Investigating Effects of BOTOX on Weight Loss and Glucose Tolerance in Obese, Type 2 Diabetic Subjects

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1. Background

Obesity currently affects more than 1 in 3 adults in the US. An alarming consequence is the astounding increase in type 2 diabetes (T2D), which has emerged as the 7th leading cause of death. Lifestyle interventions have proved to be only modestly effective and rarely sustainable. Bariatric surgery, particularly Roux-en-Y gastric bypass (RYGB) is currently the most effective treatment for robust and sustained weight loss, and results in remarkable improvements in T2D status. However, these surgeries involve extensive and permanent re-arrangement of the GI tract, can be associated with serious complications, and are expensive. Conceivably they reach less than 2% of all eligible subjects. The ability to target the gut to treat obesity and diabetes without surgical intervention would represent a major therapeutic breakthrough. But, in order to accomplish this we must identify novel physiological targets and therapies.

Gut as a therapeutic target for obesity and diabetes: evidence from bariatric surgery

The importance of the gut in nutrient metabolism and energy homeostasis is highlighted by the remarkable success of bariatric surgery. RYGB reduces stomach volume and diverts nutrient flow from the proximal intestine, which is a major site for nutrient sensing, absorption and transport to more distal segments of the small intestine. Digested nutrients absorbed by the enterocytes make their way directly to the bloodstream, or as in the case of lipids are delivered into the lacteals before entering the circulation. Nutrient sensing is mediated by enteroendocrine (EE) hormones that act locally to influence motility and digestive function, and relay nutritional status to the brain via vagal afferent nerve fibers, found in close proximity. The cell bodies of vagal afferent nerve fibers are located in the paired nodose ganglia and enter the brain stem via the tractus solitarius. They synapse onto neurons of the nucleus tractus solitarius (NTS) and dorsal motor nucleus of the vagus (DMV) where integration of sensory signals elicits cues for meal initiation, termination, and nutrient metabolism. Signaling across this gut-to-brain neuroendocrine axis is critical for regulation of feeding behavior, food intake, and energy homeostasis. We seek to exploit this neuroendocrine axis to impede nutrient uptake and manipulate the body's own sensory mechanisms in order to achieve a therapeutic end point for obesity and T2D without altering GI anatomy.

Manipulating sensory feedback from gut to influence nutrient metabolism

Sensory feedback to the brain is based on the chemical milieu of the lumen and the distension status of the gut wall. Distension and contraction of the wall relay sensory signals of nutrient entry. This rhythmic contraction of the gut wall is the consequence of neurotransmission at presynaptic motor terminals, largely mediated by release of acetylcholine (Ach). Blocking this neurotransmission would prevent contraction of the smooth muscle wall and potentially relay a false sensory signal of nutrient entry and/or satiety.

Botulinum neurotoxins (BoNT)[1-5], are some of the most potent anti-cholinergic agents that reduce muscle contraction. The most potent serotype A (available commercially as BOTOX®) has now been approved by the FDA for treatment of several neuromuscular conditions including blepharospasm, spasm, dystonia, spasticity, and even cerebral palsy in children[1, 6-9]. Botox possesses several unique pharmacological properties – it is specific, very potent, has limited diffusion from the injection site[1, 6-9] and is reversible. Based on its properties and evidence-based results, we propose to explore actions of Botox on the intestinal smooth muscle wall and its possible use in manipulating sensory feedback. If proved to be successful, this approach could provide a new treatment paradigm for T2D and metabolic diseases with or without obesity. This potential application of the drug has never been explored.

2. Rationale and Specific Aims

Botox has been approved by the FDA and successfully used for treatment of a variety of clinical syndromes related to abnormal muscle contraction. Its potential ability to impede intestinal wall contractions has never been explored. We seek to explore this novel application of the drug and its consequent effects on sensory feedback to the brain, feeding behavior, glucose tolerance, and weight gain. Based on its unique pharmacological properties, and our preliminary data in high-fat diet induced obese (DIO) mice, we hypothesize that Botox injected into the duodenal wall will (1) induce weight loss (2) improve glucose tolerance, and (3) improve duodenal nutrient sensing in obese, T2D subjects.

Specific Aim 1. Determine if Botox injected into the duodenum reduces body weight in obese, type 2 diabetic subjects
Specific Aim 2. Determine if Botox injected into the duodenum improves glucose tolerance in obese, type 2 diabetic subjects
Specific Aim 3. Determine if Botox injected into the duodenum improves nutrient sensing in obese, type 2 diabetic subjects

The information generated from the studies proposed may substantially alter our approach to tackle obesity and T2D, and lead to emphasis on the gut-brain cross talk in energy homeostasis.

3. Animal Studies and Previous Human Studies

3.1 Animal Studies

We have developed a surgical model for Botox injections in mice. Mice are anesthetized by isoflurane inhalation and laparotomy is performed to expose the intestines. Botox loaded into Hamilton syringes fitted with 30 g needles is injected into the smooth muscle layer of the duodenum at 3 sites, within 3 cm distal to the pyloric sphincter (**Fig 1A**) ensuring no drug diffusion into the lumen. High-fat (60%) diet induced obese (DIO) mice injected with

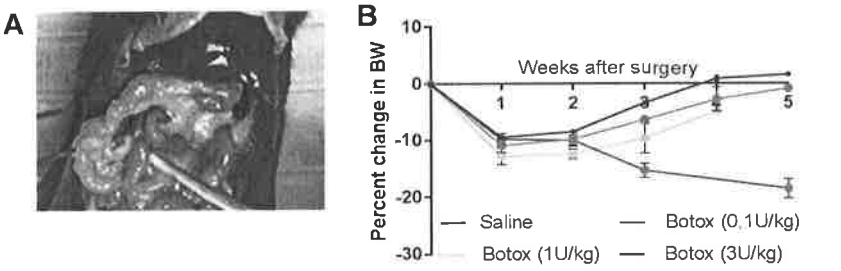

Fig. 1A: Duodenal injection site. **B**: Weight loss in DIO mice injected with saline or Botox (0.1-3U/kg body weight; *n*=3-4 mice)

Botox showed a dose-dependent decrease in body weight between 0.3-3U/kg body weight, and 3U/kg was observed to be the optimal dosage (**Fig. 1B**). The effects lasted for at least 7 weeks, following which they begin to dwindle and are almost reversed by the end of 10 weeks. Readouts analyzed to determine dosage and effective treatment time include body weight recordings, and protein expression of neurotransmitter, SNAP-25 [3, 10, 11] and smooth muscle contraction marker phospho-myosin light chain 2_{Ser19} ($P\text{-}MLC_{Ser19}$) [12-16]. We also observed distension of the muscle wall and apparent dysmotility within 48 hours of injection that persisted until 6 weeks later. SNAP-25

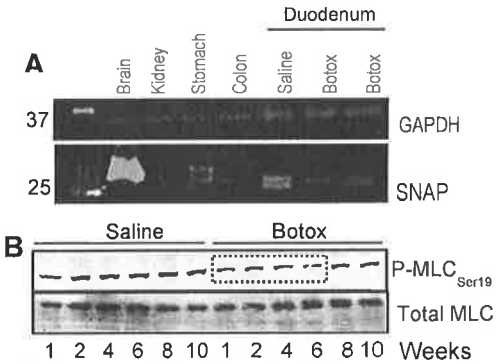

Fig. 2: Representative WB of SNAP-25 expression in mice **(A)** kidney, stomach, colon, brain (for comparison) and duodenum injected with saline or Botox, measured 6 weeks later; $P\text{-}MLC_{Ser19}$ and total MLC expression **(B)** in duodenal injection site 1-10 weeks later.

expression at the Botox injection site was lower, suggesting that *Botox induced proteolysis of SNAP-25* (**Fig 2A**). P-MLC_{Ser19}, indicator of smooth muscle contraction in the gut was down-regulated also until 6 weeks, following which expression was restored (Fig 2B). These data suggest that Botox blocks smooth muscle contraction in the intestine.

1. Botox causes weight loss in DIO mice (for up to 7 weeks post injections) without altering food intake: DIO mice injected with Botox (3U/kg body weight) in the duodenal wall lost 15-20% body weight over 7 weeks compared to saline injected controls **(Fig. 3)**. Interestingly, food intake was not significantly lower (data not shown), and thereby did not account for the robust weight loss observed.

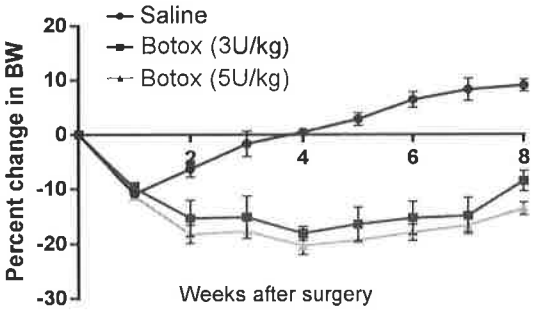

Fig 3. Weight loss in DIO mice injected with saline or Botox. Percent decrease in body weight in DIO mice injected with saline or Botox (3, or 5 U/kg) in duodenal wall (*n*=7-8).

2. Botox improves glucose tolerance in DIO mice: We observed significant improvement in glucose tolerance in Botox-treated DIO mice, measured 4 weeks after injection in the duodenal wall (3U/kg). Baseline OGTT was measured in a cohort of DIO mice (*n*=14) prior to Botox injections. Three days later, saline (*n*=6) or Botox (*n*=8) was injected in the duodenal wall, and OGTT was repeated 4 weeks later. DIO mice started out with poor glucose tolerance, as expected. No improvement was observed in saline controls after 4 weeks; however, Botox-injected group showed strong improvement in glucose tolerance **(Fig. 4)**.

3.2 Previous Human Studies

Botox for injections has been approved for treatment of overactive bladder (idiopathic or neurogenic detrusor over activity), upper and lower limb focal spasticity, cerebral palsy, primary axillary hyperhidrosis, strabismus, blepharospasm, cervical dystonia, and chronic migraine (≥15 days per month with headaches lasting 4 hours or more a day)[1, 6-9]. Adverse reactions experienced in each of these approved conditions have been listed as under. In general, adverse reactions occur within the first week of injection [17, 18].

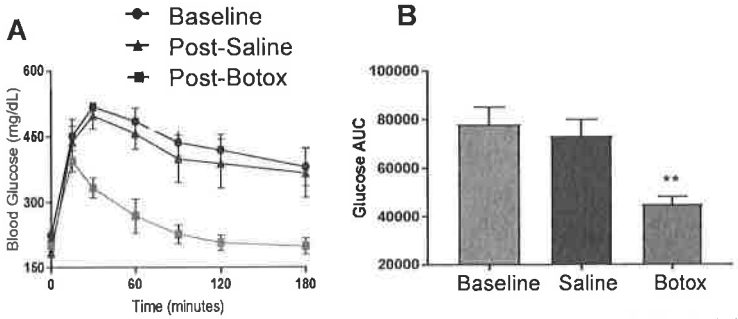

Fig 4. OGTT in DIO mice before and after Botox injection (3U/kg). (**A**) Blood glucose before (baseline, *n*=14) and 28 days after saline (*n*=6) or Botox injection (*n*=8). (**C**) Glucose AUC before and after Botox injection. *, p<0.05; **, p<0.01.

Overactive bladder (OAB):

The table below lists the most common adverse reactions occurring within 12 weeks of BOTOX injections in placebo-controlled clinical trials for overactive bladder [17, 18].

Table 1: Adverse reactions reported by patients treated for OAB in clinical trials

Adverse Reactions	BOTOX 100 Units (N=552)	Placebo (N=542)
Urinary tract infection	99 (18%)	30 (6%)
Dysuria	50 (9%)	36 (7%)

Urinary retention	31 (6%)	2 (0%)
Bacteriuria	24 (4%)	11 (2%)
Residual urine volume	17 (3%)	1 (0%)

During the entire study period, frequency of some of the above adverse reactions increased: urinary tract infections (26%), dysuria (11%), and bacteriuria (8%).

Detrusor Overactivity associated with Neurologic Condition:

The table below presents the adverse reactions reported in patients with detrusor over activity associated with neurologic condition within 12 weeks after intradetrusor Botox injections (200 Units) [17, 18].

Table 2: Adverse reactions reported by patients treated for detrusor over activity

Adverse Reactions	**BOTOX 200 Units (N=262)**	**Placebo (N=272)**
Urinary tract infection	64 (24%)	47 (17%)
Urinary retention	45 (17%)	8 (3%)
Hematuria	10 (4%)	8 (3%)
Fatigue	10 (4%)	3 (1%)
Insomnia	4 (2%)	0 (0%)

Some additional rare adverse events were reported including constipation (4%), muscular weakness (4%), dysuria (4%), fall (3%), gait disturbance (3%), and muscle spasms (2%).

Chronic Migraine:

The following adverse reactions were reported based on safety data compiled from two chronic migraine double-blind, placebo controlled phase 3 clinical trials involving 687 patients [17, 18].

Table 3: Adverse reactions reported by patients treated for chronic migraine

Adverse Reactions by System Organ Class	**BOTOX 155-195 Units (N=687)**	**Placebo (N=692)**
Eye Disorders		
Eyelid Ptosis	25 (3.6%)	2 (0.3%)
Musculoskeletal & Connective Tissue Disorders		
Neck pain	60 (8.7%)	19 (2.7%)
Musculoskeletal stiffness	25 (3.6%)	6 (0.9%)
Muscular weakness	24 (3.5%)	2 (0.3%)
Musculoskeletal pain	18 (2.6%)	10 (1.4%)
Myalgia	21 (3.1%)	6 (0.9%)
Muscle spasms	13 (1.89%)	6 (0.9%)
Nervous System Disorders		
Headache	32 (4.7%)	22 (3.2%)
Migraine	26 (3.8%)	18 (2.6%)
Facial paresis	15 (2.2%)	0 (0%)
Vascular Disorders		
Hypertension	11 (1.6%)	7 (1.01%)
Infections		
Sinusitis	28 (4.1%)	27 (3.9%)
Bronchitis	17 (2.5%)	11 (1.6%)

Others		
Pain at injection site	23 (3.3%)	14 (2.0%)

Patient dropout rate due to adverse events in these clinical trials was 3.8% for BOTOX treated patients and 1.2% for the placebo group. Most common adverse events leading to discontinuation included neck pain, muscular weakness, and migraine [17, 18].

Upper Limb Spasticity:

The most common adverse events occurring as a result of BOTOX injections in adults for treatment of upper limb spasticity are listed in the table under [17, 18]. Additional patients receiving higher doses (400 Units) or more, or four consecutive treatments for approximately 1 year reported similar type and frequency of adverse events.

Table 4: Adverse reactions reported by patients treated for upper limb spasticity

Adverse Reactions by System Organ Class	**BOTOX 251-360 Units (N=115)**	**BOTOX 150-250 Units (N=188)**	**BOTOX <150 Units (N=188)**	**Placebo (N=182)**
Gastrointestinal Disorders				
Nausea	3 (3%)	3 (2%)	1 (2%)	1 (1%)
Musculoskeletal & Connective Tissue Disorders				
Pain in extremities	7 (6%)	10 (5%)	5 (9%)	8 (4%)
Muscular weakness	0	7 (4%)	1 (2%)	2 (1%)
Infections				
Bronchitis	4 (3%)	4 (2%)	0	2 (1%)

Lower Limb Spasticity:

Adverse events reported were similar to those observed in patients that received BOTOX injections (300-400 Units) for lower limb spasticity [17, 18].

Table 5: Adverse reactions reported by patients treated for lower limb spasticity

Adverse Reactions by System Organ Class	**BOTOX (N=231)**	**Placebo (N=233)**
Musculoskeletal and connective tissue disorders		
Arthralgia	8 (3%)	2 (1%)
Back pain	6 (3%)	4 (2%)
Myalgia	4 (2%)	3 (1%)
Infections		
Upper respiratory tract infection	4 (2%)	2 (1%)
Others		
Pain at injection site	5 (2%)	2 (1%)

Cervical Dystonia:

The most frequently reported adverse reactions in cervical dystonia patients tested for safety include dysphagia, neck pain, and headaches [17, 18].

Primary Axillary Hyperhidrosis:
Based on a cohort of 346 patients that received 50 Units and 110 patients that received 75 units of BOTOX in each axilla, about 3-10% of adults reported non-axillary sweating, pharyngitis, flu syndrome, pruritus, neck or back pain following injections [17, 18].

Blepharospasm:
Patients with blepharospasm receiving an average of 33 units OF BOTOX per eye (3-5 injection sites) reported ptosis (21%), keratitis (6%), and eye dryness (6%) temporary facial paralysis and syncope were also reported [17, 18].

Strabismus:
Strabismus patients receiving higher doses of BOTOX reported vertical deviation [10, 11]. About 17% of 2058 adults that received a total of 3650 injections for treatment of horizontal strabismus reported vertical deviation. Ptosis was reported in 1%, 16%, and 38% after injections in inferior rectus, horizontal rectus, and superior rectus, respectively. Retro bulbar hemorrhage was reported in 0.3% cases [17, 18].

In general, local muscle weakness appears to be the most commonly reported and expected event following BOTOX injection in muscles. However, weakness of adjacent muscle tissues, and rarely remote tissues occurring as a result of "spread of toxin effect" have been reported in adult and pediatric patients as well [17, 18]. Events typically observed after any injection procedure including localized pain, inflammation, tenderness, bruising, erythema, and needle-related pain or anxiety have been reported in some clinical trials [17, 18].

We will recruit obese subjects with pre-diabetes or T2D for the proposed clinical study for 5 visits. After informed written consent is obtained, subjects will be admitted to the Clinical Research Center (CRC), and will undergo upper endoscopic injection of Botox into the duodenal wall. We anticipate that injections of Botox into the duodenal wall will result in significant weight loss and improvements in glucose tolerance and duodenal nutrient sensitivity. Subjects will be studied over a period of 6 months. Subjects will be asked to complete 5 study visits: On the first visit, each subject will undergo an oral glucose tolerance test (OGTT). At visit 2, subjects will undergo an esophagogastroduodenoscopy (EGD) procedure for the delivery of BtX-A to the duodenal wall. Visits 3-5 will be made 1, 3, and 6 months after EGD (Figure 1). On every study visit, body weight and body composition will be recorded and oral glucose tolerance test (OGTT) be performed. Nutrient sensing test will be performed at visits 1 and 3.

4. Inclusion/Exclusion Criteria

Inclusion Criteria

1. Age = 18-65
2. BMI ≥ 30 kg/m^2
3. Having established diagnosis of Type 2 diabetes or pre-diabetes

Exclusion Criteria

1. Ongoing insulin therapy
2. Endoscopy within past 6 weeks
3. Use of anti-obesity drugs, or less than 3 months of discontinuation
4. Serum creatinine levels higher than 1.5 mg/dL
5, Hepatic enzyme elevation> 2x the upper limits of normal

6. Pregnant
7. Abnormal ECG
8. Prior bariatric surgery
9. Prior surgery on the alimentary tract
10. Gastroparesis
11. Inflammatory bowel disease
12. Currently using "blood thinners"
13. Pre-existing cardiovascular diseases
    - Heart attack in the past 3 months
    - Cardiac stenting
    - Heart valve disorders
14. Liver failure

5. Screening and Enrollment

Obese subjects (BMI ≥ 30 kg/m^2) with prediabetes or type 2 diabetes (n = 20) will be recruited by solicitation flyers, recruitment emails using Research Match and LISTSERV that reaches all Vanderbilt employees, residents, and students, and Facebook advertisements, . Subjects who do not qualify for bariatric surgery, or subjects who are awaiting insurance approval for a bariatric procedure at the Center of Surgical Weight Loss, One Hundred Oaks (OHO) at Vanderbilt University Medical Center will also be contacted via emails and telephone calls.

Subjects will undergo an initial screen by the study coordinator or research staff to assess eligibility and ability to comply with the study requirements. Prior to each study visit, subjects on oral anti-diabetic medications will be asked to discontinue these medications 4 days prior to their study visit. Diabetic subjects will be instructed to monitor their pre-prandial blood glucose during this time and to contact the study physician if their blood glucose levels are greater than 250 mg/dL for two consecutive readings. The study physician may instruct the subject to initiate short-term insulin therapy or resume their oral anti-diabetic medications; in either case the subject will be excluded from the study. Subjects will be instructed to maintain their usual diet and physical activity levels for 1 week prior to each study visit and to arrive fasted for every visit (only water after dinner).

6. Study Procedures

The subjects will be recruited for 5 study visits (Figure 1). After informed, written consent is obtained, subjects will be admitted to the Clinical Research Center (CRC).

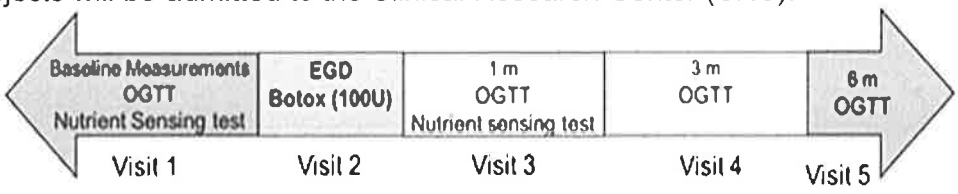

Figure 5. Study timeline

Study visit 1:
Day 1:

- Subjects will arrive to the CRC after an overnight fast.

- They will undergo standard physical examination; anthropometric measurements (height, weight, waist and hip circumference) perform body composition assessment via DEXA imaging (dual-energy x-ray absorptiometry) will be recorded.
- *Baseline glucose tolerance* will be assessed by an oral glucose tolerance test (OGTT).
- Subjects will be handed a visual analog scale (VAS) questionnaire designed to capture their perceived hunger and satiety sensations, food preferences, cravings, and feeding behavior prior to intervention. The questions will be explained by the researcher and subjects will be instructed to bring in the completed questionnaire on their second visit.
- Subjects will be provided snacks and asked to return to CRC at 7 pm for the nutrient sensing test to be performed the following day. The subject will be fed a standardized meal and restricted to water after 8:00 pm.

Day 2

- Blood will be drawn for determination of fasting plasma insulin, C-peptide, CBC, CMP, lipid profile, liver enzymes, PT, PTT, and gut hormones including ghrelin, GIP, GLP-1, pancreatic polypeptide (PP) and PYY.
- Subject will then consume a standardized 250 kcal liquid mixed meal containing 40 g carbohydrates, 6 g fat, and 9 g protein (8 oz. Ensure; Abbott Nutrition, Columbus, OH) within 10 minutes. Blood will be drawn at 15, 30, 60, and 120 minutes after consumption following which subject will be discharged.

Study visit 2 (within 2 weeks after the first visit):

- Subjects will undergo esophagogastroduodenoscopy (EGD) procedure for the delivery of BOTOX to the duodenal wall, to be performed by Dr. Patrick Yachimski at the Vanderbilt Gastrointestinal Endoscopy Suite (VGIES). We have been exempted from Investigational New Drug (IND) regulation for the proposed testing by the FDA (letter attached).
- Subjects will be monitored in the VGIES for 30 minutes and discharged with instructions for follow-up and contact information of the physicians' team (Drs. Abumrad, Yachimski).

Telemedicine visit (1 week after SV2): Study team member will contact subject by telephone to assess if there have been any adverse events from the study procedures completed thus far.

Study Visits 3-5 (1, 3, and 6 months after EGD): On every study visit, body weight, body composition via DEXA, food intake, and feeding behavior will be recorded and post-absorptive OGTT will be performed. Nutrient sensing test will be repeated at visit 3.

Oral Glucose Tolerance Test (Study visits 1 and 3-5):

- **Day 0:** Subjects will be fasting overnight (and restricted to water only after 8 pm).
- **Day 1:** Blood will be drawn for determining fasting blood glucose levels. At 8:00 am subjects will drink Trutol™ 75 (Thermo Scientific), a solution of 75g dextrose in 300 ml of water in 10 minutes. Blood will be drawn at 15, 30, 45, 60, and 120 min post ingestion.
- The subject will be fed a standardized snack and discharged around noon.

Endoscopic delivery of BOTOX via EGD (Study Visit 2):

- Subjects will arrive fasted at the GI Suite in the main hospital at Vanderbilt University for the EGD procedure.
- Under the supervision of an anesthesiologist, subjects will be given combination of intravenous medications, so they fall asleep. Dr. Yachimski will then pass the endoscope,

a long, flexible tube with light, video camera and channel for small instruments including syringes through the esophagus and stomach, pylorus into the junction of the 1st and 2nd parts of the duodenum. Botox (100 units dissolved in 200 µL of sterile, preservative-free 0.9% Sodium Chloride, USP) will be injected along the medial (mesenteric) border, into the duodenal muscle wall.

Post EGD Care:

- Following completion of EGD procedure and emergence from anesthesia, subjects will be monitored in the VGIES-dedicated post-anesthesia care unit (PACU) prior to discharge.
- Subjects will be provided written instructions regarding potential signs and symptoms of adverse events, including fever, pain, bleeding, and muscle weakness. They will be provided the 24/7 physician call number for any questions or issues that may arise.

Nutrient sensing test (study visit 1 and 3):

- **Day 1:** After OGTT subjects return to CRC at 7:00 pm. They will be fed a standardized meal and fasted overnight (restricted to water after 8:00 pm).
- **Day 2:** At 8:00 am, blood will be drawn for determination of fasting plasma insulin, C-peptide, and gut hormones stated above. Subject will then consume a standardized 250 kcal liquid mixed meal containing 40 g carbohydrates, 6 g fat, and 9 g protein (8 oz. Ensure; Abbott Nutrition, Columbus, OH) to be administered over a 10-min period. Blood will be drawn 15, 30, 60, and 120 minutes later. At the end of the study, subject will be provided a standardized meal/snack and discharged.
- All blood samples will be collected in chilled EDTA tubes with protease and DPP IV inhibitors, and immediately centrifuged at 4°C to separate plasma. The MILLIPLEX MAP Human Metabolic Hormone Magnetic Bead Panel (Millipore) will be used to simultaneously assay eight analytes. Cholecystokinin (CCK) will be measured by radioimmunoassay (RIA, ALPCO Diagnostics). Blood for CCK measurement will be collected with 500 KIU Trasylol/1 ml blood. Free fatty acids, cholesterol, and triglycerides will be assayed in the Vanderbilt Diabetes Research and Training Center Lipid Core. Glucose will be measured by the glucose oxidase method using a Beckman Glucose Analyzer.

7. Risks

EGD Procedure-related risk:

- EGD for delivery of Botox will be performed as per standard protocols and are of little risk to the subject, with minimal chance of bleeding.
- Dr. Yachimski and his group at VGIES have performed over 1000 such endoscopies with the least adverse effects.

Post EGD Care: Following completion of EGD procedure and emergence from anesthesia, patients will be monitored in the Vanderbilt GI Endoscopy Suite's (VGIES) dedicated post-anesthesia care unit (PACU) and evaluated by physician and nursing staff prior to discharge. Subjects will be provided written instructions regarding potential signs and symptoms of adverse events, including fever, pain, bleeding, and muscle weakness. They will be provided the 24/7 physician call number for any questions or issues that may arise.

Drug-related risk:

- Based on prior clinical trials targeting other neuromuscular disorders, the most commonly reported event following Botox injection is temporary local muscle weakness at the

injection site, and rarely remote tissues occurring as a result of "spread of toxin effect". Almost all of this has been reported to disappear in about 3-5 days.

- Subjects will be provided handouts providing detailed information on potential adverse events including nausea, muscular weakness, and pain in extremities and injection site.
- Subjects will be instructed to call the 24/7 number if required, for assistance in management and care of any adverse events or discussion of other potential issues.
- A specialized nurse from the VGIES will call the subject at 1 and 7 days post-procedure, and as needed.

8. Protection against risks

8.1. Recruitment and Informed Consent

- Subjects will be recruited via solicitation flyers, Research Match, LISTSERV, advertisements, and Facebook posts listing study objectives and design, inclusion criteria, remuneration, and contact information.
- Recruitment email will be sent using Vanderbilt's "Research Notification" list that reaches all Vanderbilt employees and can therefore target a large population.
- In addition, census lists from the Vanderbilt Center for Surgical Weight Loss will be screened for eligible candidates, based on inclusion/exclusion criteria. Subjects who do not qualify for bariatric surgery, or subjects who are awaiting insurance approval for a bariatric procedure will be contacted via emails and telephone calls.
- Interested subjects will attend a pre-study seminar session entailing the study's purpose, procedures, and potential risks. If subjects are still interested they will be directed to contact the recruiter for more information and additional screening. During the screening the recruiter will further review inclusion and exclusion criteria and explain the study procedures, duration of the study, benefits of participation, and potential risks, and answer any remaining questions.
- If the subject indicates interest, a copy of the informed consent will be provided (by hand, mail, or email); time will be allowed for the subject to read through the form and ask additional questions or express concerns.
- Subjects will sign the consent at the time of their screening visit to the Vanderbilt CRC. Signed consent forms will be uploaded to a secure password-protected REDCap® (Research Electronic Data Capture) database.

8.2. Protections against risk

(a) Minimizing physical, psychological, and social risks. Participation in this study is completely voluntary. Subjects will be free to drop out of the study due to any issues related, but not limited to physical discomfort, pain or other adverse events (AEs). Screening will be performed by a physician or nurse staff to ensure appropriate selection criteria are employed. Subjects will be free to refuse to respond to any question that may result in psychological disturbance. All patient care information utilized will be confidential and anonymous.

(b) Minimizing risks to confidentiality.

- All records and data will be de-identified and securely stored in Vanderbilt's HIPAA-compliant, password protected data base, REDCap®; this is a secure web application for collection and management of clinical trial data developed by Vanderbilt University in collaboration with a vast consortium of thousands of active institutional partners.
- The subject's identification is concealed, and a number is used as the identifier instead of the name. Only the PI, co-investigator, and research assistant will have access to identifiable private information. The PI will be responsible for maintaining the repository

and the identity of the samples. De-identified specimens will be released by the PI to projects after IRB approval only. Any paper documents will be stored in the PI's locked officer. All research samples (serum and plasma) obtained will be de-identified and stored in our locked freezers for an unlimited period of time. Only research personnel associated in our laboratory who are directly involved with the study will have access to research samples.

9. Reporting of Adverse Events or Unanticipated Problems involving Risk to Participants or Others

Should a study-related adverse event (AE) result in harm or injury, neither the subject nor their insurance company will be responsible for the cost of immediate medical care provided at Vanderbilt to treat the injury. There are no plans for Vanderbilt to pay for the costs of any additional care.

Adverse Event Reporting: All AEs will be graded according to the following scale:
0 = No adverse events or within normal limits
1 = Mild; did not require treatment
2 = Moderate; resolved without treatment
3 = Severe; required professional medical attention
4 = Life-threatening or disabling
5 = Death
All unanticipated, serious AEs related to the experimental procedures that are Grade 1 or above will be reported immediately to the DSMO (see below) and within 1 day to the IRB. The PIs will review AEs and notify the IRB and NIDDK of any changes needed to the protocol. The annual summary of all adverse events and any audit reports will be sent to the NIH and IRB at the time of continuing review.

(d) Data Safety Monitoring Plan (DSMP).

- Our DSMP will focus on performance, safety, and care of participants. Julia Wattacheril, MD, Columbia University, and Bettina Mittendorfer, PhD, Washington University St. Louis, will serve as the Data Safety Monitoring Board (DMSB) for this study.
- This is a pilot study. The DSMB will meet with the PIs at least 3 times. We are planning to initially enroll 3 patients and have the DSMB look at the efficacy and safety data. This will involve an initial meeting to ratify the DSMB, a meeting after the first patient undergoes EGD, and after 3 patients complete enrollment. The next meetings will be at the end of the first and second years, respectively. Progress reports will indicate patient recruitment status, adverse event occurrence, and summary of data collected. The DMSB will assess safety data including development of local or peripheral muscular weakness, bleeding, inflammation, hospitalizations, and other serious adverse events. Based on safety reports, the DMSB will advise and consent modification or termination of the study protocol or the study if it deems such actions to be warranted. Each meeting will be virtual, via zoom. We will send minutes to DSMB members for review and approval after each meeting.
- Participating subjects will be monitored by the physician and nursing staff of the VGIES PACU after recovering from anesthesia for EGD procedure and evaluated by physician prior to discharge.
- The research coordinator will be required to be present to record any AEs and report to the PI or co-investigators promptly of such events.

10. Study Withdrawal/Discontinuation

Subjects will be informed that they can discontinue or withhold participation at any time for any reason. Subjects will be compensated for their time and participation.

11. Statistical Analyses

Sample Size: We hypothesize that Botox will reduce body weight, improve glucose tolerance, and augment lipid sensing during fasting and post-prandially, in Class I-III obese subjects. Our outcome measures for the effects of Botox are: body weight; area under the curve (AUC) for plasma levels of ghrelin, CCK, GIP, and GLP-1; and glucose AUC. Power calculation based on previous studies[19-22] was performed using nQuery Advisor software. The number of subjects needed to detect a 15% decrease in body weight (n=16), 10-15% decrease in plasma levels of gut peptides (n=14-16), and a 10% decrease in glucose AUC (n=16) was determined for an estimated 90% power (two-sided type I error rate = 0.05), with effect size of 0.25. We anticipate studying 20 subjects to account for attrition.

Statistical Analysis Plan: We will analyze pre- and post-intervention measurements at 1, 3, and 6 months using one-way repeated measures ANOVA for each outcome. Correlation between visits will be determined by the first-order autoregressive (AR1) rule which assumes that correlation among repeated measures decline exponentially with time. We will also determine the most optimal (1, 3, or 6 months) and inter time point differences for each outcome.

12. Anticipated Results, Potential Pitfalls

Based on our preliminary data in mice, and assuming overlapping phenotype in humans, we anticipate that at least 60% of obese subjects recruited will lose 10-15% body weight within the first 6 months. We also anticipate at least 10% decrease in blood glucose AUC as early as 1 month after injections, which will sustain until 6 months, or as long as the actions of Botox last in human GI smooth muscle. We are aware of potential dose-dependent effects of Botox and this will be followed up in future trials. Furthermore, sustained improvements in glucose tolerance, if observed, may be a weight loss dependent effect, and dissecting out weight-independent effects will be explored in future trials as well.

13. Follow-up and Retention

Follow-up and retention of subjects is critical for the successful completion of the project. We seek to avoid and minimize high attrition to ensure validity of our results and maintain statistical power to detect true effect of the drug. This will be achieved by the following strategies[23-26].

a. Maintaining warm, welcoming environment: Research and clinical staff interacting with the subjects will be welcoming, non-judgmental and respectful to ensure that the subject's experience is as smooth, uneventful, and enjoyable as possible. Interviews and screening will be conducted in areas that afford privacy and subjects will be informed about confidentiality in all circumstances.
b. Flexibility during scheduling: Although the study requires timely follow-up visits, allowing reasonable flexibility will be critical during scheduling visits. This will avoid stress and discomfort in the participant and will keep them engaged. The importance of post-intervention visits (3-5) over the next 6 months will be emphasized and highlighted during recruitment, so that any prior commitments made by the subject for upcoming 6 months can be identified. If any of these pre-committed appointments overlap with the expected visits, it will be discussed, and adjustments made, wherever possible.

c. Efficient Tracking: Being able to locate the participant is critical for retention[23]. Subjects will be asked to provide email addresses and phone numbers, indicating the best times to call or text and whether messages can be left. The names and contact information of up to two additional people who can be contacted for assistance with locating participants will also requested. Written informed consent to contact these individuals will be obtained. All steps will be taken to ensure participants' confidentiality in all of these communications.
d. Educating subjects about their importance and role in research: In addition to informing participants about the research goal and protocol, an effort to highlight the significance of follow-up is critical to enhance retention. Subjects will also be explained how their experiences with Botox injections, both positive and negative, are so important in evaluating the treatment for a larger population and can motivate them to stay and complete the study. Efforts will be taken to resolve and work around anticipated difficulties to making study visits such as transportation, work schedules, etc.
e. Reasonable Incentives for Participation: Subjects will be provided monetary incentive for every visit ($50) and after completion of the study (additional $50). The use of incentives, especially additional remuneration at the end of the study will enhance subject retention. To avoid potential negative consequences of incentives, like motivating economically disadvantaged subjects to accept greater risks subjects will be informed that reimbursements are intended to acknowledge their efforts in completing study visits along with the expenses incurred from study participation, such as transportation, and parking.

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