Title

The Effect of the Interaction of Glucagon and Insulin on Endogenous Glucose Production in Humans

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Specific Aim: Determine the relative contribution of changes in insulin and glucagon concentration on endogenous glucose production in nondiabetic subjects.

1° Hypothesis: Hyperinsulinemia restrains the effect of rising glucagon concentrations on endogenous glucose production

2° Hypothesis: The % change in endogenous glucose production in response to rising glucagon concentrations is unchanged by hyperinsulinemia

Rationale: The experimental design we propose will enable the dissection of the relative contribution of insulin and glucagon concentrations to endogenous glucose production. Acquisition of this data will allow us to develop a model of insulin and glucagon contributions to postprandial glucose metabolism (1; 2).

A. Significance

Diabetes mellitus causes morbidity, mortality and costs in excess of ~$170 billion/year(3). Therefore preventing diabetes is important. T2DM is characterized by defects in insulin secretion & action(4), and impaired postprandial suppression of glucagon(1; 2). Recently, we have shown that diabetes-associated variation in *TCF7L2* impairs postprandial suppression of glucagon secretion – an aspect of islet function that has been overlooked in prior genotype-phenotype correlation studies and in the pathogenesis of prediabetes(5). This indicates that the abnormal regulation of postprandial glucagon secretion and glucose tolerance in the TT subjects cannot be explained by alterations in β-cell function, in keeping with recent reports where glucagon secretion is independent of β-cell function(6). Taken together, these data imply that to accurately quantify the ability of the pancreas to lower glucose concentrations in the post-prandial period, we have to account for both β-cell and α-cell function. The oral minimal model uses knowledge of C-peptide kinetics to measure insulin secretion and quantify β-cell function. In a prior series of experiments (IRB # 17-006493), we quantified the contribution of glucagon suppression to endogenous glucose production in the setting of changing insulin concentrations. This experiment will provide information of how changing glucagon concentrations alter endogenous glucose production when insulin concentrations are constant.

B. Preliminary Data

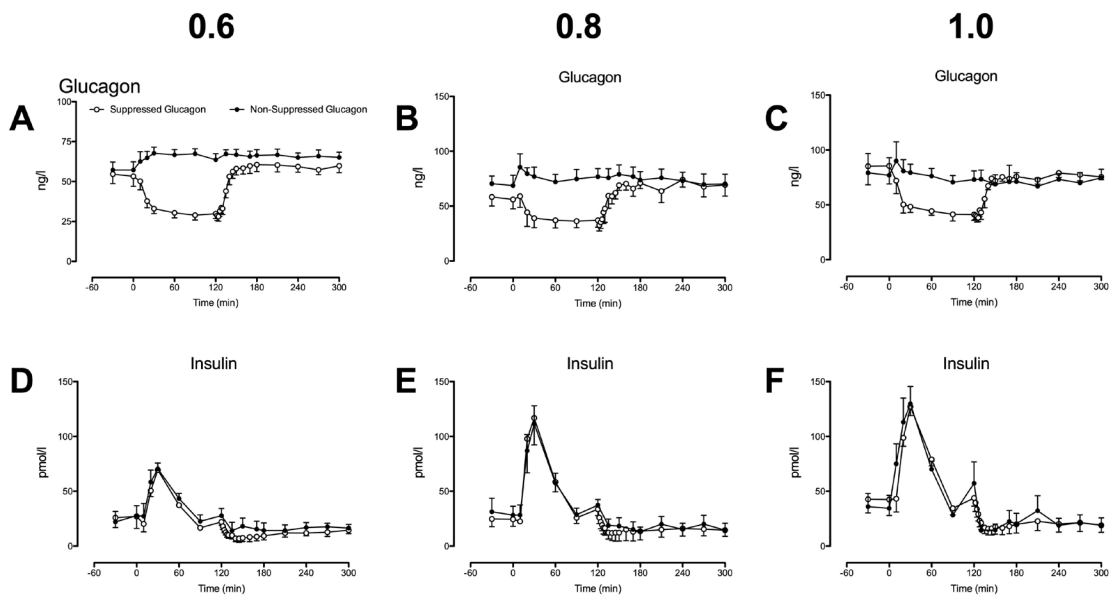

Figure 1: Prior experiment with manipulation of insulin (0.6, 0.8 and 1.0 of an insulin profile) and glucagon

1. Glucagon alters postprandial glucose metabolism (2).
2. We have expertise in the design and conduct of clamp experiments (7; 8).
3. We have experience with the use of isotopes to measure glucose turnover (9).
4. Previously we utilized data from similar in vivo experiments to develop novel models of insulin secretion (10), hepatic insulin extraction (11) and the effect of Glucagon-Like Peptide-1 (GLP-1) on insulin secretion (12).
5. Data from IRB # 17-006493 demonstrate that with decreasing postprandial insulin concentrations, the effect of a lack of glucagon suppression on endogenous glucose production becomes more marked (**Fig.1,2**)

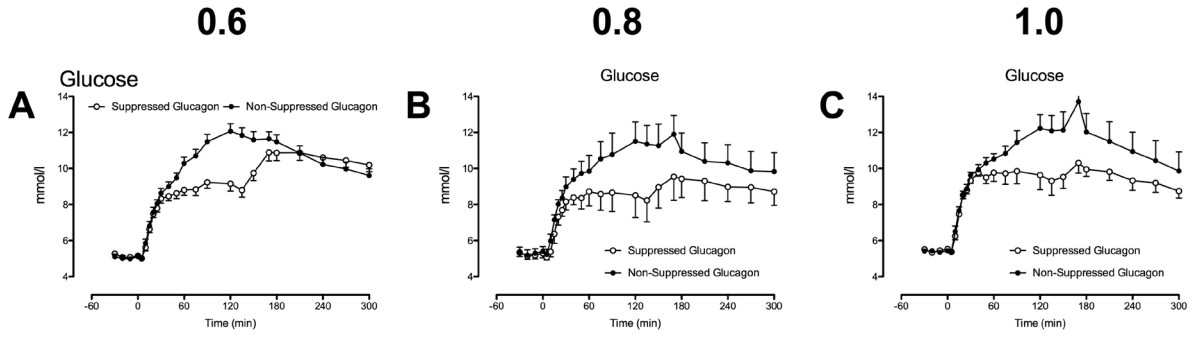

Figure 2: The glucose concentrations resulting from the interaction between a lack of glucagon suppression and progressive decreases in insulin secretion

C. Research Design and Methods

Study Subjects: We will recruit 15 otherwise healthy subjects using intramural and local extramural advertising after approval from the Mayo Clinic Institutional Review Board. Individuals who express interest in participating will be invited for a screening visit. Individuals with a BMI < 19 or > 40 kg/m^2 will be excluded from the study to avoid potential confounding effects that may result from extreme leanness or from obesity. Subjects will have no known systemic illness, taking any medication that could affect glucose metabolism and no history of upper gastrointestinal surgery.

Exclusion Criteria: Subjects < 18 years of age or > 60 years of age will not be studied to minimize the potential confounding effects of age on glucagon and insulin action. The subjects will not be taking medications that affect glucose metabolism (to be determined by PI) and have no history of chronic illness or upper gastrointestinal surgery. We are seeking to recruit subjects who do not have diabetes (fasting glucose <100mg/dL).

Screening Visit: To ensure subjects are healthy, following written, informed consent, subjects will undergo a history and physical examination, vital signs, height, weight, blood collection (after an 8 hour fast) for complete blood count, creatinine, sodium, potassium, fasting glucose and urine collection for point of care pregnancy testing for females able to become pregnant. Subjects will be instructed to consume a weight maintenance diet (55% carbohydrate, 30% fat and 15% protein, caffeine free) for at least 3 days prior to each study visit. **Body Composition:** Body composition will be measured prior to participation using dual-energy X-ray absorptiometry (iDXA scanner; GE, Wauwatosa, WI).

Experimental Design: Volunteers will participate in a total of 2 protocols. The overnight studies will be performed at least a week apart in random order.

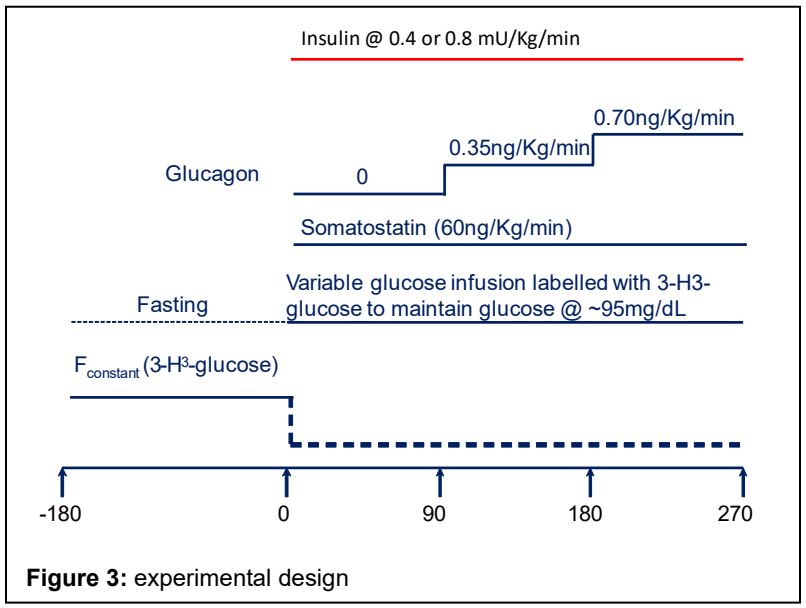

Figure 3: experimental design

Study A (Insulin 0.4mU/Kg/min): Subjects will be admitted to the CRTU at approximately 1700 on the day prior to study. They will then consume a standard 10kcal/kg meal (55% carbohydrate, 30% fat, 15% protein, caffeine free) and fast overnight. Blood will then be sampled for baseline enrichment and $^{2}H_{2}0$ (1.67g/kg of body water) will be given in 3 divided doses at 2200, 2400 and 0200 (13). The following morning (approximately 0530), a forearm vein will be cannulated to allow infusions to be performed. In addition, a cannula will be inserted retrogradely into a vein of the contra-lateral dorsum of the hand. This will be placed in a heated Plexiglas box maintained at around 120°F to allow sampling of arterialized venous blood. At approximately 0600 (-180 min), a primed, (10μCi prime, 0.1μCi/min continuous) infusion containing trace amounts of glucose labeled with [3-^{3}H] glucose will be started and continued till 0900 (0 min).

At 0900 (0 min), the infusion will be varied so as to mimic the anticipated pattern of fall of EGP. In addition, glucose also labeled with [3-^{3}H] glucose will be infused so as to maintain glucose concentrations at ~ 95mg/dL. Peripheral venous glucose concentrations will be measured every 10 minutes to allow the infusion rate of glucose to be adjusted as necessary.

Simultaneously, an infusion of somatostatin (60ng/kg/min) will be started at time 0 to inhibit endogenous islet secretion and therefore ensure identical portal insulin concentrations on the two study days(8). Insulin will be infused at a constant rate known to produce ~ 50% suppression of EGP (0.4mU/Kg/min).

From 0900 (0 min) to 1030 (90 min) no glucagon will be infused. Subsequently, a glucagon infusion will commence (91 – 180 min) at 0.35 ng/Kg/min and then increase to 0.70 ng/Kg/min (181- 270 min), a rate which will be maintained till the end of the study (1330).

Study B (0.8mU/Kg/min): Approximately 1-2 weeks after the first study visit, subjects will be asked to return to the CRTU. This study visit will be similar to Study A. However, insulin will be infused at 0.8mU/Kg/min 0 to 270 minutes.

.**Analytical Techniques:** All blood will be immediately placed on ice, centrifuged at 4°C, separated and stored at -80°C until assay. *Glucose* will be measured using a Yellow Springs glucose analyzer. *Glucagon* will be measured using an ELISA (Mercodia, Winston-Salem, NC). *C-peptide* will be measured using EMD Millipore (Billerica, MA) reagents. *Insulin* will be measured using a chemiluminescence assay with reagents obtained from Beckman (Access Assay, Beckman, Chaska, MN). [*3-^{3}H] glucose specific activity* will be measured by liquid scintillation counting following deproteinization(14). *Endogenous glucose production* (EGP) and *glucose disposal* (Rd) will be measured as before(9; 15).

Calculations: Glucose appearance and disappearance will be calculated using the steady-state equations of Steele et al., in which the actual tracer infusion rate for each interval is used in the calculation. The volume of distribution of glucose will be assumed to equal 200 ml/kg, and the pool correction factor assumed to be 0.65(16). Endogenous glucose production will be determined by subtracting the glucose infusion rate from the tracer-determined rate of glucose appearance(9). All rates of infusion and turnover will expressed per Kg/lean body mass.

	Mean ± SD	Detectable difference (0.4 vs. 0.8 insulin)	Detectable difference within each sub-group
Nadir EGP (μmol/kg/min)	3.9 ± 1.1	0.4 (10%)	0.7 (18%)
AUC EGP (μmol/kg per 5hr)	2816 ± 182	67 (2%)	120 (4%)

Table 1: Pooled Means ± SD used to estimate power. (Data from Ref. 1)

Statistical Analysis: A paired, two way student t-test (parametric) or a Wilcoxon matched-pairs signed rank test (non-parametric) will be used to examine changes of EGP at the various time points of each study day. Differences between the 3 glucagon infusion rates will be assessed using a repeated-measures ANOVA which assumes the absence of a Gaussian distribution (Friedman test). Subsequently, in the presence of significant between group differences ($p < 0.05$), a *post-hoc* Student's t-test will used to examine differences between the 3 infusion rates of glucagon. If values are not normally distributed, a Dunn's test will be used instead.

Power calculation: Assuming similar variation to that previously observed(1), 60 subjects would provide approximately 80% power (at a 2-sided 0.05 α level), to detect small, biologically significant, differences in peak glucose, glucose area above basal and EGP in the presence and absence of glucagon suppression (**Table 1, Col 3**) as would 20 per subgroup (**Table 1, Col 4**).

D. Interpretation

We will accept our 1° hypothesis if endogenous glucose production (EGP) is higher during the 0.4 vs. the 0.8 study day. This would imply that despite rising glucagon concentrations higher insulin concentrations can still restrain EGP. It also will allow us to quantify the fold changes in EGP at low or high insulin concentrations.

To test our 2° hypothesis we will express EGP at 80, 180 and 270 minutes as a percentage of fasting EGP (0 min). The relative changes with increments in glucagon will demonstrate whether the relationship is linear or asymptotic. Comparison of the two study days will enable us to determine the effect of insulin on this relationship. We will accept our 2° hypothesis if % suppression at the 80, 180 and 270 minute time points does not differ between study days.

We will calculate changes in nadir and integrated rates of EGP, as previously described(17-20), to compare suppression between the two study days. We will also undertake exploratory analysis of the rates of glucose disappearance (Rd) to determine if differences in glucagon concentrations affect Rd as well as EGP.

Limitations / Precautions/ Additional or Alternative Analyses:

i. Inhibition of glucagon secretion by somatostatin lowers portal concentrations. In the case of glucagon, an infusion that maintains peripheral concentrations of glucagon constant likely will result in a ~10% decrease in portal glucagon concentrations(21-23). However, in the current experimental design, the portal glucagon concentrations on the two study days will not differ.

E. Human Subjects

Detailed Description: Suitable volunteers will be asked to visit the CRTU on a total of 3 occasions, including screening. During the first or screening visit they will meet with a member of the study team and undergo a history and physical examination to ensure that they fulfill entry criteria. If eligible, they will be asked to undergo determination of body composition. After an overnight fast, a cannula will be placed to allow IV infusion. Infusions will be started on the study days. In addition a retrograde hand vein for blood draws will be placed. This hand will be placed in a Perspex hot-box heated to about 120 ºF. Blood will be sampled several times (via the retrograde hand vein) to obtain arterialized venous samples.

Population: Subjects will be recruited using intramural and local extramural advertising after approval from the Mayo Clinic Institutional Review Board. The racial composition of the county is outlined in the table below (%) using data from the 2000 population census. No children or prisoners will be recruited.

	American Indian or Alaskan Native	Asian or Pacific Islander	Black, not of Hispanic Origin	Hispanic	White, not of Hispanic Origin	Other	Total
Female	0.15	2.8	1.8	1.5	43.8	0	51.5
Male	0.15	2.8	1.8	1.5	43.7	0	48.5
Total	0.3	5.6	3.6	3.0	87.5	0	100.0

Research Materials: We will be obtaining blood samples to measure hormone and glucose concentrations.

Recruitment of Subjects: Subjects will be recruited using IRB-approved advertising. Eligible subjects who express interest in participating in the study will be invited to the CRTU for a screening visit.

The following are exclusion criteria:

- Age <18 or >40
- BMI < 19 or > 28 kg/m^2
- Medication that can affect glucose metabolism (determined by PI)
- Any chronic illness
- Upper GI surgery
- History of diabetes
- FBG >100 mg/dL
- For female subjects a positive pregnancy test

Potential Risks: Blood sampling. Blood samples are collected by venipuncture for this study. Bruising can occur with venipuncture, as can fainting, etc. Risk Monitoring / Risk Reduction: The samples are collected using aseptic technique in designated venipuncture areas of the CRTU where facilities are available should untoward reactions (fainting, etc.) occur. Given the aseptic nature of the sample collection and the small risk of bruising, the monitoring plan is focused on advising volunteers to call the investigators should they have unusual pain or discomfort from the venipuncture site. Blood drawn within a 12 week period will not exceed 550mL (one pint).

Vascular catheter placement. Catheter insertion, intravenous infusion and blood withdrawal are associated with a small risk of phlebitis. Risk Monitoring / Risk Reduction: This will be minimized by careful attention to sterile technique. If phlebitis occurs, it will be treated conservatively with heat and when appropriate, with antibiotics. The catheters will be cared for by experienced CRTU nurses in order to minimize the risk of these complications. In all protocols, "arterialized – venous" blood will be obtained by placing a hand in which a catheter has been inserted in a heated box during the study. The temperature inside the box is maintained at ~55°C. With prolonged exposure to continuous heat, there is a potential risk of local skin irritation or a minor burn. If this occurs, it will be treated appropriately. Catheter risks will be discussed with the volunteers prior to obtaining consent for the study.

Radiation. Subjects will be exposed to radiation in this study. Radioactive tracers will be used in the proposed studies. Lean body mass and percent body fat will be measured at the time of screening using DEXA (dual energy x-ray absorptiometry). Risk Monitoring / Risk Reduction: The lowest dose of radioactive tracers that can be reliably counted in plasma will be used. In all instances, the amount of radiation that a volunteer will receive will be well below levels that result in significant risk of harmful effects. Proposed radiation exposure will be reviewed by the Mayo Clinic Radiation Safety Board prior to initiation of any study. Women who could become pregnant will be required to have a negative pregnancy test prior to participation in each study utilizing radioactive tracers. The Mayo CRTU body composition core has a QDR4500 with fan scan technology which allows body composition to be performed in only a few minutes.

Deuterated Water. Deuterated water will be given in 3 divided doses at 2200, 2400 and 0200. Risk Monitoring / Risk Reduction: Deuterated water is sometimes associated with dizziness and vertigo which is reduced by giving the tracer in divided doses. The fact that it is given late at night while the patient is in bed will reduce the risk of falls. Prior experiments using deuterated water have suggested that the occurrence of this uncommon.

Infusion of Tracers, Hormones. All infusions carry a risk of allergic reactions, bruising, discomfort at the site of infusion, and infection. Risk Monitoring / Risk Reduction: All infusates are prepared by trained personnel (under the supervision of a research pharmacist) in a laminar flow cabinet using aseptic technique. Infusions take place on the CRTU where facilities are available should untoward reactions occur. Somatostatin infusion will be undertaken under the auspices of an IND obtained from the FDA. Somatostatin use can be associated with nausea and hypotension. In prior experience these effects are minor and disappear within minutes of cessation of somatostatin infusion. Insulin can lower the levels of glucose in the blood and cause symptoms of hypoglycemia. Plasma glucose will be monitored at regular intervals during study visits. Vital signs will be monitored hourly as per our standard practice during infusions in the CRTU.

Confidentiality. All studies expose participants to the psychosocial risks arising from any breach in confidentiality. The risks include anxiety, confusion, damage to family relationships or a compromised ability to obtain insurance or employment. These risks may not be confined to the individual but may extend to other family members. Risk Monitoring/Risk Reduction: The nature of the information obtained will be explained in detail to each participant. All information will be stored anonymously in the database and only the PI or one of his designates will have access to the data.

Data Safety and Monitoring Plan. The ultimate goal of this application is to further our understanding the role of different therapies in the management of diabetes. The DSMP utilized will adhere to the protocol approved by the Mayo Clinic IRB. We propose the following plan: -
Data quality and management: The principal investigator will review all data collection forms on an three-monthly basis for completeness and accuracy of the data as well as protocol compliance.
Adverse events grading: The common grading scale listed below will be used to grade AEs:

0 No adverse event or within normal limits or not clinical significant
1 Mild AE, did not require treatment
2 Moderate AE, resolved with treatment
3 Severe AE, resulted in inability to carry on normal activities and required professional medical attention
4 Life threatening or disabling AE
5 Fatal AE

Attribution scale: An adverse event includes both, an expected side effect that is of a serious nature, or an unexpected side effect/ event regardless of severity. All events will be graded as to their attribution (unrelated to protocol, or possibly, probably, or definitely related to protocol). Any event that is reported to either the principal investigator or his designated research associates by the subject or medical staff caring for the subject and which meets the criteria will be documented as such.

Data Monitoring. The majority of data generated from these protocols will be from analyses performed in our laboratory or the immunochemical core laboratory. Standard quality control procedures are in place for each assay. Genotyping results are examined for consistency between repeated genotypes of a given sample chosen at random. The frequency of data review for this study differs according to the type of data and can be summarized in the following table:

Data type	**Frequency of review**
Subject accrual (adherence to protocol regarding demographics, inclusion/exclusion)	Weekly
Adverse event/safety rates (injuries)	Weekly
Study data	Weekly
Annual report	Yearly for CRTU, IRB

Informed Consent. Written informed consent will be obtained from all individuals who participate in the study. The principal investigator or his co-investigators meet with each participant, review the consent form in detail and confirm the subjects understanding of the study. They answer all questions posed by the participants and when convinced that the subject verbally demonstrates understanding of the protocol obtains a signed consent. Only designated staff are authorized to obtain informed consent.
Benefits: This study exposes subjects to risks detailed above. However, as detailed it will advance our knowledge of how to treat diabetes and help make clinicians make rational therapeutic choices in the future.

F. Gender/Minority Mix

The majority of residents in Rochester, Minnesota and surrounding counties are White, recent population estimates from over the past decade have indicated that minorities make up a significantly larger segment of our communities. According to census area data such cultural groups as the Somalis, Hispanic/ Latinos, and South East Asians have increased the percentage of minorities living in Rochester and the surrounding counties from 3% in 1990 to nearly 10% in 2000. Despite the increases in the number of minorities within rural Rochester, Minnesota, it is likely that recruitment will fall short in the area of minority participation. However, we are actively working with the minority outreach specialist in the Center for Patient Oriented Research and Tribal Elders from the local minority populations, to develop complementary community-based strategies for recruitment of minorities at Mayo Clinic Rochester.

G. Bibliography

1. Shah P, Basu A, Basu R, Rizza R: Impact of lack of suppression of glucagon on glucose tolerance in humans. Am J Physiol 1999;277:E283-290
2. Shah P, Vella A, Basu A, Basu R, Schwenk WF, Rizza RA: Lack of suppression of glucagon contributes to postprandial hyperglycemia in subjects with type 2 diabetes mellitus. J Clin Endocrinol Metab 2000;85:4053-4059
3. Albright AL, Gregg EW: Preventing type 2 diabetes in communities across the U.S.: the National Diabetes Prevention Program. American journal of preventive medicine 2013;44:S346-351
4. Vella A, Camilleri M, Rizza RA: The gastrointestinal tract and glucose tolerance. Curr Opin Clin Nutr Metab Care 2004;7:479-484
5. Shah M, Varghese RT, Miles JM, Piccinini F, Dalla Man C, Cobelli C, Bailey KR, Rizza RA, Vella A: TCF7L2 Genotype and alpha-Cell Function in Humans Without Diabetes. Diabetes 2016;65:371-380
6. Faerch K, Vistisen D, Pacini G, Torekov SS, Johansen NB, Witte DR, Jonsson A, Pedersen O, Hansen T, Lauritzen T, Jorgensen ME, Ahren B, Holst JJ: Insulin Resistance Is Accompanied by Increased Fasting Glucagon and Delayed Glucagon Suppression in Individuals With Normal and Impaired Glucose Regulation. Diabetes 2016;65:3473-3481
7. Varghese RT, Dalla Man C, Sharma A, Viegas I, Barosa C, Marques C, Shah M, Miles JM, Rizza RA, Jones JG, Cobelli C, Vella A: Mechanisms Underlying the Pathogenesis of Isolated Impaired Glucose Tolerance in Humans. J Clin Endocrinol Metab 2016;101:4816-4824
8. Varghese RT, Viegas I, Barosa C, Marques C, Shah M, Rizza RA, Jones JG, Vella A: Diabetes-Associated Variation in TCF7L2 Is Not Associated With Hepatic or Extrahepatic Insulin Resistance. Diabetes 2016;65:887-892
9. Vella A, Rizza RA: Application of isotopic techniques using constant specific activity or enrichment to the study of carbohydrate metabolism. Diabetes 2009;58:2168-2174
10. Laurenti MC, Vella A, Varghese RT, Andrews JC, Sharma A, Kittah NE, Rizza RA, Matveyenko A, De Nicolao G, Cobelli C, Dalla Man C: Assessment of pulsatile insulin

secretion derived from peripheral plasma C-peptide concentrations by nonparametric stochastic deconvolution. Am J Physiol Endocrinol Metab 2019;316:E687-E694

11. Piccinini F, Dalla Man C, Vella A, Cobelli C: A Model for the Estimation of Hepatic Insulin Extraction After a Meal. IEEE Trans Biomed Eng 2015;
12. Dalla Man C, Micheletto F, Sathananthan A, Rizza RA, Vella A, Cobelli C: A model of GLP-1 action on insulin secretion in nondiabetic subjects. Am J Physiol Endocrinol Metab 2010;298:E1115-E1121
13. Adkins A, Basu R, Persson M, Dicke B, Shah P, Vella A, Schwenk WF, Rizza R: Higher insulin concentrations are required to suppress gluconeogenesis than glycogenolysis in nondiabetic humans. Diabetes 2003;52:2213-2220
14. Basu R, Di Camillo B, Toffolo G, Basu A, Shah P, Vella A, Rizza R, Cobelli C: Use of a novel triple-tracer approach to assess postprandial glucose metabolism. Am J Physiol Endocrinol Metab 2003;284:E55-E69
15. Smushkin G, Sathananthan M, Piccinini F, Dalla Man C, Law JH, Cobelli C, Zinsmeister AR, Rizza RA, Vella A: The effect of a bile acid sequestrant on glucose metabolism in subjects with type 2 diabetes. Diabetes 2013;62:1094-1101
16. Steele R, Wall JS, De Bodo RC, Altszuler N: Measurement of size and turnover rate of body glucose pool by the isotope dilution method. Am J Physiol 1956;187:15-24
17. Reed AS, Charkoudian N, Vella A, Shah P, Rizza RA, Joyner MJ: Forearm vascular control during acute hyperglycemia in healthy humans. Am J Physiol Endocrinol Metab 2004;286:E472-480
18. Sathananthan M, Farrugia LP, Miles JM, Piccinini F, Dalla Man C, Zinsmeister AR, Cobelli C, Rizza RA, Vella A: Direct Effects of Exendin-(9,39) and GLP-1-(9,36)amide on Insulin Action, beta-Cell Function, and Glucose Metabolism in Nondiabetic Subjects. Diabetes 2013;62:2752-2756
19. Vella A, Reed AS, Charkoudian N, Shah P, Basu R, Basu A, Joyner MJ, Rizza RA: Glucose-induced suppression of endogenous glucose production: dynamic response to differing glucose profiles. Am J Physiol Endocrinol Metab 2003;285:E25-E30
20. Vella A, Shah P, Basu R, Basu A, Holst JJ, Rizza RA: Effect of glucagon-like peptide 1(7-36) amide on glucose effectiveness and insulin action in people with type 2 diabetes. Diabetes 2000;49:611-617
21. Ishida T, Chap Z, Chou J, Lewis R, Hartley C, Entman M, Field JB: Differential effects of oral, peripheral intravenous, and intraportal glucose on hepatic glucose uptake and insulin and glucagon extraction in conscious dogs. J Clin Invest 1983;72:590-601
22. Ishida T, Chou J, Lewis RM, Hartley CJ, Entman M, Field JB: The effect of ingestion of meat on hepatic extraction of insulin and glucagon and hepatic glucose output in conscious dogs. Metabolism 1983;32:558-567
23. Ishida T, Lewis RM, Hartley CJ, Entman ML, Field JB: Comparison of hepatic extraction of insulin and glucagon in conscious and anesthetized dogs. Endocrinology 1983;112:1098-1109