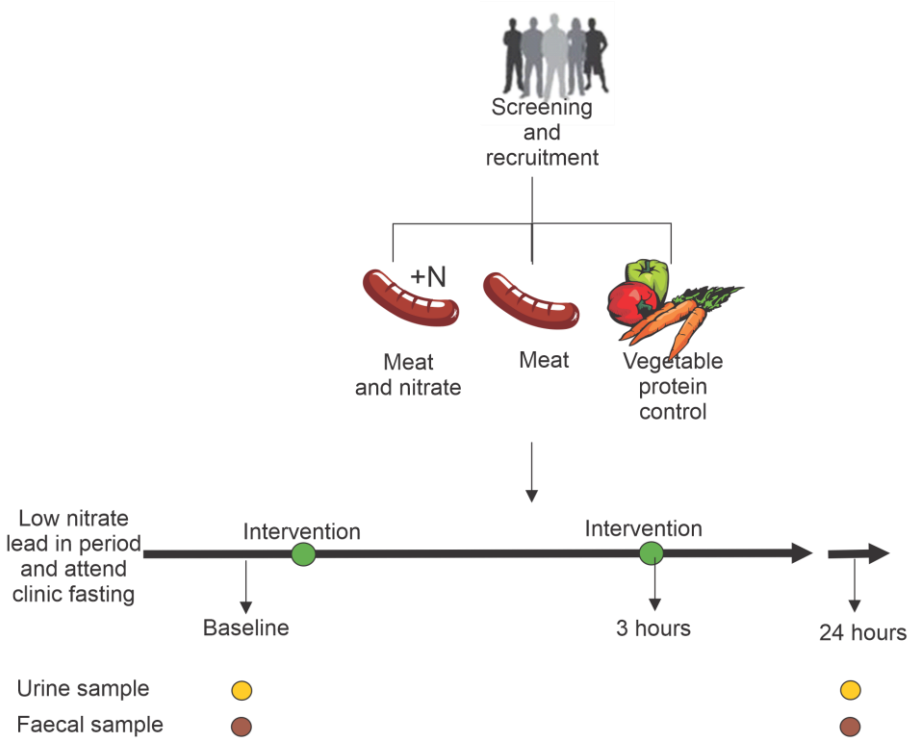

Figure 1: Schematic representation of study day timeline

Outcomes

Primary outcomes

Level of *N*-nitrosamines (*N*-Nitrosodimethylamine, NDMA; *N*-Nitrosomethylethylamine, NMEA; *N*-Nitrosodiethylamine, NDEA; *N*-Nitrosopiperidine, NPIP; *N*-Nitrosomorpholine, NMOR) in urine and faecal stool post intervention.

Assessments

Urine and faecal stool collection. A urine sample will be collected at baseline, and from intervention up until 24 hours post-intervention. For the baseline sample, participants will be provided with a sterilized container and instructions to discard the first urine sample of the day and then collect all urine until the intervention which will be brought into the clinic. Participants will be instructed to drink 250 ml water on waking, For the 24-hour urine samples, participants will be provided with sterilized containers and instructions to collect all urine until 24 hours post intervention. Urine aliquots will be frozen at -80°C until analysis. A stool sample will be collected at baseline and for the 24 hour period post the first intervention. Participants will be provided with instructions and a stool sample collection pack (collection bags, cable ties, large zip lock bags, freezer ice blocks and a designated cooler bag for transport). Collected stool samples will be weighed and frozen at -80°C until analysis.

***N*-nitrosamines**. Nitrosamines in the interventions, urine and stool samples will be measured by gas chromatography mass spectrometry (GCMS). Volatile nitrosamines will be extracted with dichloromethane and are well separated by gas chromatography. Identification will be by retention time and unique molecular ions for each of five common nitrosamines (**Table 1**). Quantitation will be made

by using deuterium labelled internal standards, *N*-nirtrosodimethylamine–D6; N-nitrosodiethylamine D10; N-nitrosomorpholine D8 (Cambridge Isotopes) and N-nitrosopiperidine D10 (Toronto Research Chemicals). The most commonly detected nitrosamines are NDMA and *N*-Nitrosopiperidine (NPIP). Control experiments in which water stored in the same containers as those used for sample collection and storage will be performed to test for any leaching of volatile nitrosamines.

Table 1: N-nitrosamines that will be analysed by GCMS

N-Nitrosamine	Abbreviation	M+ ion (m/z)
N-Nitrosodimethylamine	NDMA	74.05
N-Nitrosomethylethylamine	NMEA	88.06
N-Nitrosodiethylamine	NDEA	102.08
N-Nitrosopiperidine	NPIP	114.08
N-Nitrosomorpholine	NMOR	116.06

Sample size and power

Sample size is based on a crossover design and the primary outcome of *N*-nitrosamines, specifically *N*-Nitrosodimethylamine (NDMA) in urine. At α=0.05, 2 measures per subject (baseline and post) and a correlation of ρ=0.6 between measures, 25 participants will provide 80% power to detect a 0.6 SD increase in NDMA concentration. To allow for a 10% withdrawal rate, we plan to recruit 25 participants into the study. A rolling recruitment of participants will be performed until the sample size is reached.

Statistical methods

Statistical analyses will be performed using IBM SPSS Statistics for Windows, version 25 (IBM) and STATA/IC 17.0 (StataCorp LLC). Descriptive statistics of normally distributed continuous variables will be expressed as mean (± standard deviation, SD), non-normally distributed continuous variables as median (interquartile range, IQR) and categorical variables as number (proportion, %). Data will be assessed for outliers and normality prior to analysis. Non-normally distributed data will be log transformed if necessary. Treatment effects for outcomes will be obtained using linear mixed models including baseline measurements, treatment order, period, and time (as a categorical variable) as fixed effects. We will also include a treatment X period interaction term to assess for possible treatment-period interactions and we will separately assess for carryover effects with dummy variables to indicate the previous treatment. Treatment effects for outcomes with a single post-intervention measurement will be obtained using linear mixed models including baseline measurements, treatment order, and period as predictors. The subject ID number will be included as a random intercept in each model. An overall 2-sided type-1 error rate of $P<0.05$ will be used to assess statistical significance for all hypothesis testing.

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