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Effect of dietary prebiotic supplementation on advanced glycation, insulin resistance and inflammatory biomarkers in adults with pre-diabetes: a study protocol for a double-blind placebo-controlled randomised crossover clinical trial

Nicole J Kellow[1*], Melinda T Coughlan[2,3], Gayle S Savige[1] and Christopher M Reid[1]

Abstract

Background: Advanced glycation endproducts (AGEs) contribute to the development of vascular complications of diabetes and have been recently implicated in the pathogenesis of diabetes. Since AGEs are generated within foodstuffs upon food processing, it is increasingly recognised that the modern diet is replete with AGEs. AGEs are thought to stimulate chronic low-grade inflammation and promote oxidative stress and have been linked to the development of insulin resistance. Simple therapeutic strategies targeted at attenuating the progression of chronic low-grade inflammation and insulin resistance are urgently required to prevent or slow the development of type 2 diabetes in susceptible individuals. Dietary modulation of the human colonic microbiota has been shown to confer a number of health benefits to the host, but its effect on advanced glycation is unknown. The aim of this article is to describe the methodology of a double-blind placebo-controlled randomised crossover trial designed to determine the effect of 12 week consumption of a prebiotic dietary supplement on the advanced glycation pathway, insulin sensitivity and chronic low-grade inflammation in adults with pre-diabetes.

Methods/Design: Thirty adults with pre-diabetes (Impaired Glucose Tolerance or Impaired Fasting Glucose) aged between 40–60 years will be randomly assigned to receive either 10 grams of prebiotic (inulin/oligofructose) daily or 10 grams placebo (maltodextrin) daily for 12 weeks. After a 2-week washout period, study subjects will crossover to receive the alternative dietary treatment for 12 weeks. The primary outcome is the difference in markers of the advanced glycation pathway carboxymethyllysine (CML) and methylglyoxal (MG) between experimental and control treatments. Secondary outcomes include HbA_{1c}, insulin sensitivity, lipid levels, blood pressure, serum glutathione, adiponectin, IL-6, E-selectin, myeloperoxidase, C-reactive protein, Toll-like Receptor 4 (TLR4), soluble receptor for AGE (sRAGE), urinary 8-isoprostanes, faecal bacterial composition and short chain fatty acid profile. Anthropometric measures including BMI and waist circumference will be collected in addition to comprehensive dietary and lifestyle data.

Discussion: Prebiotics which selectively stimulate the growth of beneficial bacteria in the human colon might offer protection against AGE-related pathology in people at risk of developing type 2 diabetes.

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* Correspondence: nicole.kellow@monash.edu
[1]Department of Epidemiology & Preventive Medicine, School of Public Health & Preventive Medicine, Monash University, The Alfred Centre, Melbourne, Victoria 3004, Australia
Full list of author information is available at the end of the article

Background

Advanced Glycation Endproducts (AGEs) are formed via the Maillard reaction, which consists of a complex network of non-enzymatic reactions involving the carbonyl groups of reducing sugars which react with the amino groups of proteins [1]. AGEs are generated *in vivo* as a normal consequence of metabolism, but their formation is accelerated under conditions where blood glucose is chronically elevated such as poorly controlled diabetes [2]. AGE formation is also increased in the presence of oxidative stress, which is frequently observed in individuals with the metabolic syndrome [3,4]. Overproduction of reactive oxygen species (ROS) can result in maladaptive responses including interruption of cellular glycolysis, which can generate highly reactive dicarbonyl compounds capable of rapid AGE formation [5].

Excessive AGE accumulation can lead to several pathophysiological consequences. AGE-modification of proteins results in changes in structure and/or function. For example, the AGE-modification of extracellular collagen reduces its elasticity and solubility, and results in increased stiffness, disturbed cellular adhesion and reduced turnover contributing to basement membrane thickening [6]. Intracellularly, AGE-modification of mitochondrial proteins is associated with suppression in the activity of respiratory chain enzymes and overproduction of ROS [7,8]. Indeed, glycated proteins provide stable active sites for catalysing the formation of free radicals [9]. Finally, AGEs are able to bind and activate a range of receptors, which then trigger a downstream cascade of pathogenic mediators. Interaction of AGEs with the Receptor for AGEs (RAGE) promotes activation of the transcription factor nuclear factor kappa-B (NF-κB), with subsequent upregulation of chemokines, such as MCP-1 and profibrogenic mediators such as TGFβ in addition to proinflammatory cytokines which are known to be involved in thrombogenesis, vascular inflammation and pathological angiogenesis. These RAGE-mediated events contribute to many of the long-term complications of diabetes [10]. AGE/RAGE ligation also promotes overproduction of ROS which can then activate NF-κB [11], a key driver of inflammation.

More recently, AGEs have been implicated in the pathogenesis of both type 1 and type 2 diabetes. Several studies have shown that AGEs are associated with insulin resistance [12,13], and can induce low-grade inflammation [14] and pancreatic beta cell dysfunction [15,16].

In contrast to endogenous AGE formation, AGEs are also absorbed by the body from exogenous sources such as cigarette smoke and through consumption of processed foods [17]. Since AGEs are generated within foodstuffs upon heating and food processing, it is increasingly recognised that the modern diet is replete with AGEs [18]. AGE-restricted diets can arrest the development of type 2 diabetes in animal models [19]. A recent study found that excess consumption of AGE-precursors in mice over several generations led to the development of insulin resistance [20]. Human trials have found that dietary AGE restriction can improve insulin sensitivity [21,22] and decrease markers of oxidative stress [23] or inflammation [24]. Further studies are required to confirm the long-term benefits of dietary AGE-restriction in humans [25]. However, simple, safe and effective interventions which prevent or minimise excessive AGE accumulation and subsequent AGE-related pathology in people with diabetes and/or in those at risk of developing the condition are warranted.

Interventions which influence the human intestinal microbiota are worthy of further investigation given that specific micro-organisms have the ability to significantly affect host metabolism. Gut bacteria play an important role in the host immune system, modulation of inflammatory processes, extraction of energy from the host's diet, fermentation of dietary fibres to produce short-chain fatty acids, alteration of human gene expression, regulation of intestinal permeability, production of some vitamins and promotion of mineral absorption by the host [26-31]. Furthermore, the total quantity and relative proportions of distinct bacterial species found in the colon differ between lean and obese individuals as well as between individuals with and without diabetes [32,33].

It is thought that certain dietary AGEs are largely undigested by human gut enzymes and eventually enter the colon, where they may act as a growth substrate for detrimental bacteria such as some Clostridium and Bacteroides species [34]. Therefore it is conceivable that individuals who consume highly processed diets (which contain large quantities of AGEs) may adversely alter their colonic microbial composition, potentially enhancing their risk for the development of metabolic diseases such as obesity and type 2 diabetes [35].

Therapeutic manipulation of the gut microbiota and restoration of normobiosis could potentially reduce circulating AGE levels and improve the metabolic health

of individuals at risk for the development of type 2 diabetes. Regular consumption of prebiotics to promote the growth of beneficial gut bacterial flora is one such avenue currently under investigation. Prebiotics are non-digestible plant-derived carbohydrates which confer health benefits to the host by acting as a fermentation substrate in the colon, stimulating the preferential growth and activity of a limited number of beneficial microbial species [36]. Supplementation of the human diet with prebiotic fructans such as inulin or fructo-oligosaccharides alters the bacterial composition of the large intestine by favouring the selective proliferation of beneficial lactic acid-producing species such as bifidobacteria and lactobacilli. Prebiotic-stimulated increases in intestinal Bifidobacterium species have been shown to attenuate the production of ROS and markers of inflammation in individuals consuming high fat diets [37].

While the complex interactions between diet, intestinal microbiota and host metabolism are still being elucidated, no studies have investigated the effect of dietary prebiotics on circulating AGE concentrations. This trial was designed to investigate the effect of a prebiotic dietary supplement on AGE accumulation and explore changes to the growth and activity of specific gut microbiota in adults diagnosed with prediabetes.

Methods/Design

Study design and setting
This is a 6.5-month randomised crossover controlled clinical trial (RCT) in which adults aged 40–60 years with diagnosed pre-diabetes will be enrolled. Potential study participants will be identified from General Practice (GP) clinics throughout South Gippsland, Victoria. The study design is presented in Figure 1.

Inclusion criteria
Individuals aged between 40–60 years and diagnosed with prediabetes (Impaired Fasting Glucose or Impaired Glucose Tolerance) within the previous 12 months. Diagnosis will have been made at each individual's local GP clinic after undertaking an Oral Glucose Tolerance Test (OGTT). Prediabetes was defined as a fasting plasma glucose concentration ≥ 6.1 and < 7.0 mmol/L followed by a 2-hour post glucose load glucose concentration < 7.8 mmol/L, or a fasting plasma glucose < 7.0 mmol/L followed by a 2-hour post glucose load glucose concentration ≥ 7.8 and < 11.1 mmol/L [38].

Exclusion criteria
Individuals previously diagnosed with type 1, type 2 diabetes or impaired renal function (eGFR <90 mL/min/1.73 m^2), individuals with known gastrointestinal pathology (coeliac disease, inflammatory bowel disease), pregnant women, smokers, individuals who have taken antibiotics, dietary prebiotic or probiotic nutritional supplements within the previous three months, individuals taking aspirin or Vitamin B, individuals who have made major dietary or lifestyle changes in the previous three months, individuals who are unwilling to provide blood, urine and stool samples or are unable to attend their local pathology collection centre.

Ethics
The trial has received ethical approval from the Monash University Human Research Ethics Committee.
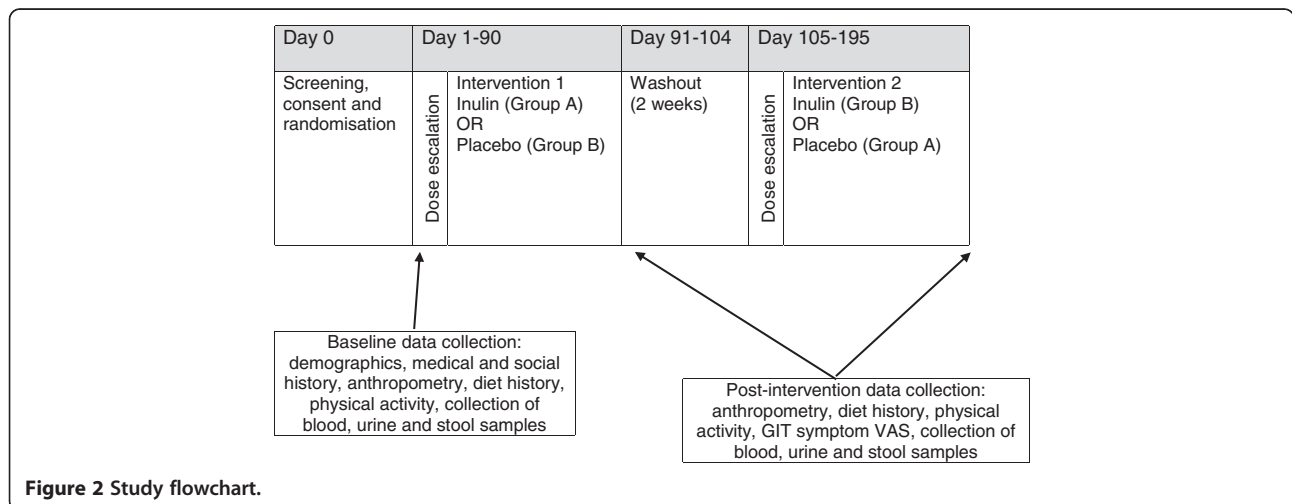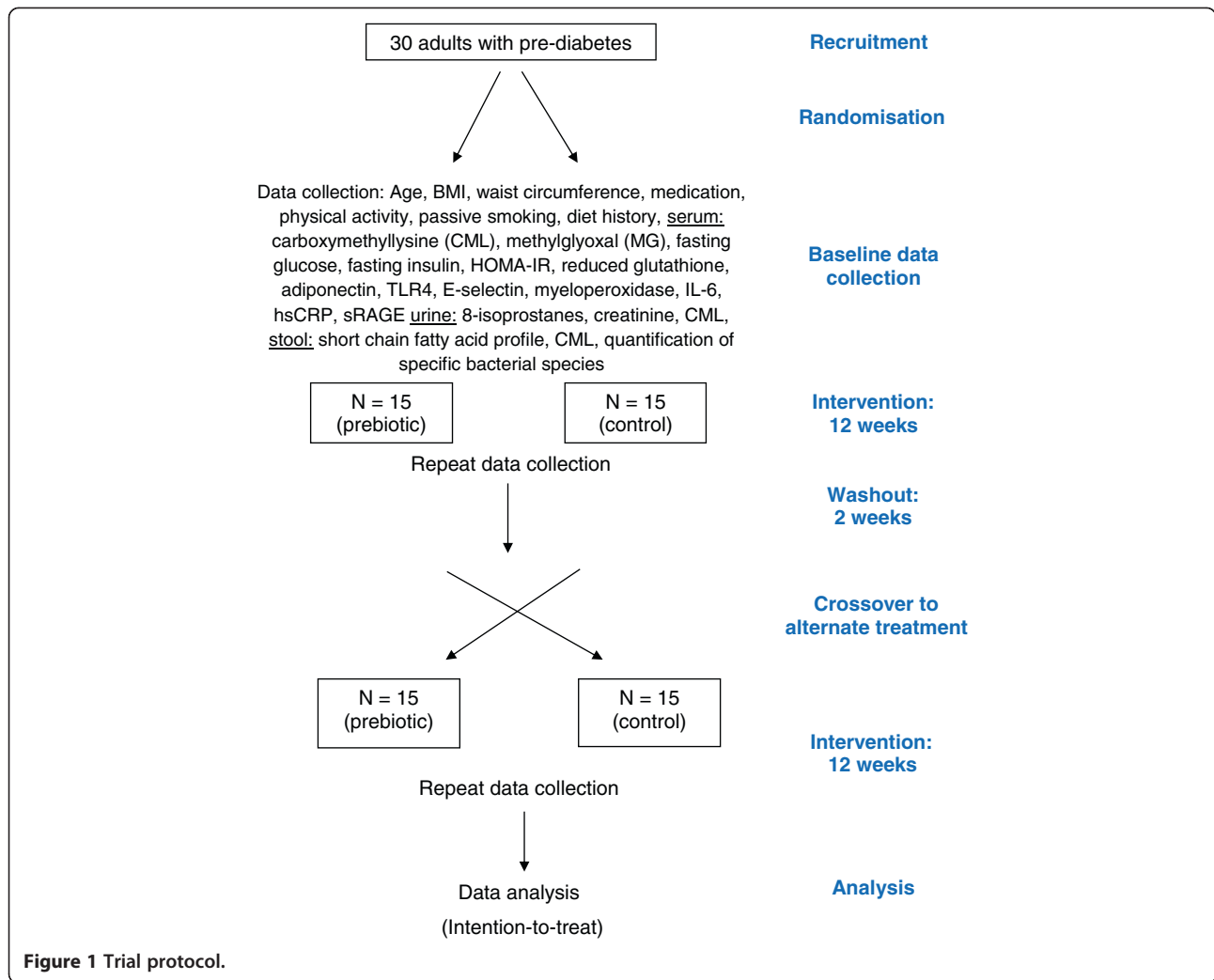
Sample size calculation
The minimum difference we wish to detect is 0.4 micromol/L serum CML (20% reduction in CML), with a standard deviation of 0.4 [39] with 5% Significance and 80% Power. This calculates as a total sample size of 18 individuals, plus 12 individuals to allow for withdrawals = 30 subjects required. Epidemiological studies have demonstrated a positive correlation between serum CML and all-cause and cardiovascular mortality, cardiovascular disease, glucose intolerance, impaired insulin secretion, renal impairment and diabetic vascular complications [40,41]. A 0.4 micromol/L increase in serum CML concentration represented a 68% increased risk for all-cause mortality over seven years in a large prospective cohort study [42].

Baseline assessment
The study timeline is presented in Figure 2. Following recruitment and screening, consenting participants will undergo a baseline assessment at their local GP clinic. The baseline assessment will be undertaken by the research dietitian, who will collect demographic details, medical and social history (living situation, marital status, current occupation), physical activity questionnaire, dietary intake assessment, anthropometric measurements including Body Mass Index (BMI) and waist circumference. Participants will also be instructed to attend the local pathology centre to provide a stool sample, 24-hour urine collection, and have blood taken for analysis.

Randomisation
Randomisation of participants to intervention/placebo sequence will be completed by a local pharmacist using a permuted-block randomisation stratified by gender via the web site www.randomization.com. The pharmacist will distribute the experimental and placebo nutritional supplements to participants in the appropriate sequence. Other than provision of supplements, the pharmacist will have no contact with study participants or involvement in data collection or analysis. Dietary supplements will be provided in sealed opaque packages which will be weighed at the conclusion of the study in order to assess compliance. The supplements will be packaged

30 adults with pre-diabetes — Recruitment

Randomisation

Data collection: Age, BMI, waist circumference, medication, physical activity, passive smoking, diet history, serum: carboxymethyllysine (CML), methylglyoxal (MG), fasting glucose, fasting insulin, HOMA-IR, reduced glutathione, adiponectin, TLR4, E-selectin, myeloperoxidase, IL-6, hsCRP, sRAGE urine: 8-isoprostanes, creatinine, CML, stool: short chain fatty acid profile, CML, quantification of specific bacterial species — **Baseline data collection**

N = 15 (prebiotic)   N = 15 (control) — **Intervention: 12 weeks**

Repeat data collection

Washout: 2 weeks

Crossover to alternate treatment

N = 15 (prebiotic)   N = 15 (control) — **Intervention: 12 weeks**

Repeat data collection

Data analysis (Intention-to-treat) — **Analysis**

Figure 1 Trial protocol.

Day 0		Day 1-90	Day 91-104		Day 105-195
Screening, consent and randomisation	Dose escalation	Intervention 1 Inulin (Group A) OR Placebo (Group B)	Washout (2 weeks)	Dose escalation	Intervention 2 Inulin (Group B) OR Placebo (Group A)

Baseline data collection: demographics, medical and social history, anthropometry, diet history, physical activity, collection of blood, urine and stool samples

Post-intervention data collection: anthropometry, diet history, physical activity, GIT symptom VAS, collection of blood, urine and stool samples

Figure 2 Study flowchart.

off-site by an external pharmaceutical packaging agency. Each package will contain a participant's study identification number and will be labelled as either Supplement 1 or Supplement 2 corresponding to the first and second intervention periods. This will ensure all investigators and participants are blinded to the treatment. Blinding will cease only after statistical analysis of the data has been completed.

Intervention

Participants will be randomly assigned to receive either 10 g of chicory-derived inulin/oligofructose powder (kindly provided by Beneo-Orafti Active Food Ingredients, Belgium) (intervention) or 10 g of maltodextrin powder (placebo) daily. Previous studies have demonstrated the bifidogenic effect of daily consumption of 5 g and 8 g dietary inulin supplements [43]. The inulin and maltodextrin powders to be consumed are both tasteless and can be mixed into hot or cold liquids or semi-solid foods. Participants will be instructed how to incorporate their supplement into their usual diet, and advised to gradually increase their dose over ten successive days until the target dose is reached. This stepped escalation in supplement dose aims to minimise gastrointestinal discomfort for participants, as a sudden increase in dietary prebiotic intake may result in increased stool frequency, abdominal bloating and flatulence until the bowel adapts to the increased fibre intake [44]. Written instructions will also be provided. Participants will be advised to consume each dietary supplement daily for 90 days, and otherwise maintain their usual dietary intake and level of physical activity. Gastrointestinal tolerance to the dietary supplements will be indicated by each study participant using a visual analogue scale as described below.

Follow up visits

Visits to the GP clinic will be scheduled for each participant at the conclusion of both Intervention 1 and Intervention 2 treatment periods, in order to provide follow-up data. Information collected will include anthropometric measurements, dietary intake assessment and physical activity assessment. Each participant will complete a self-administered questionnaire designed to assess gastrointestinal tolerance to the dietary supplement. Completed questionnaires will be placed into sealed opaque envelopes in order to maintain blinding of the researcher collecting data during the follow-up visits. Participants will also be instructed to attend the local pathology centre to provide urine and stool samples, and have blood taken for analysis.

Safety considerations

All adverse events will be documented.

Outcome measurements

The primary outcome of interest is the difference in serum AGE and AGE-precursor concentration (measured as CML and MG respectively) between experimental and control treatments. Secondary outcomes include HbA1c, insulin resistance (measured indirectly by homeostasis model assessment), antioxidant capacity (reduced glutathione), markers of oxidative stress on lipid molecules (urinary 8-isoprostanes), inflammatory biomarkers (serum IL-6, high sensitivity C-reactive protein, MCP-1, sRAGE), adhesion molecules (E-selectin), gut barrier integrity (TLR4), 24-hour urine and faecal CML and MG concentrations, faecal bacterial composition (*Bifidobacterium* spp., *Lactobacillus* spp., *Roseburia* spp., *Faecalibacterium prausnitzii* and *Akkermansia muciniphila*) and faecal short chain fatty acid concentrations. Lipid levels (serum total cholesterol, LDL, HDL, TG) and blood pressure will also be measured. Anthropometric measurements including BMI and waist circumference will be collected, in addition to comprehensive dietary and lifestyle data. Gastrointestinal side-effects will be assessed using a visual analogue scale adapted from Lecerf *et al.* [45]. The scale rates nine items including flatulence, bloating, rumbling, abdominal cramps, a global digestive tolerance score calculated from the five previous items, stool consistency, stool frequency and general well-being through disturbances in usual and professional activities as well as disturbances in personal and social activities. Items are scored on a 10 cm linear scale.

Data collection
Dietary intake

An experienced research dietitian will obtain a comprehensive dietary history from each study participant at baseline and at the completion of each intervention period. A dietary history is a structured interview method consisting of questions regarding habitual food intake. It involves a 24-hour diet recall followed by a food frequency questionnaire to clarify information about usual consumption over the previous month. Usual portion sizes will be obtained in household measures and with the use of photographic aids. The dietitian will review the dietary history with each participant, probing for details on portion sizes and cooking methods in order to improve data accuracy. Dietary AGE content will be estimated from an open source database which lists the AGE concentration of foods using validated analytical techniques [46]. Dietary macro and micronutrient intakes will be estimated using the Foodworks nutrient software program (Xyris Software, NSW, Australia). Each participant will also be randomly contacted by telephone during the study and asked to provide a 24-hour diet recall, and changes in urinary urea excretion will be monitored in order to validate the dietary history data collected [47].

Physical activity will be estimated by asking participants to complete the International Physical Activity Questionnaire (Short Form) prior to and at the completion of experimental and placebo intervention periods [48].

Anthropometry

Body weight will be measured in participants wearing light clothes without shoes using a digital scale (Seca, Germany) to the nearest 0.1 kg. Height will be measured using a portable stadiometer (Seca, Germany) to the nearest 0.1 cm. BMI is calculated by dividing weight (kg) per height (m) square. Waist circumference will be measured at the midpoint of the lowest rib and iliac crest using a measuring tape to the nearest 0.1 cm. Body composition (total body water, fat mass, fat-free mass) will be determined using Bioelectrical Impedance Analysis (BodyStat-1500, Bodystat, Douglas, Isle of Man, United Kingdom). Ambulatory blood pressure will be measured using an electronic blood pressure machine (Omron Corporation, Kyoto, Japan), with subjects at rest in a seated position. All anthropometric measurements will be conducted in duplicate, with the mean measurement recorded.

Laboratory investigations

Biological samples will be collected at baseline and at completion of each three-month supplement intervention period and stored at −80°C immediately after collection. Twenty ml of fasting venous blood will be collected from each participant by phlebotomy into a sodium fluoride EDTA tube, a heparin-lined vacuum tube and a clean glass test tube. Twenty-four hour urine collections and morning stool samples will be collected in sterile containers.

Serum, urinary and faecal CML will be measured using a competitive ELISA (AGE-CML ELISA, Microcoat, Penzberg, Germany) [49]. This assay has been validated [50], is specific, and shows no cross-reactivity with other compounds [49]. The within assay and between-assay co-efficient of variation are both less than 5%, respectively. Methylglyoxal will be measured by HPLC. Serum total cholesterol and triglyceride concentrations will be determined by enzymatic colorimetric assay (Technicon Instruments, Ltd., New York, N.Y., USA), while HDL cholesterol will be determined enzymatically in the supernatant after precipitation of other lipoproteins with dextran sulphate-magnesium. LDL-cholesterol will be calculated using the Friedewald formula. Plasma glucose levels will be determined by using an automated glucose oxidase method (Glucose analyser 2, Beckman Instruments, Fullerton, California). Insulin will be measured by enzymatic colorimetry (WAKO Pure-Chemical Industries, Osaka, Japan). Insulin Resistance (IR) will be estimated by the homeostasis model assessment (HOMA) index as [FI × (fasting glucose/ 22.5)], where FI is insulin in microunits per millilitre and fasting glucose is in millimoles per litre [51,52]. HbA1c will be measured by autoanalyser (Roche Diagnostics, Mannheim, Germany). Plasma IL-6, MCP-1, E-selectin, hsCRP, TLR4, glutathione (GSH), and myeloperoxidase will be measured by commercial ELISA kits (Biosource International, Camarillo, CA, USA). Urine 8-isoprostanes will be measured by ELISA (Oxford Biomedical Research, MI, USA).

Stool samples will be homogenised in a blender and stored at −20°C for SCFA analysis. Samples will be thawed and 5 g aliquots placed in Centriprep fluid concentrators, MWCO 30,000 kDa (Amicon Inc., Beverly, MA, USA). Samples will be centrifuged for 30 minutes at 1000 × g, room temperature and supernatants placed in 15 ml polypropylene tubes. 0.3 ml of 25% m-phosphoric acid will be added to each tube, samples will be vortexed and incubated at room temperature for 25 minutes. Samples will be centrifuged at 5000 × g for 15 minutes at room temperature. Supernatants will be decanted and frozen overnight. The following day, samples will be thawed and the pH of each sample adjusted to 6.5 using 4 N KOH. Oxalic acid will be added at a final concentration of 0.03% and SCFA concentrations determined by gas chromatography with use of a Hewlett-Packard 5880A gas chromatograph (Hewlett Packard, Palo Alto, CA, USA) containing an 80/120 Carbopack B-DA/4% Carbowax 20 M column (Supelco Inc., Bellefonte, PA, USA).

Quantitative Real-time PCR will be used to determine faecal concentrations of *Bifidobacterium* spp., *Lactobacillus* spp., *Roseburia* spp., *Faecalbacterium prausnitzii* and *Akkermansia muciniphila*. The primers used will be based on the following 16S rRNA gene sequences: *Bifidobacterium* spp: F-CTCCTGGAAACGGGTGG and R-GGTGTTCTTCCCGATATCTACA [53], *Lactobacillus* spp: F-AGCAGTAGGGAATCTTCCA and R-CACCGCT ACACATGGAG [54], *Roseburia* spp: F-CGKACTAGAG TGTCGGAGG and R-GTCATCTAGAGTGTCGGAGG [55], *Faecalbacterium prausnitzii*: F-GGAGGAAGAAGG TCTTCGG and R-AATTCCGCCTACCTCTGCACT [56], and *Akkermansia muciniphila:* F-CAGCACGTGAA GGTGGGGAC and R-CCTTGCGGTTGGCTTCAGAT [57]. PCR amplification and detection will be achieved with an ABI 7300 Real-time PCR system (Applied Biosystems, Foster City, CA, USA) using Mighty Amp for Real-time (SYBR Plus) and Rox Reference Dye (Invitrogen, Carlsbad, CA, USA). Each assay will be performed in duplicate in the same run. The cycle threshold of each sample will then be compared with a standard curve (performed in duplicate) made by diluting genomic DNA (tenfold serial dilution). Prior to isolating the DNA, the cell counts will be determined in culture and expressed as "colony forming units" (CFU). Data will be expressed as log CFU/g of faeces.

Statistical analysis

Outcome analyses will be undertaken on an intention-to-treat basis. Data will be presented as means ± SD. The Kolmogorov-Smirnov goodness-of-fit test will be used to test for normal distribution, and data not normally distributed will be log-transformed. Correlation analyses will be performed using the Pearson correlation coefficient. Significance of changes during the study will be assessed by comparing change of means between placebo and prebiotic treatment periods by paired sample t-tests. Trial data will be analysed using a linear mixed model design based on repeated measures to account for fixed factors such as treatment sequence (inulin – placebo vs placebo - inulin) and treatment period (intervention 1 vs intervention 2) in addition to participants as a random factor. Significant differences will be defined as a value of $P < 0.05$ based on two-sided tests. Any differences in physical activity levels, anthropometry, energy or nutrient intake during the course of the trial will be identified using ANOVA. Gastrointestinal symptom data obtained by visual analogue scale will be analysed using the Wilcoxon signed rank test. Effect sizes including 95% confidence intervals will be calculated for all significant outcomes. Data analysis will be performed using SPSS 20.0 software (SPSS, Chicago, IL).

Discussion

AGEs are derived from both exogenous and endogenous sources, and the rate at which AGEs accumulate in the body is dependent to a large extent on the chronological age, lifestyle and metabolic health of an individual. Smoking cigarettes and consuming foods containing high concentrations of AGEs (and their precursors) increases the accumulation of AGEs from exogenous sources. Endogenous AGE formation is accelerated under conditions of hyperglycaemia, dyslipidaemia and increased oxidative stress, conditions that are common in individuals with diabetes and in those at risk of developing type 2 diabetes (such as those with prediabetes and the metabolic syndrome) [58]. Moreover, in individuals with impaired renal function, urinary AGE excretion may be diminished resulting in a greater accumulation of AGEs in the body [59].

Risk factors for the development of type 2 diabetes include obesity, hypertension and cardiovascular disease; conditions that are commonly associated with unhealthy lifestyles including poor food habits. Restricting the intake of foods high in AGEs might potentially reduce AGE accumulation, but adherence to such diets can be challenging given that foods high in AGEs are very palatable due to their enhanced flavour, colour and aroma [60].

Supplementation of the diet with bifidogenic prebiotic fibres (such as inulin) may reduce or retard the accumulation of AGEs in individuals at risk of developing type 2 diabetes. Prebiotics have been shown to improve and restore optimal microbial balance within the gastrointestinal tract, potentially reducing AGE absorption and/or production by the human host. Preliminary investigations indicate that consuming a high-AGE diet is sufficient to favour the proliferation of potentially pathogenic colonic bacteria over more beneficial species. Consumption of glycated proteins [34], fried meats [61] and toasted wheat flakes [62] encouraged the preferential growth of greater numbers of detrimental gram negative and sulphate-reducing colonic micro-organisms when compared to control diets. Short Chain Fatty Acids (SCFAs) produced as a bacterial by-product of prebiotic fermentation act to lower the intestinal pH, inhibiting the growth of protein-degrading micro-organisms capable of producing potentially toxic metabolites. SCFAs also stimulate colonic smooth muscle contractions, speeding intestinal transit and limiting the time available for protein fermentation and putrefaction to occur in the gut [63]. Therapeutic manipulation of the gut microbiota with prebiotics may restore gut normobiosis and reduce AGE accumulation in humans at risk for type 2 diabetes development by the following mechanisms:

Maintenance of gut barrier function

Kinetic studies have estimated that up to 30% of dietary AGEs consumed are intestinally absorbed [64]. Under circumstances of increased intestinal permeability, it is likely that greater quantities of dietary AGEs and their reactive dicarbonyl precursors may be able to gain entry into the systemic circulation. Elevated levels of circulating proinflammatory cytokines and ROS frequently observed in individuals with prediabetes are known to compromise tight junctions between cells, disrupting the integrity of the intestinal barrier and enabling the absorption of larger, potentially deleterious compounds [65]. Prebiotic fermentation products may reduce gastrointestinal permeability and as a result limit the absorption of exogenous AGEs.

The SCFAs acetate and butyrate are intricately involved in the maintenance of gut epithelial integrity. Acetate increases colonic blood flow and butyrate is the principal fuel for colonocytes, assisting to optimise epithelial cell health [66]. Butyrate reduces gastrointestinal permeability by enhancing the activation of the peroxisomal proliferator-activated receptor gamma (PPARgamma) gene, a nuclear receptor involved in the attenuation of inflammation in colonic epithelial cells [67]. Butyrate also upregulates the expression of mucin-associated genes important in maintaining the integrity of the intestinal mucosal barrier [68]. Oligofructose supplementation in mice has been shown to increase the expression of zonula and occludin, proteins important in the maintenance of tight junctions between gastrointestinal cells [69], and a dietary inulin intervention reduced markers of intestinal permeability in healthy adult males [70]. Butyrate is a histone deacetylase inhibitor and is likely to promote the transcription of these tight junction

proteins. Prebiotic-induced changes in gut microbiota also increase endogenous production of Glucagon-like peptide 2 (GLP-2), which enhances gut barrier function by promoting the proliferation of crypt cells [71,72].

Reduction of oxidative stress, inflammation and insulin resistance

Increased production of ROS stimulate endogenous AGE formation by oxidising glucose and unsaturated fatty acids to generate reactive dicarbonyls. Experimental drug treatments which attenuate oxidative stress have previously demonstrated reductions in serum AGE levels [73]. Cytokine production at sites of inflammation stimulate immune cell activation of NADPH oxidase (NOX) and production of myeloperoxidase, enzymes involved in the oxidation of amino acids to form AGE precursors [74]. Activated immune cells can also secrete the high-mobility group box 1 (HMGB-1) protein which is capable of binding to RAGE, thereby inducing further inflammation [75].

A high fat diet (independent of the level of obesity in the host) is associated with negative changes in bacterial communities within the colon [76]. In response to a high fat meal, bacterial lipopolysaccharide (LPS) translocates from the intestine into the host circulation, resulting in 'metabolic endotoxemia' [77,78]. LPS is a major component of the outer membrane of gram negative bacteria, and is a potent activator of the mammalian immune system. LPS interaction with immune cells stimulates macrophage over-production of ROS, enhances the secretion of pro-inflammatory cytokines, and contributes to weight gain and development of insulin resistance [79,80]. Individuals with type 2 diabetes have been found to possess endotoxemia levels 2-fold higher than people without diabetes [81]. Metabolic endotoxemia also positively correlates with total energy intake and fasting insulin levels in the general population [82]. In mice with high fat diet-induced metabolic endotoxemia, nutritional supplementation of the diet with prebiotics restored intestinal levels of gram positive bacteria (particularly Bifidobacterium species) and subsequently improved glucose tolerance and reduced circulating concentrations of LPS and pro-inflammatory cytokines [83]. Human trials involving dietary prebiotic supplementation have successfully reduced serum LPS levels [45] and markers of lipid peroxidation (a process which generates AGE precursors), possibly through the reduction of ROS production or the direct antioxidant ability of some Bifidobacterium and Lactobacillus bacterial species [84,85].

SCFAs produced as a bacterial by-product of prebiotic fermentation are absorbed into the host circulation, effecting the expression of a wide range of genes in distal tissues associated with cell proliferation, differentiation and apoptosis. SCFAs are ligands for the G-protein coupled receptors (GPRs) GPR41 and GPR43 [86] on immune cells. These receptors are involved in down-regulating inappropriate immune cell production of pro-inflammatory cytokines, chemokines and ROS [87]. The SCFAs acetate, proprionate and butyrate exhibit a variety of anti-inflammatory actions through inhibition of NFκB activation, prevention of LPS-stimulated TNFα production in neutrophils and suppression of cytokine production [31,88-90].

Numerous other immune modulating effects have been observed secondary to gut bacterial activity including the production of anti-inflammatory compounds such as polysaccharide A [91], peptidoglycan [92] and conjugated linoleic acid [93], and the induction of T-regulatory cells [26].

Promotion of weight reduction

An energy-restricted diet resulting in weight loss has been shown to reduce serum AGE levels in overweight and obese individuals [94]. The consumption of prebiotics in human clinical trials has promoted self-reported satiety [95], weight reduction, reduced production of the orexigenic hormone grehlin and stimulated expression of the appetite-reducing hormone peptide YY (PYY) [96]. Interaction with GPR41 by the SCFAs proprionate and butyrate increases satiety [97], upregulates PYY production and modulates the expression of leptin, a hormone important in controlling energy intake and expenditure [31].

In mice, the selective growth of certain lactobacillus species in the colon reduced body fat storage through the up-regulation of fiaf (fasting induced adipose factor) gene expression and inhibition of lipoprotein lipase (LPL) [98-100]. These findings may have the potential to affect weight reduction in humans and subsequently reduce circulating AGE levels.

Enhanced antioxidant capacity

Inulin enhances the proliferation of lactic acid producing bacteria capable of synthesising B-group vitamins, some of which have an antioxidant capacity [101]. These vitamins can be utilised by the human host to neutralise ROS. Vitamins B1 and B6 trap the carbonyl groups of highly reactive AGE precursors before they can react with proteins [102]. Some Lactobacillus and Bifidobacterium species are efficient scavengers of the lipid peroxidation product malandialdehyde, protecting the host from excessive accumulation of this toxic AGE precursor [84]. Inulin also exhibits antioxidant properties independent of altering gut bacterial growth and is able to scavenge a number of ROS, which may help to reduce lipid peroxidation in the stomach [103].

The SCFA butyrate, produced as a bacterial by-product of inulin fermentation, has been shown to increase colonic glutathione production [104]. Glutathione is an antioxidant co-factor required for glyoxalase I activity, an enzyme which degrades the AGE precursor methylglyoxyl. Increased production of ROS is also thought to deplete

glutathione levels. Through the reduction of oxidative stress, prebiotics may assist in the maintainance or up-regulation of the glyoxylase pathway.

Reduction of hyperglycemia

Both transient and chronic elevations in blood glucose increase endogenous AGE generation. Activation of GPR43 in adipocytes by proprionate inhibits lipolysis and lowers glycemia in healthy individuals [105]. Butyrate has been shown to reverse diet-induced insulin resistance in animal studies [106], possibly by enhancing PPARgamma expression which increases fatty acid oxidation in muscle.

Glucagon-like peptide 1 (GLP-1) is an incretin hormone released from intestinal L-cells in response to consumption of carbohydrates and fats. GLP-1 potentiates glucose-induced insulin secretion, reducing post-prandial blood glucose levels. GLP-1 also enhances satiety and slows gastric emptying. Prebiotic feeding in rats promoted L-cell differentiation in the colon and increased GLP-1 production [107,108], probably through an increase in bacterial production of butyrate.

The consumption of inulin as a dietary supplement may also influence circulating AGE concentrations independently of its prebiotic function. High glycemic index (GI) diets and their resultant hyperglycemic effect have been shown to enhance AGE formation in healthy individuals [109]. Inulin is a soluble fibre which when consumed daily may play a role in the reduction of the GI of the diet. Many soluble fibres are known for their ability to delay gastric emptying and slow the rate of intestinal nutrient absorption, reducing the GI of the carbohydrates in the meal. Regular consumption of prebiotic soluble fibre reduces fasting and post-prandial serum glucose levels in people with impaired glucose tolerance [110] and type 2 diabetes [111].

Study strengths

- To our knowledge, this is the first trial to investigate the potential effects of gut bacterial modulation on advanced glycation.
- Random allocation of participants to treatment sequence and intention to treat analysis will ensure study bias is minimised.
- Double-blind crossover placebo-controlled trial.
- Dietary data will be obtained to determine dietary patterns as well as estimates of total energy intake, fat and AGE consumption.
- CML in this study will be measured using a validated ELISA method.
- Dietary AGE consumption will be estimated from an open-source food AGE database, containing AGE values obtained using validated measurement techniques.

Study limitations

Type 2 diabetes is a chronic condition which often develops over decades, making it difficult to conduct intervention studies using the presence or absence of diabetes as the primary biological end-point. This study will measure surrogate biochemical markers of early type 2 diabetes pathogenesis, which could be considered a limitation of the trial. Long-term studies will need to be conducted in order to confirm the results of this research.

This study will measure serum CML as an indicator of AGE concentration in blood samples. Multiple other forms of AGEs exist, many of which have not yet been characterised, so the findings of this trial cannot be applied to all members of the AGE family. However, serum CML concentration shows a moderate to high correlation with other known circulating AGEs [39].

Applicability of research findings

In Australia, conservative estimates predict that at least 2 million adults will have been diagnosed with type 2 diabetes by 2025 [112]. The burden of disease associated with diabetes has a substantial impact on costs associated directly with health care as well as loss of productivity and decreased quality of life. The widespread consumption of high fat, heat processed foods and the increasing prevalence of obesity in Australia warrant simple interventions including those that prevent AGE-mediated damage. If dietary treatments aimed at altering the gut microbiota prove to be effective strategies for preventing or slowing the development of type 2 diabetes, they could become mainstream therapies for individuals with diabetes risk factors.

Author details
[1]Department of Epidemiology & Preventive Medicine, School of Public Health & Preventive Medicine, Monash University, The Alfred Centre, Melbourne, Victoria 3004, Australia. [2]Glycation, Nutrition & Metabolism Laboratory, Baker IDI Heart & Diabetes Institute, Melbourne, Victoria 8008, Australia. [3]Department of Medicine, Central Clinical School, Monash University, Alfred Medical Research & Education Precinct, Melbourne, Victoria 3004, Australia.

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