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Postprandial Hypoglycemia in Patients after Gastric Bypass Surgery Is Mediated by Glucose-Induced IL-1β

Matthias Hepprich,[1,3,6] Sophia J. Wiedemann,[1,6] Benjamin L. Schelker,[1] Beckey Trinh,[1] Alessandra Stärkle,[2] Marco Geigges,[4] Jordan Löliger,[5] Marianne Böni-Schnetzler,[1] Gottfried Rudofsky,[3] and Marc Y. Donath[1,7,8,*]

[1]Clinic of Endocrinology, Diabetes and Metabolism University Hospital Basel, and Department of Biomedicine, University of Basel, Basel, Switzerland
[2]Swiss Federal Institute of Technology Zurich, Department of Health Sciences and Technology, Zurich, Switzerland
[3]Endocrinology and Metabolic Diseases, Cantonal Hospital Olten, Olten, Switzerland
[4]Department of Biosystems Science and Engineering, ETH Zürich, Basel, Switzerland
[5]Immunobiology Laboratory, Department of Biomedicine, University and University Hospital of Basel, Basel, Switzerland
[6]These authors contributed equally
[7]Senior author
[8]Lead Contact
*Correspondence: marc.donath@usb.ch
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SUMMARY

Postprandial hypoglycemia is a disabling complication of the treatment of obesity by gastric bypass surgery. So far, no therapy exists, and the underlying mechanisms remain unclear. Here, we hypothesized that glucose-induced IL-1β leads to an exaggerated insulin response in this condition. Therefore, we conducted a placebo-controlled, randomized, double-blind, crossover study with the SGLT2-inhibitor empagliflozin and the IL-1 receptor antagonist anakinra (clinicaltrials.gov NCT03200782; n = 12). Both drugs reduced postprandial insulin release and prevented hypoglycemia (symptomatic events requiring rescue glucose: placebo = 7/12, empagliflozin = 2/12, and anakinra = 2/12, $pval_{likelihood\ ratio\ test\ (LRT)}$ = 0.013; nadir blood glucose for placebo = 2.4 mmol/L, 95% CI 2.18–2.62, empagliflozin = 2.69 mmol/L, 95% CI 2.31–3.08, and anakinra = 2.99 mmol/L, 95% CI 2.43–3.55, $pval_{LRT}$ = 0.048). Moreover, analysis of monocytes *ex vivo* revealed a hyper-reactive inflammatory state that has features of an exaggerated response to a meal. Our study proposes a role for glucose-induced IL-1β in postprandial hypoglycemia after gastric bypass surgery and suggests that SGLT2-inhibitors and IL-1 antagonism may improve this condition.

INTRODUCTION

Postprandial hypoglycemia is a serious complication of gastric bypass surgery affecting up to a third of the patients (Capristo et al., 2018; Gribsholt et al., 2016). With bariatric surgery being the most effective treatment for obesity (Cheng et al., 2016; Courcoulas et al., 2017), the number of patients with this condition is set to increase (English et al., 2018; Finkelstein et al., 2012; Finucane et al., 2011). Typically, post-gastric bypass surgery hypoglycemia occurs 1–3 h after carbohydrate ingestion and presents with disabling neuroglycopenic symptoms, such as altered cognition, seizures, and loss of consciousness. In the long term, it may result in increased food intake and subsequent weight regain (Varma et al., 2017). It is characterized by a hyperglycemic peak shortly after carbohydrate intake followed by an exaggerated hyperinsulinemic response. Several underlying mechanisms for this phenomenon have been proposed, including changes in incretin hormones, gut microbiota, and bile acid composition (Goldfine et al., 2007; Nannipieri et al., 2016; Roslin et al., 2011; Salehi et al., 2014; Tack et al., 2009; Vaurs et al., 2016). Clinical studies aiming to test these hypotheses, however, remain inconclusive (Salehi et al., 2018). Therefore, no approved medical therapy exists so far (Salehi et al., 2018).

Context and Significance

Weight loss surgery is an effective treatment of obesity. A serious long-term complication of this procedure is a rapid fall in blood sugar to levels below normal following a meal. So far, no therapy exists, and the underlying mechanisms remain unclear. Researchers at the University Hospital Basel suggest that this is due to an early peak in blood sugar leading to an inflammatory response with a subsequent exaggerated rise in insulin secretion. Accordingly, treatment with either empagliflozin, which reduces the peak of blood sugar, or with anakinra, which blocks the inflammatory cytokine IL-1β, effectively improves this disabling complication of weight loss surgery.

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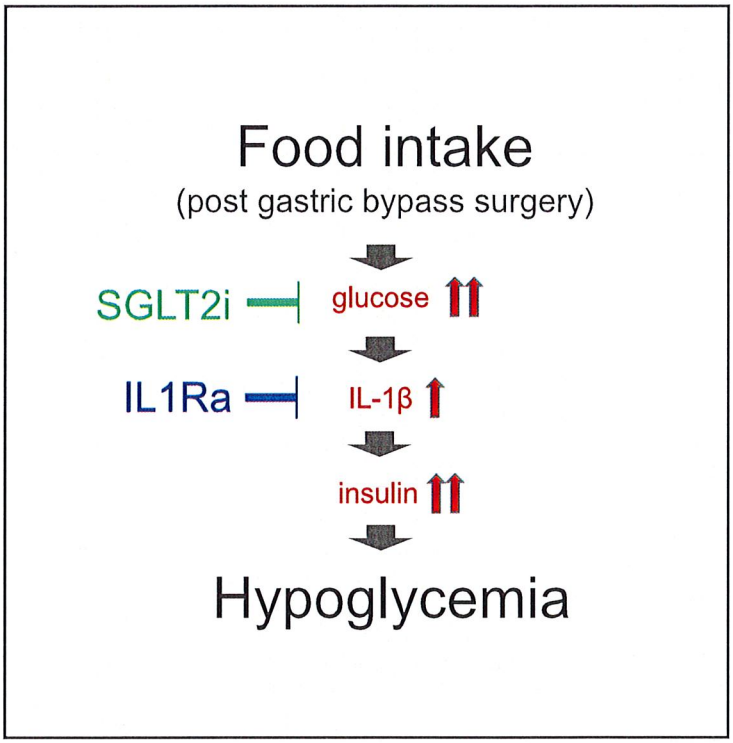
Graphical Abstract



Authors

Matthias Hepprich,
Sophia J. Wiedemann,
Benjamin L. Schelker, ...,
Marianne Böni-Schnetzler,
Gottfried Rudofsky, Marc Y. Donath

Correspondence

marc.donath@usb.ch

In Brief

Postprandial hypoglycemia is a disabling complication of bariatric surgery. Hepprich et al. show that both the SGLT2-inhibitor empagliflozin and the IL-1 receptor antagonist anakinra reduced postprandial insulin release and prevented hypoglycemia. The study therefore proposes a role for glucose-induced IL-1β in postprandial hypoglycemia after bariatric surgery, along with two treatments.

Highlights

- Post gastric bypass surgery, glucose-induced IL-1β triggers insulin and hypoglycemia
- SGLT2-inhibition prevents glucose-induced IL-1β effects
- SGLT2 or IL-1β inhibition prevents postprandial hypoglycemia after gastric bypass
- Monocytes after gastric bypass surgery are in a hyper-reactive inflammatory state

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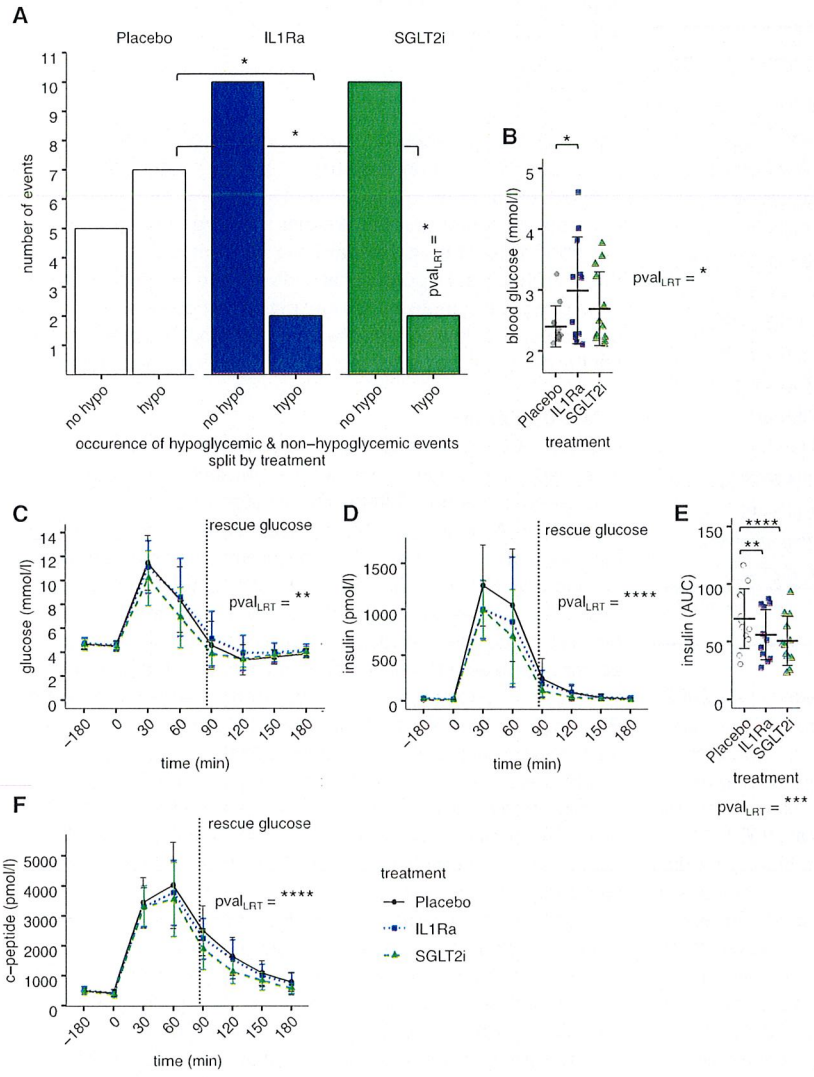

Figure 1. Metabolic Parameters in Patients after Gastric Bypass Surgery Undergoing a Mixed Meal Test upon Treatment with Empagliflozin or Anakinra

(A) Total number of visits without (no hypo) and with hypoglycemic (hypo) events, split according to treatment condition.

(B) Nadir glucose measurements in patients after gastric bypass surgery undergoing a mixed meal test upon treatment with empagliflozin or anakinra.

(C–F) Plasma glucose (C) and insulin (D) with corresponding area under curve (AUC) (E) and c-peptide levels (F). Of note, placebo-treated patients required more often rescue glucose administration; therefore, glucose levels after 60 min are artificially increased compared with the those in empagliflozin- and anakinra-treated groups. Data are presented as arithmetic mean; horizontal bars in (B) and (E) and points in time series analysis in (C), (D), and (F) ± SD (errorbars). Vertical dotted lines in (C), (D), and (F) indicate the earliest time point that was biased through rescue glucose administration (earliest administration = 84 min). Statistical analysis was performed using linear and generalized linear additive mixed-effect models as described in the STAR Methods section unless otherwise indicated ($pval_{LRT}$ = overall p values generated by likelihood ratio test (LRT) comparison of full versus reduced model). p values for individual comparisons were obtained by a subsequent Benjamini and Hochberg posttest for multiplicity adjustment. For details regarding LRT results and effect size estimates, see Table S4; *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001. See also Figures S2–S4, as well as Tables S1, S2, and S4.

were significantly increased by treatment ($pval_{LRT}$ = 0.048, Figure 1B). The reduction of the severity of the symptoms by treatment was also documented on the Edinburgh hypoglycemia scale (Figure S3A; Table S2).

Secondary Outcomes

Empagliflozin reduced peak postprandial glucose levels (Figure 1C). Of note, the majority of placebo-treated patients required rescue glucose administration; therefore, glucose levels at later time points (>60 min), i.e., following rescue glucose administration, were artificially increased in the placebo-treated group as compared with the empagliflozin- and anakinra-treated groups.

Anakinra and empagliflozin treatment reduced insulin release (Figures 1D and 1E), as well as c-peptide secretion over time (Figure 1F) most likely thereby preventing hypoglycemic events. Furthermore, anakinra reduced the insulin secretion index (p = 0.005), whereas empagliflozin showed a trend in same direction (p = 0.08) (placebo average 108.0 pmol/mmol [95% CI 84.4 to 132]; effect estimate for anakinra −18.5 pmol/mmol [95% CI −29.3 to −7.66]; effect estimate for empagliflozin −10.8 pmol/mmol [95% CI −21.6 to 0.0433]). Serum GLP-1 and glucagon levels were not affected by either treatment (Figure S4). Of note, due to the rapidity and severity of the hypoglycemic symptoms the Mini-Mental Status Test did not prove feasible in practice. The Stanford Sleepiness Scale as well as Sigstad Score did not show significant differences between treatments but were equally confounded by imbalanced rescue glucose application between treatment groups (Figures S3B and S3C).

Exploratory Outcomes

Monocytes of Patients, Isolated on Days with Hypoglycemia, Display Increased Inflammatory Gene Expression. Following our hypothesis of an overactivation of the innate immune system in patients experiencing hypoglycemia after gastric bypass surgery, we assessed transcriptional changes in innate immune cells from our patient population using an unbiased RNA sequencing (RNA-seq) approach. Therefore, we isolated monocytes from peripheral blood samples immediately before and 60 min (i.e., before rescue glucose application when needed) after ingestion of the mixed meal on all 3 treatment days. For details on sample isolation and gating strategy, see STAR Methods and Figure S5.

Table 1. Patient Baseline Characteristics

Baseline Characteristics	Values
Sex	
Female, n (%)	9 (75)
Male, n (%)	3 (25)
Age (years)	43.5 (35.3–52.5)
Time since gastric bypass surgery (months)	41.6 (26.2–67)
Height (cm)	165 (160.8–171)
Preoperative weight (kg)	118.5 (105.5–128)
Current weight (kg)	75.7 (69.8–82.2)
Preoperative body mass index (kg/m^2)	42.1 (39.5–45.6)
Current body mass index (kg/m^2)	26.9 (24.2–29.7)
Systolic blood pressure (mm Hg)	109 (97–127)
Diastolic blood pressure (mm Hg)	75 (65–80)
Heart rate (/min)	76 (64–89)
Glycated hemoglobin A1c (%)	5.1 (4.67–5.2)
Fasting glucose (mmol/L)	4.5 (4.3–4.9)
Leucocytes (G/L)	6.72 (5.25–8.09)
C-reactive protein (mg/L)	0.6 (0.37–2.3)

Baseline characteristics of the 12 study participants. Given are median values and interquartile ranges.

Glucose induces the production of IL-1β (Maedler et al., 2002). Accordingly, lowering glucose in an animal model of diabetes via phlorizin, an inhibitor of the sodium-glucose-co-transporters (SGLTs) 1 and 2, normalized plasma glucose by inducing glucosuria and prevented the induction of IL-1β expression and associated damage within the islets (Maedler et al., 2002). These findings paved the way for further animal and human studies indicating that IL-1β plays a critical role in the pathogenesis of type 2 diabetes and its complications (Donath et al., 2019; Donath and Shoelson, 2011; Larsen et al., 2007; Ridker et al., 2017). More recently, it was shown that IL-1β also displays physiologic effects in the context of food intake and in the adaptation to a high-fat diet (Dror et al., 2017; Hajmrle et al., 2016). Thus, a postprandial rise in glucose leads to an acute elevation of myeloid cell-derived IL-1β, which contributes to postprandial insulin secretion (Dror et al., 2017). This effect depends on food- and gut-derived microbial products, which prime macrophages to produce more IL-1β.

Against this background, we hypothesized that food intake in patients post-gastric bypass surgery would rapidly reach the lower part of the gastro-intestinal tract, leading to an early peak in glycemia. This, in turn, would entail an exaggerated induction of IL-1β, leading to an overshooting insulin response, followed by eventual hypoglycemia. Based on this hypothesis, we designed two different strategies to further understand and possibly prevent postprandial hypoglycemia after gastric bypass surgery (see Graphical Abstract): first, the SGLT2-inhibitor empagliflozin that may reduce postprandial peak glycemia, which in turn may decrease IL-1β- and glucose-stimulated insulin secretion. Second, the recombinant IL-1 receptor antagonist (IL-1Ra) anakinra that may lower the postprandial rise in IL-1β-induced insulin secretion (Dror et al., 2017). Both empagliflozin and anakinra have been shown to improve glycemic control in type 2 diabetes (Larsen et al., 2007; Zinman et al., 2015) and are approved for the treatment of type 2 diabetes and rheumatoid arthritis, respectively.

RESULTS

We conducted a placebo-controlled, double-blind, randomized, crossover proof-of-concept study in patients with postprandial hypoglycemia after gastric bypass surgery. Patients were randomly assigned to receive either anakinra, empagliflozin, or placebo followed by a standardized liquid mixed-meal test on three study days. Hypoglycemia was defined as (1) the appearance of typical symptoms, (2) low plasma glucose, and (3) relief of symptoms following glucose administration (Whipple's triad). For the details of the protocol, see STAR Methods and Figure S1.

Trial Population

Between June 2017 and September 2018, 14 patients were screened for the trial, 13 of whom met inclusion criteria and completed the study (Figure S1C, dates of enrollment: June 22, 2017 to August 27, 2018). To assure a more homogeneous trial population, the original study protocol was amended to include only patients after gastric bypass surgery. Importantly, this was done before any of the study investigators were unblinded. Thus, one patient was excluded per the updated protocol for having an alternative bariatric procedure (gastric sleeve). Data pertaining to this patient are reported separately (Figure S2). Baseline characteristics of the 12 participants included in the final analysis are presented in Table 1. Briefly, on average, participants were 44 years old and weighed 76 kg (preoperative average body weight 119 kg). Nine were women and 3 were men. One subject was not able to ingest the full-specified liquid mixed meal (carbohydrate load of 40 g instead of 60 g) on the first study day and thereafter received the same portion of liquid mixed meal on subsequent study days. Individual body weights remained stable throughout the study period. On days where patients received an injection of anakinra, serum levels of IL-1Ra increased from an average of 272 pg/mL (95% CI 244.16–299.19) to 43,836 pg/ml (95% CI 43,707–43,965), confirming correct application and resorption of the drug. Leukocyte and C-reactive protein (CRP) levels were not elevated (Table 1) and remained stable throughout the trial (data not shown).

Both Anakinra and Empagliflozin Reduced Hypoglycemia by Decreasing Postprandial Insulin Secretion

Primary Outcome

On the day of placebo administration, 1–3 h following the ingestion of the mixed meal, 7 of 12 patients developed severe symptomatic hypoglycemia requiring glucose administration (Figure 1A). All patients responded to 10 g glucose administration with immediate and complete resolution of their hypoglycemic symptoms, thus fulfilling the Whipple's triad. One patient on placebo developed another hypoglycemic episode after blood glucose normalization that again resolved immediately after administration of an additional 10 g of glucose (Table S1). In contrast, pre-treatment with either anakinra ($p = 0.037$) or empagliflozin ($p = 0.037$) significantly reduced the severity of hypoglycemic events with only two events requiring rescue glucose administration (Figure 1A). Moreover, nadir glucose values

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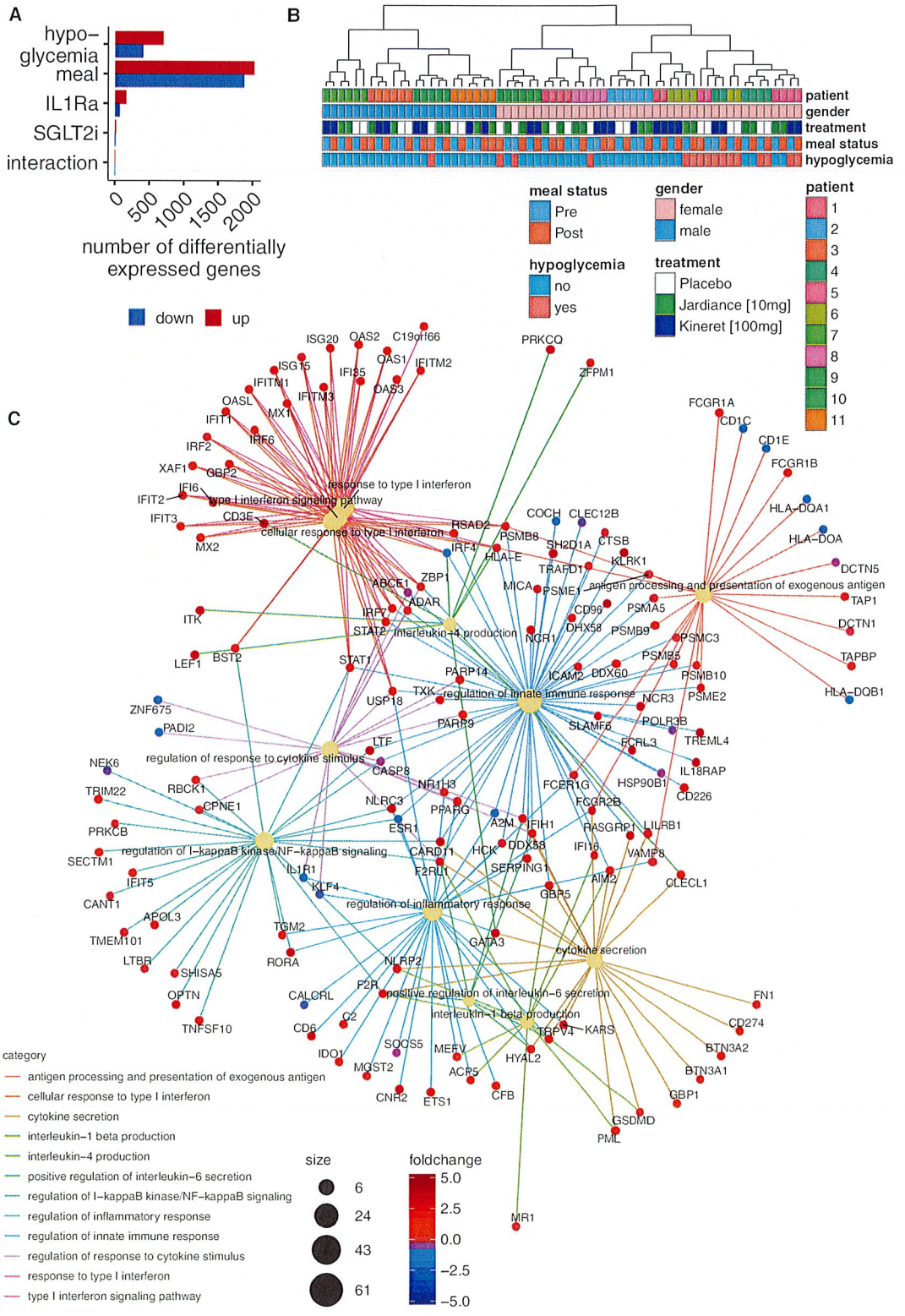

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Samples collected during study days with hypoglycemic events differed profoundly from samples taken on study days that did not require glucose rescue. Indeed, a total of 1,111 genes were differentially regulated in this comparison, with 702 genes being upregulated and 409 downregulated (Figure 2A). Next, we performed unsupervised hierarchical clustering of the 20 most variably expressed genes across all samples. As expected, gender- and patient-specific effects accounted for large differences between samples (Figure 2B). However, whether hypoglycemia occurred during the visit or not explained more of the variance than either meal or treatment status, as indicated by clustering of these samples at the right edge of the dendrogram (Figure 2B).

Subsequently, we investigated the gene expression signature that distinguished study visits with hypoglycemic episodes from those without hypoglycemia. Gene Ontology (GO) pathway analysis of the differentially expressed genes obtained in this comparison revealed that pathways for "interleukin-1β production," "positive regulation of interleukin-6 secretion," "cytokine secretion," "regulation of inflammatory response," "regulation of innate immune response," and "regulation of response to cytokine stimulus" were significantly enriched in these samples (Figure 2C). To test the robustness of these results, we additionally performed weighted gene correlation network analysis with our dataset. Pathway analysis revealed "interleukin-1-mediated signaling" and "positive regulation of interleukin-1-mediated signaling" among the top 5 significantly enriched pathways in the thistle2 module significantly correlating both with whether hypoglycemia happened on a study day or not and status pre- or post-mixed-meal test (data not shown).

Next, we assessed the effect of the meal on monocyte transcriptomes. Samples taken pre-meal differed vastly from samples taken post-meal (total number of differentially regulated genes = 3,901, number of upregulated genes = 2,025, and number of downregulated genes = 1,876). Specifically, we saw marked signs of immune cell activation. Most prominently, there was a broad activation of gene expression encoding for Toll-like receptors (TLRs). Indeed, *TLR 2*, *4*, *5*, *7*, and *8* were all significantly upregulated (Figure 3A). Likewise, pathway analysis of differentially expressed genes equally showed upregulation of various TLR cascades, MyD88, and NF-kB pathways (Figure 3B). Similar to our previously published data in mice (Dror et al., 2017), we could observe upregulation of genes encoding for *HK2*, *CXCL1*, and *CCL2* and downstream targets of the insulin receptor (*IRS1* and *IRS2*) upon mixed-meal ingestion (Figure 3B).

We then compared whether the response to food intake itself differed between days with and without hypoglycemia. An interaction analysis revealed barely any difference between the response to food intake in samples taken on study days where hypoglycemia occurred as compared to days where it did not (number of upregulated genes = 1 and number of downregulated genes = 2; Figure 2A). This was confirmed by analyzing the mean trend in gene expression for genes differentially expressed in both conditions (number of overlapping genes = 151). Unsupervised hierarchical clustering separated these 151 genes into 2 large clusters (accounting for 85% of overlapping genes) (Figure 4A). Herein, cluster 1 (70/151 genes) represented genes that were significantly upregulated, whereas cluster 2 (57/151 genes) represented genes being downregulated in the postprandial state. Analysis of the mean trend in gene expression confirmed an average increase in gene expression from before and 60 min after meal ingestion in cluster 1 (Figure 4B) as well as the respective downregulation in cluster 2 (Figure 4C). Moreover, on average, genes in cluster 1 that were upregulated upon food intake (representative example *IRF2*; Figure 4D) were further upregulated in samples taken during visits that required glucose rescue (Figure 4B). Vice-versa, genes that were downregulated upon food intake in cluster 2 (Figure 4C) decreased even more in samples taken during visits where hypoglycemia occurred (representative example IL1-R1; Figure 4E). Therefore, the gene signature defining a predisposition to hypoglycemia in monocytes had the characteristics of an exaggerated response to food intake.

Finally, we assessed the impact of treatment with either anakinra or empagliflozin on gene expression of monocytes from our patients. Treatment with anakinra had little impact when compared with placebo (total genes differentially regulated = 234, number of upregulated genes = 163, and number of downregulated genes = 71) and empagliflozin barely changed gene expression at all (total genes differentially regulated = 14, number of upregulated genes = 14, and number of downregulated genes = 0; Figure 2A).

Overall, these data show that on days when patients developed hypoglycemia in response to a mixed-meal test, their monocytes displayed a distinct gene expression pattern characterized by upregulated IL-1β, IL-6, and other cytokine pathways. Furthermore, ingestion of a mixed meal equally enhanced expression of pro-inflammatory genes.

Postprandial Ex Vivo Secretion of Inflammatory Cytokines by Monocytes Is Prevented by Anakinra and Empagliflozin. In order to evaluate if changes at RNA level translated into protein expression, we cultured monocytes isolated as described for RNA sequencing for a period of 18 h and measured pro-inflammatory cytokines in cell culture supernatants. Compared with monocytes isolated before food intake, monocytes harvested post liquid mixed-meal test showed significantly increased secretion of key inflammatory cytokines including IL-1β, IL-6, and tumor necrosis factor (TNF)-α (Figures 5A–5C). As expected, treatment with both, anakinra and empagliflozin, significantly inhibited this postprandial increase in

Figure 2. Monocytes Taken from Patients on Days with Hypoglycemia Show Upregulation of Inflammatory Pathways

(A) Number of genes differentially expressed (up- or downregulated) in patients presenting with hypoglycemia, between patient samples pre- and post-meal (meal), and samples treated with anakinra (IL1Ra) and empagliflozin (SGLT2i), as well as the interaction between the factor hypoglycemia and meal status.

(B) Dendrogram produced by unsupervised hierarchical clustering of the 20 most highly variably expressed genes across all sequenced samples. Patient, gender, treatment, pre- or post-meal and hypoglycemia for each sample are indicated in the colored squares below the dendrogram.

(C) Gene-concept network plot (cnet plot) of significantly enriched GO-pathway terms and corresponding differentially expressed up- (red) or downregulated (blue) genes in the comparison of hypoglycemia with no hypoglycemia.

See also Figures S5 and S6A.

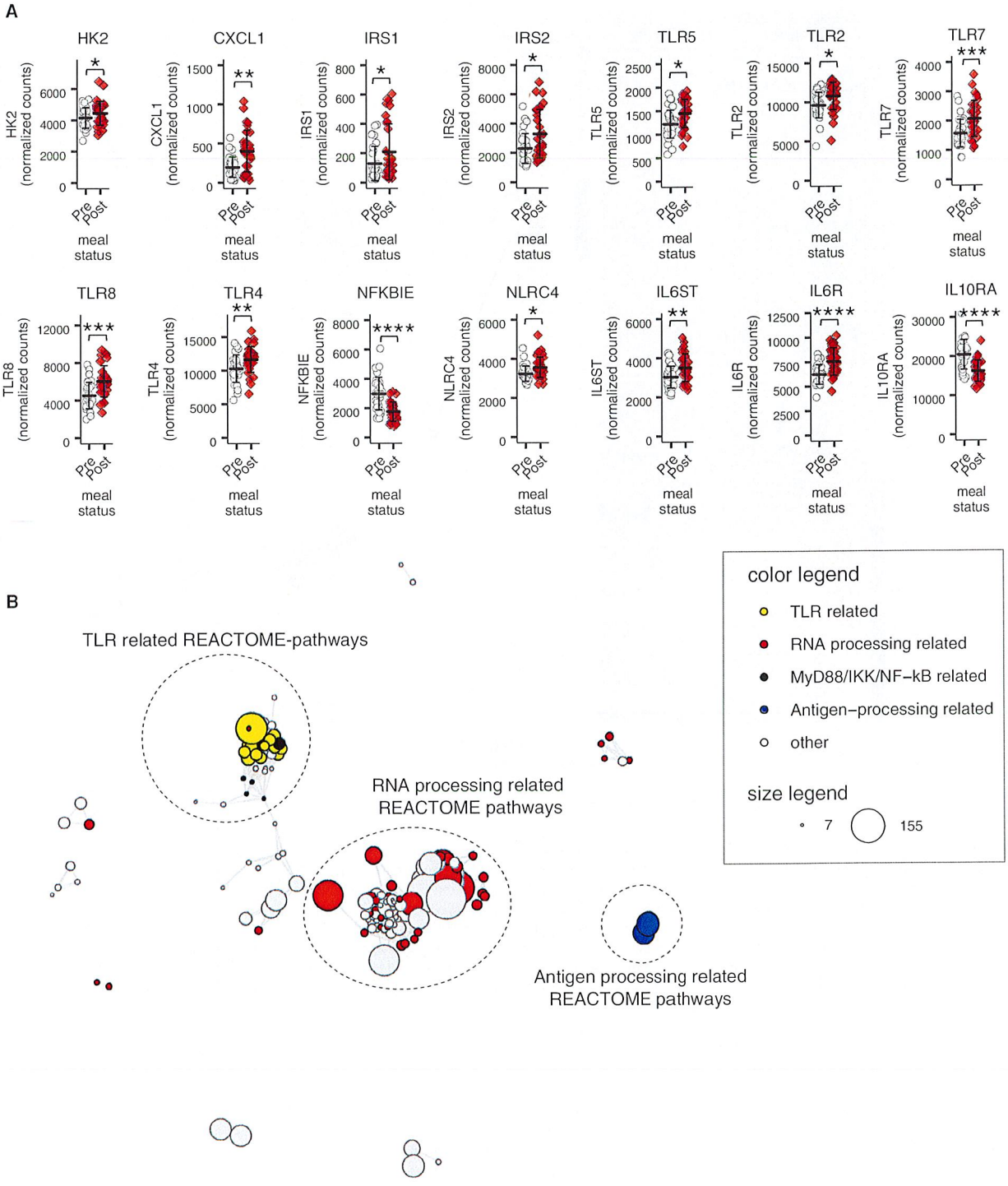

Figure 3. Food Intake Induces Gene Expression of Pattern-Recognition Receptors, as well as Immune and Metabolic Pathways in Patients after Gastric Bypass Surgery

(A) Selection of differentially expressed genes in monocytes isolated from patients post-gastric bypass bariatric surgery before (pre) and after (post) a mixed meal test. Data are presented as overall arithmetic mean of normalized count data for each gene (horizontal bars) ± SD (error bars).

(B) Network of all significantly enriched REACTOME-pathway terms in monocytes isolated from patients post-gastric bypass surgery in the comparison before (pre) and after (post) a mixed-meal test. Individual terms are represented as single points. Distance and line connections between points indicate more (closer

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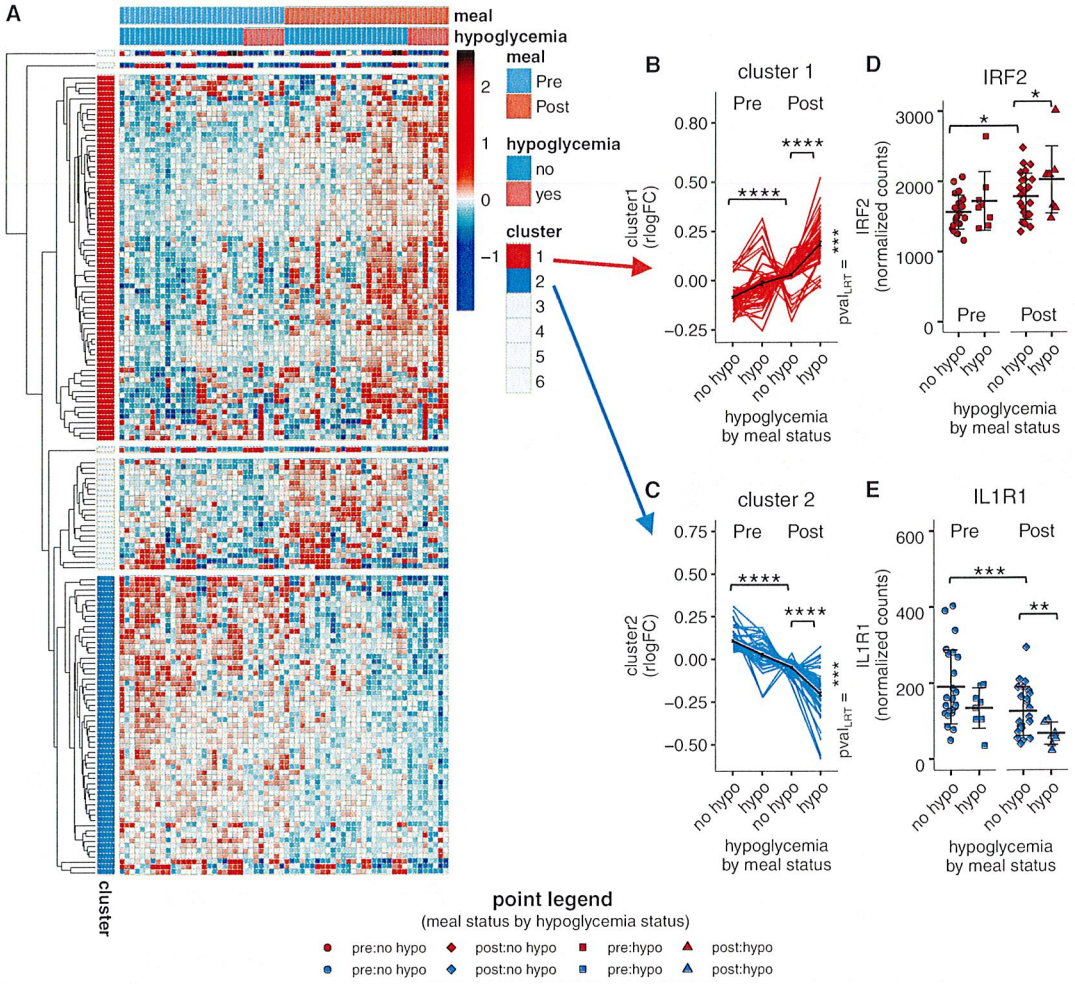


Figure 4. Gene Expression in Response to Hypoglycemia Appears to be an Exaggeration of the Response to Food Intake

(A) Heatmap of differentially expressed genes overlapping in the gene expression level in response to hypoglycemia and to food intake. Displays regularized log-fold changes from base mean and the result of unsupervised hierarchical clustering, separating these genes into two large clusters (1 and 2).

(B and C) Analysis of the mean trend in gene expression within cluster 1 (B) and 2 (C). Displayed are plots of regularized log-fold change from base mean for each gene found in both the comparison hypoglycemia (hypo), no hypoglycemia (no hypo), and before and after food intake.

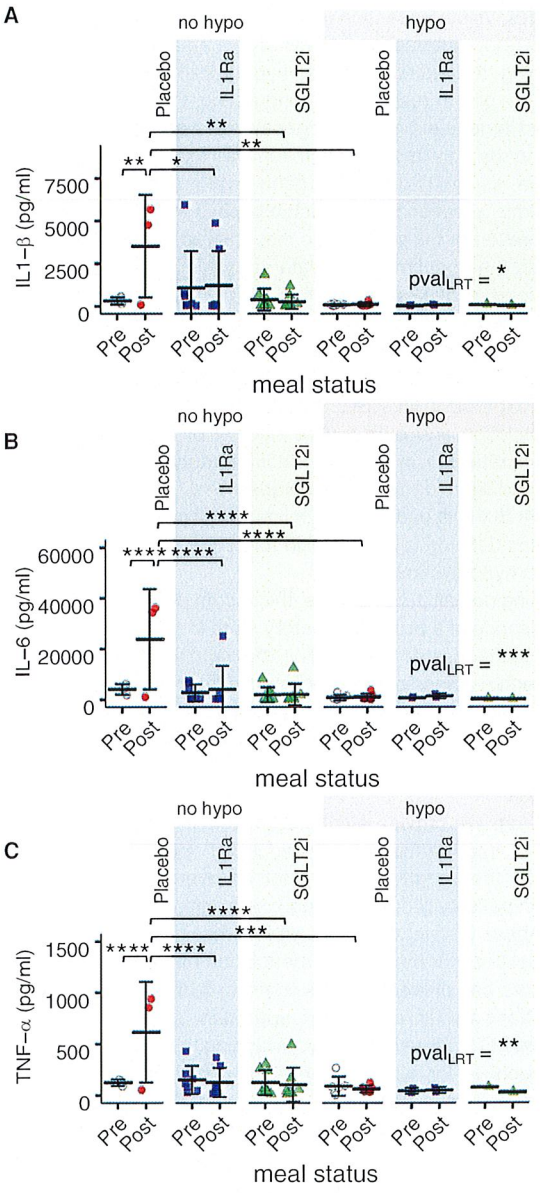
(D and E) Plots displaying normalized counts of 2 representative genes in clusters 1 (D) and 2 (E). Data are presented as gene-specific arithmetic mean (individual colored lines in plots C (red) and E (blue), and overall arithmetic mean—black lines in plots (B) and (D), horizontal bars in plots (D) and (E) ± SD (error bars). Statistical analysis was performed using linear mixed-effect models as described in the STAR Methods section ($pval_{LRT}$ = overall p values generated by LRT comparison of full versus reduced model). p values for individual comparisons were obtained by a subsequent Benjamini and Hochberg posttest for multiplicity adjustment. For details regarding LRT test results and effect size estimates, see Table S4; *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

See also Figures S5 and S6A, as well as Table S4.

inflammatory cytokine secretion as compared with placebo (Figures 5A–5C). Surprisingly, in samples isolated on study visits where hypoglycemia occurred, no postprandial increase could be observed in the *ex vivo* secretion of inflammatory cytokines under any condition (Figures 5A–5C), potentially because of previous *in vivo* cytokine release. To support this hypothesis, we additionally treated cultured monocytes with lipopolysaccharide (LPS) and observed that on study days where hypoglycemia

and/or more connections) or less (farther and/or less connection) shared genes contributing to the pathway. Closely related terms are grouped by color, e.g., yellow for TLR-related pathways and red for RNA processing related ones. Point size represents the number of differentially expressed genes contributing to the pathway. Selected clusters of related terms are encircled. Statistical analysis was performed using the R package "DESeq2" as described in the STAR Methods section. p values for individual comparisons represent multiplicity-adjusted FDR values as implemented in the DESeq2 package (Benjamini and Hochberg posttest for multiplicity adjustment). *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

See also Figures S5 and S6A.



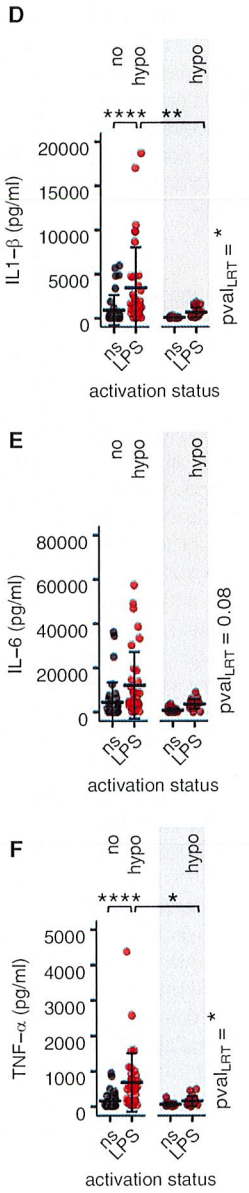


Figure 5. *Ex Vivo* Cytokine Secretion in Meal- and Hypoglycemia-Preconditioned Monocytes

(A–C) Protein measurements of IL-1β (A), IL-6 (B), and TNF-α (C) in cell supernatants taken from non-stimulated bulk monocytes isolated from patients that subsequently developed hypoglycemia (hypo) or not (no hypo) and treated with either placebo, anakinra, or empagliflozin, pre- and post-liquid mixed-meal test.

(D–F) Protein measurements of IL-1β (D), IL-6 (E), and TNF-α (F) in cell supernatants taken from bulk monocytes isolated from patients post-gastric bypass surgery that either did (hypo) or did not (ho hypo) respond with hypoglycemia to a mixed-meal test. Cells were isolated and subsequently stimulated with LPS or left unstimulated (ns = non-stimulated condition, LPS = LPS stimulated condition). Data are presented as arithmetic mean (horizontal) ± SD (error bars). Statistical analysis was performed using linear mixed-effect models as described in the STAR Methods section ($pval_{LRT}$ = overall p values generated by LRT comparison of full versus reduced model). p values for individual comparisons were obtained by a subsequent Benjamini and Hochberg post-test for multiplicity adjustment. For details regarding LRT results and effect size estimates, see Table S4; *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

See also Figure S5 and Table S4.

occurred monocytes failed to respond adequately to LPS stimulation (Figures 5D–5F). In line with our reasoning, it has been shown that monocytes harvested from an inherently pro-inflammatory milieu (septic shock), expressed increased mRNA levels of various inflammatory cytokines and chemokines (IL-1β, IL-1α, IL-6, and CCL2) but released markedly lower levels of cytokines following *ex vivo* stimulation with LPS (Hudspith et al., 1984; Shalova et al., 2015). Accordingly, taking from the publicly available microarray dataset (GEO: GSE46955) the top 200 differentially expressed genes found in monocytes taken from patients in acute septic shock compared with those post-recovery as input, we found that this "endotoxin-tolerant gene signature" was significantly enriched in monocyte samples collected on study days where hypoglycemia occurred (Figure S6A).

To summarize, monocytes taken from patients post-gastric bypass surgery experiencing hypoglycemia display increased levels of pro-inflammatory gene expression. Notably, the release of meal-induced cytokines by these same monocytes can be prevented by treatment with either anakinra or empagliflozin.

Sensitivity Analyses

We found no correlation between the amount of weight loss after gastric bypass surgery and the severity of hypoglycemia (Table S4). Moreover, we performed additional analyses to see whether the gender of our patients had an influence on hypoglycemia-specific gene expression. Testing for differentially expressed genes between the factor symptomatic hypoglycemia and gender revealed a total of 229 genes differentially regulated. However, there were no genes contributing to the IL-1 or IL-6 pathway among them, and we found no pathways in the GO analysis influenced by gender.

Follow-up

As an anecdotal note, 2 trial participants were continued to be treated, and 4 more patients were initiated on off-label therapy with empagliflozin after completion of the study. This led to relevant subjective improvement of their life quality and a lower frequency of hypoglycemic episodes in each of the 6 patients. One

severely affected patient, who had to ingest 8–12 small meals (less than 20 g of carbohydrates each) a day to reduce the number of hypoglycemic episodes, could significantly reduce the frequency of meals. More than half a year after initiating this off-label therapy, the treatment is still effective and well tolerated by all patients. See Figure S6B for a representative continuous glucose monitoring report of a 31-year-old female patient before and during treatment with 10 mg empagliflozin daily.

Safety

We observed no drug-related side effects following these single dose drug applications. Adverse events are outlined in Table S3.

DISCUSSION

Previous studies established glucose as a driver of postprandial IL-1β-induced insulin secretion in rodents (Dror et al., 2017). Here, we could translate these observations from mouse physiology into human pathophysiology. We showed that both, reduction of postprandial peak-hyperglycemia with empagliflozin and blockade of glucose-induced postprandial IL-1 signaling with anakinra, reduced postprandial insulin secretion and subsequent hypoglycemia in predisposed patients after gastric bypass surgery. These effects were independent of changes in either glucagon or GLP-1. However, this does not rule out additional mechanisms involving incretin hormones (Almby et al., 2019; Salehi et al., 2018; Smith et al., 2018) and meal composition. We can also not exclude that empagliflozin acted by reducing intestinal glucose absorption via SGLT1 inhibition, although this was unlikely due to its high selectivity for SGLT2 (Grempler et al., 2012). Moreover, all our patients had failed to respond to dietary measures.

Next, we identified a dysregulation of glucose-induced postprandial inflammatory signaling as an important driver of overshooting insulin secretion in these patients. In accordance with our initial hypothesis of overactivation of the innate immune system, and specifically IL-1 signaling as a key driver of disease-pathology, our RNA-seq data showed a broad upregulation of IL-1 and related cytokine pathways in monocytes taken from patients experiencing hypoglycemia. Of note, our gene expression analysis only showed a limited response to the two treatments in monocytes. A possible explanation is that both treatments work at a post-transcriptional level, rather than influencing the underlying diseased transcriptional response. Furthermore, changes at gene expression level may take more time to resolve.

Because food intake can provoke episodes of hypoglycemia in patients after gastric bypass surgery (Salehi et al., 2018; Tack et al., 2009), we equally assessed the transcriptional response to a meal. We saw marked signs of immune cell activation after a mixed meal. Genes encoding for various TLRs and subsequent inflammatory cascades were upregulated in the post-meal condition. In addition, we could confirm data previously gathered in mice (Dror et al., 2017) that showed key genes involved in metabolism and chemotaxis to be upregulated in our dataset (*HK2*, *CXCL1*, *CCL2*, *IRS1*, and *IRS2*). Moving on to see how gene expression changes elicited by food intake would overlap with changes seen in samples collected on study days where hypoglycemia occurred, we plotted genes differentially expressed in both conditions in a heat map of regularized $\log_2$ fold changes. Visual inspection of this heatmap confirmed the results of the above interaction analysis. The magnitude of expression defining a predisposition to hypoglycemia looked very similar to the one in response to food intake. Furthermore, the mean trend in gene expression of genes that were up- or down-regulated in the post-meal comparison was exaggerated in hypoglycemia, suggesting that the gene level response defining hypoglycemia in patients post-gastric bypass surgery might be an exaggeration of the physiologic response to food intake.

We could then confirm these data at protein level. Similar to data obtained in mice (Dror et al., 2017), monocytes isolated from our patients had increased secretion of key inflammatory cytokines after food intake. Interestingly, cultured cells that showed an overactivation of innate immune response pathways on a gene expression level reacted inadequately to LPS. In fact, an overactivation and subsequent inability of cultured monocytes to secrete IL-1 in other pro-inflammatory settings, such as sepsis and sarcoidosis, has been described before (Hudspith et al., 1984; Shalova et al., 2015). In line with this phenotype, we found genes defining an "endotoxin tolerance signature" to be enriched in hypoglycemia.

Supporting our initial hypothesis, these data provide evidence for the presence of a pro-inflammatory state in patients with hypoglycemia after gastric bypass. Together with the fact that anakinra prevented episodes of hypoglycemia, this might indicate a re-establishment of proper physiologic immune responses in the postprandial setting (Del Rey et al., 2006; Winkler et al., 2019).

Conclusion

In conclusion, we provide evidence toward the presence and therapeutic importance of dysregulated pro-inflammatory signaling as the underlying cause for an overshooting insulin response in patients prone to hypoglycemia after gastric bypass surgery. Above all, we show that either limiting peak glycemia with empagliflozin or therapeutic modulation of the IL-1 system with anakinra can prevent hypoglycemia in these patients. This could be translated in a two-step approach, starting with the widely used SGTLT2 inhibitors and switching to an IL-1 inhibitor in non-responder, thereby re-establishing a physiologic immune response after meal. Finally, we provide new insights into mechanisms governing meal-induced hyperinsulinemic hypoglycemia in patients after gastric bypass surgery. Importantly, we offer a sound therapeutic basis for future long-term trials to base their experimental protocols on.

Limitations of Study

Limitations of our study include potential carryover effects inherent in the crossover design, questionable generalizability due to the uniqueness of our small trial population (12 patients), use of a liquid mixed-meal test, fixed carbohydrate loads (as opposed to body-weight adapted loads), and our inability to provide data exceeding the acute effects examined within the study. Moreover, we only analyzed patients after gastric bypass surgery. Reactive hypoglycemia also occurs after sleeve gastrectomy, although it is reportedly less severe than after gastric bypass (Capristo et al., 2018). Other procedures, such as biliopancreatic diversion, omega loop bypass, or Scopinaro duodenal switch, are less associated with hypoglycemia (Lazar et al., 2019; Nilsen et al., 2019), possibly due to differences in

patient characteristics, such as diabetes and higher BMI. Finally, while we only analyzed circulating monocytes in this trial, we cannot preclude a similar activation of other immune cells.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.cmet.2020.02.013.

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AUTHOR CONTRIBUTIONS

M.H., B.T., and M.Y.D. conceived the trial. M.H., B.L.S., B.T., and A.S. did on-site trial management and data entry. S.J.W., J.L., M.B.-S., and M.Y.D. designed laboratory procedures. S.J.W. and J.L. performed laboratory procedures. S.J.W. and M.H. performed inferential statistics. Library preparation for RNA-seq analysis was done by M.G. Computational analysis of RNA-seq data was done by S.J.W., M.H., and S.J.W., and M.Y.D. wrote the manuscript. All authors interpreted the data, revised the paper critically for important intellectual content, approved the final version, and agreed to be accountable for all aspects of the work.

DECLARATION OF INTERESTS

M.Y.D. is listed as an inventor on a patent submitted by University Hospital Basel covering the use of SGLT-2 inhibitors or IL-1R antagonists for reduction of hypoglycemia after bariatric surgery (EP19151525.3). All others declare no competing interests.

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Human FcR TruStain FcX	BioLegend	Cat# 422302; RRID: AB_2818986
APC Mouse anti-Human CD3 [HIT3a alpha]	BD Biosciences	Cat# 555342; RRID: AB_398592
FITC Mouse Anti-Human CD19 [HIB19 RUO]	BD Biosciences	Cat# 560994; RRID: AB_10563406
PE-Cy5 Mouse Anti-Human CD56 [HCD56]	BioLegend	Cat# 318308; RRID: AB_604105
PE Mouse Anti-Human CD16 [3G8 RUO]	BD Biosciences	Cat# 560995; RRID: AB_10562387
APC-Cy7 Mouse Anti-Human CD14 [MφP-9 RUO]	BD Biosciences	Cat# 561709; RRID: AB_10893806
Chemicals, Peptides, and Recombinant Proteins		
Anakinra (Kineret®), recombinant human IL-1ra; 100 mg/0.67ml solution for subcutaneous injection	Swedish Orphan Biovitrum AB	NA
Empagliflozin (Jardiance)®, 10 mg tablet	Boehringer Ingelheim GmbH	NA
DNA-binding dye DAPI	BioLegend	Cat# 422801
LPS	InvivoGen	Cat# tlrl-smlps
ATP	InvivoGen	Cat# tlr-atp
Critical Commercial Assays		
point of care testing (Contour XT®)	Ascensia Diabetes Care, Switzerland	NA
insulin ELISA	Mercodia AB, Uppsala, Sweden	Cat# 10-1113-01
c-peptide ELISA	Mercodia AB, Uppsala, Sweden	Cat# 10-1136-01
glucagon ELISA	Mercodia AB, Uppsala, Sweden	Cat# 10-1271-01
GLP-1	Mercodia AB, Uppsala, Sweden	Cat# 10-1278-01
V-PLEX Human Proinflammatory Panel II (4-Plex) kit	Mesoscale Discovery	Cat# K15053D-2
Nucleo Spin RNA II Kit	Machery Nagel	Cat# 740955.250
Arcturus PicoPure RNA Isolation Kit	Thermo Fisher Scientific, USA	Cat# KIT0204
Quant-iT RiboGreen RNA Assay Kit	Thermo Fisher Scientific, USA	Cat# R11490
TruSeq Stranded mRNA HT Sample Preparation Kit	Illumina, USA	Cat# RS-122-2101
Deposited Data		
bulk RNAseq data of human circulating blood monocytes isolated from patients post-gastric bypass surgery undergoing a liquid-mixed meal test upon treatment with either placebo, anakinra or empagliflozin	This manuscript	GSE132781
publicly available microarray dataset of the top 200 differentially expressed genes found in monocytes taken from patients in acute septic shock compared to those post-recovery	https://doi.org/10.1016/j.immuni.2015.02.001	GSE46955
Software and Algorithms		
liquid mixed-meal (300 ml Ensure plus®, 60 g carbohydrates, 450 kcal)	Abbott	NA
Other		
R (versions 3.5.2 and 3.5.3)	www.r-project.org	NA
Prism8 (version 8.0.1)	Graphpad Prism	http://www.graphpad.com; RRID: SCR_002798
lme4 (version 1.2.21)	https://cran.r-project.org/web/packages/lme4/index.html	RRID: SCR_015654

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
multcomp (version 1.4-10)	http://multcomp.R-forge.R-project.org	NA
emmeans (version 1.3.4)	https://github.com/rvlenth/emmeans	NA
FastQC (version 0.11.4)	www.bioinformatics.babraham.ac.uk/projects/fastqc/	RRID: SCR_014583
Trimmomatic (version 0.36)	https://doi.org/10.1093/bioinformatics/btu170	http://www.usadellab.org/cms/index.php?page=trimmomatic; RRID: SCR_011848
STAR (version 2.7.0)	https://doi.org/10.1093/bioinformatics/bts635	http://www-stat.stanford.edu/~tibs/SAM/; RRID: SCR_010951
HTSeq (version 0.6)	http://htseq.readthedocs.io/en/release_0.9.1/	RRID: SCR_005514
DESeq2 (version 1.20.0)	http://doi.org/10.1186/s13059-014-0550-8	http://bioconductor.org/packages/release/bioc/html/DESeq2.html; RRID: SCR_000154
WCGNA (version 1.66)	https://doi.org/10.1186/1471-2105-9-559	http://www.genetics.ucla.edu/labs/horvath/CoexpressionNetwork/; RRID: SCR_003302
clusterProfiler (version 3.10.1)	https://doi.org/10.1089/omi.2011.0118	RRID: SCR_016884; http://bioconductor.org/packages/release/bioc/html/clusterProfiler.html
pathfindR (version 1.3.0)	https://doi.org/10.1101/272450	NA
pheatmap (version 1.0.12)	https://github.com/raivokolde/pheatmap, https://cran.r-project.org/web/packages/pheatmap/pheatmap.pdf (Kolde, 2019).	https://www.rdocumentation.org/packages/pheatmap/versions/0.2/topics/pheatmap; RRID: SCR_016418
FlowJo 10.6.1 software	Tree Star	https://www.flowjo.com/solutions/flowjo/downloads; RRID: SCR_008520
ggplot2 (version 3.1.0)	https://www.springer.com/de/book/9783319242750	https://cran.r-project.org/web/packages/ggplot2/index.html; RRID: SCR_014601

LEAD CONTACT AND MATERIALS AVAILABILITY

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Marc Y Donath (marc.donath@usb.ch). This study did not generate new unique reagents.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Participants

Eligible patients needed to be at least 18 years of age and have documented episodes of postprandial hypoglycemia after gastric bypass surgery. Hypoglycemia was defined (Whipple's triad) as (1) appearance of typical symptoms (Edinburgh Hypoglycemia Scale), (2) plasma glucose < 2.5mmol/l and (3) relief of symptoms following administration of 10 g of glucose (per os or intravenously). Key exclusion criteria were clinically significant infections or inflammatory diseases, major cardiac, renal and hepatic disorders. For full inclusion and exclusion criteria see *clinicaltrials.gov* (NCT03200782). For patient baseline characteristics see Table 1. Of note, the initial protocol included also patients after sleeve gastrectomy but was subsequently modified to allow for a more homogenous trial population including only patients after gastric bypass surgery. Importantly, this was done before unblinding. Furthermore, sensitivity analysis including this patient still leads to a significant difference in the incidence of severe episodes of hypoglycemia between treatment groups (pvalLRT= 0.0125). The study was conducted in compliance with the protocol, the current version of the Declaration of Helsinki, the ICH-GCP or ISO EN 14155 as well as national legal and regulatory requirements. The trial protocol was reviewed and approved by the local authorities and ethics committee (EKNZ 2017-00689) on 08.05.2017. The trial was registered at Clinicaltrials.gov (NCT03200782) and has been completed. The trial was registered on 30.05.2017 and was first publicly available on clinicaltrials.gov on 27.06.2017. Sample collection was completed between the 07.07.2017 and the 29.08.2018. For more details on and the number of trial participants see the Trial Population section of our manuscript. For details regarding sex-specific analyses of gene expression data, see the Sensitivity Analyses section of our manuscript. Further sex-specific analyses were neither prespecified in our statistical plan, nor powered for and thus not performed.

Randomization and Masking

Patients were randomly assigned to receive either anakinra, empagliflozin or corresponding placebos on three individual study visits with a minimum wash-out phase of four days and a maximum interval of seven days between visits. Patients and investigators were

masked to treatment assignment using a double-dummy design. Allocation was done by an independent researcher at the University Hospital Basel (Fahim Ebrahimi, MD) who ensured equal distribution of the study interventions to study days using block randomization (six blocks of two subjects respectively). For each randomized subject, a separate file was prepared on which each study day and respective intervention was double-checked and confirmed by unblinded accessory study personnel who was solely responsible for administration of study drugs to the blind-folded patient and had no role in any other capacity of the trial. The blinded study personnel was not present nor informed about the preparation and administration of the study drugs. The unblinded accessory study personnel handed-over a non-transparent, closed envelope to the blinded personnel in case of emergency unblinding.

METHOD DETAILS

Study Design

This study was designed as a double-blind, double-dummy placebo controlled, randomized, cross-over trial and was performed at the Clinical Trial Unit of the University Hospital of Basel, Basel, Switzerland.

Procedures

On each individual study day, patients received either anakinra and placebo or empagliflozin and placebo or double placebos. Anakinra (Kineret®; recombinant human IL-1ra, Swedish Orphan Biovitrum AB) was applied subcutaneously at a dose of 100 mg (corresponding placebo 0.67 ml 0.9% saline solution, applied subcutaneously). Empagliflozin (Jardiance®; Boehringer Ingelheim GmbH) was applied orally at a dose of 10 mg (corresponding placebo Winthrop tablets, applied orally). Treatments were applied three (anakinra or corresponding placebo) and two (empagliflozin or corresponding placebo) hours before ingestion of a standardized liquid mixed-meal (300 ml Ensure plus®, Abbott, 60 g carbohydrates, 450 kcal).

Patients were regularly assessed for changes in vital signs, clinical signs of hypoglycemia (Edinburgh Hypoglycemia Scale), circulating glucose, insulin, c-peptide, glucagon-like peptide 1 (GLP-1), glucagon, inflammatory parameters and leukocytes as depicted in Figure S1B. In case of symptomatic hypoglycemia, immediate glucose measurement and blood sampling was performed followed by the administration of 10-g glucose (per os or intravenously). For details regarding symptoms of hypoglycemia see Table S2. Adverse events were regularly documented at each study visit (see Table S3 for further information).

Outcomes

Primary outcome was to assess whether empagliflozin or anakinra reduce the severity of hypoglycemia following a standardized mixed-meal test, as compared to placebo in patients after gastric bypass surgery. *Secondary outcomes* were insulin secretion and sensitivity, levels of c-peptide, GLP-1, glucagon, IL-6, IL-1Ra, C-reactive protein, Edinburgh Hypoglycemia Scale, Stanford Sleepiness Scale, Sigstad Score and length of time and amount of glucose needed for restoring normoglycemia under anakinra or empagliflozin as compared to placebo. Of note, since there is no validated questionnaire for postprandial hypoglycemia after bariatric surgery, we used the Edinburgh Hypoglycemia Scale with a threshold score of ≥ 6 to better differentiate the symptoms during hypoglycemia from lighter dumping symptoms. Some symptoms were consistently present throughout all treatment groups and differed from the stronger hypoglycemia symptoms. *As exploratory outcomes*, we performed RNAseq analysis and cytokine-release assays with circulating blood monocytes. Since insulin secretion was significantly changed by treatment, HOMA-Insulin is no longer a valid readout for changes in insulin sensitivity and was thus not reported.

Laboratory Analysis

Immediate bed-side glucose measurement was performed with point of care testing (Contour XT®, Ascensia Diabetes Care, Switzerland) and then confirmed for the analyses in the central laboratory of the University Hospital Basel, where also routine blood count and chemistry analyses were done. Plasma levels of insulin, c-peptide, glucagon, and GLP-1 were measured with ELISAs by Mercodia AB, Uppsala, Sweden (assay # 10-1113-01, 10-1136-01, 10-1271-01, and 10-1278-01 respectively) according to the manufacturer's instructions.

Purification of Blood Monocytes

Blood samples were taken immediately before and 60 minutes after meal intake. Due to scheduling conflicts, samples from two patients could not be processed. These samples are thus assumed to be missing at random. Of note, samples from patient 3 (sleeve gastrectomy) had already been processed for RNA sequencing before the final change of protocol. Sequencing data obtained from this patient were thus kept for the purpose of these analyses. Peripheral blood mononuclear cells were obtained by Ficoll density gradient centrifugation (Lymphoprep Fresenius Kabi, Norway). For 9 out of 11 patients, an additional step was added after density gradient centrifugation to pre-enrich monocytes by negative selection with CD3 human MicroBeads (#130-050-101; Miltenyi Biotec). The obtained cellular monocyte fractions were then incubated with FC-Block for 15 minutes at room temperature [(Human FcR TruStain FcX (Biolegend) REF 422302, *compatible with flow cytometric staining with anti-human CD16 (clone 3G8)*], and subsequently labeled with the following antibodies: APC Mouse anti-Human CD3 [HIT3a alpha] (#555342; BD Biosciences), FITC Mouse Anti-Human CD19 [HIB19 RUO] (#560994; BD Biosciences), PE-Cy5 Mouse Anti-Human CD56 [HCD56] (#318308; BioLegend), PE Mouse Anti-Human CD16 [3G8 RUO] (#560995; BD Biosciences), APC-Cy7 Mouse Anti-Human CD14 [MφP-9 RUO] (#561709; BD Biosciences).

RNAseq Computational Analysis

Initial quality checks were performed with FastQC version 0.11.4 (www.bioinformatics. babraham.ac.uk/projects/fastqc/). Adaptor clipping and quality-trimming of sequences was performed using Trimmomatic version 0.36(Bolger et al., 2014) and reads were aligned to the GRCh38 reference genome using the splice aware aligner STAR version 2.7.0(Dobin et al., 2013). Count-tables were produced using HTSeq version 0.6.1(Anders et al., 2015). Subsequent analyses were performed in R (v3.5.3, www.r-project.org) using the DESeq2 package version 1.20.0(Love et al., 2014) and WCGNA package version 1.66(Langfelder and Horvath, 2008). GO- and REACTOME pathway analysis was performed using the R package clusterProfiler version 3.10.1(Yu et al., 2012). Custom input pathway analysis testing the enrichment of a gene signature defining an "Endotoxin Tolerance phenotype" was analysed using the R package "pathfindR" version 1.3.0(Ulgen et al., 2018). Unsupervised hierarchical clustering of the 20 top variably expressed genes and visualization thereof was performed using the R package pheatmap version 1.0.12(Kolde, 2019).

Analysis of Mean Trend in Gene Expression

Applying linear mixed effect models using the genes themselves as random effects, we were able to analyze the mean trend of regularized log fold changes within each cluster identified by the pheatmap function's unsupervised hierarchical cluster algorithm (R «pheatmap» package v1.0.12(Kolde, 2019)). To obtain regularized log fold changes, we transformed count values with the rlog function included in the previously mentioned R package «DESeq2»(Love et al., 2014). Regularized log fold changes were then obtained by subtraction of the base mean for each gene. Generation of a dummy variable combining meal and hypo status allowed us to estimate beta-coefficients and p-values for the pre-meal-no-hypo, pre-meal-hypo, post-meal-no-hypo and post-meal-hypo state.

DATA AND CODE AVAILABILITY

All Sequencing data reported in this study have been deposited at the NCBI's public functional genomics data repository Gene Expression Omnibus (the accession number for the data reported in this paper is: GEO: GSE132781, processed data are available on Series record, raw data are available in SRA) and will be made available upon publication. Other trial data cannot be made freely available to safeguard informed patient consent. Reasonable requests can however be submitted for review by our local institutional review board. For details, please contact the corresponding author. The publicly available dataset used for enrichment analysis can be found at GEO: GSE46955. All major software and code used to analyze this dataset are referenced above.

ADDITIONAL RESOURCES

The trial was registered at clinicaltrials.gov (NCT03200782, https://www.clinicaltrials.gov/ct2/show/NCT03200782?cond=hypobear&draw=2&rank=1).