Memory Advancement by Intranasal Insulin in Type 2 Diabetes (MemAID) RCT Trial

Study Protocol and Statistical Analysis Plan

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Hypotheses and Aims:

Memory Advancement by Intranasal Insulin in Type 2 Diabetes (MemAiD)

Type 2 diabetes mellitus (DM) accelerates brain aging(1), alters neurovascular coupling(2-6) and increases the risk for dementia and Alzheimer's disease (AD)(7-9). Insulin is a key neurotrophic factor in the brain, where it modulates cognition through regulation of energy metabolism, neurovascular coupling, and neuroprotection (10-13). Intranasal insulin (INI) binds to the receptors in the limbic system and cognitive networks including the hippocampus, hypothalamus, and insular cortex(14),(13),(15). Short-term administration of INI improved learning and memory in both healthy and cognitively impaired non-diabetic adults.(16-24) Our pilot randomized, cross-over, placebo-controlled study (5R21-DK-084463-02, IND 107690, NCT01206322)(24, 25) has shown that a single 40 IU dose of INI improved visuospatial memory in DM and controls. In the DM group, INI improved perfusion and resting state functional connectivity(25), and better visuospatial memory was associated with greater vasodilatation. No serious adverse events (AEs) or hypoglycemic episodes occurred. This study provided proof-of-concept supporting the potential usefulness and safety of INI and laid the groundwork to expand on these findings to determine the long-term effects and safety of INI treatment.

We propose a randomized controlled trial determining the long-term effects of INI on cognition and memory in DM and non-DM groups. We hypothesize that: 1) INI-treated adults with DM have better memory and functioning of specific cognitive domains and faster walking during a dual task than those treated with placebo and the control group; 2) Glycemic and insulin resistance and genetic markers for AD (ApoE4) may serve as predictors of positive responses to INI therapy; 3) INI treatment neither adversely affects systemic glycemic levels or the cardiovascular system nor causes weight gain.

Aim 1: To determine whether INI-treated type 2 DM adults have a) better memory and functioning in specific cognitive domains and b) faster dual-task gait speed and better daily living functioning than the placebo-treated and non-DM groups.

We will conduct a randomized, double-blinded, placebo-controlled study in 120 older adults with type 2 DM and 90 non-DM controls examining whether 40 IU INI once daily over a 24-week period improves:

- Specific domains of visuospatial attention and memory, verbal learning (primary outcomes);
- Gait speed during a dual task (which is an excellent predictor of overall health (26)), daily living functionality, and depression as compared to the DM group receiving sterile saline and the non-DM groups. The non-DM groups will provide reference of INI effects in a clinical phenotype of cognitive decline and insulin resistance that occurs with normal aging.

Aim 2: To identify a phenotype and long-term trajectory predicting clinically relevant response to INI therapy based on glycemic control, insulin resistance, endothelial and genetic markers.

a. We will determine a phenotype predicting a clinically relevant response to INI therapy and identify time-dependent trajectories of INI effects on cognition in the DM group vs. the placebo and the non-DM groups. Clinical predictors will be based on associations between cognitive function and/or gait and demographic, glycemic control, insulin resistance, endothelial and genetic (ApoE*4*) measures.
b. We will evaluate the dose-escalating trajectory of cognition, gait speed, and functionality during the 24 weeks of therapy and 24 weeks post-treatment and their dependence on the above- mentioned factors, and determine the time point when maximum effect was reached. INI therapy response is defined as a clinically relevant improvement on cognitive tests or in gait speed (as a continuous variable) or as responders vs. non-responders as compared to placebo within DM and non-DM groups (as a categorical variable).
c. MRI substudy: We will explore the long-term INI effects on regional perfusion, vasodilatation, and resting functional connectivity in 40 DM subjects pre- and post- INI/placebo treatment at baseline and at the end of intervention and their relationships to cognitive outcomes. Regional perfusion and vasodilatation will be measured by pseudo-continuous arterial spin labeling (PCASL) MRI at 3 Tesla, and resting-state functional connectivity will be quantified from low-frequency (0.01–0.08 Hz) fluctuations (LFF) of the whole-brain blood-oxygen-level dependent (BOLD) fMRI.

Aim 3: To determine the long-term safety of intranasal insulin vs. placebo with regard to glycemic control (fasting glucose, Hb1Ac, hypoglycemic episodes), vital signs, body mass, appetite feelings and food intake.

a. We will obtain measurements of fasting glucose, insulin, vital signs, body mass measurements, waist and hip circumference, appetite feelings using visual analogue scales (VAS) at baseline, 2-months, 4-months, and 6-months follow-up and keep weekly logs monitoring glucose and AEs.
b. Safety substudy: In the first 20 DM patients (ICF signed, enrolled) treated with subcutaneous insulin, we will conduct safety substudy with five glucose measurements per day using finger sticks (FS) for one week during baseline and during the first week of INI or placebo treatment, to evaluate the INI effects on glycemic control and hypoglycemic episodes. The enrollment has stopped per Amd #21 9/25/2017.

This study may pave the way to potential treatment and/or cure of DM- and age-related cognitive decline.

Primary Endpoints:

Aim 1: To determine whether INI-treated type 2 DM adults have a) better memory and functioning in specific cognitive domains and b) faster dual-task gait speed and better daily living functioning than the placebo-treated and non-DM groups.

- Specific domains of visuospatial attention and memory, verbal learning, normal and dual task gait speed (Primary outcomes).
- Gait speed during a dual task (which is an excellent predictor of overall health (26), daily living functionality, and depression as compared to the DM group receiving sterile saline and the non-DM groups.
- These measures will be collected at baseline, during 4 on-treatment (at V2-intervention week 1, V4 –week 8, V6 week 16, V8 week 24) and 4 post-treatment visits (V9 week 25, V10 week 32, V11 week 40 and V12 week 48). V9 will be a virtual visit conducted over the phone with an abbreviated assessment.

Aim 2: To identify a phenotype and long-term trajectory predicting clinically relevant response to INI therapy based on glycemic control, insulin resistance, endothelial and genetic markers.

- Clinical predictors will be based on associations between cognitive function and/or gait and demographic, glycemic control, insulin resistance, endothelial and genetic (ApoE*4*) measures.

The dose-escalating trajectory of cognition, gait speed, and functionality during the 24 weeks of therapy and 24 weeks post-treatment and their dependence on the above-mentioned factors, and determine the time point when maximum effect was reached. INI therapy response is defined as a clinically relevant improvement on cognitive tests or in gait speed (as a continuous variable) or as responders vs. non-responders as compared to placebo within DM and non-DM groups (as categorical variable).

Secondary End Points

Aim 3: To determine the long-term safety of intranasal insulin vs. placebo with regard to glycemic control (fasting glucose, Hb1Ac, hypoglycemic episodes), vital signs, body mass, appetite feelings and food intake.

We will obtain measurements of fasting glucose, insulin, vital signs, body mass measurements, waist and hip circumference, and appetite feelings using VAS at baseline, on-treatment and post-treatment and keep weekly logs monitoring glucose and AEs (V1-V8).

METHODS

DESIGN AND RATIONALE

Design: We propose a prospective double-blind, placebo-controlled study with 210 subjects with or without type 2 DM randomized into 4 groups (60 DM–INI, 60 DM–PLACEBO, 45 CONTROL–INI, and 45 CONTROL–PLACEBO) examining whether 40 IU INI (Novolin®R Novo Nordisk, Bagsvaerd, Denmark) once daily over 24 weeks improves cognition, daily functionality and walking speed, as compared to placebo (Aim 1). We aim to identify a phenotype and predictors of best response (Aim 2) and investigate long-term safety (Aim 3).

Rationale: Outcome measures were selected based on positive reports of INI effects on cognition.(16-24, 27) The INI dose of 40 IU has shown promise for long-term improvement of cognition in DM and non-DM people.
The 24-week INI treatment extends beyond previous studies (11, 16, 20, 22, 28) to maximize potential benefits, optimize duration and dose-escalating effect, and evaluate long-term safety.
Study Groups: Broader inclusion criteria permit to enroll a diverse DM and non-DM population to generalize the results. The matched design of DM vs. non-DM is essential to elucidate: 1) Long-term safety and INI effects; 2) INI impact on specific cognitive domains that may differ in DM vs. normal aging; 3) The effects of insulin resistance and glucose levels on brain aging and cognitive decline where longitudinal data are lacking; 4) The role of demographics (age, sex), cardiovascular risks (hypertension, obesity), and genetic markers (ApoE4) for prediction of an INI-responsive phenotype; 5) Sex-specific outcomes; 6) Furthermore, our Aim 3 will also explore the impact of INI on weight, waist and hip circumference, appetite feelings and food intake as another potentially beneficial mechanism of INI on food and brain reward system and potential benefits for weight management.

Rationale for Study Population
Diabetes mellitus affects >44 million people and 27% of older adults in the U.S. It accelerates brain atrophy and promotes earlier onset of cognitive impairment (5, 29, 30), vascular dementia (31) and AD (1, 32-35).
Currently, there is no cure for DM-related cognitive impairment. Even a tight glycemic control did not improve cognitive function in participants of the large clinical trials (36, 37). Insulin resistance, altered insulin transport and signaling in the brain may mediate DM-related cognitive decline. Older adults are the most rapidly growing segment of T2DM population, which is also at greater risk for cognitive decline. **Recently, intranasal insulin (INI) therapy has emerged as a potential solution to deliver insulin safely to the brain and treat DM-related cognitive impairment.**

DM management: Insulin/placebo will be added to participants' medication regimen for diabetes, and for any co-morbidities DM participants will receive the usual care from their providers. Primary care providers will receive the study description, and laboratory results upon enrollment and after each intervention visit during the treatment period (V2, V4, V6, V8), and if an adverse event occurs to adjust medication regimen. They will be required to inform the investigators about any changes in their regimen or health status during the follow-up visits. Glycemic control and other potential confounders will be monitored, and their effects will be tested and accounted for in the analyses.
Insulin/placebo will be administered once daily in the morning before breakfast. Participants will receive two or three 10-mL vials containing 100 units/mL of insulin (Novolin® R Novo Nordisk, Bagsvaerd, Denmark) or sterile saline that will be used over three to four weeks. Insulin/saline will be delivered over two minutes using a ViaNase electronic atomizer (Kurve Technology, Inc., Seattle, WA). The device is easy to operate, and it allows precise preprogrammed dosing and targeted delivery into the olfactory region with minimal peripheral deposition.

Compliance: Participants will be asked not only to bring their devices and vials to the follow-up visits every 3-4 weeks, but also to keep detailed diaries of drug usage and of any AEs daily and weekly measures of glucose before and after INI. The volume remaining in the vial will be measured at each visit to document medication usage.

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Compliance: Participants will be asked not only to bring their devices and vials to the follow-up visits every 3-4 weeks, but also to keep detailed diaries of drug usage and of any AEs daily and weekly measures of glucose before and after INI. The volume remaining in the vial will be measured at each visit to document medication usage.

Recruitment & retention & enrollment:
We plan to screen up to 800 subjects over the phone. We anticipate that we will need to recruit (sign ICF) in 360 subjects, to allow for attrition during the longitudinal study assuming 33% drop out in the DM and control groups. However, the insulin treated DM subjects have 66.7% drop-out rate which negatively affected the overall recruitment goals. Subjects will be recruited consecutively. We aim that 210 participants (90 controls and 120 DM) will complete the Visit 8 (end-of treatment) upon the study completion (May 31, 2020) (Aim 1). As of 8.1.2017, 55 participants completed V8 and 36 completed also the follow-ups (V12) at BIDMC. We plan to enroll 50 subjects per year in years 3 and 4 and 24 in year 5 across both sites (30 or more at BIDMC and up to 20 subjects at BWH).Aim 3b- safety substudy enrollment of IDDM participants was closed for futility in October 2017.

We will also attempt to complete the six-month follow-up (V12) in as many participants as possible (Aim 2), we estimate that 168 participants may complete the six month follow up. However, we may need to shorten the duration of the follow-up and further reduce the number of follow-up visits to meet our primary recruitment goal. We will leverage the existing resources at Harvard Catalyst and our experience with successful recruitment and retention in the greater Boston area for previous studies (PIs CREED, TRACT, Look Ahead). We will use community recruitment within a 3-mile radius of BIDMC greater Boston area and Joslin Diabetes Clinic. We will use BIDMC records, city censuses, senior centers and TrialX databases. Emphasis on recruitment of minorities:
Type 2 DM is common in African Americans and Hispanics, who are underrepresented in clinical trials. We will use community advertisement and activities, e.g., targeting barbershops and beauticians, churches and educational sessions. The PI has established a recruitment repository with >300 records of participants with T2DM older than 50 years and controls who are eligible and potentially interested in participating.

Retention strategies: 1) The PI or the study physicians are present during the consent process and are available to further answer any questions or concerns. 2) Participants who previously failed phone screening can be re-screened with the new inclusion/exclusion criteria. 3) Participants have 24/7 contact for investigators phone numbers and pager with contact for the PI and MDs; 4) Skipping visits: Participants will be allowed to miss two assessment visits during the treatment period and two during the long-term follow up for serious reasons. Minimum requirements: assessment visits V2, V8 or V10, V12 to complete the study due to serious reasons. Participants will be allowed to skip visits V4, V6, V9 or V10, V11. Post-treatment 4 (Visit 9) will be replaced by a phone interview. The follow-up visits 3,5,7 may be replaced by a phone interview of drug supply, device functionality, FS measures and calendar if necessary due to significant reasons. In special circumstances, functional assessments (WHODAS and GDS) can be done over the phone (V9 or skipped assessment or post-intervention visits). Interim visits can be scheduled if the procedures cannot be completed or re-training on the study procedures is needed. For patients running out of

medication that cannot come to the hospital for refill the BIDMC/BWH pharmacies will dispense the filled prescriptions to the study team to mail them out to subjects. 5) Appointment reminders: reminders are mailed a week before the visits; appointment reminder phone calls are made on the night before each visit); After V2- a phone call is made within one week to welcome participants to the study and meet our team cards. 6) Prior to V1 "Welcome on board" packages are mailed (study brochures and materials, overview, ICF, directions and instructions etc.). 7) New incentives: gift cards, key chains, mugs, totes, pens, etc. will be provided. 8) Meals are provided. 9) Transportation is reimbursed (parking and MBTA reimbursement, taxi in special circumstances, Uber/Lyft, pilot Lyft concierge service). 10) Visit 8 assessment will be performed even after treatment end date if patient cannot make it on time for that visit. Study team will ask the patient to stop study medication on the treatment end date and come for the assessment visit as soon as possible after that date.

FDA assurance: Current active FDA-IND 107690 has been amended to follow FDA regulations; ViaNase is a minimum risk device and therefore IDE is not required. We will obtain renewed authorization to cross-reference Novo Nordisk IND 29089 Novolin® R, Regular, Human Insulin Injection (rDNA origin).
Data and Safety Monitoring Board was established and the trial was registered at Clinical Trials.gov (NCT02415556).

STUDY PROTOCOL

Studies will be conducted at the SAFE Laboratory at the BIDMC and Brigham and Women Hospital (BWH) Harvard Catalyst Clinical Research Center (CRC). Joslin Diabetes Center will serve only for advertisement. Harvard Medical Center will serve for cognitive training.
The PI and co-investigators have extensive experience in clinical studies in older vulnerable adults and all procedures that we propose. The protocol involves 24 weeks of treatment and 24 weeks of long-term follow-up, and it includes 12 visits (**Table 1**). There are 9 assessment visits (baseline, 4 on-treatment assessments , and 4 post-treatment assessments) and 3 short follow-up visits during the on-treatment . An interim visits can be scheduled in case participants are not able to come fasting for visit, or physical exam cannot be scheduled, or all activities for a given visit cannot be completed for any reason or for participants in the MRI and safety substudies. Participants in MRI substudy may be required to complete MRI exam on a special visit. Potential participants will be screened by a phone interview.

Screening –V1 will include: informed consent, medical history, medications, vital signs, ECG, MMSE, metabolic panel (fasting glucose, HbA1c, lipids, CBC panel, C-peptide for insulin-treated diabetic subjects), height, weight, and waist and hip circumferences (HWC), demonstration of the ViaNase device. Participants of the MRI substudy will complete the MRI safety checklist and their head circumference and shoulder width will be measured.
Participants of the safety substudy will begin baseline glucose monitoring for one week.
The physical and neurological exam has to be completed on V1 or during in the interim visit or at the V2 baseline prior to beginning of intervention (V2 Intervention 1).
Enrollment, Randomization: The final eligibility and approval for participation is determined after completion of V1 procedures or during the V2 (baseline) prior to V2 (Intervention 1).
Baseline, Intervention 1-V2 will include: vital signs, physical and neurological exam, Toronto Neuropathy Scale (if not done before), medical history, fasting glucose, insulin and metabolic panels, endothelial and DNA markers, HWC, VAS for appetite feelings. Two blood draws will be obtained during this visit, one at baseline assessment and one during intervention 1.

Visit 2 should be completed within 3 months after screening visit 1. The assessment visits should be completed within 2 weeks of the scheduled visit, unless there are serious reasons to justify longer period. The end of treatment visit must be <168 days after the treatment started. The study completion visit must be on or before the study date.

BASELINE ASSESSMENT will include: a neuropsychological assessment, a short WTAR IQ test (http://www.pearsonclinical.com/psychology/products/100000256/wechsler-test-of-adult-reading-wtar.html), 6-min normal walk and performance of a dual task (a 6-min walk while counting backward), 30-second balance test with

eyes open, 30-second balance test with eyes closed, functional measures and depression scale. INTERVENTION 1 will be done during V2, and will begin after ~30-min rest. INI or placebo will be administered and followed by blood draws for glucose and insulin, neuropsychological, depression and functional assessments, normal walk and walk with a dual task.

ASSESSMENT VISITS – V2, V4, V6, V8, (V9*), V10, V11, V12 – will include the same laboratory panels, anthropometric measurements, VAS, cognitive, functional, and gait testing procedures as V2. One week after V2 the study team will call patients to check compliance and assess any problems with device usage and other study procedures. Visit 9 (V9*) will be a virtual visit that will be done over the phone and will include updates on medical history, medications, AEs and one fasting glucose finger stick measurement at one week post treatment and functional assessments (WHODAS and GDS), and complete Visual analog scale (VAS). Participants will receive the functional assessment forms, VAS at V8 and will be asked to mail the forms back.

There will be a baseline assessment and 4 assessments during on-treatment (ON-TREATMENT V2 week 1, V4 week 8, V6 week16, V8 week 24 and 4 assessments during post-treatment (POST-TREATMENT V9 week 25, V10 week 32, V11 week 40 and V12 week 48) On-treatment, there will be 3 medication refill follow-ups (V3 week 4, V5 week 12 and V7 week 20) which will include: vital signs, glucose measurements using a finger stick, drug inventory and refill, compliance checks, AEs monitoring. Subjects will continue their usual daily activities and treatment plans, will measure fasting glucose with a finger stick once a week and when symptomatic for hypoglycemia, and keep a detailed diary of physical activities (38). V3 will be 3 weeks after enrollment (V2) as a run-in period to ensure compliance and understanding of procedures and proper device use.
If patients are diabetic treated with medications or insulin and they take their medications in the morning, they will be asked to hold their morning dose of sugar control medications before the visits that require to come fasting (V2, V4, V6, V8,V10, V11, V12). They should bring their medications with them for the visit and they will take them after the fasting blood draw. If they cannot come fasting to these visits, they will be asked to come early morning next day for fasting blood draws only.

INTERIM VISIT- The subject may be asked to come on a separate visit if study procedures cannot be completed during any of the study visits for any reason. For example, if they are not able to come fasting for their first visit or other study visits, we will ask them to come back for an extra visit; or any scheduling issues for physical exam. Safety substudy, MRI study walk test, cognitive tests or other reasons.
Safety Substudy: A subset of 20 insulin-treated subjects will also complete FS glucose monitoring five times per day for 1 week before and 1 week after INTERVENTION 1 (V2) using our established protocol.(39). C-peptide levels will be measured in patients enrolled in this substudy in order to select type 2 DM subjects with greater beta cell reserve and thus lesspropensity to experience hypoglycemic events improving subject's retention. Patients with type 2 DM pts on insulin with a C-peptide of <0.8 nmol/L and a corresponding blood glucose > 150 mg/dl should be excluded from the substudy, even without history of hypoglycemia during FS measurements or otherwise. Insulin-treated type 2 diabetics with a C-peptide of <0.8 nmol/L and blood glucose >150 mg/dl will be excluded even without history of hypoglycemia during finger stick measurements. The study was terminated for futility in 2017).

MRI Substudy: 40 DM subjects will also complete an MRI scan at baseline (within ten days before V2) and at or within ten days before V8 INTERVENTION 4.

Table 1: Protocol and procedures

Table 2: Study Visits and Procedures

WEEK	DESCRIPTION	VISITS	Assessment	INI vs. PL	doses	Drug Refill	Drug Inventory	Labs	Med Hx	Phys Exam	Vital Signs	EKG	Height, Weight, Waist & Hip Circumference	Visual analogue Scales for Appetite Feelings	Body Composition-BIA	Glucose FS	Cogn test	Gait	Functional	3d Food logs	MRI*	AE,SE	Time (min)
n/a	Phone Screen																						15
0	SCREEN	V1	ICF					X	X		X	X				X	X*						90
1	ENROLL, RANDOM, INTERV1	V2	BASELINE	X	1	X	X	X	X	X	X		X	X	X	X	X	X	X	X	X	X	240
			INTERV 1	X	1	X	X	X	X		X		X	X		X	X	X	X				
2				X	7											X							
3				X	7											X						X	
4	FOLLOW-UP	V3	F1	X	7	X	X		X		X					X						X	45
5				X	7											X							
6				X	7											X						X	
7				X	7											X						X	
8	8W-ASS	V4	INTERV2	X	7	X	X	X	X		X		X	X	X	X	X	X	X	X		X	180
9				X	7											X						X	
10				X	7											X						X	45
11				X	7											X							
12	FOLLOW-UP	V5	F2	X	7	X	X		X		X					X						X	45
13				X	7											X						X	
14				X	7											X							
15				X	7											X						X	
16	16W-ASS	V6	INTERV3	X	7	X	X	X	X		X		X	X	X	X	X	X	X	X		X	180
17				X	7											X						X	
18				X	7											X						X	
19				X	7											X							
20	FOLLOW-UP	V7	F3	X	7	X	X		X		X					X						X	45
21				X	7											X						X	
22				X	7											X							
23				X	7											X						X	
24	24W-ASS	V8	INTERV4	X	1		X	X	X		X		X	X	X	X	X	X	X	X	X	X	180
25	25W-PTASS	V9	PT ASSES 5						X							X			X	X		X	phone interview
32	32W-PTASS	V10	PT ASSES 6					X	X		X		X	X	X	X	X	X	X	X		X	180
40	40W-PTASS	V11	PT ASSES 7					X	X		X		X	X	X	X	X	X	X	X		X	180
48	48W-PTASS	V12	PT ASSES 8					X	X		X		X	X	X	X	X	X	X	X		X	180

Table Abbreviations: ICF: informed consent; INI vs. PL: insulin vs. placebo; Med Hx: Medical. History; Phys: Physical; d: day Cogn test; Cognitive tests * MMSE only; **FS: Finger Sticks; AE, SE: Adverse and Serious Events. Ass: Assessment, PTASS: post-treatment assessment.

Insulin/placebo administration

Insulin/saline will be delivered using ViaNase electronic atomizers (Kurve Technology, Inc. Lynnwood, WA). The ViaNase electronic atomizers allow precise electronic dosing for each administration and will be pre-programmed by the company. They also allow for targeted delivery into the olfactory region with efficient and effective nasal cavity saturation; and with minimal peripheral deposition to the lungs and stomach. Clinical results showed that the average area of intranasal deposition is almost 300 percent greater than with spray pumps. Each administration device will contain a pre-programmed daily dose of 40 IU insulin (Novolin® R Novo Nordisk, Basvearg, Denmark) or sterile saline to be administered once daily in the morning before breakfast, a process that takes about 1 minute. The device dispenses 0.1ml per 20 seconds, so participants will have to press the devices twice for each nostril to receive 0.4ml dose. Each vial will contain 10 ml of insulin (100 IU/mL) or placebo and will be used over a 3-week period, to be refrigerated between daily treatments. Placebo, the sterile saline, will be stored in identical vials. Participants will be required to bring the device with the INI/placebo vial for each study visit. During the study visit the device usage will be reviewed and the vial will be replaced. The volume of remaining insulin or placebo will be measured to quantify usage and assess compliance with the study. Research participants will stay on intranasal insulin/placebo treatment for a maximum of 168 days. Participants that use the device 65% of the days will be considered compliant. If this compliance rule is not met, participants will we excluded from the study and analyses.

Packaging and Labelling of Study Medication(s)
BIDMC research pharmacy will handle insulin and placebo storage, labelling and dispensing, randomization documentation and tracking of medications shipment and packaging using the BIDMC online tracking system for the BIDMC site.

BWH hospital pharmacy will handle insulin and placebo storage, labelling and dispensing, randomization documentation and tracking of medications shipment and packaging using their online tracking system for the BWH site.

Storage and Drug Accountability of Study Medication(s)
Insulin/placebo will be stored at the BIDMC research pharmacy and refrigerated at 2-8^{O}C. To ensure blinding study participants, insulin/ placebo will be placed in the sterile vials extended stability dating to will be performed according to USP. Participants will be required to store the devices with the insulin/placebo vials in the refrigerator.
Depending on the site, BIDMC and BWH pharmacy online tracking systems will be used to track medications. Participants will be requested to bring their devices with used and un-used vials to the study visits. BIDMC/BWH pharmacy will measure the residual vial volumes as a part of the compliance procedures. Un-used insulin will be returned to Novo Nordisk. Unused placebo-sterile saline will be disposed using the disposal guidelines.

Auxiliary Supply
Insulin/placebo will be administered using the ViaNase device (Kurve technology, Inc Lynnwood WA).

Potential limitation
Meta-cresol which is used as a preservative in insulin has a distinct smell that may be recognizable by the subjects using insulin, and therefore diabetic subjects treated with SC insulin may be familiar with the smell. However, the ViaNase device itself has a distinct odor that is noticeable during device operation (air only) and even during the saline administration. Participants will be trained with a device that operates on air only, and there is noticeable distinct smell due to the device operation that appears to mask other odors. The investigators are present during the first INI/placebo administration during V2 intervention. Participants record any unusual events and observations in the home diaries, which are reviewed at each visit.

EXPERIMENTAL PROCEDURES AND MEASURES
A neurocognitive assessment battery will be administered using the Cambridge Cognition computerized system (CANTAB) http://www.cambridgecognition.com). CANTAB enables simplified data collection, standardized administration, and computerized grading that increases reliability of the data collected (see http://www.cantab.com/cantab-tests.asp for test details). CANTAB provides multiple validated parallel versions of these tests to minimize practice effects in a complex repeated measures design. The CANTAB brief neuropsychological battery correlates with traditional measures that showed on-insulin improvement (24) and has been extensively used in cognitively impaired(40),(41) and AD populations(42). The CANTAB will be administered at BASELINE, during ON-TREATMENT (V2, V4, V6, V8) and POST-TREATMENT VISITS (V9,V10,V11,V12) (**Table 2**).

Paired Associates Learning (PAL): PAL, a measure of visuospatial memory and learning, uses a pattern recognition task useful for assessing older adults with MCI or AD. *Outcome Measure:* PAL Total Errors (adjusted): is the total number of errors across all assessed problems and all stages.

Spatial Working Memory (SWM): SWM that tests the participant's ability to retain spatial information in the working memory by identifying the boxes with and without tokens, is a sensitive measure of executive dysfunction in DM.(43) The SWM strategy includes errors and latency measures (44). SWM showed that DM patients have slower processing speed and worse spatial planning than controls (45),(43).
Outcome Measures: Between Errors are number of times the subject revisits a box to find a token.

Rapid Visual Information Processing (RVP): RVP is a test of sustained attention and a sensitive measure of general cognitive performance. Slower RVP speed detected DM-related cognitive impairment in the frontal and parietal lobes.(29, 46) Task: Participants are asked to detect and register target sequences of digits. *Outcome Measures:* A′ (A prime) and Response Latency. A′ is the signal detection measure or sensitivity to the target, regardless of response tendency (range 0.00 to 1.00; worse to better).
Verbal Recognition Memory (VRM): The VRM assesses immediate and delayed verbal memory (a list of words) information under free recall and forced choice recognition conditions. *Outcome measures:* VRM Free Recall: total correct of a word list, which is a measure of immediate verbal learning. VRM Recognition: total correct is a measure of delayed verbal learning.

Executive function composite z score is a sum of individual z scores of PAL, SWM-total errors and strategy). Verbal memory composite z score is a sum of individual VRM z scores (immediate free recall and immediate and delayed recognition).

WTAR- premorbid verbal IQ test. http://www.pearsonclinical.com/psychology/products/100000256/wechsler-test-of-adult-reading-wtar.html. Brief (under 5 minutes) measure of premorbid verbal IQ, which provides a correlate of verbal intelligence and is well-normed. The manual provides conversions from verbal IQ (VIQ) to full scale IQ (FSIQ).

The Mini Mental State Examination (MMSE) will be administered at V1 to screen for clinically significant dementia,(47-51) using age- and education-adjusted norms. Therefore, we will adopt relatively liberal criteria for inclusion of individuals with MCIs, as these are likely to be prevalent and representative of the targeted cohort, particularly at the higher age range, and exclude only individuals with MMSE ≤20 (total score =30).

Functional measures: Geriatric Depression Scale (GDS) is a self-report measure of overall mood, activity, sadness, and worry. *Outcome measure*: a total score (up to 30 points) that reflects level of depression.

WHO Disability Assessment Schedule 2.0 (WHODAS 2.0) (http://www.who.int/classifications/icf/whodasii/en/) is a short, easy-to-administer and widely used self-report measure of function and disability that is valid across a broad age range and across all diseases. *Outcome measures*: Standardized disability levels and profiles for: Cognition, understanding and communicating; Mobility, moving and getting around; Self-care, hygiene, dressing, eating, and staying alone; Getting along, interacting with other people; Life activities, domestic responsibilities, leisure, work, and school; Participation, joining in community activities. The summary score will be converted into a percentage score – WHODAS Complex score.

Gait speed and step characteristics will be measured with a Mobility Lab System (APDM, Inc., Portland, OR) during 6-min walking at usual speed and 6-min walking with a dual task counting backwards subtracting 7.

Balance will be measured with a Mobility Lab System (APDM, Inc., Portland, OR) during a 30-second standing position with eyes open and 30 seconds with eyes closed.

Laboratory measures: routine blood and urine panels including fasting glucose, HbA1c, fructosamine, insulin, CBC, lipid panels and genetic markers (ApoE4), markers of endothelial function (soluble intracellular adhesion molecule, and vascular adhesion molecule, C-reactive protein, and matrix metallopeptidase 1-10) that have a demonstrated relationship to gray matter atrophy and cognition (5). Insulin resistance will be quantified using an updated HOMA-2 model (http://www.dtu.ox.ac.uk/homacalculator/)(52). C-peptide will be measured only in insulin-treated DM type 2 participants.

DNA samples: Serum samples will be obtained to measure genetic markers (ApoE4), that have been linked to greater risk for Alzheimer's disease. Genetic and DNA markers (ApoE4) collection was requested by NIH, and was

approved by BIDMC IRB in the protocol and ICF. Blood samples will be stored, and analyzed upon the study completion. The results will not be available until un-blinding of the study. Acutely, INI enhances verbal memory in memory-impaired ApoE4-adults (20, 27, 53). All of the above is contained in the ICF with an option to opt from providing a sample for DNA analyses.

Anthropometric measurements: Participants' body weight, height, waist and hip circumferences will be measured by the study team at V1 and assessment visit V2, 4, 6, 8,10, 11 and 12.

Visual Analogue Scales (VAS): Appetite feelings will be assessed with the use of VAS at V2, 4, 6, 8, 9, 10, 11 and 12. Participants will be asked to rate their appetite feeling on 10 cm visual analogue scales before and after meal and/or INI/placebo administration.

Magnetic resonance imaging (MRI) substudy: anatomical, perfusion, vasoreactivity, functional MRI.
We will conduct an exploratory substudy in 40 randomly selected DM subjects to determine the long-term INI effects on cerebral perfusion, vasodilatation, and functional connectivity. MRI will be done at baseline (before treatment initiation) and at end of treatment (during or within 10 days before V8. Brain scans will be acquired using a GE 3 Tesla MR750 scanner with 32-head coil in 3–D plane. Subjects for the MRI substudy should have a head circumference less than 65 cm and shoulder width less than 60 cm in order to fit to the machine. Pseudo-continuous arterial spin labeling (PCASL)
and 3D stack of spirals Rapid Acquisition with Relaxation Enhancement (RARE) sequences will be used to measure cerebral blood flow (CBF) and vasoreactivity (mL/100g/min) and (54-56) resting-state fMRI blood-oxygen-level dependent (BOLD) will be used to measure intrinsic brain activity.
Protocol: High-resolution anatomical images will include T1-weighted anatomical images were acquired with a **3D BRAVO** sequence and T2 and fluid attenuation inversion recovery (**FLAIR**) to improve image registration and to review images to detect brain lesions. Subjects will complete PCASL (6 min) and BOLD (8 min) during supine rest in normocapnia, and PCASL during hypercapnia (rebreathing of a mixture of 5% CO_2 and 95% air for about 2 min), hypocapnia (hyperventilation for 2 min), with vital signs and CO_2 monitoring. **3DCASL** is a noninvasive technique utilizing electromagnetic labeling of water in arterial blood. Reference images will be acquired with the same PCASL sequence for quantification of CBF brain map. CBF images will be automatically reconstructed and stored in the scanner database. **Quantification of CBF*:*** CBF will be quantified from PCASL during normocapnia, hypercapnia, and hypocapnia, as reported.[57-61] Regional CBF will be quantified via a voxel-based approach complemented with region-based analyses using a template of anatomical regions (e.g., insular cortex).The average difference between control and label images and the intensity in the reference image acquired with the same sequence will be used to calculate

CBF (Formula 1), $$CBF = \frac{\rho_b (S_c - S_l)}{2\alpha\rho_a T_{1a} C \exp\left(-\frac{w}{T_{1a}}\right)\left(1 - \exp\left(-\frac{\tau}{T_{1a}}\right)\right)} \quad [1]$$

where ρ_b is the density of brain tissue, 1.05 g/ml (62), α is the labeling efficiency, assumed to be 70% due to efficiency of background suppression and labeling, w is the post-labeling delay of 1.5 seconds (57), τ is the labeling duration of 1.5 seconds, T_{1a} is the T1 of arterial blood(63), ρ_a is the density of water in blood, 0.85 g/ml,(62) and S_l and S_c are the signal intensities in the labeled and control images, respectively, and C is the reference image. The equation assumes that the labeled blood remains in the arterioles and capillaries and does not reach the tissue.

Vasodilatation reactivity: will be calculated as a regional perfusion difference between hypercapnia and baseline divided by the difference in CO_2 between hypercapnia and baseline.

Vasoconstriction reactivity will be calculated as perfusion difference between hypocapnia and baseline divided by the difference in CO_2 between hypocapnia and baseline.
For vasoreactivity we will use only the first 2 min of PCASL data during baseline to match the signal-to-noise ratio at the other 2 conditions.

Functional connectivity: Resting state functional connectivity will be measured using fractional amplitude of low frequency fluctuation (fALFF) from BOLD fMRI data (see **Analyses Section**).

SAFETY:

DM management: Insulin/placebo will be added to participants' medication regimen for diabetes and any co-morbidities DM participants will receive a usual care from their providers. They will be required to inform the investigators about any changes in their regiment or health status during the follow-up visits. Glycemic control and other potential confounders will be monitored and their effects will be tested and accounted for in the analyses. Primary care providers will receive study description and laboratory results upon enrollment and after each intervention visit during the treatment period (V2, V4, V6, and V8) and if an AE occurs.

Medical Oversight: All patients are seen by a physician (study physician or CRC medical research officer (MRO)) during screening visits. The study physicians review all materials from the screening visit (including the physical exam by MRO) and provide approval for the study. CRC nurse will perform medication reconciliation at each visit. In accordance with the study procedures subject will be asked if they experience adverse events at all visits post baseline measurements. During the study visits the CRC nurse meeting the patient will identify adverse events and will discuss these with the study MD, while the patient is present.

Concomitant illnesses and medications:

INI/placebo will be used as add on medication to current medications for treatment of concomitant illnesses per definition below. Concomitant illness: any illness that is present at the start of the trial *(i.e. at the first visit).* Concomitant medication: any medication other than the trial product(s) that is taken during the trial, including the screening and run-in periods.

Safety substudy: DM participants treated with subcutaneous insulin will be required to complete one week of glucose finger stick monitoring five times per day prior to V2 baseline and one week after beginning of V2 intervention. Participants will be requested to document the time of INI/placebo administration the time and doses of medications administration, meals and activities in diaries (39). Finger stick glucose will be measured in the morning before breakfast after INI/placebo administration, 2 hours after breakfast, before lunch, dinner and at bedtime. The C-peptide blood levels will be measured as an additional measure to select type 2 DM subjects with greater beta cell reserve and thus less propensity to experience hypoglycemic events improving subject's retention.

Glucose measurements using a finger sticks: All study Participants will be required to measure fasting glucose once a week using a finger sticks including one week post-treatment (V9).

DATA MANAGEMENT AND STATISTICAL ANALYSES PLAN
Data Management: The electronic data capture system ScienceTRAX (FDA, HIPAA compliant) will be used for the questionnaires, assignment of variable names and coding and verification. Cognitive and gait data will be captured electronically (CANTAB, Mobility Lab), and laboratory data will be uploaded. The data will be converted to SAS datasets for analysis**.**

Table 3 lists **o**utcome variables and analytical methods**.**

Table 3: DEPENDENT AND INDEPENDENT OUTCOME VARIABLES, TIME POINTS, AND ANALYSES				
Aim	Dependent Variable	Independent Variables	Data Collection Times	Method of Analysis
Aim 1a	Visuospatial learning and memory; Learning and sustained attention (all continuous variables) PAL : Total errors - adjusted SWM: Between errors and Strategy RVP: A′ (A prime) responses & latency VRM: Recognition - total correct VRM: Free recall – total correct	TREATMENT_ARM, TIMEG, TREATMENT_ARM*TIME	Baseline, 4 On-treatment (V2,V4,V6,V8) Post-treatment 4 Post-treatment (V9,V10,V11,V12) *V9 no Cantab data	Linear Mixed Effects Models
Aim 1b	Gait speed, WHODAS, GDS (all continuous variables)	TREATMENT_ARM, TIMEG, TREATMENT_ARM*TIME	Baseline 4 On-treatment (V2,V4, V6, V8) 4 Post-treatment (V10, V11,V12) • V9 has only WHODAS and GDS	Linear Mixed Effects Models
Aim 2a	Responses to INI therapy (binary) INI_RESPONSE	Age, sex, adiposity Fasting glucose, insulin, HbA1c, Insulin resistance (HOMA-2), DM_duration Endothelial function Genetic markers (ApoE4)	VBaseline 4 On-treatment (V2,V4, V6, V8) 4 Post-treatment (V10,V11,V12) V9* only glucose FS	Generalized Linear Model with Log Link and Binary Error term
Aim 2b	Trajectory of INI therapy response (all continuous variables from Aim 1a)	TREATMENT_ARM, TIMEG	V2,V4, V6, V8, V10,V11,V12 V9* limited dataset	Mixture Modeling Generalized Logit Model & General Linear Models
Aim 2c	MRI –Perfusion, vasodilation reactivity Functional connectivity	TREATMENT_ARM* TIME ,	V2 baseline, V8 end of treatment	Mixture Modeling Generalized Logit Model & General Linear Models
Aim 3a, 3b	Safety of INI therapy (all continuous variables) fasting glucose*; HbA1c, insulin resistance, blood pressure, number of hypoglycemic episodes	TREATMENT_ARM, TIMEG, TREATMENT_ARM*TIMEG	V2,V4, V6, V8,V10,V11,V12 weekly glucose finger stick V2-V9 V9*limited dataset IDDM baseline and post V2 one week FS 5xdaily	Linear Mixed Effects Models

Aim 1a and b: To determine whether INI improves long-term cognition and memory in specific domains and normal and dual task gait speed in type 2 DM subjects as compared with the placebo and non-DM groups.
Primary efficacy variables are cognitive outcomes and gait (executive function and verbal learning composite scores, NW and DTW). We will use 5 summary measures of the 4 computerized CANTAB tests. PAL Total errors (adjusted) and SWM -Between errors and Strategy are 2 assessments of visuospatial learning and memory, previously tested using a Brief Visuospatial Memory test (BVMT_Trial 2 and BVMT_Total recall). We will also use 3 measures of learning and sustained attention: RVP: A′ (A prime) and responses latency, VRM Recognition - total correct, and VRM Free recall – total correct, for which we previously used Verbal Fluency Category and FAS. These new variables are highly correlated with standard measures, but computerized systems allow for more accurate and higher-quality data collection. The Executive function composite score will be a sum of z scores from Paired Associates Learning (PAL, total errors) and Spatial Working Memory (SWM, total errors and strategy to complete tasks; lower score better). Verbal memory composite score will be calculated from z scores of verbal immediate free recall, immediate and delayed Verbal Recognition Memory (VRM; higher score better). The primary variables will be collected at baseline, during 4 intervention and 4 post-treatment visits (V2-baseline, V2 intervention 1, V4, V6, V8, V9-replaced by phone interview, V10, V11 and V12) (Table 3). Primary cognitive outcomes will be converted to scaled z-scores and summed to create composite cognitive measures. Primary efficacy measures will be compared between DM-INI and DM-Placebo and between Control-INI and Control placebo groups at baseline, on-treatment and post-treatment. The end-of-treatment 4 (V8) is the primary time point of interest in the assessment of the efficacy of the INI treatment at the end of intervention (Tables 2 and 3). Functional measures (GDS and WHODAS) will be also collected at these time points.

Linear mixed-effects models will be to estimate the effects of INI in the DM and Control groups.
The INI efficacy for each of the variables listed above are the differences D1, D2, D1-D2 where D1=DM_INI-DM_PLACEBO, and D2=CONTROL_INI-CONTROL_PLACEBO. We will estimate D1 and D2 two different ways: first, at each of the 8 time points; second, the D1 or D2 differences at baseline, on-treatment and post-treatment. Statistically, we will use a 2-tailed test and will test for the hypotheses that $D1 \neq 0$, $D2 \neq 0$, $D1\text{-}D2 \neq 0$. To estimate D1, D2, and D1-D2, we will use linear mixed-effects models (LMM),(64) 1 for each of the outcome variable. The dependent variable will be the outcome variable, the independent variable will be 4-level indicator variable (TREATMENT_ARM), 3-level indicator variable TIMEG, and the interaction TREATMENT_ARM*TIMEG. The randomization scheme would balance the background variables or baseline data, so that adjusting for these potential measured confounders may not be necessary; however, we will make sure that their distributions among the treatment arms are indeed similar. We will review the potential confounders such as baseline differences in cognition, years of education, and potential effects of age, sex, and race and use normative values for cognitive tests that account for these variables. Because the measurements for each subject are longitudinal and repeated, the within-subject data are correlated, we will use either an autoregressive model with lag such as AR(1) or compound symmetry to model the within-subject correlation. We will use the Akaike information criterion (AIC) computed from the maximized restricted log likelihood value to select the appropriate variance-covariance structure for the LMMs. We will use linear contrasts formed from the estimates of the main effects TREATMENT_ARM and TREATMENT_ARM*TIMEG to estimate quantities D1, D2, D1-D2, and 95% confidence intervals at each of the 8 time points.

Power analyses
We computed the required sample size based on the expected estimated mean difference at the end of treatment between the DM-Placebo and DM-INI group for CANTAB cognitive variables SWM errors, SWM strategy, VRM free recall. We set type-I error rate at 0.05, power of 0.80 or above, effect size of 15% improvement due to INI, and obtained n=120 for the DM group (60 DM-INI; 60 DM-Placebo) and n=90 for the Control group (45 Control-INI; 45 Control-Placebo) yielding 210 patients with data at the end of the treatment period.

Analysis for Aim 2a: Identify a clinical phenotype predictive of a positive response to INI therapy based on demographics, glycemic control measures, endothelial function, and genetic markers (ApoE4). For Aim 2a, we define a positive response to INI therapy as D1=DM_INI-DM_PLACEBO for PAL (Total errors (adjusted) and SWM -Between errors and Strategy ≥1.5 at end-of-treatment 4 (V8) or at any time from week 24 to week 48 (V9*-V12). For V9 only GDS and WHODAS, glucose FS will be available The outcome is a binary variable (INI_RESPONSE) with a value of 1 for positive response or 0 otherwise. We have potential phenotype variables predictive of INI_RESPONSE: 3 demographic characteristics (AGE, SEX, ADIPOSITY), 5 glycemic control measures (FASTING_GLUCOSE, INSULIN, HbA1c, HOMA2_INSULIN_ RESISTANCE, DM_DURATION), endothelial function (ENDOTHELIAL), genetic markers (APOE4). These potential predictive variables will be used in a generalized linear model (GLM) with log link and binary error. The Akaike information criterion (AIC) will be used to select the optimum model predictive of INI_RESPONSE. We will first carry out a univariable GLM model and will retain those predictors that have a p-value of ≤0.20. We will then perform an all-subset model selection procedure by generating all possible main-effects models. For example, if 5 variables meet the univariable model criterion of having p-value ≤0.20, then there will be 2^5-1 = 31 possible main-effects models. Each of these will yield an estimate of AIC value. We will rank these models by ascending order of AIC estimates and will examine the first 5 models with the smallest AIC estimates. We will consider models whose AIC estimates are within 1% of each other to be equivalent statistically, and will also take into account the clinical significance and relevance of the chosen model. We will compute and report the estimated area under the receiver-operating-characteristic curve (aROC) by obtaining the model's c-statistic. This is a check of the discriminatory value of the predictors. Models will be calibrated using the Hosmer-Lemeshow statistic that will allow us to estimate the predicted probability of positive response to INI for any patient with a given set of predictors' values, and it will indicate the magnitude of association of the predictors in relation to the likelihood of a positive response to INI.

Power analyses revised (9.11.2017) for reduced sample size of 120 DM and 90 Controls
As determined in Aim 1, we will have a sample size of 210 subjects. With an estimated attrition rate of 20%, we will have 168 subjects by the end of the study. We estimate that 30% will have a positive response (INI_RESPONSE=1). We plan to split the sample of 168 into a validation set and a testing set. With N = 84 for testing, and an expected response rate of up to 30%, we will have 25 positive outcomes (INI_RESPONSE=1). We need about 5 outcomes per predictor in the selected multivariable GLM model. Thus, a sample size of 84 with 25 expected positive outcomes would be sufficient for the estimation. We will validate the model using the validation set of 84 subjects. In both the test and validation model, we expect to have a c-statistic of at least 0.70.

Aim 2b: We will determine different types of longitudinal trajectories that allow prediction of escalating dose-effects and maximum response to INI therapy. It is likely that trajectories based on the profile of baseline and 7 assessments for each of the 5 cognition outcomes, functional outcomes, and gait speed will form a finite number of distinct groups. For WHODAS and GDS we will have 8 assessments. Some subjects may experience the INI benefit earlier (e.g., at week 8 or earlier), while others may experience it later (up to week 24), and others may not respond to the INI (thus, a flat or declining line through follow-up time). Therefore, we will estimate the number and shape of the trajectories, using a mixture modeling technique of the longitudinal data for each subject. We will plot the data for each subject over time to understand the shape of the observed trajectories and how the individual trajectories cluster. We will assume that the number of clusters ranges from 1 to 6, but we expect the optimal estimate of the number of clusters is 3 or 4. The marginal density of a subject having the outcome variable cognition/memory/gait speed measurement at a particular time point is modeled as a function of the product of 2 densities: the probability of the outcome belonging to a particular trajectory, and the probability of observing such a measurement belonging to a particular trajectory. The first probability is modeled using a generalized logistic model since the outcome is categorical (from 1 to 6 categories/types of trajectories), and the second probability is a polynomial linear regression of the outcome variable as a function of time (linear, quadratic, cubic). To select the optimal number of trajectories, we will compare the Bayesian information criterion value from the 6 models (corresponding to 1 to 6 types of trajectories). The algorithm and the SAS macro for this type of modeling has been published (65).

Power analyses includes reduced sample size of 120 DM and 90 Controls. We will use data from all subjects for this analysis. Given that we will have up to 6 data points per subject, estimating up to the cubic function for the trajectories would be acceptable with our sample size of 210 subjects.

Aim 2c: To determine the INI effects on regional vasodilation and functional connectivity.
Regional perfusion and vasoreactivity (vasodilation, vasoconstriction responses) will be quantified using Formula [1] in anterior circulation (e.g., the MCA territory and insular cortex) by 3D-PCASL MRI. Resting state brain activity and functional connectivity will be measured using fMRI fractional amplitude of low frequency fluctuations (fALFF) measured by BOLD MRI. MRI outcome variables (MCA_PERFUSION, VASODILATATION_RESPONSE, fALFF) will be obtained at baseline (before treatment initiation) and at the end of intervention (during or within 10 days before V8). For each subject, perfusion images will be registered to the corresponding high-resolution anatomical images and aligned to baseline (V2 pre-INI). The anatomical images in each subject will be normalized to the standard brain T1 template using statistical parametric mapping (SPM version 8, http://www.fil.ion.ucl.ac.uk/spm/, Wellcome Department of Imaging Neuroscience, London, England) toolbox. The alignment parameters will transform the perfusion maps to the space of the baseline perfusion image, the registration parameters will transform those images to the space of high-resolution anatomical images, and the normalization parameters will further transfer those images to the space of standard brain template. In this way, the perfusion maps from all subjects will be co-localized with the standard brain template. These maps will be smoothed by using an isotropic Gaussian kernel filter of 8-mm full width at half maximum to meet the assumption of the Gaussian field theory for SPM analysis.

Low-frequency (0.01–0.08 Hz) fluctuations in resting-state BOLD signals are considered to reflect intrinsic neural activity in humans (66),(67). Recent studies proposed a reliable measure of intrinsic neural activity using Falff (68)(69). fALFF has proven to be sensitive to detect change in physiological and pathophysiological states of brain(68, 70). An fALFF map will be calculated based on the power spectrum of each voxel's time series, i.e., from the power in the low frequency range (0.01-0.08 Hz) and the power of the whole frequency range (0-0.25 Hz). BOLD image processing and statistical analysis will be carried out using SPM8 toolbox. BOLD image volumes will be aligned to the first BOLD image volume for the baseline and 4 week intervention of each subject. A created mean BOLD image from the alignment will be co-registered to the T1 structural image in the same session, and the co-registration parameters will be used to transform the fALFF map from the original BOLD space to T1 space. The T1 structural image will be normalized to the standard brain Montreal Neurological Institute T1 template, and the resulting normalization parameters will be used to further transform the fALFF map to the standard space. The normalized fALFF maps will be smoothed with isotropic Gaussian kernel filter of 6-mm full width at half maximum. The BOLD data will be high-pass filtered with a cut-off of 128 s (0.008 Hz) to remove low frequency noise. We will use same LLM approach as in Aim 1 to calculate differences between TREATMENT_ARM*TIME to compare INI treatment to placebo using SPM8. Only the clusters exceeding the whole-brain false discovery rate (FDR) of $p < 0.05$ will be reported as significant clusters. Regional analysis will be performed on the clusters to rule out the effects from potential confounders, such as age, sex, and race. Region-based analysis will be also performed in anatomical regions of specific interest, e.g., insular cortex and MCA territory, regressing out the above potential confounders. We have reported (24, 25) that acute positive cognitive response to INI therapy depends on vasodilation in the MCA territory (R^2 =0.58, p=0.0098). We will also use our LMM approach described in Aim 2a.

Power analyses revised use sample size of 120 DM and 90 Controls. We have based our power estimate on our data (24, 25) that showed an increase in perfusion on INI in right insular cortex in the DM group as compared to placebo (44.5±12.28 vs.37.7±11.8, p=0.028; paired t -test). With 60 subjects we will have a power 0.81 to detect a difference between insulin and placebo at this observed level of mean difference of 6.8, and SD of 13.

Aim 3a: To determine the long-term safety of intranasal insulin vs. placebo.
For this Aim we have 5 continuous safety outcomes: FASTING_GLUCOSE, Hb1AC, HOMA-2 INSULIN RESISTANCE, NUMBER_HYPO_EPISODES, BLOOD_PRESSURE. We will evaluate the D1 and D2 for these outcomes at each of the on-treatment and post-treatment 8 visits (V2, V4, V6, V8,V10, V11 and V12; V9 will have

only AEs, and glucose using FS) using the LMM and interaction term (as described in Aim 1). We will track the weekly glucose and AE monitoring logs. Based on our data with no AEs and studies with infrequent mild AEs, we do not expect significant differences from placebo.

Aim 3b: Safety substudy. In the first 20 DM patients treated with subcutaneous insulin, we will conduct measure FS glucose five times per day for one week during pre-treatment and one week after V2 on INI or placebo treatment. We will calculate the number of symptomatic and asymptomatic hypoglycemic episodes (defined as glucose ≤70mg/dL on plasma glucose or finger stick measurement) and compare them using LMM before and after the first week of treatment to assess the overall glycemic trends and variability, and to assess the time-dependencies between INI administration, glucose control medications administration, meals and activities.

Power analysis 3 a, b: For 5 safety outcomes, we expect that the 95% confidence interval of D1 and D2 will include zero, indicating no differences between INI and PLACEBO for both the DM and control groups.

MISSING DATA

Subjects and data will be included as described above in the Statistical section. Since this is a longitudinal study of 48 weeks with data collected as frequently as weekly, and every 3 weeks (safety), and at 8 different time points for Labs, Cognitive tests, Functional tests, and Gait, we expect to have missing data that could occur for both the outcome (dependent) and exposure (independent) variables resulting from patients dropping out due to AEs or other reasons. We expect the key independent variable of TREATMENT and TIME used in Aims 1a, 1b, 2b, 2c, and 3a, 3b to have no missing data. Potentially we may have minimal missing data in model covariates in Aim 2a (< 5%).
Any missing data of these covariates for Aim 2a would cause the entire observation for that patient to be dropped from the analysis; therefore, we will make sure that these variable distributions are monitored carefully during collection. Other aims have continuous outcomes and their distributions are assumed to be random. The documentation of any exclusions of data will be stored with the remaining trial documentation. LMMs that will be used for the analysis are designed to handle missing data in dependent variables. Therefore, a missing data point would not cause estimation issues since the distribution of the data (or its transformation using, for example, log function) is assumed to be normally distributed, and the missing data point for the dependent variable is implicitly imputed by the LMM model. However, even if LMM could impute the missing data points, when more than 70% of a participant's data points are missing, that subject's data may not be used in the LMM. We will attempt to use the multiple imputation technique via the Markov-Chain-Monte-Carlo algorithm in sensitivity analyses only to check for the effect of missing data on model inference.
Interim Analysis Not planned.

SUBJECT SELECTION

We anticipate that we will screen 800 subjects by phone and enroll 360 subjects (ICF signed) in order to meet our enrollment goals of 210 subjects complete V8 treatment upon the trial's completion; with 60 subjects in each of the four study groups (DM-INI, DM-placebo, control-INI, control-placebo).

Randomization and matching

A computer-generated random allocation sequence (blocks of 2,4,8 and 12) will be used for randomization into 4 groups. Our RCT design includes a non-DM group with cardiovascular risk factors, e.g., age, hypertension, obesity, and insulin resistance. In this RCT we expect the distribution of patient-specific confounding variables to be similar across groups, and we will adjust for differences in the analyses. We expect that we will have to randomize 360 subjects (signed ICF) subjects (200 DM and 160 controls) in order to meet our goal to complete treatment (V8) with 210 individuals (60 DM-INI, 60 DM placebo and 45 controls INI and 45 controls placebo).We expect that similar distribution of age, sex, education, and co-morbidities between the groups will be achieved through randomization. We will use frequency quota sampling at 25% increments to ensure that distribution of these variables is indeed similar.

Inclusion criteria: 1) Men and women aged 50-85 yr old; 2) able to walk for 6 min 3) DM group: diagnosis and treatment for type 2 DM with non-insulin oral or injectable agents or insulin; 4) non-DM group with similar age range as the DM group, non-diabetic fasting plasma glucose (<126 mg/dL) and HbA1c (<6.5%)(71). We'll aim for race and gender. Subjects capable of providing informed consent

Exclusion criteria: 1) Insulin-treated type 2 DM; 2) Type 1 DM; 3) intolerance to insulin; 4) history of severe hypoglycemia (Severe hypoglycemia is an event requiring assistance of another person to actively administer carbohydrates, glucagon, or take other corrective actions. Plasma glucose concentrations may not be available during an event, but neurological recovery following the return of plasma glucose to normal is considered sufficient evidence that the event was induced by a low plasma glucose concentration (72); 5) participants will be asked to keep a detailed diary about hypoglycemic episodes or other AEs that will be reviewed every 3-4 weeks. ADA clinical practice guidelines will be used for identification and documentation of hypoglycemic episodes as follows (symptomatic or asymptomatic hypoglycemia documented on plasma or finger stick measurements (glucose $\leq$ 70 mg/dL) (72). After enrollment, participants who have more than one asymptomatic and/or symptomatic episode of hypoglycemia (glucose $\leq$ 70 mg/dL) through the entire duration of the study documented on plasma glucose or finger stick will be excluded. 6) Acute medical condition that required either hospitalization or surgery within the past 6 months (e.g., severe hypoglycemia, malignancies, myocardial infarction, stroke); 7) liver or renal failure or transplant; 8) dementia. We will use age- and education-adjusted norms to screen for the presence of clinically significant dementia, but will include individuals who have MCI. Therefore we will adopt relatively liberal criteria by excluding only individuals with MMSE scores ≤20; 9) current recreational drug or alcohol abuse;) 10) serious systemic disease that would interfere with conduction of clinical trial (mild forms of neurological conditions e.g. PD, autonomic neuropathy etc. would be allowed); 11) MRI substudy: claustrophobia and implants incompatible with 3 Tesla MRI. 12) Safety substudy: Insulin-treated type 2 diabetics with a C-peptide of <0.8 ng/mL and fasting blood glucose >150 mg/dL will be excluded even without history of hypoglycemia during finger stick measurements.

Withdrawal Criteria: 1) The subject may withdraw at will at any time; 2) pregnancy or intention of becoming pregnant; 3) non-compliance or inability to follow the details of the protocol; 4) hypoglycemic episodes; 5) serious adverse events; 6) medication(s)/treatment(s) not permitted during the trial.

We anticipate that we will screen 800 subjects by phone and enroll 360 subjects (ICF signed) in order to meet our enrollment goals of 210 subjects complete V8 treatment upon the trial's completion; with 60 subjects in each of the four study groups (DM-INI, DM-placebo, control-INI, control-placebo).

Randomization and matching

A computer-generated random allocation sequence (blocks of 2,4,8 and 12) will be used for randomization into 4 groups. Our RCT design includes a non-DM group with cardiovascular risk factors, e.g., age, hypertension, obesity, and insulin resistance. In this RCT we expect the distribution of patient-specific confounding variables to be similar across groups, and we will adjust for differences in the analyses. We expect that we will have to randomize 360 subjects (signed ICF) subjects (200 DM and 160 controls) in order to meet our goal to complete treatment (V8) with 210 individuals (60 DM-INI, 60 DM placebo and 45 controls INI and 45 controls placebo).We expect that similar distribution of age, sex, education, and co-morbidities between the groups will be achieved through randomization. We will use frequency quota sampling at 25% increments to ensure that distribution of these variables is indeed similar.

Subject Replacement: We will also implement frequency quota sampling to ensure similar distribution of demographic and other potentially confounding variables during recruitment at 25% sample increments. Therefore, the subject's replacement will follow distribution of these variables within each group. We plan for a similar distribution of men and women in the study population. All women in the study will be required to be post-menopausal or practice adequate birth control. All efforts will be made to assure that the study population includes a sample of minorities that is representative of the racial distribution of the population in Massachusetts. A special

effort to recruit Latinos and African Americans will be made so as to reflect the high nationwide distribution of stroke in these populations. Cost and sample size will prohibit us from studying each racial group separately.

POSSIBLE BENEFITS

Our previous experience, the noninvasive nature of the proposed studies, the general acceptance by practicing physicians of the procedures to be used, and the close supervision and monitoring of subjects, all minimize the potential risks. Based on previous research with this and similar protocols, we judge the risks associated with this study to be minimal. Individual subjects may benefit from the proposed study by learning more about their cardiovascular health and diabetes control that would not have been routinely indicated.

Importance of the knowledge to be gained

This translational study will address an important area—the long-term effects of intranasal insulin on cognition in older diabetic people—that has not been studied, and may provide a novel therapeutic target for prevention and treatment of cognitive decline and dementia. This prospective study will provide data about long-term effects of insulin on cognition in a larger population of older diabetic and control subjects. Identification of a clinical phenotype of people who would be more likely to respond positively to INI therapy would allow introduce new benefits to a larger population at an earlier disease stage, before cognitive decline manifests clinically. Therefore, INI could be a promising method for the treatment of disorders that involve abnormal insulin signaling within cognitive and reward networks, such as obesity, Alzheimer's disease, and type 2 DM. If successful, knowledge gained from this study may be used for a design of large-scale trials.

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