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Mannose is an insulin-regulated metabolite reflecting whole-body insulin sensitivity in man

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Abstract

Mannose is a glucose-associated serum metabolite mainly released by the liver. Recent studies have shown several unexpected pleiotropic effects of mannose including increased regulatory T cells (Tregs), prevention of auto-immune disease and ability to reduce growth of human cancer cells. We have previously shown in large cohorts that elevated serum mannose levels are associated with future development of type 2 diabetes (T2D) and cardiovascular disease. However, potential direct effects of mannose on insulin sensitivity in vivo or in vitro are unknown.

We here show that administration of mannose (0.1.g/kg BW twice daily) for one week in man did not elicit negative effects on meal-modified glucose tolerance, markers of inflammation or insulin levels. Tregs number and insulin signaling in human liver cells were unchanged. These data suggest that mannose is a marker, and not a mediator, of insulin resistance. To verify this, we examined serum mannose levels during long-term euglycemic hyperinsulinemic clamps in non-diabetic and T2D individuals. Mannose was reduced by insulin infusion in proportion to whole-body insulin sensitivity. Thus, mannose is a biomarker of insulin resistance which may be useful for the early identification of diabetic individuals with insulin resistance and increased risk of its complications.

Introduction

Mannose, a C2 epimer of glucose, constitutes the main residue of glycoconjugates (Sharma et al., 2014). Once taken up into various organs – through sugar transporters of the GLUT (*SLC2A*) class – mannose is phosphorylated in a reaction catalyzed by hexokinase (HK); mannose-6-phosphate can then be isomerized to fructose-6-phosphate (in a reversible reaction catalyzed by phosphomannose isomerase, PMI) or channeled toward N-glycan synthesis following conversion to mannose-1-phosphate by phosphomannose mutase (PMM). The rate of disappearance of mannose thus depends on the ratio of PMI to PMM activity (Sharma and Freeze, 2011). Conversely, intracellular mannose is exported into the bloodstream following N-glycan degradation; most of this process is believed to occur in the liver (Davis and Freeze, 2001). In humans, plasma mannose concentrations are roughly 100 fold lower than those of glucose.

These glucose-linked pathways have long been known to be associated with glycation of α-dystroglycan, which is important for normal development and skeletal muscle function (Godfrey et al., 2011). However, recent studies have identified novel enzymatic pathways, unrelated to PMI or PMM, that are important for mannose-induced protein glycation of the broad family of cadherins and plexins (Larsen et al., 2017). Additionally, mannose has shown to be an important regulator of Tregs as adding mannose to drinking water prevented development of autoimmune diabetes in NOD mice and asthma in mice immunized with ovalbumin (Zhang et al., 2017). Mannose may also have effects on the gut microbiome to protect young mice from becoming obese (Sharma et al., 2018). Importantly, it was recently reported that mannose impairs tumor cell growth by reducing glycolysis, particularly in colorectal cancer cells, and also enhances the effect of chemotherapy (Gonzalez et al., 2018). Thus, mannose is considerably more pleiotropic in its biological effects than previously recognized.

In previous work – using cell-specific integrated network analysis of genome-scale metabolic models, transcriptional regulatory networks, and protein-protein interaction networks – we found raised plasma mannose emerging as a strong marker of insulin resistance in non-diabetic individuals (Lee et al., 2016). In the liver of obese, insulin-resistant subjects, hexokinase (HK) activity was reduced while the activity of PMM, glucokinase, and phosphofructokinase were increased in a co-regulated manner. It was therefore hypothesized that downregulation of genes responsible for mannose disposal accounted for the elevated plasma mannose concentrations associated with obesity and insulin resistance.

Moreover, in large clinical studies we found elevated serum mannose levels to be associated with future development of incident type 2 diabetes (T2D), cardiovascular disease (CVD) and diabetic kidney disease (Mardinoglu et al., 2017). Thus, mannose is a circulating biomarker of insulin resistance- associated complications but if it also contributes to the development of insulin resistance is currently unclear. Irrespective of mechanisms, there is currently a clinical need for the identification of biologically plausible, reliable biomarkers of insulin resistance and associated incident diseases. This is particularly important in T2D where we lack good clinical biomarkers of insulin resistance and where recent large studies have shown that individuals with T2D and genetic or other indirect markers of insulin resistance are at considerably higher risk of developing both CVD and severe diabetic kidney disease (Ahlqvist et al., 2018; Udler et al., 2018). Thus, an urgent clinical need is to identify these individuals to initiate early preventive therapy.

Whether insulin has any effect on mannose metabolism is not, to our knowledge, known as neither PMI nor PMM has been reported to be insulin-regulated. Here, we here measured the effect of insulin on circulating mannose levels in healthy insulin-sensitive subjects and in patients with T2D using the euglycemic hyperinsulinemic clamp technique. In addition, we performed a clinical study administering mannose to non-diabetic individuals for one week to examine potential effects on glucose tolerance and inflammation. We also examined whether mannose treatment increased CD4+ Tregs in these subjects, and performed experimental studies in human liver cells to examine effects on cellular insulin signaling. Overall, we conclude that mannose appears to be causally an insulin-regulated biomarker of whole-body insulin sensitivity in man, and that oral administration does not exert any negative effects on glucose tolerance and inflammation nor does it impair insulin signaling in human liver cells *in vitro*.

Methods

Mannose supplementation study

Subjects were recruited at the University of Gothenburg/Sahlgrenska University Hospital, Gothenburg, Sweden and included individuals with normal glucose tolerance (NGT) or impaired glucose tolerance (IGT). In total, 10 subjects participated and their characteristics are shown in Table 1. Inclusion criteria were healthy men and women, 50-65 years of age and exclusion criteria were subjects smoking or using snuff, subjects with diabetes or other serious chronic disease.

All three visits were conducted after an overnight fast. At each visit, body weight in light clothing and height were measured and BMI calculated. Waist circumference was measured with the subjects standing up, arms along their sides, between their lowest rib and hip. Blood pressure and pulse rate were measured in the right arm after 5 min of rest in the sitting position (Omron M7 blood pressure monitor, Omron Healthcare Europe, The Netherlands). The first visit included medical history and samples were drawn for the measurement of mannose, glucose, insulin, HbA_{1c}, hemoglobin, high-sensitive C-reactive protein (hs-CRP), leukocytes, liver and kidney function, electrolytes, and cholesterol. The second visit, around one week after the first, included a mixed meal tolerance test (MMTT)/modified oral glucose tolerance test (OGTT). A urine sample was collected before the onset of the test for creatinine and albumin analysis. The breakfast meal consisted of one egg (50 g), 40 g parmesan cheese, and 75 g glucose (glucose powder for oral solution, ALP, Stockholm, Sweden) dissolved in 250 ml of water (509 kcal; 16% protein, 28% fat, 56% carbohydrates). Subjects were told to finish the meal within 15 min. Blood samples for glucose and insulin were taken before the meal and after 15, 30, 45, 60, 90, 120, 150 and 180 min. Subjects were given mannose powder (0.1g/kg body weight) to dissolve in 150 ml of water. They were instructed to consume one dose every morning and evening for seven consecutive days, starting on the evening of the second visit. The last dose was to be consumed on the morning of the third visit, approximately one hour before the visit. At the third visit, blood samples were taken according to the two previous visits and MMTT and OGTT were performed as described. Following analyses, glucose and insulin levels are reported and total area under the glucose and insulin curves were calculated using a standard trapezoidal method. Routine blood samples were analyzed at the SI-approved Central Laboratory. Serum and plasma were then aliquoted and stored at -70°C. Plasma for analysis of mannose concentrations were stored frozen and subsequently analysed as described when all subjects had completed all visits.

Euglycemic hyperinsulinemic clamp study

Healthy volunteers (n=10) and patients with T2D (n = 20) were recruited at the Department of Clinical and Experimental Medicine, University of Pisa, Italy. Their clinical and metabolic characteristics are shown in Table 4. The healthy subjects had negative family history of diabetes and normal glucose tolerance on a standard oral glucose tolerance test (OGTT). The T2D patients were on metformin (860 mg twice daily), which was withheld 3-5 days before the metabolic study. All studies were conducted according to the guidelines laid down in the Declaration of Helsinki and were approved by the Regional Ethics Committees in Gothenburg, Sweden and in Pisa, Italy. Healthy subjects participated in

2 consecutive studies, one with insulin infusion and one with saline infusion (control), in random order within 1-2 weeks of one another; T2D subjects only underwent the clamp study. The euglycemic hyperinsulinemic clamp was carried out using an exogenous insulin infusion rate of 240 $pmol \cdot min^{-1} \cdot m^{-2}$ for 180 min, with timed blood sampling, as previously described (Ferrannini and Mari, 1998).

Plasma mannose measurement

Plasma mannose concentrations were measured by liquid chromatography/mass spectrometry (LC-MS-MS); the analytical method has been reported (Campi et al., 2019). In brief, high-performance liquid chromatography (HPLC) was optimized to separate mannose from glucose, which is present at a 100-fold higher concentrations in human plasma; LC-MS-MS was then used to quantify mannose concentrations.

Insulin signaling in human liver cells

Immunoblotting was performed as descried earlier (Hammarstedt et al., 2013) using primary antibodies for AKT and pS473-AKT (Cell Signalling, Danvers, MA, USA). Human IHH hepatocytes (ATCC, Manassas, VA, USA) were cultured in DMEM (Lonza, Basel, Switzerland), supplemented with 10% FBS and antibiotics. To study the effect of mannose on insulin signaling, the cells were incubated with different concentrations of mannose for 24 h or 14 days, followed by insulin stimulation (10 nM) for 5 min. Cellular proteins were then extracted followed by immunoblotting.

Isolation and stimulation of regulatory T cells

Peripheral blood mononuclear cells (PBMC) were isolated from fresh heparinized blood by density gradient using Ficoll-Paque (GE Healthcare). 50 µL of PBMC were used for flow cytometry, the remaining cells were washed twice in PBS and then resuspended in 2% foetal bovine serum (Sigma-Aldrich, Saint Louis, MO, USA) in PBS complemented with 1 mM EDTA to a concentration of 50 million cells/mL. The Tregs were then isolated from the samples using EasyStep Human CD4+CD127lowCD25+ Regulatory T Cell Isolation Kit (EasySep#18063, StemCell Technologies, Vancouver, BC, USA). The purity of the isolated cells was tested by flow cytometry in two samples.

After isolation of CD4+ T cells, the cells were resuspended to a concentration of 1 million cells/mL in RPMI medium 1640 + GlutaMAX (Gibco) supplemented with 10% foetal bovine serum (Sigma-

Aldrich), 50 µM β-mercaptoethanol (Gibco) and 50 µg/mL gentamycin (Sanofi-Aventis, Paris, France) in addition to 30 nM phorbol 12-myristate 13-acetate (PMA, Sigma-Aldrich) and 500 nM ionomycin (Sigma-Aldrich) to activate the cells. 500 µL of the cell suspension were added to each well of a 24-well plate and the cells were cultured for 2 h at 37°C and 5% CO_2. At the end of incubation time, cells were pelleted, supernatants collected for further analysis and the cells lysed followed by RNA extraction using Total RNA Purification Micro Kit (Norgen Biotek corp., Thorold, ON, Canada).

Quantitative PCR was performed on a ViiA™ 7 Real-Time PCR (Applied Biosystems) using SYBR Green qPCR Mastermix (SA Biosciences, Qiagen) and primer pairs for the genes FOXP3 (5´-TGGTGCTGGAGAAGGAGAAG-3´ and 5´-AGCCCTTGTCGGATGATG-3´) and IKZF2 (5´-GAGAAGCCGTTCAAATGTCC-3´and 5´-TTGCACTTGTGAGGTTTACCC-´3). Expression levels of target genes were normalized to reference genes to obtain dCt. Relative quantity (RQ) was calculated using the ddCt method.

Flow cytometry was performed on a Becton Dickinson (BD) FACS Canto II as described previously (Wasén et al., 2017). Single cell suspensions in FACS buffer (PBS, 10% FBS, 0.09% NaN3, 0.5 mM EDTA) were pre-incubated with Fc-block (BD). Cells were stained with specific antibodies to CD4-V450 (clone RM4-5, BD), CD25-FITC and Foxp3-APC with secondary antibody (clone AF647, Cell Signaling). For staining of intracellular antigens, the FoxP3/Transcription Factor staining buffer set (eBioscience) was used according to manufacturer's protocol. Analysis was performed with FlowJo using fluorochrome minus one (FMO) staining as a negative control.

Statistical analyses

Data are presented as mean ± SD or median [interquartile range] for non-normally distributed variables. In the mannose supplementation study, the differences in blood values before and after intake of mannose in the supplementation study were analyzed by the Wilcoxon signed ranks test. Two-tailed, paired student's t-test was adopted when comparing the difference between two groups during OGTT and the trapezoidal method was used to calculate AUC. In the Tregs study the group analysis of continuous data was performed using non-parametric Mann-Whitney U test for 2 groups or paired *t*-test. In the euglycemic hyperinsulinemic clamp study, group comparisons were carried out using the Wilcoxon test. Changes in plasma mannose concentration during the clamp were analysed by the Wilcoxon signed rank test. General linear models were used to test the association of variables of

interest. The analyses were conducted using SPSS Statistics software (version 25.0, IBM) or the Prism version 7 for windows (GraphPad Software, Inc.). A p value ≤ 0.05 was considered statistically significant.

Results and Discussion

Administration of mannose did not induce insulin resistance or enhance CD4+ Tregs in man

Oral administration of mannose in nondiabetic subjects: Mannose at the dose used was well tolerated and only minor gastrointestinal effects were reported (looser stools or slight nausea). Following one-week ingestion, plasma mannose levels rose 5-6 fold (Table 2). The MMTT showed no significant changes in plasma glucose or insulin levels as also evident from AUC of glucose and insulin curves (Figure 1A & B). We also separately analysed the response in individuals with or without known heredity for diabetes. Individuals with diabetes heredity were somewhat less glucose-tolerant and had a lower post-prandial insulin response than individuals without known heredity but they showed a similar lack of response to mannose (data not shown). No differences in lipids or hs-CRP were seen (Table 2).

Tregs: The number of Tregs, defined as $CD4^{+}CD127^{lo}CD25^{+}$, and isolated from the peripheral blood was not different the day before mannose administration and 7 days later. The activated Tregs before and after mannose administration had no significant difference in the expression of Treg-specific transcription factors Foxp3 and IKZF2 (Table 3). Thus, mannose administration in this amount for one week does not elicit any evidence of impaired glucose tolerance, increased insulin resistance or a change in $CD4^{+}$Tregs.

Insulin signaling in human liver cells: We incubated liver cells with different concentrations of mannose for 24 h or 14 days but saw no difference in pAkt activation under any condition (Figure 2). Thus, mannose does not directly affect insulin signaling in human liver cells.

Collectively, these data suggest that mannose as a biomarker of insulin resistance is unlikely to directly contribute to this state. We also did not see any direct increase in Tregs in contrast to recent data in mice (Zhang et al., 2017). However, the concentration of mannose used in these cell-based mouse

studies were high (25 mM *in vitro*) and *in vivo* mannose was administered long-term as 2% mannose in the drinking water, which may account for the differences from the present experiments.

Mannose is an insulin-regulated biomarker of insulin sensitivity in man

Clamp study: T2D patients were older and heavier than the healthy controls. They also showed higher fasting plasma insulin and triglyceride levels and marked insulin resistance in the clamp (Table 4). Fasting plasma mannose concentrations, which were measured at time 0 before starting the clamp, averaged 53 [20] µmol/L in the controls and 128 [49] µmol/L in T2D subjects (p<0.0001) (Figure 3A). In univariate analysis of the pooled group data, fasting mannose levels were directly related to age (r = 0.75, p<0.0001), BMI (r = 0.59, p<0.0001), percent fat mass (r = 0.53, p=0.0004), triglycerides (r = 0.55, p=0.0004), and fasting glucose (r = 0.76, p<0.0001), and inversely related to serum HDL cholesterol (r = -0.39, p=0.0170). In the bivariate analyses reported and plotted in Figure 4, age was not independently (of insulin-mediated glucose disposal) related to either fasting mannose levels (p=0.11) or the insulin-induced change in mannose levels (p=0.80).

During the clamp, plasma mannose declined in both controls and T2D subjects as fasting insulin levels were raised and maintained (at 552 ± 71 and 424 ± 53 pmol/L, p=ns) by the exogenous insulin infusion, while no changes in fasting mannose (Figure 3A) or insulin were seen during the control saline infusion. Insulin-mediated glucose disposal (the M value) was reduced by ~60% in T2D as compared to healthy subjects (Table 4). Although the absolute drop in fasting mannose concentration during the last 40 min of the 3-h clamp was not significantly different in T2D and controls (-43 [15] *vs* -48 [42] µmol/L, p=0.25), the percent change from baseline mannose levels averaged 70 [27] % in healthy subjects and 37 [20] % in T2D patients (p=0.001) (Figure 3B). In the pooled data from the two groups, bivariate analysis adjusting for BMI showed that both fasting plasma mannose concentrations and the insulin-induced drop in mannose levels were independently related to insulin-mediated glucose disposal (Figure 4).

Taken together, these data show that mannose is an insulin-regulated metabolite; it is considerably higher in the fasting state in T2D than in insulin-sensitive individuals, and remains much higher also after insulin infusion for several hours. These data clearly demonstrate that insulin lowers plasma mannose concentrations in direct, close proportion to the insulin sensitivity of glucose disposal and this effect is independent of the effect of obesity *per se* (as the BMI). Whether the insulin-induced drop in

plasma mannose levels is due to augmented disposal or reduced production *in vivo* cannot be firmly decided unless endogenous mannose appearance is traced using a labelled mannose analogue. However, the following considerations can be made. In human liver, hexose transport is facilitative (mainly through GLUT2) with apparent affinity constant of 17 mM and high transport rate, allowing extremely rapid equilibration of hexose concentrations across the hepatocyte plasma membrane. Glucokinase (GCK), the predominant HK isoform in the liver, is induced by insulin, and binds both glucose and mannose (Iynedjian, 2009). Therefore, extracellular and intracellular mannose concentrations are very likely to be virtually identical under most physiological circumstances. In T2D, hepatic GCK activation is impaired, leading to reduced glucose uptake (Basu et al., 2001). Because of the non-specific hexose affinity of GCK, this abnormality would also result in reduced mannose phosphorylation and raised intra- and extracellular mannose concentrations. By the same token, elevated plasma mannose concentrations should also be detectable in genetic conditions of GCK deficit, such as maturity-onset diabetes of the young (MODY2) (Watanabe et al., 2018). The reverse reaction, namely dephosphorylation of glucose-6-phosphate back to glucose *via* glucose-6-phosphatase (G6Pase), also reconstitutes free mannose from mannose-6-phosphate (Crane, 1955). To the extent that G6Pase activity is upregulated in T2D (Pajvani and Accili, 2015), both free glucose and free mannose levels should be increased in the liver of diabetic and, presumably, also in insulin-resistant subjects.

Using parameters such as hexose uptake or phosphorylation, respiration, lactic acid production, or stimulation of lipogenesis, the metabolism of mannose and glucose has been found to be similar in preparations of rodent brain, rat diaphragm, adipose tissue and other tissues (Wood and Cahill, 1963). The vast difference in the circulating concentrations of mannose and glucose can be easily accounted for by the fact that the rate of intestinal absorption of exogenous mannose is only one tenth that of glucose while urinary mannose excretion is much greater than that of glucose (Wood and Cahill, 1963) due to the hexose specificity of sodium-glucose co-transporters (Wright et al., 2011). Therefore, in all tissues that respond to insulin, acceleration of glycolysis might deplete fructose-6-phosphate concentrations, thereby shifting the PMI reaction towards predominant mannose-6-phosphate isomerization; the result would be a joint reduction in both free glucose and free mannose. In the presence of insulin resistance, the sequence would be reversed, and the plasma mannose response to insulin would be blunted in proportion to the degree of insulin resistance as also found here (Figure 4). This ‘pull’ mechanism does not require, but does not rule out, changes in the expression or activity of specific enzymes in the pathway in states of insulin resistance. On the other hand, higher intracellular

mannose availability might also 'push' mannose-6-phosphate through the N-glycan synthesis pathway, especially if PMM is upregulated, as indicated by the network analysis (Lee et al., 2016).

The finding that the insulin-induced drop in plasma mannose was also reciprocally related to BMI independently of insulin sensitivity matches our previous findings from large cohorts of individuals (Lee et al., 2016). The reason for this apparently specific effect of obesity is not clear. However, like the association of fasting mannose levels with triglycerides and HDL-cholesterol that with BMI may simply reflect the clustering of these variables with insulin resistance.

In summary, our data show that mannose at the dose used is well tolerated, does not impair glucose tolerance or alter insulin signaling in human cells. Instead, we show that mannose is insulin-regulated, and that circulating levels are closely associated with whole-body insulin sensitivity also in T2D. Thus, mannose can be regarded as a useful marker to identify insulin-resistant, high-risk groups, including T2D patients who are at a markedly increased risk of developing both cardiovascular and diabetic kidney complications (Ahlqvist et al., 2018; Udler et al., 2018).

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Conflict of interest

The authors declare no competing financial interests.

Author's contribution

EF, MB and US designed the studies. All authors contributed to the experiments and data analysis. EF and US wrote the paper with input from all authors. All authors have approved the manuscript.

Legend to the figures

Figure 1. Changes in plasma (A) glucose and (B) insulin levels during MMTT/OGTT in subjects before and after 7 days of mannose administration. The right panel of each figure represents the area under curve (AUC). Data are expressed as mean ± SEM. a.u: arbitrary units.

Figure 2. Western blot analysis showing insulin-induced AKT phosphorylation (serine 473) and total AKT protein in IHH hepatocytes, treated with different levels of mannose for 24h or 14 days

Figure 3. A) Time-course of plasma mannose concentrations in healthy controls and patients with type 2 diabetes (T2DM) during euglycemic hyperinsulinemia or saline infusion (in controls only). Plots are mean ± SEM. B) Boxplots of percent changes from baseline of plasma mannose concentrations during the last 40 min of a 3-hour euglycemic hyperinsulinemic clamp.

Figure 4. Reciprocal BMI-independent association of fasting plasma mannose concentrations (*top*) percent changes in plasma mannose from baseline during the clamp (*bottom*) and insulin-mediated glucose disposal (plots are average of log-transformed M values adjusted for BMI)

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Highlights

What is known

-Experimental studies have shown that mannose administration can prevent auto-immune conditions, increase number of T-regulatory cells and reduce growth of cancer cells

-Serum mannose levels are increased in obesity and insulin resistance

-Elevated mannose levels are associated with increased risk of future development of T2D and CVD

New Findings

-Administration of mannose to non-diabetic individuals did not increase markers of insulin resistance, inflammation or change number of T-regulatory cells

-Mannose did not reduce insulin signaling in human liver cells in vitro

-Long-term hyperinsulinemic euglycemic clamps showed that serum mannose levels were reduced in relation to degree of insulin sensitivity in both non-diabetic and T2D individuals

Impact on clinical practice

-Mannose is an easy biomarker of degree of insulin resistance and can be useful for identifying patients with T2D at high risk of developing diabetic renal complications and CVD

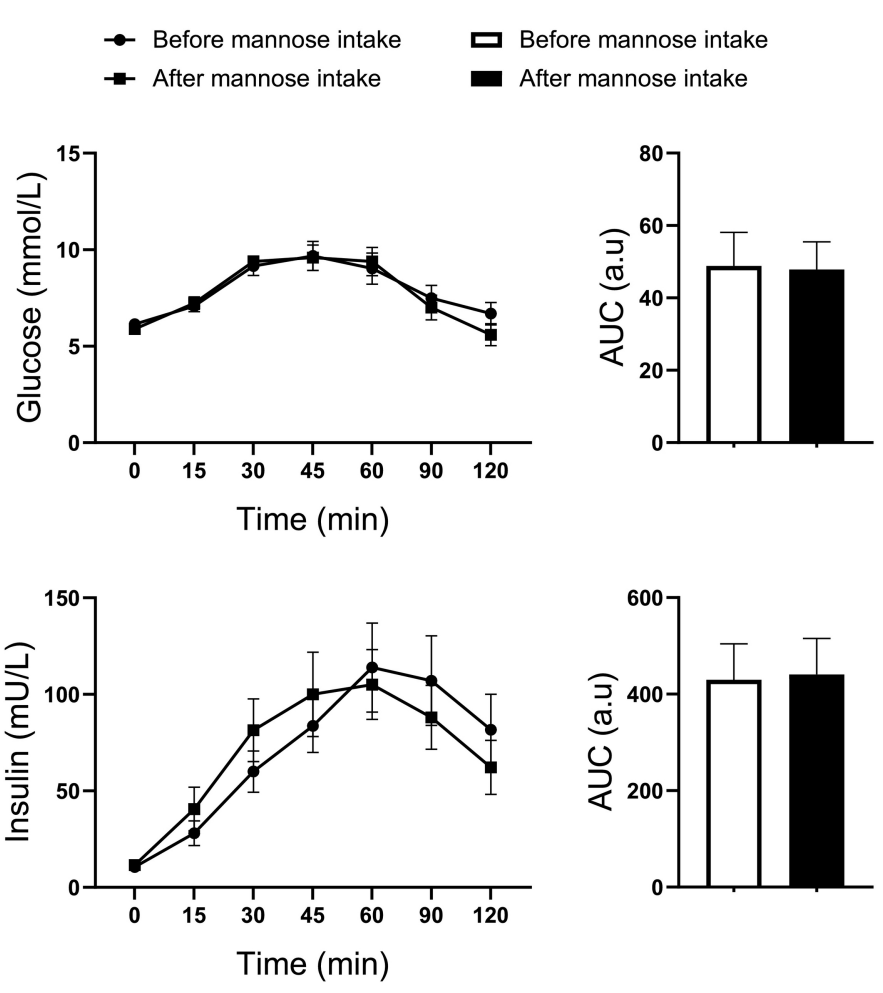
Before mannose intake
After mannose intake
Before mannose intake
After mannose intake
Glucose (mmol/L)
15
10
5
0
0
15
30
45
60
90
120
Time (min)
AUC (a.u)
80
60
40
20
0
Insulin (mU/L)
150
100
50
0
0
15
30
45
60
90
120
Time (min)
AUC (a.u)
600
400
200
0

Figure 1