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Intra-individual variation in markers of intestinal permeability and adipose tissue inflammation in healthy normal weight to obese adults1

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Abstract

Background: Intestinal permeability and adipose tissue inflammation are considered mechanistic links in the relationship between diet, obesity, and chronic disease. However, methods to measure both are not well standardized, and the reliability of commonly used measures is not known.

Methods: We calculated the intraclass correlation coefficient (ICC) for several common measures of intestinal permeability and adipose tissue inflammation from a randomized clinical trial of cross-over design in which normal weight (n=12) or overweight/obese (n=12) individuals each completed three 8-day dietary intervention periods.

Results: For biomarkers of intestinal permeability, plasma zonulin and lipopolysaccharide binding protein, ICCs were 'excellent' (i.e., > 0.9). The direct measure of intestinal permeability, the lactulose/mannitol test, exhibited 'fair' reliability (ICC=0.53). A wider range of ICCs (0.6 – 0.9), suggesting 'good' to 'excellent' reliability, were obtained for measures of adipose tissue expression of genes encoding major mediators of inflammation. Similarly, individual immune cell populations isolated from adipose tissue, expressed as a percentage of all $CD45^+$ cells, also had 'good' to 'excellent' ICCs. However, when these populations were expressed as number of cells per gram of tissue, ICC values were 'fair', falling below 0.6.

Conclusion: Due to the repeated measures design, our study offered a unique opportunity to assess reliability of commonly used biomarkers of intestinal permeability and adipose tissue

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inflammation. Our findings suggest that these measures were generally highly reliable in the short-term.

Keywords

Intestinal permeability; biomarker reliability; adipose tissue inflammation; lactulose mannitol; intraclass correlation coefficient

Introduction

Although the exact physiological processes governing the development of obesity and type 2 diabetes remain elusive, existing evidence identifies inflammation as a key etiological factor in the pathology of insulin resistance [1–3]. For example, plasma concentrations of pro-inflammatory cytokines such as tumor necrosis factor (TNF), interleukin (IL)-6, and C-reactive protein (CRP) are elevated among individuals that are both obese and insulin resistant, and further predict the development of metabolic disease [2]. In addition, the composition of the gut microbiota and an increase in intestinal permeability may be linked to obesity and insulin resistance in part through endotoxemia-induced inflammation [4–6]. In adipose tissue, immune cell infiltration and increased pro-inflammatory cytokine expression are strongly associated with obesity-induced insulin resistance [3, 7–9].

Lack of standardization and variability, and perhaps validity, in many of the measures used to assess adipose tissue inflammation represents a significant hurdle in fully unmasking the relationship between inflammation and metabolic disease. There likely also exists variation both within and among individuals and this has not always been well characterized in studies. With regard to systemic inflammation, several studies have assessed the reliability of plasma biomarkers of inflammation within individuals and have reported a high degree of reliability in short-term assessments of up to one year [10, 11]. For adipose tissue inflammation, however, there is a paucity of such literature. Similarly, no data exist on the reliability of common measures of intestinal permeability, including direct measures such as the lactulose-mannitol test, as well as more indirect measures of plasma biomarkers like zonulin and lipopolysaccharide-binding protein (LBP) assessed in this study. We therefore sought to conduct a secondary analysis using data from a recently completed study in which 24 healthy normal weight or overweight/obese adults underwent a controlled intervention of three separate 8-day dietary exposures to sugar-sweetened beverages (SSB), in random order, with identical clinical measures carried out following each dietary period. A strength of this study is that the diet consumed in the eight days prior to each clinical assessment was standardized, with the only difference being the type of sugar used in the SSB. Because the intervention did not affect most of the biomarkers of intestinal permeability and adipose tissue inflammation (i.e. the results were null), it provides an opportunity to assess their reliability.

The aim of this secondary analysis was to characterize the intra-individual variability in repeated measures of the adipose tissue mRNA expression of key mediators of inflammation and leukocyte cell populations in human subcutaneous adipose tissue, as well as biomarkers of intestinal permeability associated with short-term exposures.

Methods

Research Design and Study participants

This study represents a secondary analysis of a completed dietary intervention, the 'Diet and Systemic Inflammation' (DASI) study, carried out at the Fred Hutchinson Cancer Research Center (FHCRC), Seattle, WA, as described previously [12, 13]. Briefly, 24 healthy individuals aged between 18 and 65 years, were recruited into two body mass index (BMI) categories: normal weight (20–24.9 kg/m^2, n=12) and overweight to obese (25–39.9 kg/m^2, n=12). Upon enrollment, participants were randomized into a controlled double-blinded cross-over dietary intervention to compare the effect of high-dose SSB consumption on energy intake, low-grade chronic inflammation, and intestinal permeability. Each of the three dietary periods lasted for eight days and an identical menu of solid foods, patterned after the average American diet (50% carbohydrate, 34% fat, and 16% protein) was provided. An additional 25% of estimated energy intake was provided in the form of a SSB, sweetened with 100% fructose, 100% glucose, or high-fructose corn syrup (HFCS; 55% fructose, 41% glucose, 4% higher saccharides). While the solid foods were to be consumed *ad libitum*, consumption of the SSB each day was mandatory. Each subject completed each of the three SSB phases in random order and a 21-day washout period separated each of the three phases. Subject eligibility was restricted to those individuals who had been weight stable to within 4.5 kg of their lifetime maximum weight and who would comply with study diets and clinical procedures. Exclusion criteria included smoking, use of recreational drugs, consumption of more than two alcoholic beverages per day, presence or history of cardiovascular disease, diabetes mellitus, or any other chronic inflammatory, autoimmune or metabolic disease, phenylketonuria, anemia, fructose intolerance or other malabsorption syndrome, recent pregnancy or current breast feeding, or use of insulin, antidiabetics, β-blockers, glucocorticoids, anabolic steroids, warfarin, antibiotics, probiotics, or non-steroidal anti-inflammatory drugs (daily use). Written informed consent was obtained from all subjects and the FHCRC Institutional Review Board approved this study.

Specimen Collection

At the end of each of the three 8-day dietary periods, fasting blood was collected by venous puncture with plasma stored at −70°C. All participants underwent a lactulose/mannitol test to assess intestinal permeability [14], and urine was collected over five hours and stored at −70°C. Subcutaneous adipose tissue was collected from 14 of the 24 study participants, as described previously [15]. Approximately 1–2 g of adipose tissue was collected from a the subcutaneous depot lateral to the umbilicus, using a different location following each dietary period. A portion of the tissue was frozen immediately on dry ice and the remainder was used to quantify and characterize tissue leukocytes by flow cytometry.

Specimen Analysis

Lactulose and mannitol concentrations in urine were measured by gas chromatography (columns supplied by Agilent Technologies) using a previously published method [16]. Briefly, pooled urine from healthy volunteers was spiked with lactulose and mannitol to create a 6-level standard curve with concentrations ranging from 0.02 to 5.00 mg/mL. The average recovery rate was 96.0% when pure sugar standards were added to pooled urine and

the intra- and inter-assay coefficients of variation were 4.2% and 5.6%, respectively. In addition, two indirect measures of intestinal permeability, plasma zonulin (ALPCO, Salem, NH) and LBP (Cell Sciences, Newburyport, MA), were measured by ELISA. Intra- and inter-assay coefficients of variation were 1.9% and 9.2% for zonulin, and 8.3% and 32.2% for LBP, respectively. Because of the high inter-assay variability for LBP, we normalized across plates with the use of internal standards that were run twice in triplicate on each plate.

Total RNA was extracted from whole adipose tissue using RNeasy Lipid Tissue kit (Qiagen, Hilden, Germany) and quantified using RiboGreen (Invitrogen Corp., Carlsbad, CA). cDNA synthesis was carried out on ~1 mg of total RNA using the RETROscript® Kit (Ambion/Applied Biosystems, Foster City, CA) and PCR performed using pre-designed TaqMan® Gene Expression Assays (Applied Biosystems) on an ABI Prism® 7900HT Sequence Detection System. Gene targets included adiponectin (*ADIPOQ)*, *IL1B*, *IL6*, *IL10*, *TNFA*, C-C motif chemokine ligand 2 (*CCL2*), and interferon-γ (*IFNG)*. We measured β-glucuronidase (*GUSB*) as a housekeeping gene as this is stably expressed in adipose tissue across different conditions [17]. A normalization factor was then calculated using this housekeeping gene and applied to all target genes.

Stromal vascular cells (SVC) were isolated from fresh adipose tissue and assayed by multi-parameter flow cytometry as described previously [18]. Characterized cell populations were then normalized as a percentage of the $CD45^+$ cell fraction (total leukocytes) and as number of cells per gram of adipose tissue. This normalization strategy allowed us to capture any changes in cell populations by calculating both the absolute change in cell population number (per g of adipose tissue) as well as the change relative to other immune cell populations (as a percentage of total leukocyte cell fraction).

Statistics

All statistical analyses were performed using the Statistical Package for the Social Sciences for Macintosh (version 20.0; IBM Corporation, Armonk, NY). Distribution of variables was analyzed by checking histograms and normal plots of the data, and normality was tested by means of Kolmogorov-Smirnov and Shapiro-Wilk tests. All non-normally distributed variables were log-transformed prior to analysis. All data are presented as means ± SD or median (25^{th}, 75^{th} percentiles). Random effects analysis of variance was used to estimate the intraclass correlation coefficient (ICC) and 95% confidence interval across all three diet periods. Values between 0.4 and 0.75 were interpreted as providing ‘fair’ to ‘good’ correlation, while those greater than 0.75 were indicative of ‘excellent’ correlation [19].

Results

The detailed baseline characteristics for the 24 participants who completed all study procedures have been described previously [12]. Subjects were either normal weight (n = 12; 3 women and 9 men) with a mean ± SD age of 33 ± 11 y, BMI of 23.7 ± 1.0, fasting glucose of 87 ± 10 mg/dL, and baseline CRP of 1.1 ± 1.0 mg/L or overweight/obese (n = 12; 6 women and 6 men) with a mean ± SD age of 39 ± 12 y, BMI of 31.0 ± 4.3, fasting glucose of 96 ± 8 mg/dL, and baseline CRP of 2.5 ± 1.8 mg/L [12].

Intraclass correlation coefficients for biomarkers of gut permeability are presented in Table 1. Coefficients for plasma zonulin and LBP, as well as urinary excretion of lactulose and mannitol, were ‘excellent’ (ICC ≥ 0.75). Estimates of the lactulose/mannitol (L/M) ratio and lactulose recovery were ‘good’ (ICC ≈ 0.60). Of note, there was a significant difference in the mean L/M ratio by repeated measures (RM)-ANOVA [13]. Post-hoc tests revealed that the L/M ratio obtained for HFCS was significantly different from both glucose and fructose while glucose and fructose L/M ratios were not different from each other. Therefore, we recalculated the ICC value for the L/M ratio by using only the data obtained at the end of the glucose and the fructose periods, and the ICC decreased slightly from 0.59 (0.16 – 0.82) to 0.53 (−0.2 – 0.79). Similarly, lactulose recovery between the glucose and HFCS diet periods was also significantly different [13]. After adjusting for these findings, ICCs based on only the fructose and glucose phase, as well as the fructose and HFCS phase, were 0.57 (0.07 – 0.81) and 0.46 (−0.15 – 0.76), respectively. Thus, the reliability for the L/M ratio and the lactulose recovery can be rated as ‘fair’.

Intraclass correlation coefficients for markers of adipose tissue inflammation are presented in Table 2. The values for adipose tissue mRNA expression ranged from ‘good’ to ‘excellent’ for all key mediators of inflammation studied (ICC ≥ 0.60). After excluding data from the fructose period that differed significantly from the HFCS and glucose periods, the recalculated ICC for adipose tissue expression of ADIPOQ mRNA was 0.74 (0.19 – 0.92), based only on the glucose and HFCS diet periods. Among cell populations assessed, ICCs were typically ‘good’ to ‘excellent’ (ICC ≥ 0.64) when expressed as cell number per gram of adipose tissue or as a percentage of total leukocytes ($CD45^+$ cells). However, ‘fair’ and ‘poor’ ICCs (values < 0.52) were obtained for the number of adipose tissue macrophages (ATM) and dendritic cells per g of adipose tissue, and the ICC for $CD4^+$T cells was uninterpretable (ICC = −1.2) (Table 2). The mean number of dendritic cells, as a percentage of all leukocytes ($CD45^+$ cells), was significantly different following the fructose compared to the glucose and HFCS diet periods. Therefore, the ICC was recalculated to determine the reliability of the marker based on the glucose and HFCS diet periods only (Table 2).

Discussion

In this study, we took advantage of available data from a well-controlled randomized, crossover intervention study to assess the intra-individual variability in measures of adipose tissue inflammation and intestinal permeability in healthy individuals. While the intervention was not intended *a priori* for this purpose, the design provided repeated measures in up to 24 participants approximately one month apart, following an 8-day dietary period that was largely standardized. The one factor that differed between the three dietary periods (the type of sugar used to sweeten the SSBs) did not affect intestinal permeability or adipose tissue inflammation in a consistent manner, as discussed in the original publication [13]. Therefore, this study provided an ideal opportunity to assess the reliability of repeated measures of these biomarkers.

Overall, ICCs fell within the ‘good’ to ‘excellent’ range of reliability for plasma and urinary markers of intestinal permeability as well as gene expression in subcutaneous adipose tissue. To the best of our knowledge, we are the first group to publish data on the reliability of these

commonly used biomarkers. Our study shows that at least in the short-term, that among healthy individuals these measures provide reliable measures of intestinal permeability and gene expression of key mediators of inflammation in subcutaneous adipose tissue. However, over a much longer time frame, one likely of greater relevance in the etiology of chronic human disease, the test-retest reliability of these measures remains unclear and warrants further study. Importantly, there were some measures that did not exhibit comparable reliability. Specifically, for immune cell populations isolated from adipose tissue, there was greater reliability when cell numbers were normalized to the percent of total leukocytes in the sample rather than the total cell number per gram of adipose tissue. This discrepancy similarly warrants further assessment and consideration for future studies.

Interestingly, the L/M test is currently considered the best non-invasive method by which to measure intestinal permeability [14, 20]. Median L/M ratios of 0.038 and 0.080 have previously been reported in humans free from chronic inflammatory or GI diseases [20, 21]. Our reported median L/M ratios of 0.03–0.05 [13] are in agreement with these previously published data. Based on the estimated ICCs of 0.53–0.59, we infer that among healthy subjects, the L/M-ratio has ‘fair’ reliability. However, it stands to reason that the ICC for the L/M-ratio would be higher if our study included individuals with certain GI disorders associated with substantially elevated L/M-ratios, given that any error will(would) likely be inflated relative to a low baseline in the healthy individuals studied here. As for the other commonly used measures of intestinal permeability, plasma zonulin and LBP, the ICCs calculated from our repeated measures were both ‘excellent’ (ICC ≥ 0.95). However, the validity of both zonulin and LBP for the assessment of intestinal permeability is not clear. While an initial study showed a modest correlation between fasting serum zonulin concentrations and the L/M-ratio in type 1 diabetic patients ($r = 0.36$, $p = 0.0004$) [22], suggesting that zonulin may be a biomarker for intestinal permeability, our study could not confirm this correlation in our healthy population ($r = 0.033$, $p = 0.789$) [13]. Furthermore, a recent analysis suggests that not only is serum zonulin concentration highly variable from day to day and even within a single day [23], but a widely-used, commercially available ELISA kit from Immundiagnostik (supplied by ALPCO) does not actually detect the zonulin protein alone, but rather several structurally similar proteins [24]. All studies, including our own, which utilized this assay to measure plasma zonulin most now be interpreted with this in mind. Similarly, no correlation between fasting plasma LBP concentrations and the L/M-ratio was observed in either our study ($r = 0.009$, $p = 0.940$) [13], or that of Vogt et al. ($r = 0.207$, $p = 0.168$) [25]. Furthermore, zonulin and LBP both are clearly affected by a number of other factors including age, sex, and adiposity [26–28]. Taken together, while the reliability of fasting plasma zonulin and LBP in this population was ‘excellent’ while that of L/M-test based measures was only ‘fair’, it is important to emphasize that numerous factors inform the decision for or against any particular assay and the reliability data presented here are only one such factor. Other factors to consider include the validity of the measure (i.e. how closely the assay measures what the investigator intends to measure), subject burden, and study costs.

Among measures of adipose tissue inflammation, we observed ‘very good’ to ‘excellent’ reliability for subcutaneous adipose tissue expression of *ADIPOQ*, *TNFA*, *IL10*, *CCL2*, and *IFNG* mRNA, and ‘good’ reliability for *IL1B* and *IL6* mRNA expression. This suggests that

these markers are stably expressed within an individual and that the use of a single time-point measurement provides a good assessment of adipose tissue inflammation in healthy individuals.

We also identified and quantified leukocyte populations isolated from the SVF of adipose tissue to characterize adipose tissue inflammation. Normalization of the individual leukocyte populations as a percentage of the entire $CD45^+$ leukocyte fraction resulted in 'good' to 'excellent' reliability across time points. However, ICCs decreased substantially when cell populations were normalized to the total gram amount of the adipose tissue sample. The poor reliability estimates in these instances likely reflects variability associated with adipose tissue digestion and processing, along with inter-rater differences in cell numeration, as previously described [18]. However, we have argued before [18] that normalizing numbers of individual immune cell populations solely to the number of all $CD45^+$ cells is suboptimal, because an increase in one cell type will then appear to be associated with a reduction in all other leukocyte cell types even if this is not the case (for examples, see [29, 30]). Thus, while leukocyte numbers reported as a percentage of total $CD45^+$ cells have better reliability than those expressed as number of cells per g of tissue, it is fair to say that the former, if used exclusively, may provide a distorted view. That is, leukocyte numbers reported as a percentage of total $CD45^+$ cells may have low validity given that this measure by itself poorly reflects what is actually occurring in the tissue. While this would be a rationale to normalize tissue cell populations both relative to all $CD45^+$ cells and to the number of cells per gram of tissue, the relatively high measurement error introduced by the latter method needs to be taken into consideration in the design of studies in this field as well as in the reporting and interpretation of results.

The major strength of this study was the design that allowed for each biomarker to be assessed in each study participant at three different time points, approximately one month apart, as part of a randomized, crossover intervention. Each subject was provided with an identical diet at(for each intervention phase) each of the three time points, overall energy intake did not differ among the dietary periods, and subjects remained weight stable over the course of the study. Only the exposure (i.e., the type of sugar used to sweeten the SSB provided during each dietary period) varied. Because the intervention did not have a consistent effect on intestinal permeability or adipose tissue inflammation, this study offered a good opportunity to carry out this secondary analysis.

Nevertheless, there are a few limitations to consider. The study population was small, and several of the assessments (adipose tissue inflammation analyses) were completed on only a subset of the total study population (n=12). While the type of sugar used to sweeten the SSB did not impact the measures of intestinal permeability and adipose tissue inflammation in a consistent manner, there was an SSB effect on adipose tissue expression of *ADIPOQ* mRNA and on the lactulose-related measures of intestinal permeability. We addressed this issue by recalculating the ICCs after excluding the diet period that differed significantly ($p < 0.05$ based on *post hoc* paired t-tests, after Bonferroni correction for multiple testing) from the other diet periods. Therefore, the ICCs presented for *ADIPOQ*, L/M ratio, lactulose recovery, and urinary lactulose were calculated from two, rather than three, of the diet periods and represent minimal estimates of the ICC for these biomarkers. In all cases in

which we excluded a diet period that differed significantly from one of the other two, the ICC was slightly lowered. Even though the degree of this reduction tended to be small, this finding is counterintuitive, as the reliability should be higher, not lower, when a diet period with differential effect is excluded. This observation raises the possibility that minor differential effects of the three diet periods on individual study endpoints that we were not powered to detect may have slightly inflated the reported ICCs. Another potential limitation of our study is that the participants consumed standardized identical solid foods in the eight days prior to each clinic visit, which may have led to better reproducibility measures than if the diet had not been standardized. At the same time, the fact that dietary intakes were standardized could also be seen as a strength, because a potentially major determinant of study endpoints was standardized, thereby reducing random variability due to non-standardized and hard to measure dietary intakes.

Because low-grade inflammation is associated with several chronic diseases and cancer, and because an increase in intestinal permeability may be a key contributor to low-grade inflammation, it is imperative that the reliability of methods used to measure inflammation is known. Our study provides data to show that, at least over a relatively short time frame, that the commonly used indirect measures of intestinal permeability, fasting plasma zonulin and LBP provide ‘excellent’ reliability and the L/M-ratio ‘fair’ reliability. Among commonly used measures of adipose tissue inflammation, the transcript levels of key mediators of inflammation also exhibit ‘good’ to ‘excellent’ reliability. With regard to flow cytometry-based data on the cellular composition of adipose tissue, and particularly the types and numbers of specific leukocyte populations, the reliability was ‘very good’ to ‘excellent’ when cell populations were expressed relative to all $CD45^+$ cells. However, the reliability was only ‘fair’ for most cell populations when expressed as number of cells per gram of tissue. In conclusion, our study suggests that most measures of intestinal permeability and low-grade chronic adipose tissue inflammation have ‘good’ to ‘excellent’ reliability in the short-term, similar to many blood-based biomarkers indicative of both cancer and chronic disease risk.

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Impact: Along with other factors, particularly validity, the demonstrated reliabilities can help inform the choice of endpoints in studies of intestinal permeability and adipose tissue inflammation.

Table 1.

Intraclass correlation coefficients of gut permeability biomarkers (n=24).[1]

Biomarker	ICC
Fasting plasma LBP (ug/mL)	0.95 (0.89 – 0.97)
Fasting plasma zonulin (ng/mL)	0.96 (0.91 – 0.98)
Urinary lactulose (mg/mL)	0.75 (0.44 – 0.89)[2]
Urinary mannitol (mg/mL)	0.85 (0.71 – 0.93)
L/M ratio	0.53 (−0.2 – 0.79)[2]
Lactulose recovery	0.57 (0.07 – 0.81)[2]; 0.46 (−0.15 – 0.76)[3]

[1]Values are intraclass correlation coefficients (95% confidence interval).

LBP: lipopolysaccharide-binding protein, L/M: lactulose/mannitol.

[2]Based on the glucose and fructose diet periods

[3]Based on the fructose and HFCS diet periods

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Table 2.

Intraclass correlation coefficients of biomarkers of adipose tissue inflammation.[1]

Biomarker	ICC
mRNA (n=14)	
ADIPOQ	0.74 (0.19 – 0.92)[2]
TNFA	0.79 (0.49 – 0.93)
IL1B	0.60 (0.01 – 0.86)
IL6	0.69 (0.24 – 0.89)
IL10	0.91 (0.79 – 0.97)
CCL2	0.76 (0.43 – 0.91)
IFNG	0.92 (0.80 – 0.97)
Myeloid lineages (n=12)	
ATM per g	0.52 (−0.32 – 0.85)
ATM %CD45$^+$	0.69 (0.20 – 0.90)
CD11c ATM per g	0.42 (−0.67 – 0.82)
C11c ATM %CD45$^+$	0.73 (0.31 – 0.92)
Neutrophils per g	0.64 (0.09 – 0.89)
Neutrophils %CD45$^+$	0.70 (0.23 – 0.91)
DC per g	0.33 (−0.63 – 0.78)
DC %CD45$^+$	0.70 (−0.15 – 0.92)[2]
Lymphoid lineages (n=11)	
CD4$^+$ T cells per g	−1.2 (−6.01 – 0.39)
CD4$^+$ T cells %CD45$^+$	0.72 (0.2 – 0.92)
CD8$^+$ T cells per g	0.83 (0.53 – 0.95)
CD8$^+$ T cells %CD45$^+$	0.91 (.075 – 0.97)
CD4$^+$CD8$^+$ T cell ratio	0.97 (.092 – 0.99)

[1] Values are intraclass correlation coefficients (95% confidence interval).

IL: interleukin, CCL2: C-C motif chemokine ligand 2, ATM: adipose tissue macrophage, DC: dendritic cells.

[2] Based on the glucose and HFCS diet periods