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Serum zonulin measured by enzyme-linked immunosorbent assay may not be a reliable marker of small intestinal permeability in healthy adults

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Abbreviations

BMI; body mass index

CV; coefficient of variation

DXA; dual-energy X-ray absorptiometry

hs-CRP; high-sensitivity C-reactive protein

IP; Intestinal permeability

L-R; lactulose-rhamnose

LPS; lipopolysaccharide

VAT; visceral adipose tissue

WC; waist circumference

WHO; World Health Organization

Abstract

The association between intestinal permeability (IP) and body composition remains unclear. The gold standard differential sugar-absorption test is arduous to complete, with zonulin being increasingly used as an independent biomarker of IP. This pilot study aimed to explore the association between small IP, zonulin concentrations and body composition in healthy adults. The urinary lactulose-rhamnose ratio was used to measure small IP. Serum zonulin, lipopolysaccharide (LPS) and high-sensitivity C-reactive protein (hs-CRP) were analyzed in serum. Body composition was measured using dual-energy X-ray absorptiometry and anthropometric measurements were collected. In total, 34 participants were included (12 males, median age 28 years, body mass index 24kg/m^2, waist circumference 77cm). No correlation was observed between the lactulose-rhamnose ratio and zonulin (*r* -0.016, *p* 0.929). The lactulose-rhamnose ratio displayed a strong positive correlation with LPS (n 22, *r* 0.536, *p* 0.018) but did not correlate with body composition measures. Conversely, zonulin displayed a moderate positive correlation with waist circumference (*r* 0.437, *p* 0.042) in female participants and hs-CRP (*r* 0.485, *p* 0.004) in all participants. These findings raise important considerations for the measurement of small IP, warranting exploration in larger powered studies that address the limitations of the present study.

Keywords: Intestinal permeability; L-R ratio; zonulin; endotoxemia; inflammation; body composition.

1. Introduction

The importance of intestinal barrier homeostasis is being increasingly recognized in the scientific literature [1 2]. The differential sugar-absorption test is considered the current non-invasive *in vivo* gold standard method of measuring intestinal permeability (IP). Non-digestible sugars that provide a measure of transcellular (e.g. L-rhamnose) and paracellular (e.g. lactulose) permeability are ingested and subsequently measured in urine. The higher the ratio of paracellular to transcellular permeability, the more severe the IP [3]. Increased IP, a reflection of impaired intestinal barrier function, has been associated with endotoxemia and inflammation in clinical populations [1]. There is emerging evidence to suggest that IP may be altered in obesity in otherwise healthy populations [4 5].

The association between obesity and increased IP was first elucidated in animal studies [4]. One of the first studies to explore this relationship in healthy adults found no differences in the lactulose-mannitol (a measure of small IP) and lactulose-sucralose (a measure of colonic permeability) ratios in 13 obese and 11 control participants [6]. Subsequent studies completed in healthy female adult participants have reported a positive association between small and colonic IP and waist circumference (WC) [7 8], as well as colonic IP and visceral adiposity [7]. These findings suggest that central and in particular visceral adiposity, independent of weight status, may contribute to increased IP [7 8]. This may be explained in part by the association between visceral adipose tissue (VAT) and inflammation, irrespective of body mass index (BMI) [5 9]. These findings are important as increases in IP,

weight and abdominal adiposity are associated with inflammation, insulin resistance and liver steatosis [4 10].

Although the differential sugar-absorption test is widely used to measure IP, it is onerous and expensive to complete. Zonulin, more recently identified as prehaptoglobin-2, is a ~47-kDa protein that is capable of modulating intercellular tight junctions in the small intestine, with increased concentrations in blood indicative of increased small IP [11 12]. Zonulin is being used independently of the differential sugar-absorption test as a measure of small IP, owing to the ease of analyzing concentrations of zonulin in blood.

However, limited studies have explored the association between zonulin concentrations and the differential sugar-absorption test [13]. Furthermore, zonulin concentrations are associated with obesity in healthy adults and have also been found to be higher in individuals with higher WCs, waist: hip ratios, fat mass and total fat percentage [14-16]. To our knowledge limited studies have explored the association between small IP, zonulin concentrations and body composition measures in healthy populations. The hypothesis for this prospective pilot study was that small IP would display a positive and significant association with zonulin concentrations and measures of abdominal adiposity. The study aimed to explore the relationship between small IP, zonulin and body composition. Specifically, the objectives of the study were twofold; (1) to explore whether small IP measured using the differential sugar-absorption test is associated with zonulin concentrations and (2) to explore the association between small IP, zonulin concentrations and body composition measures in healthy adults. The findings of this study will advance our

understanding of the measurement of small IP using gold standard methods and its relationship with body composition measures and may assist in informing the management of increased small IP and/or abdominal adiposity in healthy adults in future.

2. Methods and materials

This prospective pilot study was conducted at La Trobe University in Melbourne, Australia. The study was approved by the La Trobe University Human Ethics Committee and written consent was obtained from all participants prior to study commencement. The study was not registered.

Adults aged 18 years or older with no current or past history of diabetes, heart disease and gastrointestinal conditions (including gastrointestinal intolerances) were invited to take part in the study. Adults who were pregnant or those that had taken antibiotics or nonsteroidal anti-inflammatory drugs within the fortnight prior to study commencement were excluded. Screening processes were conducted via email and/or telephone, with eligibility reassessed prior to obtaining written consent at the first study appointment. The study was advertised on social media platforms and study flyers were distributed throughout the La Trobe University Bundoora campus, General Practitioner clinics in the City of Darebin and The Alfred Hospital, Melbourne, Australia with recruitment taking place between July 2014 and April 2016.

Participants meeting the eligibility criteria and consenting to take part in the research study attended La Trobe University, Bundoora campus on three separate occasions for study appointments (Figure 1).

2.1 Demographics and health information

Participant demographics and health information, including current and past medical history, smoking status, alcohol intake, weekly physical activity (minutes per week of total, moderate [e.g. walking] and vigorous [e.g. jogging]), the use of medication and nutrition supplements were collected using a questionnaire at the initial appointment. Smoking and alcohol intake were assessed using questions from the 2013 National Drug Strategy Household Survey [17], while physical activity was assessed using questions from the Active Australia Survey [18].

2.2 Intestinal permeability measurements

Small IP was measured using 0.5 g L-rhamnose and 1 g of lactulose mixed together and dissolved in potable water. This pilot study formed part of a research project measuring segmental IP, however this manuscript reports the results relating to small IP only as measured using the lactulose-rhamnose (L-R) ratio as the zonulin system is not operative in the large intestine [19 20]. The sugar dosages used were based on the novel and sensitive analytical method published by van Wijck and colleagues [21].

As the optimal sampling time for IP tests is debatable and dependent on individual participants' intestinal transit times [22 23], the percentage urinary recovery of each sugar was calculated in 24-hour urine samples in order to capture complete urinary sugar excretion along the gastrointestinal tract.

Prior to commencing urine collections, participants were asked to empty their bladders. A baseline non-fasted 24-hour urine collection was conducted to determine the concentrations of sugars in urine samples prior to the administration of the sugar solution. Following completion and return of the baseline urine collection, participants consumed a 50 mL solution containing the sugar probes dissolved in potable water. Participants collected urine for a subsequent 24-hour period, referred to as the 'test' collection.

Once returned, the weight of the 24-hour urine collections were recorded and participants were asked whether all urine produced during the 24-hour collection period was collected, including reasons for incomplete collections. The weight of 1 ml of urine was determined in triplicate (3 x 1 ml aliquots) for each participant 24-hour urine sample to allow for conversion of urine collection weight (kg) to volume (L). Samples were vortexed to achieve a homogeneous solution and aliquots were stored at -80°C until analysis.

Urinary concentrations of sugars in baseline and test samples were analyzed using gas chromatography- mass spectrometry. Samples were extracted and analyzed by staff at Metabolomics Australia at The University of Melbourne, a National Collaborative Research Infrastructure Strategy initiative under Bioplatforms Australia

Pty Ltd. In brief, a 20-microliter aliquot of homogenised urine sample was treated with 20 µL of urease (1 mg of urease [V7752-VL]) and dissolved in 1 ml of Milli-Q water) following 15 minutes incubation at 30°C with a mixing speed of 950 rpm. Subsequently, 150 µL of 100% cold methanol containing 1% (v/v) 13C6 sorbitol was added to the sample and vortexed before being incubated on ice for 10 minutes. The sample was then centrifuged for 15 minutes at 13,000 rpm (4°C). A 50 µL aliquot of supernatant was dried down in glass insert in vacuo using a Rotational Vacuum Concentrator (RVC 2-33 CD plus, John Morris Scientific, Pty Ltd) set at ambient temperature, prior to the derivatisation. The dried sample was prepared in 20 µL of 30 mgmL-1 methoxyamine hydrochloride in pyridine followed by two hours at 37°C with mixing at 500 rpm. The sample was then derivatised with 20 µL of N,O-bis (Trimethylsilyl) trifluoroacetamide with trimethylchlorosilane (BSTFA with 1% TMCS, Thermo Scientific) for 30 minutes at 37°C. The sample was then left for 1 h before 1 µL was injected onto the gas chromatography column using a hot needle technique. Sugar concentrations present in each sample were quantified by acquiring 9 points calibration series: 160, 80, 40, 20, 10, 5, 2.2, 1.25, 0.625 µM containing L-rhamnose and lactulose. Data was processed using Agilent MassHunter Quantitative Analysis software (B.07.00).

The concentrations of sugars in baseline and test urine samples were analyzed in a non-fasted state using the method described above and reported in µmol/L. Participants were advised to adhere to their normal diet during the study period.

When calculating the (1) urinary excretion of each sugar and (2) urinary recovery of each sugar in relation to the orally administered dose, the following formula were

used. Urinary excretion of sugar (µmol) = concentration of sugar in urine (µmol/L) x total 24-hour volume of urine (L). Percent urinary recovery of sugar = (urinary excretion of sugar [µmol]/ quantity of sugar ingested [µmol]) x 100.

Baseline sugar concentrations were subtracted from test sample concentrations to increase IP test accuracy [21]. Values were excluded from analysis if sugar concentrations were higher in baseline samples in comparison to test samples. The L-R ratio was used to measure small IP, as both L-rhamnose and lactulose undergo degradation by bacteria in the large intestine [21 24]. The percentage recovery of L-rhamnose was used to measure small intestine transcellular permeability, while the percentage recovery of lactulose was used as a measure of small intestine paracellular permeability.

2.3 Zonulin measurements

Blood samples were collected at the first study appointment in a non-fasted state. Serum zonulin concentrations were analyzed in duplicate using a 96 well plate ELISA kit (K5601, Immundiagnostik AG®, Bensheim, Germany), with the absorbance measured at 450 nm. The lower limit of detection for the kit was 0.225 ng/mL. The intra- and interassay coefficient of variation (CV) for the ELISA kit was between 3.4-6.0% and 13.3-13.6%, respectively. Based on information included in the analysis kit manual, the median value of zonulin in the serum of 40 healthy individuals included in Immundiagnostik studies was reported to be 34 ng/mL (±14 ng/mL) (results unreferenced).

2.4 Body composition measures

Anthropometric measurements were completed at the first study appointment. Participants were asked to empty their bladders, remove their shoes and any heavy items from their pockets prior to the completion of measurements. Measurements were completed in duplicate by the same researcher, with the mean of two measures recorded to the nearest tenth of a centimeter/ kilogram (0.1 cm/ 0.1 kg). Anthropometric measurements included weight, height, waist and hip circumference. BMI, WC and waist-hip ratio were calculated and cut-offs were categorized in accordance to World Health Organization (WHO) classifications [25 26].

Body composition was measured using the Hologic Discovery W QDR 4500A fan beam Dual-energy X-ray absorptiometry (DXA) device (Hologic, Inc., Bedford, MA). Prior to each scan, calibration was performed using a spine phantom, according to the manufacturer instructions. The DXA measurements included total body fat (%), trunk fat (%) and VAT (cm^2). VAT was estimated by the DXA analysis software based on a patented method developed by Hologic [27].

2.5 *Analyses of C-reactive protein and lipopolysaccharide*

High-sensitivity C-reactive protein (hs-CRP) and lipopolysaccharide (LPS) were measured in blood samples as markers of inflammation and bacterial translocation, respectively. The analysis of hs-CRP in serum samples was performed by Dorevitch Pathology using particle enhanced turbidimetric assay. The lower detection limit for the assay was 0.1 mg/L and the intra- and interassay CV was 0.6-1.3% and 2.2-3.5%, respectively. Concentrations of hs-CRP <3mg/L were considered to be normal

[28 29]. Concentrations between ≥3 and ≤10 mg/L were considered to be indicative of low-grade inflammation, whilst concentrations of hs-CRP >10mg/L were considered to be reflective of an acute infection [28 29].

The concentration of LPS was analyzed in a subset of participants. Serum samples were analyzed in duplicate using a 96 well plate ELISA kit (Abbexa®, Cambridge, UK), with the absorbance measured at 450nm. The range of detection for the kit was 0.015-1 EU/mL, with a sensitivity of <0.0078 EU/mL. The intra- and interassay CV for the ELISA kit was reported as ≤4.3% and ≤5.5%, respectively.

2.6 Sample size

This pilot study formed part of a research project determining the utility of a sensitive multi-sugar test in measuring segmental IP in healthy participants in a non-fasted state. As differences in the dosage of sugar probes, urine sampling times and analysis techniques can all influence IP measurements, a sample size calculation was not performed for the broader research question and aims explored in this paper due to lack of data available in this area utilizing comparable methods.

2.7 Statistical analyses

All statistical analyses were conducted using IBM® SPSS® Statistics for Windows, Version 25.0 (Armonk, NY: IBM Corp). Normality tests were first performed to determine the distribution of data points of interest using the Shapiro-Wilk test. Descriptive statistics such as means and standard deviation (SD) were used to

describe normally distributed data, whilst medians and interquartile range (IQR) were used to describe data that violated the assumption of normality. For independent variables, the Independent-Samples *t* Test was used for normally distributed data and the Mann-Whitney U test was used for data that was not normally distributed. Correlation analyses was used to explore relationships between small IP, zonulin and body composition parameters. The Pearson correlation coefficient was used for normally distributed data, whilst a Spearman Rho Test was used to explore associations for variables that were not normally distributed. A two-tailed *p*-value less than 0.05 was considered statistically significant.

3. Results

During the recruitment period, 35 participants met the eligibility criteria and were included in the study. One participant did not return urine samples, leaving 34 participants with IP results (Figure 2). Participant characteristics are displayed in Table 1. Participants had a median age of 28 (IQR 17) years and were predominantly female (64.7%). The majority of participants were non-smokers (91.2%) and consumed alcohol between 1-2 days weekly (29.4%) or 1-3 days a month (29.4%). Six (17.6%) of the 34 participants listed a past medical history that has been linked to increased IP in the literature, such as asthma, dermatitis and depression/anxiety but were not taking prescription medication related to these conditions [30-32]. All of the remaining participants either had no past medical history or unrelated history such as orthopedic surgeries completed several years prior to participation. Six (17.6%) participants were taking regular medications including the oral contraceptive pill (n = 4), implanon (n = 1) and medication to treat

male pattern hair loss (n = 1). Three participants (8.8%) had taken antibiotics within two months of participation but not within the two-week timeframe outlined as part of the eligibility criteria. Nutrition supplements, including nutrition ergogenic aids, were consumed by 19 (55.9%) participants. The most commonly consumed supplements included fish oil, multivitamins, individual vitamin and mineral supplements and nutrition ergogenic aids such as pre-workout, protein, amino acid and creatine supplements. No differences were observed in age between male and female participants. However, weekly physical activity and body composition measures, excluding hip circumference, differed significantly between males and females (Table 1). The L-R ratio and zonulin concentrations did not differ according to the patient characteristics mentioned above (data not shown).

3.1 Intestinal permeability measurements

The median urinary volume for baseline and test collections was 2.0L (IQR 1.3L) and 2.3L (IQR 1.3L), respectively. For baseline collections, 24 (71%) participants reported complete urine collections, with ten participants (29%) reporting incomplete collections during the measurement period. For test collections, 19 (56%) participants reported complete urine collections, with 15 (44%) reporting incomplete collections during the measurement period. The most common reasons for incomplete collections were forgetting to collect urine samples overnight and omitting to collect samples at social outings. There were no significant differences in the L-R ratio between participants who reported complete versus incomplete collections (median L-R ratio 0.032 [0.018] versus 0.025 [0.024], respectively, *p* 0.321), with the decision made to include all IP data in the analysis and manuscript.

Concentrations of sugars prior to the consumption of the sugar solution were analyzed in 24-hour baseline urine collections. L-rhamnose and lactulose were detected in 18 (53%) and 16 (47%) baseline urine collections, respectively. Concentrations of sugars in baseline 24-hour urine collections were compared with concentrations in test 24-hour urine collections in order to determine if IP measurements could be reliably interpreted in a non-fasted state. The concentration of L-rhamnose increased substantially following consumption of the sugar solution [4.8 (8.1) and 139.3 (147.0) μmol/L and interquartile range, respectively for baseline and urine collection p <0.001], with no overlap between baseline and test concentrations at the participant level. Although the group median concentration of lactulose in baseline samples increased significantly following consumption of the sugar solution from 0 to 12 μmol/L (p <0.001) [values 0.0 (8.3) and 11.9 (6.2) μmol/L and interquartile range, respectively for baseline and urine collection p <0.001], the concentration of lactulose in one participant's baseline sample exceeded the concentration in the test sample. For this reason, the L-R ratio has been reported in 33 participants (Table 2).

3.2 Intestinal permeability and zonulin measurements

Zonulin concentrations are displayed in Table 2. No correlation was observed between zonulin concentrations and the L-R ratio (r -0.016, p 0.929), the percentage recovery of L-rhamnose (r -0.298, p 0.087) or the percentage recovery of lactulose (r -0.304, p 0.086).

3.3 Intestinal permeability, zonulin, hs-CRP and LPS

LPS concentrations were measured in a subgroup of participants (n = 20) (Table 2). A strong positive correlation was observed between LPS concentrations and the L-R ratio (*r* 0.536, *p* 0.018). No correlation was observed between LPS and zonulin concentrations (*r* 0.047, *p* 0.845).

Median hs-CRP concentrations for the group were 0.32 (IQR 0.86) mg/L, with three participants considered to have an elevated hs-CRP concentration indicative of low-grade inflammation and one participant with an elevated hs-CRP concentration indicative of acute elevation (Table 2). Participants with elevated hs-CRP concentrations did not differ from remaining participants in characteristics and there were no indicators as to why hs-CRP concentrations were elevated in these participants. The L-R ratio did not correlate with hs-CRP concentrations (*r* 0.039, *p* 0.831). Similarly, the L-R ratio was not higher in participants with elevated versus normal hs-CRP concentrations (0.044 versus 0.028, respectively, *p* 0.184). In contrast, zonulin concentrations displayed a significant and moderate positive correlation with hs-CRP concentrations (*r* 0.485, *p* 0.004). Similarly, mean zonulin concentrations were significantly higher in participants with elevated (n = 4) versus normal (n = 30) hs-CRP concentrations despite the small participant numbers (60.2 versus 43.7 ng/mL, respectively, *p* <0.001). No correlation was observed between hs-CRP and LPS concentrations (*r* -0.246, *p* 0.297).

3.4 Intestinal permeability and body composition

Body composition measures are presented in Table 1. The median BMI of participants was 23.2 (IQR 5.6) kg/m^2. Based on the WHO BMI classifications, 22 participants (65%) had a BMI within the healthy range, eight participants (24%) were classified as overweight and four participants (12%) were classified as obese. Of the 12 participants (35%) classified as either overweight or obese, five participants (15%, n = 1 male, n = 4 females) had a WC indicative of "substantial risk" (>102 cm for males and >88 cm for females) [25]. Similarly, three participants (9%, n = 1 male, n = 2 females) had a waist: hip ratio indicative of "substantial increased risk" based on WHO guideline classifications (≥0.90 cm for males and ≥0.85 cm for females) [25]. There were no differences in the L-R ratio in participants classified as healthy weight versus overweight using BMI and in those with increased WC or waist: hip ratios in comparison to values within recommended references ranges (data not shown) [25 26].

Correlation analyses between the L-R ratio, zonulin concentrations and body composition measures are displayed in Table 3. No associations were observed between small IP (L-R ratio) and body composition measures. Due to the differences in body composition between male and female participants, data was split by gender and reanalyzed. Although the findings were not statistically significant, the L-R ratio displayed a strong positive correlation with total body fat (*r* 0.566, *p* 0.055) and trunk fat (*r* 0.552, *p* 0.063) in male participants that was nearing statistical significance. No other significant correlations or trends were observed between body composition measures and the L-R ratio.

3.5 Zonulin and body composition

There were no differences in zonulin concentrations in participants according to BMI, WC or waist: hip ratios classifications. No associations were observed between zonulin concentrations and body composition measures when explored in all participants (Table 4). However, zonulin concentrations displayed a statistically significant and moderate positive correlation with WC (*r* 0.437, *p* 0.042) and a moderate positive correlation that was nearing statistical significance with VAT (*r* 0.418, *p* 0.053) in female participants. Furthermore, zonulin concentrations in female participants displayed a moderate positive correlation with BMI (*r* 0.368, *p* 0.092), whole body fat (*r* 0.391, *p* 0.072) and trunk fat (*r* 0.382, *p* 0.079), but findings were not statistically significant. Due to these findings, the association between zonulin, hs-CRP and LPS concentrations was explored in female participants. Zonulin concentrations displayed a strong positive correlation with hs-CRP concentrations (*r* 0.571, *p* 0.005), with no correlation observed with LPS concentrations (*r* 0.131, *p* 0.669). There were no associations between zonulin concentrations and body composition measures in male participants

4. Discussion

In this study, the associations between small IP (L-R ratio), zonulin concentrations and body composition measures were explored in healthy adult participants. No significant association was observed between the L-R ratio and zonulin concentrations, both used as measures of small IP in the literature. The L-R ratio displayed a positive association with LPS concentrations but no significant associations were observed with body composition measures and hs-CRP

concentrations. Conversely, zonulin concentrations displayed a positive association with WC and hs-CRP concentrations in female participants, with no association observed with LPS concentrations. These findings do not support the study hypothesis but should be interpreted with consideration of the sample size and study limitations.

4.1 Intestinal permeability and zonulin measurements

Increasingly, zonulin concentrations are being used to measure small IP in lieu of the more arduous, but comprehensively researched, differential sugar-absorption test [14 15]. To our knowledge, the correlation between small IP and zonulin was first assessed in humans by Sapone and colleagues [13]. In this study, a statistically significant and moderate positive association (*r* 0.36, *p* 0.0004) was observed between the L-M ratio and zonulin concentrations in a subset of type I diabetic patients (n = 36) and their relatives (n = 56) [13].

In our study, we did not observe a significant association between the L-R ratio, percentage recovery of lactulose and zonulin concentrations in healthy adult participants. This is in accordance with recent studies that have reported no significant associations between zonulin and small IP in a grouped analysis of patients with diarrhea-predominant irritable bowel syndrome, coeliac disease and healthy controls [33], frequent migraine sufferers [34], adults undertaking very intense sport activity (zonulin measured using stool assays) [35] and healthy participants in a crossover randomized controlled trial exploring the effects of inulin-enriched pasta on IP [36]. A recent paper by Wegh and colleagues reported a

significant positive correlation between serum zonulin and urinary sucrose excretion in ulcerative colitis patients in remission, although this was similarly not observed between serum zonulin and the L-R ratio [37]. Nonetheless, there is limited research that has investigated the association between zonulin concentrations and the differential sugar-absorption test in healthy populations. This study adds to findings suggesting that the L-R ratio, a measure of small IP, may not be associated with serum zonulin concentrations in healthy populations. These findings are important in light of the increasing use of zonulin as a surrogate biomarker of IP in research studies involving healthy populations and warrants exploration on a larger scale [14 15]. The view that zonulin concentrations alone may not be a reliable biomarker of IP is mirrored in a recent paper, which reinforces that more than 50 different proteins are involved in the regulation of intestinal tight junctions, highlighting the complex nature of tight junction regulation [16].

4.2 Small intestinal permeability and body composition

The association between the L-R ratio and body composition in healthy participants was explored in the present study. The initial intentions of the study were to investigate IP in participants of varying body compositions, however only 12 (35%) of the recruited participants were classified as either overweight or obese. Of these participants, five and three had an elevated WC and waist: hip ratio according to WHO classifications, respectively. Furthermore, in comparison to the National Health and Nutrition Examination Survey (NHANES) population-based dataset acquired with Hologic fan beam DXA scans across the United States from 1999 through to 2004, participants in the present study had a lower total body fat percentage in

comparison to 25-year-old white males and white females [38]. Interpreted together with physical activity data and nutritional supplement usage, recruited participants overall had healthy body compositions, were exceeding recommended weekly physical activity targets and may have been more health conscious [39].

The relationship between IP and body composition in healthy adults has not been researched extensively. In past studies, BMI was not found to correlate with measurements of IP [6-8], which is consistent with the findings of the present study. Despite not being related to BMI, a study by Gummesson and colleagues found that the 6-12 hour sucralose-mannitol ratio, used to reflect colonic IP, displayed a positive association with WC, VAT and liver fat measured using computed tomography in 55 healthy female participants [7]. No significant associations were observed between small IP and body composition measures in the present study, which may in part be due to small participant numbers, although total body fat and trunk fat displayed a positive association with the L-R ratio in male participants that was nearing statistical significance. The majority of studies exploring IP and body composition have been completed in healthy female participants [7 8], with limited studies completed solely in male participants. Furthermore, data relating to colonic permeability, whole-gut permeability and body composition measures are not explored in this paper. The relationship between segmental IP and body composition, in particular visceral fat, needs to be measured in a larger powered study using gold standard methods, including a greater representation of varying body compositions in order to confirm or refute these findings.

LPS concentrations displayed a positive correlation with the L-R ratio, suggesting that increases in small IP are associated with bacterial translocation in the small intestine in healthy populations. The findings from this study suggest that LPS concentrations in blood may be more reliable measures of increases in small IP in healthy populations, in comparison to zonulin concentrations. This needs to be further explored with consideration of limitations associated with the use of LPS concentrations as an indirect measure of bacterial translocation, including the short half-life of LPS in blood and variable detection rates [40].

Inflammation can be a consequence of increased IP and can similarly contribute to ongoing increases in IP [5]. In this study, hs-CRP concentrations did not correlate with the L-R ratio. However, only four participants were found to have elevated hs-CRP concentrations above 3.0 mg/L, with a lack of heterogeneity in sample concentrations. Therefore, these findings may have been due to tests being completed in healthy participants who as a group were not considered to have increased inflammation.

4.3 Zonulin and body composition

Zonulin concentrations displayed a statistically significant positive correlation with WC and a positive correlation with VAT in female participants that was nearing significance. The inclusion of four females and only one male with a WC classified as “substantial risk” and the higher total body fat and trunk fat percentage observed in female participants may partly explain this finding. However, findings were not reflected in the L-R ratio or in LPS concentrations. The association between zonulin

concentrations, waist: hip ratio [14] and WC [16 41] have been previously reported. However, none of these studies included direct measurements of IP, using zonulin as an independent biomarker of IP. Zonulin release is not restricted to enterocytes but also occurs in several tissues including, but not limited to, the liver and adipose tissue [16]. In this study, a positive correlation was also observed between zonulin concentrations and hs-CRP. Previous studies have proposed that zonulin can activate the complement system and that concentrations are associated with inflammatory markers [14 15 42].

It is plausible that serum zonulin concentrations in the present study were reflective of release from extra-intestinal tissues as opposed to providing an indication of small IP. Although this needs further exploration, it is possible that serum zonulin may be used as a risk marker of low-grade inflammation, visceral adiposity and/or metabolic syndrome in the future. The lack of an association between zonulin concentrations and body composition measures in males may have been due to the smaller number of males included in the study and the low abdominal fat content observed in these participants. Nonetheless, based on the results of the present study, zonulin concentrations may not reflect IP in healthy adults and levels may be elevated due to variations in abdominal adiposity and low-grade inflammation. Whether fecal zonulin concentrations are more reflective of small IP needs to be explored.

4.4 Strengths and limitations

The strengths of this study include the exploration of associations between small IP and body composition using a sensitive IP test, which has not been completed extensively in healthy populations. The measurement of VAT using DXA, represents

a study strength, with WC commonly used as a surrogate marker of abdominal adiposity in past studies. Furthermore, the comparison of the L-R ratio with zonulin concentrations has not been previously completed in healthy populations, despite zonulin being used as a biomarker of IP in research and selected clinical laboratories.

The measurement of IP in a non-fasted state and deduction of baseline sugar concentrations from test concentrations may be viewed as a limitation as participants were not on standardized meal plans and due to the potential temporal variation in baseline sugar concentrations. However, baseline sugar concentrations in the present study were comparable to those detected in the urine samples of fasted participants [21], L-rhamnose and lactulose are not found abundantly in the diet and baseline concentrations of sugars can act as test confounders but are rarely tested and reported in IP studies. The measurement of zonulin in a non-fasted state may have similarly impacted serum concentrations. The small sample size is a limitation of the present study, which may have resulted in type II errors, therefore findings should be interpreted with caution. Although participants were excluded if they had GI conditions, diabetes or heart disease, a small number of participants had conditions associated with increased IP such as asthma and depression, nonetheless these participants were not found to have elevated L-R ratios or zonulin concentrations. Insulin resistance was not measured in the present study representing a limitation, with previous studies suggesting a possible link between increased IP, zonulin and insulin resistance [10 14]. An additional limitation relates to the completeness of urine collections. Approximately half of the participants reported incomplete test urine collections. Although the L-R ratio did not vary between

complete versus incomplete collections, this represents a limitation of the study that may have impacted analysis and study findings. All studies employing urine collections in free-living populations should put methods in place to minimize the likelihood of incomplete collections occurring including frequent reminders and explanation of the importance of compliance for findings. Lastly, a number of recent studies have voiced significant concerns regarding the measurement of zonulin using commercial ELISA kits [43-45]. Findings suggest that currently available kits may not be specific to the detection of zonulin (prehaptoglobin-2) and that measurements are subject to high intra-participant variation [43-45]. These findings raise concerns regarding the use of presently available ELISA kits for the measurement of zonulin and question the interpretation of studies utilizing these analysis methods.

In conclusion, this paper contributes important findings to the limited evidence base on the association between small IP, serum zonulin concentrations and body composition measures in healthy adults. LPS, but not zonulin concentrations, were related to small IP measurements. LPS may be a more reliable biomarker of small IP in this population. The use of serum zonulin as an independent biomarker of small IP in healthy populations should be carefully considered in future studies until more data is available. Conversely, zonulin concentrations were associated with hs-CRP, a marker of inflammation, and abdominal adiposity in healthy female participants. Further research studies are needed to explore the association between small IP, zonulin and body composition, including sex-specific relationships, using reliable measurement methods in larger powered studies in healthy populations.

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Table 1. Characteristics of healthy participants

Variable	All participants	Males	Females	*p*-value[a]

Age, years	28.0 [8.0]	27.5 [7.0]	28.5 [10.0]	0.219
Weight, kg	72.3 ± 17.4	87.5 ± 13.2	64.1 ± 13.5	<0.001
Height, m	1.71 ± 0.11	1.82 ± 0.07	1.65 ± 0.07	<0.001
BMI, [kg/m^2]	23.7 [5.7]	25.8 [3.9]	21.2 [4.4]	0.006
Waist circumference, cm	77.2 [16.9]	85.8 [7.1]	73.5 [8.0]	0.002
Hip circumference, cm	100.9 ± 11.8	103.9 ± 5.6	99.3 ± 11.4	0.204
Waist: hip ratio	0.79 ± 0.06	0.84 ± 0.05	0.77 ± 0.06	0.001
Total body fat, %	27.6 ± 8.6	19.9 ± 5.3	31.7 ± 7.1	<0.001
Trunk fat, %	23.5 [10.0]	18.4 [9.4]	25.6 [11.4]	0.006
Visceral fat area, cm^2	47.9 [41.1]	67.5 [25.5]	37.6 [32.8]	0.014
Nutritional supplements				
No	15 (44.1)	5 (41.7)	10 (45.5)	1.000
Yes	19 (55.9)	7 (58.3)	12 (54.5)	
Smoking				
No	26 (76.5)	6 (50.0)	20 (90.9)	
No, but have smoked in the last 12 months	5 (14.7)	4 (33.3)	1 (4.5)	
Yes, but less often than weekly	3 (8.8)	2 (16.7)	1 (4.5)	
Alcohol				
3-6 days a week	7 (20.6)	4 (33.3)	3 (13.6)	
1-2 days a week	10 (29.4)	2 (16.7)	8 (36.4)	
1-3 days a month	10 (29.4)	5 (41.7)	5 (22.7)	

<1 day a month	7 (20.0)	1 (8.3)	6 (27.3)	
Physical activity, minutes/week[b]				
Total	500 [326]	600 [413]	435 [301]	0.048
Moderate	203 [178]	180 [218]	233 [230]	0.543
Vigorous	240 [338]	450 [383]	180 [240]	0.002

All participants, n=34; males, n=12; females, n=22. Data are analyzed by Independent-Samples *t* test for parametric variables, Mann-Whitney U test for non-parametric variables and Chi-Square Test for Independence for categorical variables. Data are presented as means ± standard deviation for parametric variables, medians [interquartile range] for nonparametric variables and numbers (percentage) for categorical variables. BMI, body mass index.

a. *p* values for the differences between male and female participants, *p* values <0.05 are considered statistically significant.

b. Physical activity was self-reported and assessed using questions from the Active Australia Survey [18]. Examples of moderate physical activity included walking, gentle swimming and social tennis. Examples of vigorous physical activity included jogging, cycling and competitive tennis.

Table 2. Small intestinal permeability, zonulin, hs-CRP and LPS concentrations in healthy participants

Variable	All participants	Males	Females	*p*-value[a]
L-R ratio[b]	0.030 [0.020]	0.033 [0.020]	0.028 [0.022]	0.626
L-rhamnose recovery, %	10.5 ± 4.4	9.5 ± 3.0	11.1 ± 5.0	0.297
Lactulose recovery, %[b]	0.7 [0.5]	0.6 [0.6]	0.7 [0.4]	0.721
Zonulin, ng/mL	45.7 ± 9.3	45.9 ± 6.9	45.5 ± 10.6	0.899
hs-CRP, mg/L	0.32 [0.86]	0.17 [0.55]	0.55 [0.99]	0.217
Endotoxins (LPS), EU/mL[c]	0.51 ± 0.15	0.53 ± 0.11	0.50 ± 0.18	0.683

All participants, n=34; males, n=12; females, n=22. Data are analyzed by Independent-Samples *t* test for parametric variables and Mann-Whitney U test for non-parametric variables. Data presented as means ± standard deviation for parametric variables and medians [interquartile range] for nonparametric variables. EU, endotoxin units; hs-CRP, high-sensitivity C-reactive protein; L-R, lactulose/rhamnose; LPS, lipopolysaccharide.

a. *p* values for the differences between male and female participants, *p* values <0.05 are considered statistically significant.

b. n=33 (12 males, 21 females).

c. n= 20 (7 males, 13 females).

Table 3. Association between the lactulose-rhamnose ratio (measure of small intestinal permeability) and body composition measures in healthy participants

			Weight	Height	BMI	WC	W:H ratio	Total body fat	Trunk fat	VAT area
L-R ratio	**All participants**	r	0.151	0.008	0.091	0.135	0.039	0.113	0.162	0.226
		p	0.400	0.967	0.615	0.453	0.828	0.531	0.367	0.206
	Males	r	0.175	0.126	0.116	0.399	-0.119	0.566	0.552	0.420
		p	0.587	0.697	0.721	0.199	0.712	0.055	0.063	0.175
	Females	r	0.038	-0.126	-0.004	0.018	-0.018	0.122	0.081	0.173
		p	0.869	0.586	0.987	0.938	0.939	0.600	0.728	0.454

All participants, n=33; males, n=12; females, n=21. Data are analyzed by the Pearson Correlation test for parametric variables and the Spearman Rho test for non-parametric variables. BMI, body mass index; L-R, lactulose/rhamnose; VAT, visceral adipose tissue; WC, waist circumference; W:H ratio, waist: hip ratio.

Table 4. Association between zonulin and body composition measures in healthy participants

			Weight	Height	BMI	WC	W:H ratio	Total body fat	Trunk fat	VAT area
Zonulin, ng/ml	**All participants**	r	0.173	-0.021	0.147	0.210	0.265	0.188	0.205	0.264
		p	0.327	0.905	0.408	0.233	0.129	0.288	0.246	0.131
	Males	r	-0.199	-0.169	-0.217	0.042	0.327	-0.202	-0.147	0.077
		p	0.535	0.600	0.498	0.897	0.299	0.529	0.649	0.812
	Females	r	0.350	-0.025	0.368	0.437[a]	0.298	0.391	0.382	0.418
		p	0.111	0.912	0.092	0.042[a]	0.178	0.072	0.079	0.053

All participants, n=34; Males, n=12; Females, n=22. Data are analyzed by the Pearson Correlation test for parametric variables and the Spearman Rho test for non-parametric variables. BMI, body mass index; VAT, visceral adipose tissue; WC, waist circumference; W:H ratio, waist: hip ratio.

a. Statistically significant (*p* value <0.05) correlation.

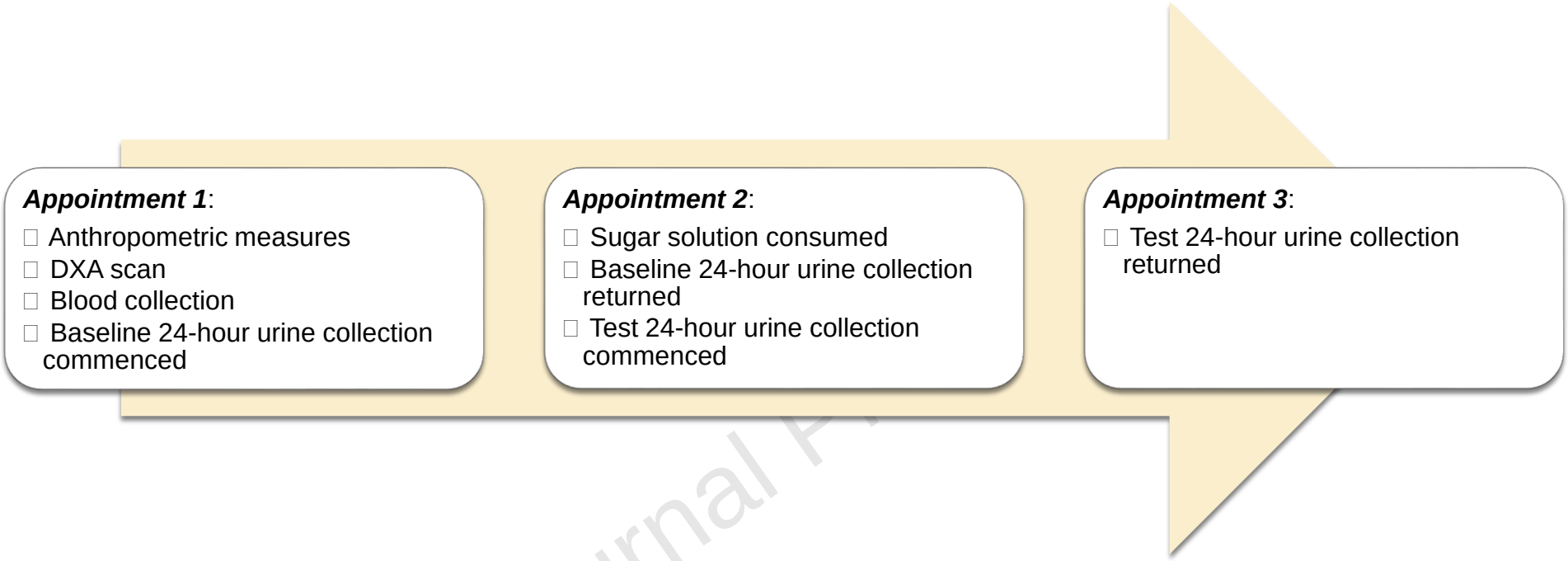

Figure 1. Overview of participant appointments
DXA, Dual-energy X-ray absorptiometry.

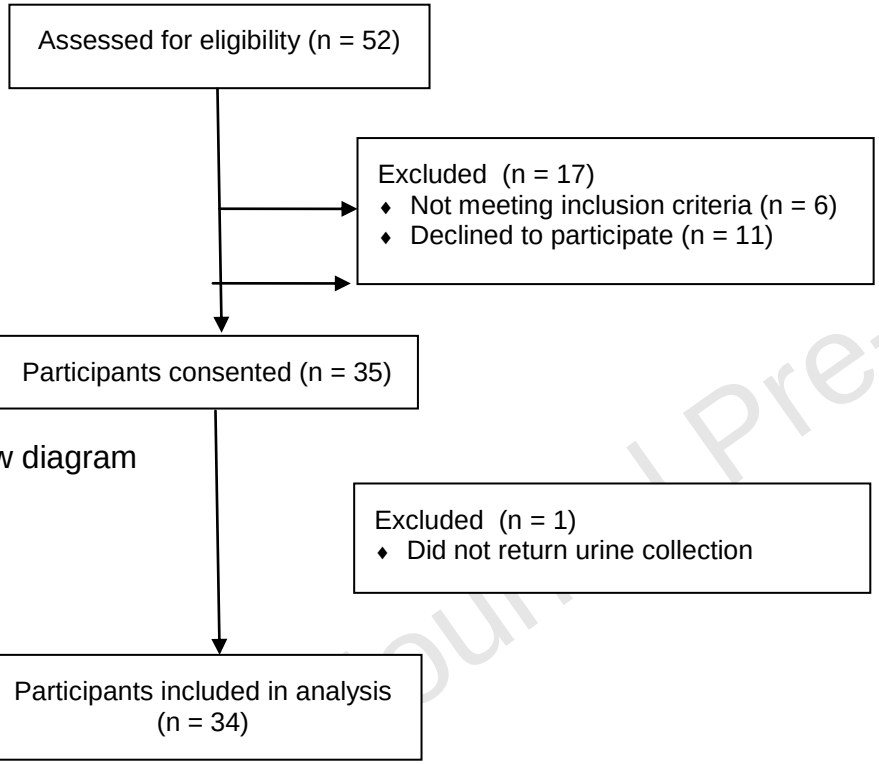

Figure 2. Study flow diagram

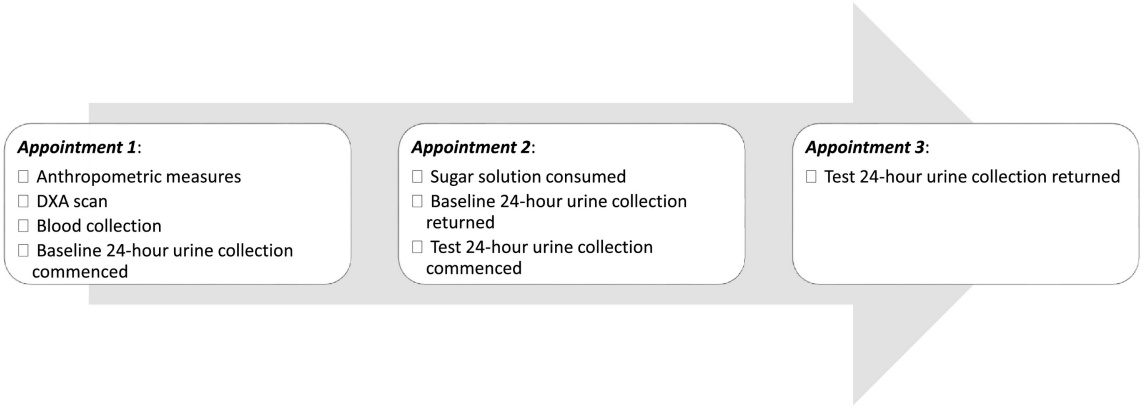
Appointment 1:
Anthropometric measures
DXA scan
Blood collection
Baseline 24-hour urine collection commenced
Appointment 2:
Sugar solution consumed
Baseline 24-hour urine collection returned
Test 24-hour urine collection commenced
Appointment 3:
Test 24-hour urine collection returned

Figure 1

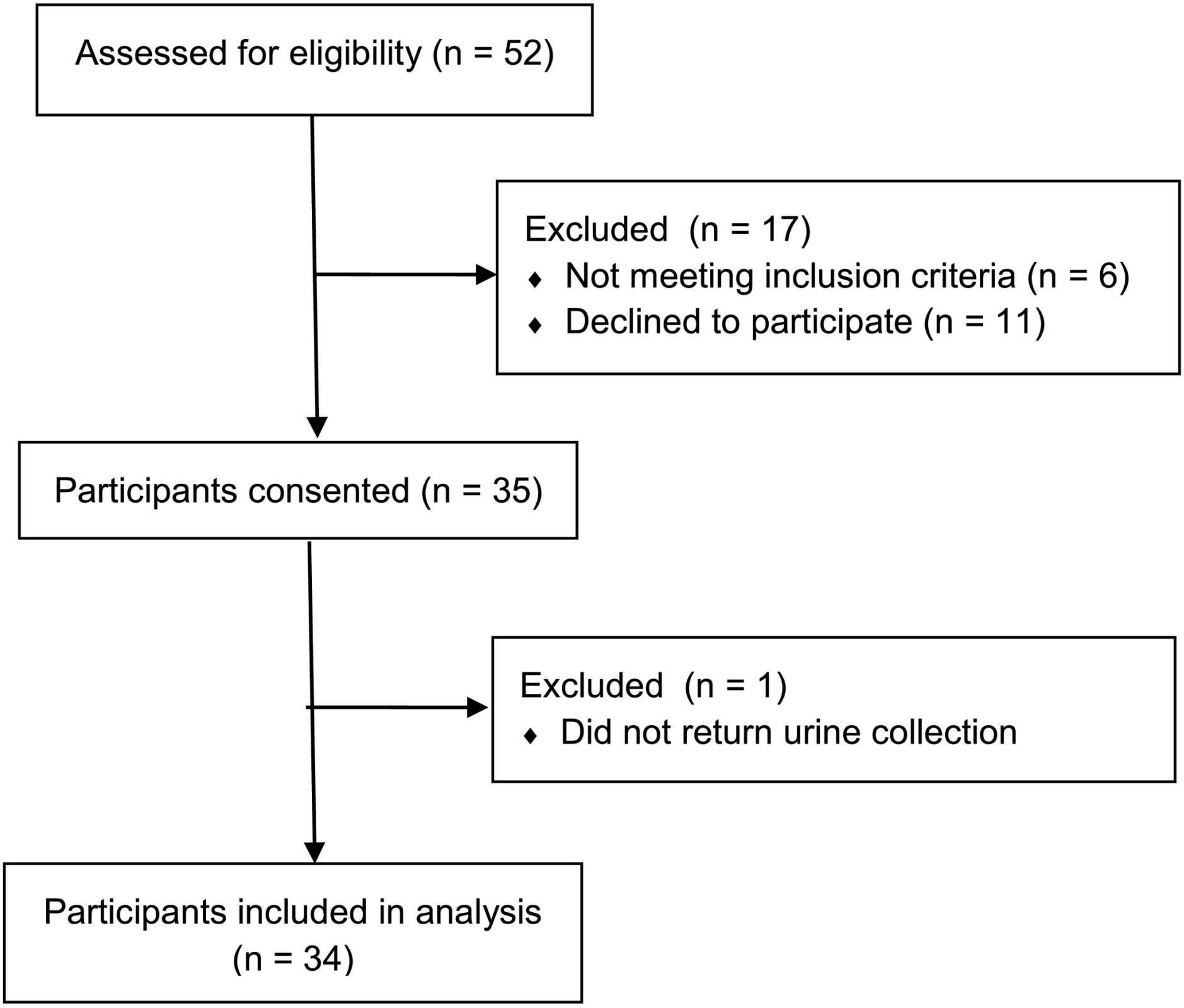
Assessed for eligibility (n = 52)
Excluded (n = 17)
Not meeting inclusion criteria (n = 6)
Declined to participate (n = 11)
Participants consented (n = 35)
Excluded (n = 1)
Did not return urine collection
Participants included in analysis (n = 34)

Figure 2