Protocol – The effect of age and timing of protein consumption on energy intake and appetite.
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Materials and Methods

Study Population and Ethics

Twenty-four, healthy, community-dwelling participants, aged 50–75 years were recruited from Sheffield, UK and surrounding areas between April–June 2019. Recruitment was via both an electronic and physical poster advertising campaign targeting university staff, local networks and community groups. Inclusion criteria were: adults aged 50–75 years old. Exclusion criteria were: a BMI of <18 or >30 kg/m^2, the presence of kidney issues or diabetes, dietary restrictions or the anticipation of a disruption to their habitual diet, e.g., as a result of holiday. Furthermore participants needed to be able to respond to the recruitment campaign (via email) and have no known cognitive difficulties. Written informed consent for participation was collected during a one-to-one enrolment discussion prior to the study.

Ethical approval for this study was granted by the University of Sheffield's Ethics Committee (ethical approval number: 024856). This trial was registered on the ISRCTN registry database (registry number: ISRCTN99945020).

Design and Intervention

The study design was a randomised cross-over trial, which was comprised of 3 phases (**Figure 1**). Following recruitment of participants, height (cm) (Seca 217 portable height measure, model number: 217 1821 009) and body mass (kg) (Seca 761 floor scales, model number: 761 7019004) were measured. Participants were asked to record everything they ate or drank during a 24 h period, 3 times a week (Monday, Wednesday and Friday) using a physical food diary booklet. Participants were also provided with a food portion booklet, containing photographs from the Ministry of Agriculture, Food and Fisheries (MAFF) food atlas [20] to aid with their assessment of food portion sizes. Phase 1 (baseline) consisted of participants completing the 3-day food diary in addition to answering a validated visual analogue scale (VAS) appetite questionnaire, consisting of nine questions relating to feelings of hunger and appetite [21]. Participants were asked to complete the questionnaires three hours after the first meal of the day.

In the second and third phases, participants consumed a whey protein hydrolysate gel (containing 20 g protein (7 g of which are branched chain amino acids) and 376 kJ of energy) for 4 days, either in the evening (before bed) or in the morning (after breakfast) and completed the same tasks as phase 1. Morning protein supplementation required participants to consume the supplement 30–60 min after consumption of their first meal of the day, on Monday, Tuesday, Wednesday and Thursday. Evening supplementation took place 30–60 min before participants went to bed on Sunday, Monday, Tuesday and Wednesday. Thus, the timing of supplementation fell in line with the participant's daily routine, and they were asked to note when they consumed this supplement in their study documentation. A 1-week wash-out period separated phase 2 and 3 before crossing over to the alternative time point. The protein supplement was a strawberry or lemon flavoured 78 mL gel (based on participant's preference), containing 376 kilojoules of energy, 20 g of

protein (whey), 1.8 g of carbohydrate (1.4 g of which sugars) and 0.1 g of fat (Science in Sport, Whey20, Nelson, Lancashire.)

All participants were given an identification code number, to keep their identity anonymous, and an online randomisation generator (**http://www.randomization.com/**, accessed: 15 April 2019) was used to assign participants to their study sequence. Stratified block randomisation, in block sizes of four, was utilised to ensure equal numbers of participants, in relation to their age category (50–64 years and 65–75 years), were receiving each treatment arm in all phases for the trial. Following completion of the trial, participants were invited to attend a verbal feedback session with the researcher. During this session food diaries and questionnaires were assessed for clarity and any anomalies or missing data were gathered. Compliance was also checked by participants completing a daily tick sheet, as part of their study documentation, to indicate if they had consumed their supplement.

Data Analysis

Dietary intake data from the food diaries was analysed using Dietplan7 nutritional analysis software (Forestfield Software Ltd., Horsham, UK). Protein intakes were also further analysed by adjusting for the participant's body mass. This data was used to explore if participants were achieving recommended RNI for protein (0.75 g per kg body weight/d), in addition to higher values in line with literature recommendations for older adults to maintain physical function: 1–1.2 g per kg body weight/d for healthy older adults and 1.2–1.5 g per kg body weight/d for older adults with acute or chronic disease [8].

Histograms were investigated to examine normality in the continuous variables and were summarised using median/Inter-Quartile Range (IQR) due to the modest participant numbers. Categorical data were summarised with frequency and percentages. The Wilcoxon signed-rank test was used to explore differences between treatment arms: Baseline week (no protein supplementation) vs. morning supplementation vs. evening supplementation. A McNemars test was used to compare categoric paired data for information presented as percentages. Statistical significance was regarded as $p < 0.05$. All statistical analyses were undertaken in SPSS software (version 25).

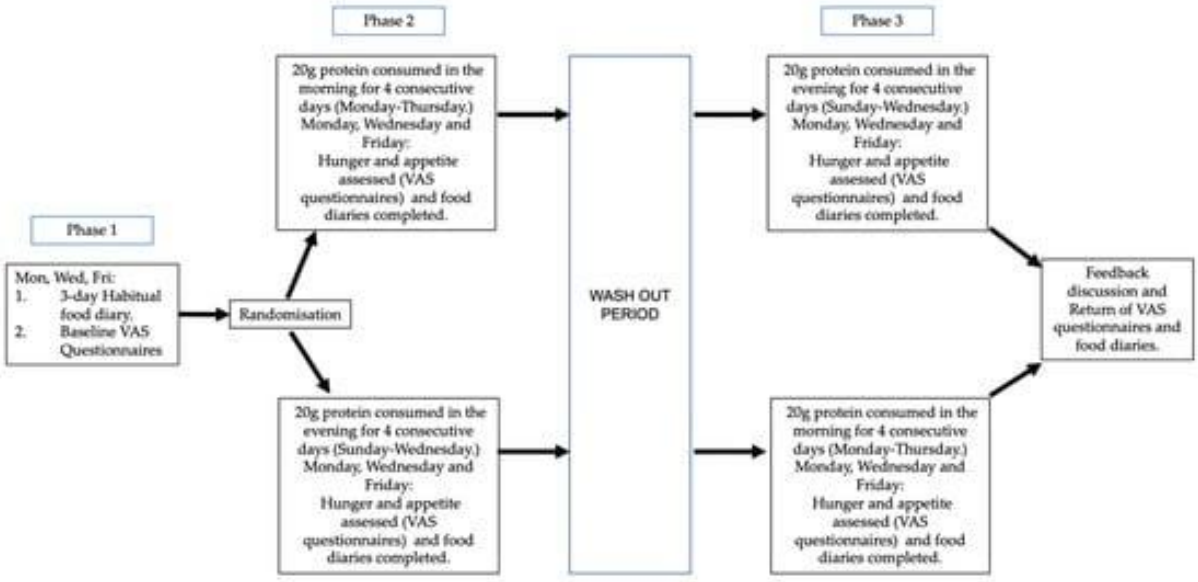

Figure 1. Graphical summary of trial workflow.