**Application no.: \_\_\_\_\_\_\_\_;**

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**Early predictors of age-related brain atrophy, cerebrovascular pathologies, and cognitive decline: Five to 16-year follow-up of post-intervention clinical trials**

**מנבאים מוקדמים לאטרופיה מוחית מקושרת גיל, פתולוגיות כלי דם במוח והידרדרות קוגניטיבית; 5-16 שנות מעקב לאחר ניסויים קליניים התערבותיים**

**Abstract and Program**

**Keywords:**

Aging; Age-related brain atrophy; Clinical trials, Follow-up; MRI measurements; Neurodegeneration.

מילות מפתח:

הזדקנות, אטרופיה מוחית מקושרת גיל, מחקרים קליניים, מעקב, מדידות MRI, נוירודגנרציה.

**A. Scientific Abstract**

**Background:** With increasing life expectancy and aging, cognitive performance or biological brain age may vary with chronological age. Deviation of magnetic resonance imaging (MRI)-assessed biological brain age from chronological brain age may be an early biomarker for cognitive decline. Without effective treatment, it is estimated by 2050 that dementia and Alzheimer’s disease (AD) prevalence will be tripled and quadrupled, respectively. Recently, we found in the DIRECT PLUS trial (n=294 participants) that whereas brain atrophy is age-related, there is great variation within individuals. During 18 months of intervention, the decline in MRI-assessed hippocampal occupancy score (HOC) accelerated ominously above 50 years of age, and the lifestyle intervention-induced attenuation associated with the HOC was mediated mainly by glycemic control.

**Gap of knowledge:** Predictors of age-related brain atrophy and vascular-related brain pathologies indicating cognitive decline are mostly unidentified, requiring further investigation.

**Overarching goal**: Our goal is to identify individual retrospective predictors of age-related brain atrophy based on MRI-assessed deviations from chronological age, cerebrovascular pathology, and a cognitive test. We will integrate our results into long-term lifestyle interventions and follow-ups.

**Specific aims**:

*1.* ***Identify specific******predictors of age-related brain atrophy and cerebrovascular pathology.*** We will explore multi-omics (epigenetics, metabolomics), brain-related biomarkers such as brain-derived neurotrophic factor (BDNF), phosphorylated at threonine (p-tau) 217 and 181, apolipoprotein E4 (APOE4), the presenilin (PSEN) 1/2 genes, other blood biomarkers, and clinical, lifestyle, demographic, and anthropometric parameters.

2***. Understand the potential predictive roles of individual responses.*** We will study individual responses induced by randomized lifestyle intervention trials, such as weight loss, visceral and hepatic fat dynamics, specific biomarkers, and microbiome, with brain status at follow-up.

3. ***Gain insight into the dynamic pattern and trajectory of anatomical brain atrophy.*** We will specifically explore the evolution of atrophy five years after completing the DIRECT PLUS trial.

**Hypothesis**: We hypothesize that specific early parameters will predict brain age deviation. Furthermore, individual responses induced by lifestyle intervention will predict brain health status further in life.

**Methods**: We propose brain MRI follow-up measurements for 1000 participants five to 16 years after completion of their intensive lifestyle randomized control trial (RCT). We will perform MRI-based brain anatomy via quantitative volumetric imaging and assess brain age deviation from chronological age, calculated by Neuroquant software. We will use MRI to determine cerebrovascular morbidities, such as cerebral microinfarcts (CMIs) and cerebral microbleeds (CMBs), and assess cognitive function by Montreal Cognitive Assessment (MoCA). Follow-up measurements will include blood, urine, feces, and clinical tests. We propose integrating the measurements with past data from four dietary RCTs (DIRECT, CASCADE, CENTRAL, DIRECT PLUS) (18-24 months, 85-89% adherence rates). The dataset will include 1,000 participants with obesity or type-2 diabetes mellitus (T2DM) (2000+ person-years of intervention), including a vast dataset of potential dynamic predictors, such as biomarkers, metabolomics, genetics, microbiomes, MRI-derived fat deposits, and brain measurements for some trials. We will then test past interventions with current individual responses. We will use machine learning to examine past and follow-up data for predictors of age-related brain atrophy.

**Feasibility:** In the last four months, we started approaching the participants, and meanwhile, 42% agreed to register for the follow-up measurements. **Significance:** The detailed vast retrospective, dynamic, and MRI-based follow-up measures of our 1000 participants should shed light on potential drivers of brain age deterioration and variation, which advance our understanding of potential strategies toward healthy brain aging.

**B. Research Program**

**Scientific Background**

**Age-related cognitive decline**

In recent decades, Western life expectancy has increased constantly, resulting in an older population with a wide prevalence of aging-related concerns [2, 3]. Cognitive decline is a common complication associated with aging [2] and may progress to dementia, specifically Alzheimer’s disease (AD). Dementia and AD increase exponentially with age [1], and it is estimated that by 2050, the prevalence of dementia and AD will triple and quadruple, respectively [4, 5]. Deterioration starts in the third or fourth decade of life [6, 7], and there is no effective treatment [8]. Metabolic, lifestyle, heritable, and related risk factors are linked to cognitive decline. For example, cardiovascular risk factors (CvRF) such as type 2 diabetes, hypertension, cholesterol, and inflammation markers increase the risk of dementia, AD, mild cognitive impairment (MCI), and cognitive decline. Diabetes is the most consistent factor associated with decreased cognition. Additionally, genetic factors such as specific variants of apolipoprotein E (*ApoE*), brain-derived neurotrophic factor (*BDNF*), presenilin1 (*PSEN1*), and presenilin2 (*PSEN2*) are linked to young-onset dementia within a familial setting of the disease [9, 10]. Even without disease-linked variants, some biomarkers, like BDNF levels in plasma, decrease significantly with age [11, 12]. Furthermore, plasma-phosphorylated at threonine (p-tau) 217 and 181 proteins are excellent diagnostic performance for differentiating AD patients from patients with other neurodegenerative diseases [13, 14]. Plasma p-tau181 is a noninvasive diagnostic and prognostic biomarker for AD. Elevated plasma P-tau181 levels are associated with AD development in cognitively unimpaired and MCI subjects [15], and changes in the mentioned factors alone may lower age-related cognitive deterioration by about 40% [16, 17]. Reducing specific risk factors (diabetes mellitus, midlife hypertension, midlife obesity, physical inactivity, depression, smoking, low educational attainment) by 10% or 20% per decade may also reduce AADworldwide by 8-15% (8.8-16.2 million cases) by 2050 [7].

***Increased life expectancy highlights the critical need for predictors of health and quality decline in later decades of life. Discovering new risk-associated biomarkers to prevent or attenuate age-related cognitive decrease is essential to respond to this need.***

**Age-related gray matter (GM) volume**

Brain structure deteriorates with age [18], with an approximate volume decrease of 3-7 cm3 per year after age 65 [19]. GM volume decreases in early adulthood and continues linearly throughout life [20, 21]. Intervention trials suggest that age-related brain atrophy is attenuated by polyphenol consumption [22, 23]. Specifically, the Mediterranean diet (MED) is associated with reduced atrophy, similar to the subtraction of several years [24].

Furthermore, risk factors such as hypertension [25], diabetes mellitus [26-28], alcohol [29], hyperlipidemia [30], cigarette smoking [31], gender and menopause [32], salivary cortisol levels, and elevated plasma homocysteine (Hcy) [33] may accelerate brain atrophy. Also, obese individuals have lower GM volume than control lean individuals [34]. Many studies associate obesity with lower global and regional GM volume, particularly within the hippocampal cortex, prefrontal, and other regions in the frontal and temporal lobes [ ]. Obese individuals present a lower GM volume in the cerebellum, parietal and occipital cortex, basal ganglia, insula, thalamus, amygdala, and limbic lobes, among other regions [35-43]. In support, individuals with two anthropometric markers of abdominal or central obesity, higher waist circumference (WC) or waist-to-hip ratio (WHR), have lower brain volume relative to individuals with only a higher body mass index (BMI), a marker of global obesity [44, 45]. Insufficient sleep length and quality are additional risk factors associated with accelerated brain atrophy [ ]. Moreover, self-reported sleep of fewer than 7 hours per night results in a significant longitudinal decrease in cortical thickness in frontotemporal regions [46, 47]. Participants with type 2 diabetes mellitus (T2DM) had significantly reduced total brain volume, with a difference of 20.50 cm3 (1.81% of normal) attributable to T2DM and a significantly smaller GM volume (−17.43 cm3 2.88%) [48]. High Blood Pressure (BP) is also associated with, and predictive of, global and regional volume reduction with preferential localization in the hippocampus and prefrontal cortex. Some reports indicate an association of GM loss with high or low BP [39], whereas others find no association [49-51]. Physical activity and exercise are related inversely to age-related brain atrophy [52, 53] and can change hippocampal structure [54]. Higher aerobic fitness is associated with greater hippocampal volume and better spatial memory performance [55]. Increments in physical activity (categorized in quintiles) are associated with ~2-2.5% greater average tissue volume after controlling for age, sex, and education [56].

**Age-related cerebrovascular pathologies**

Cerebrovascular disease is a spectrum of conditions affecting the brain’s blood vessels. Cerebral microbleeds (CMBs) and cerebral microinfarcts (CMIs) are common in cerebrovascular disease, and this “silent pathology” is responsible for a high portion of dementia cases [57]. CMBs (i.e., cerebral micro-hemorrhages) are tiny blood degradation deposits detected by susceptibility-weighted imaging (SWI) [58, 59]. Over the years, CMB has increased in longitudinal studies of native aging [60, 61] and dementia, and the relevance and incidence of CMBs are significantly greater in AD compared to normal aging. The prevalence of CMBs in MCI (20%) and AD groups (18%) is also twice that of the healthy group (10%)[62]. CMIs are microscopic regions of cellular death or tissue necrosis invisible to the naked eye [63], with a mean size of ~1 mm [64]. CMIs are common in brain autopsies of individuals with dementia [65-68], and their presence is recognized as causal [69]. CMI prevalence was 62% in pathological assessments of vascular dementia patients compared to 24% in undiagnosed patients [70] [63, 71]. Furthermore, larger infarct volume and number are associated with decreased cognitive performance and increased dementia risk [69, 72-74]. Besides aging, the brain, like the heart, is affected by risk factors for cerebrovascular diseases such as hypertension [75, 76], smoking [61], heavy alcohol consumption [61, 77], and T2DM. Obesity status is a clinical condition associated with CMBs [78]; however, lower BMI, fat mass (FM), and WC are associated with microbleeds in MCIs [79]. Gene variants significantly increase the risk of lobar CMBs, which are prevalent in carriers of the *APOE ε4* allele compared to ε3/ε3 genotype carriers [75]. Age was an independent risk factor for CMBs in all locations in a large healthy population. Male gender and *APOE4* are positively associated with lobar CMBs, whereas a higher BMI is negatively associated. Hypertension, smoking, and alcohol are associated with deep CMBs but not lobar CMBs [75, 77]. In addition, plasma homocysteine is an independent predictor of CMBs [80], and there is a strong association between CMIs, age, and race [81]. Atrial fibrillation is a significant risk factor for cortical CMIs [82-84], and there is a neuropathological association between diabetes and large infarcts, but not CMIs [85, 86]. ***Despite existing metabolic, genetic, medical, fat stores, microbiome, and hormonal predictors, there is a critical need for individual responses following long-term interventional trials and their effects on cerebrovascular diseases in later life.***

**Integration** **of brain anatomy and** **cerebrovascular disease with age-related cognition**

Brain atrophy is an early predictor of cognitive impairment, especially in vascular disease patients [87, 88]. Hippocampal atrophy is an initial neuropathological change associated with MCI and predictive of further cognitive decline [89-91]. Medial temporal lobe variance also significantly improves the discrimination of normal aging from MCI and predicts future decline [92]. Furthermore, increased learning impairment combined with increased atrophy is associated with a significantly greater risk of developing AD [93]. Individuals with CMBs display more executive dysfunction than those without CMBs [94]. CMB location is associated with cognitive task performance, and the number of CMBs correlates with reduced cognitive scores [95]. In one study, multiple CMBs or mixed microbleeds in deep and lobar locations are associated with increased dementia risk [96]. In contrast, in another study, lobar CMBs are associated with accelerated cognitive decline [97]. Other studies reveal no correlation between CMBs and cognitive symptoms [ ]. Recently, the impact of CMIs on cognitive function and the risk of dementia has been recognized. Cortical CMIs at baseline are associated with the accelerated decline of memory and language domains, and cortical CMIs at baseline in patients without dementia are signiﬁcantly lower than in those with dementia [74, 98].

***Identifying clinically significant biological markers strongly associated with early-stage brain deterioration is essential to treat or prevent accelerated cognitive decline.***

**Association of age-related brain anatomy with cerebrovascular disease**

The Montreal Cognitive Assessment (MoCA) test is a screening tool to detect early mild cognitive impairment (MCI) and is a better global assessment tool than the Mini-Mental State Examination (MMSE), particularly for earlier stages of cognitive decline [99]. A significant positive relationship exists between hippocampal volume and total MoCA score, especially with delayed recall, attention, and visual-spatial/executive function. As hypothesized, delayed recall is associated with hippocampal volume [100]. MoCA scores are also associated with cortical GM atrophy and may estimate structural brain damage in less educated elders [101]. The total MoCA score correlates with posterior cingulate volume in younger individuals (under age fifty) and hippocampal and precuneus volumes in older adults [102]. Additionally, a decreased MoCA performance is associated with increased age, CMI variation, and CMB [103, 104]. Blood biomarkers, such as increased serum BDNF levels, are independently associated with a decreased decline in total MoCA score. Increased serum BDNF also significantly reduces the odds of a decline in executive function within the study population [105]. Several miRNAs correlate significantly with MoCA scores [106].

**Brain and multi-omics (genetics, epigenetics, proteomics)**

Several genes are linked to or increase the risk of cognitive decline and brain structural changes. *APOE* (rs429358) is the most investigated polymorphism related to cognitive decline, and it has three common allelic variants, *epsilon 2* (*ε2*), *epsilon 3* (*ε3*), and *epsilon 4* (*ε4*), with *ε3* most prevalent. Long expectancy is positively associated with *APOE ε2*; however, a single copy of ε4 is the strongest genetic risk factor. Studies indicate that *APOE-ε4* exerts additive effects on GM volume in regions relevant to Alzheimer’s disease pathophysiology in healthy individuals [107], and *APOE ε4* carrier status (rs769449), and *APOE ε44* genotype are associated with increased CMBs, especially lobar ([108]. *APOE ε2* alleles show no association with CMB counts [109]. Another variant in brain function is a polymorphism of *BDNF* val66met (rs6265). BDNF is a brain-derived neurotrophic factor gene. Individuals carrying the methionine-encoding A allele display poorer delayed episodic memory, altered hippocampal activation patterns, and reduced hippocampal neuronal integrity [110]. Other reports indicate associations between the A allele and hippocampal volume decline [111, 112]. The catechol-O-methyltransferase (*COMT*) gene (rs4680) encoding the primary enzyme responsible for dopamine clearance in the prefrontal cortex is also relevant. A common functional polymorphism resulting in amino acid exchange at position 158 (val158met) is extensively studied concerning cognitive function [113]. A negative effect of the G allele is observed for cognitive tasks assessing executive functioning [114, 115], episodic memory [116, 117], and semantic memory [116]. Furthermore, genome-wide association studies (GWAS) reveal loci associated with hippocampal volume (rs11979341, rs7020341, rs2268894, rs2289881, rs7492919, rs17178006 ) [118] correlated with decreased hippocampal volume and increased the risk for AD [119].

Unlike genotypes, epigenetics results in altered gene expression without changes in the DNA sequence [120]. DNA methylation (CpG5) modifies cytosine residues in cytosine/guanine-rich regions, such as CpG islands. *BDNF* promoter methylation and a tag SNP (rs6265) have a significant role in MCI etiology and its evolution to AD [121]. Increased DNA methylation is associated with the APOE 𝜀4 allele and APOE 𝜀3 carriers [122]. MicroRNAs (miRNAs) are small noncoding RNAs that play a major role in epigenetic gene regulation and as markers of cognitive function. The two biomarker pairs, miR-132 [123] and miR-134 can discriminate MCI from HC with high sensitivity and specificity [124], and the three miRNAs, miR-140-5p, miR-197-3p, and miR-501-3p, are top-ranked predictors of cognitive outcomes [125]. Lower expression of miR-384 is found in participants with MCI compared to controls, and three miRNAs, miR-384, miR-135a, and miR-193b, have a combined predictive value for MCI with an AUC of 0.997 [117]. Additionally, miR-146a is positively associated with the Apo ε4 allele and smaller hippocampus volume [126]. Metabolomic research with over 600 individuals having MRI-confirmed small vascular disease indicates that metabolites, such as glycerophospholipids, sphingolipids, and paraxanthine, are significantly associated with imaging markers for small vessel disease and cognition [127]. Significant associations by proteomics between 377 proteins and hippocampal volume are also known [128].

***Understanding brain status and the effects of lifestyle in later life is a critical diagnostic need. Exploring the association of cognitive decline and brain structural changes with specific brain-related genes, metabolomics, and methylation changes offer a novel approach to address this need.***

**Brain and the human microbiome**

Evidence is accumulating that gut microbiota are associated with neurodegeneration [129, 130]. In one study, AD participants had decreased microbial diversity in their gut compared to age- and sex-matched controls and distinct gut microbial communities characterized individuals with and without a clinical diagnosis of dementia due to AD [131]. Dialister and SMB53 bacteria are correlated with reduced AD pathology in non-demented participants and reduced Bifidobacterium in AD participants, suggesting that Bifidobacterium may be protective in AD development and progression [131]. Also, multivariable logistic regression analysis indicates a greater prevalence of specific bacteria independently associated with MCI (Bacteroides) [132] and cerebral small vessel disease [133, 134]. Such a preliminary study [135] will provide novel insights that gut microbiota composition is altered in individuals with cognitive decline.

***We propose to identify microbial metabolite profiles that are associated with or predict brain aging. Addressing the role of the human microbiome and its dynamics by MRI-assessed biological brain analysis will contribute to a novel understanding in this field.***

**Brain and visceral abdominal tissue (VAT)**

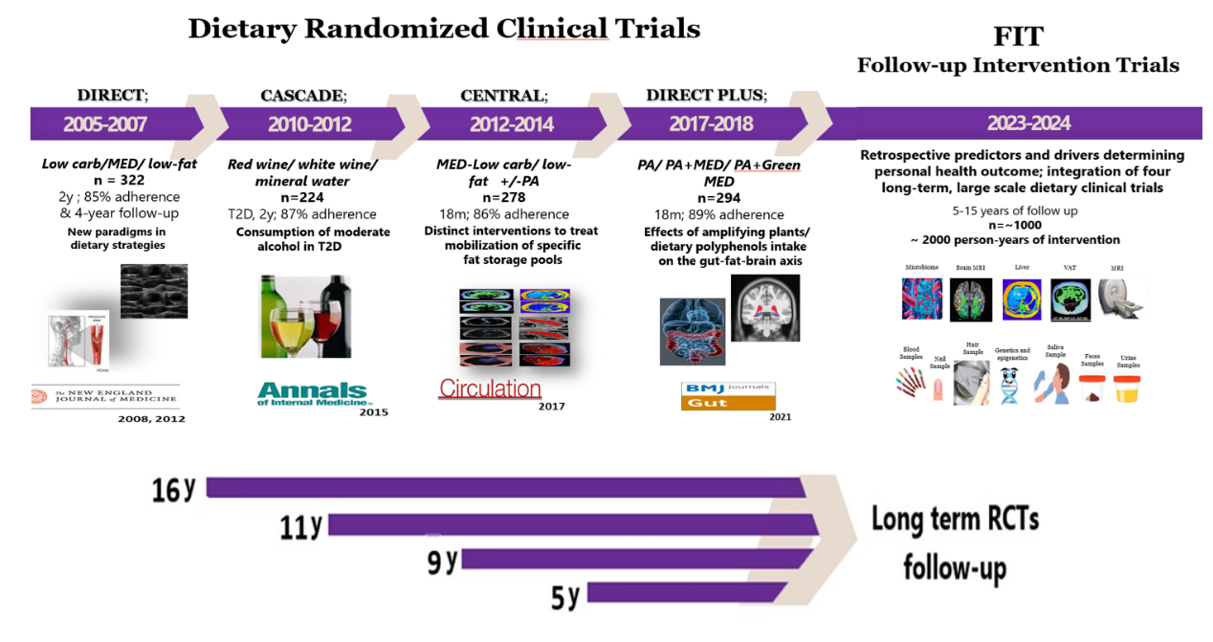
VAT is an important measure of obesity known for its high cardiometabolic risk. For instance, central obesity in midlife increases the risk of dementia independent of diabetes and cardiovascular comorbidities [45]. Increased VAT is associated with reduced verbal memory, attenuated attention, and lower executive function [136]. Moreover, VAT is positively correlated with lower brain volume in otherwise healthy middle-aged adults [44], and higher VAT is explicitly associated with larger ventricular and smaller hippocampal volumes [136]. Recently, it has been reported that VAT is negatively associated with brain network structure, as assessed by MRI, and increased blood estradiol in women (not men) attenuates the negative association of VAT with brain structure [137]. VAT is also a risk factor for cerebrovascular disease [138, 139], and one study indicated that VAT is an independent predictor of CMBs in healthy people [140].

***Although a correlation exists between VAT and brain structure, the association must be strengthened. Thus, we propose an in-depth analysis of the correlation of VAT with cognitive function and brain structure, brain atrophy, and especially cerebrovascular diseases.***

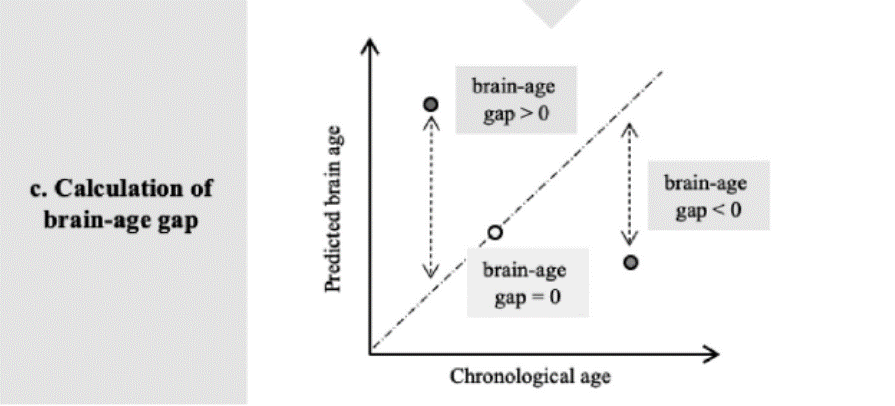
**Interventional dietary RCTs as a platform for individual predictors of brain health**

Our group has conducted four major RCTs: DIRECT, CASCADE, CENTRAL, and DIRECT PLUS (before 16y/11y/9y/5y) with more than 1,000 participants having obesity, T2DM, or abdominal obesity, for a total of over ~2000 person-years of intervention with an 85-89% compliance rate (Figure 1). These RCTs explored the effect of lifestyle intervention strategies on metabolic health, such as intrahepatic fat, cardiovascular markers, VAT, and brain health status. In the 24-month DIRECT trial (n=322) from 2005, we revealed the innovation that the Mediterranean and low-carbohydrate diet is superior to a low-fat diet for weight loss, carotid atherosclerosis [141], and improvement of cardiometabolic parameters [142]. After a four-year direct follow-up, we found better lipid marker outcomes in Mediterranean low-carbohydrate diets regardless of weight regain [143]. In the second 24-month CASCADE trial (n=224) in 2010, we discovered that moderate wine consumption (specifically red wine) is beneficial for cardiometabolic risk [144]. In 2012, we conducted the third 18-month CENTRAL trial (n=278), showing how distinct lifestyle interventions affect the mobilization of fat storage pools (atherogenic and diabetogenic fat depots) [145]. In our final 18-month DIRECT PLUS RCT in 2017 (n=294), we found that a green Mediterranean (MED) diet rich in polyphenols benefits health and the gut-fat-brain axis. We observed changes in cardiometabolic parameters [146], fatty liver [147], gut microbiome [148, 149], and brain atrophy [150].

Based on our positive outcomes, we propose an intervention trial (FIT) with 5-16 years of follow-up. We will include participants from our four previous dietary RCTs (DIRECT, CASCADE, CENTRAL, DIRECT PLUS; 18-24-month intervention; 85-89% adherence rates), including ~1,000 participants with abdominal obesity or T2DM, representing more than 2000 person-years of intervention. We will evaluate the participants for their clinical status (morbidity/ mortality), pharmacological usage, biomarkers from blood/urine/saliva/hair/nails, fecal and genetics samples, basic clinical and anthropometric measurements, MRI-derived brain and specific organ fat storages, lifestyle, and functional questionnaires. ***Our proposal will result in a unique metadata set integrating our four RCTs.***

**Figure 1:** Visual summary of our four previous RCTs and the FIT follow-up.

Our integration of this extensive dataset will result in a platform to predict brain biological age compared to chronological age. The differences we discover between predicted brain age based on brain volumes and chronological age of individuals is known as the “brain age gap” [151] (Figure. 2). This value may serve as a marker for overall brain health, support differential diagnoses, prognoses, and treatment choices.

**Figure 2.** Calculation of brain age gap for each subject as predicted age – chronological age, adapted from [151].

***In our FIT study, we propose to utilize our unique metadata set for follow-up trials to predict brain health, focusing on cognitive function, brain anatomy, and cerebrovascular pathologies.***

**C. Research Objectives and Expected Significance**

**Gap of Knowledge:** Predictors of age-related brain atrophy, vascular-related brain pathologies, and cognitive decline are mainly unknown and need further investigation.

**Overarching Goal:** Our goal is to identify individual retrospective predictors of brain age that deviate from chronological age using MRI-assessed age-related brain atrophy, cerebrovascular pathology, and cognitive tests by integrating long-term lifestyle interventions and follow-up.

**Specific Aims**:

1. ***We will identify specific*** ***predictors of age-related brain atrophy and cerebrovascular pathology.*** We will retrospectively collect age-related atrophy data from past and present follow-ups, which will be compared to expected chronological age. We will explore multi-omics (epigenetics, metabolomics), specific brain-related biomarkers such as BDNF, p-tau 217 and 181 protein levels, APOE4, PSEN 1/2 genes, and further blood biomarkers. We will also examine clinical, lifestyle, demographic, and anthropometric parameters. We propose to predict healthy brain aging by integrating distinct layers of omics (metabolome, microbiome) with biomarkers from the follow-up data. We hypothesize that combined biomarkers for individual biological patterns will better predict healthy brain aging, which will be assessed by differences between biological and chronological brain age.
2. ***We will study the predictive role of individual responses.*** Responses induced by randomized lifestyle intervention trials (such as weight loss, the dynamics of specific biomarkers, microbiome, fat depots., etc.) will be compared to brain status at follow-up.
   1. We will explore distinct subgroups based on changes in biomarkers from the pre- and post-intervention data points.
   2. We will predict brain health status at the follow-up timepoint for subgroups in 2.1 which will be assessed by MRI-based brain anatomy and pathologies.
3. ***We will specifically dissect the dynamic pattern and trajectory of anatomical brain atrophy.*** Five years after our DIRECT PLUS trial completion, this specific aimwill allow us to identify potential subtypes for healthy brain aging dynamics.
   1. We will characterize healthy brain aging trajectories from pre-, post-intervention, and follow-up time points in sub-groups demonstrating individual changes of anatomical brain atrophy evolution.
   2. We will link multi-omics and other biomarkers to subgroups of anatomical brain atrophy.

**Expected Significance:** Our detailed retrospective and follow-up measures should shed light on potential drivers of brain age deterioration and variation and may lead to possible strategies for healthier brain aging. Our FIT is an innovative research platform that will utilize metadata from multiple randomizedlifestyle intervention trials with long-term follow-ups to identify individual predictors. These predictors may promote novel preventive treatment and attenuation of age-related brain deterioration. Scientifically, the proposal will improve our understanding of the role of biological molecules in the development and progression of age-related neurodegeneration. Beyond its scientific merit, our proposal is significant for public health by identifying predictors that may lead to early detection of age-related diseases, reducing disease burden and patient costs.

**D. Detailed description of the proposed research**

**Working hypothesis:** We hypothesize that specific biomarkers will predict brain age deviation. Furthermore, individual responses induced by lifestyle intervention will predict brain health status further in life.

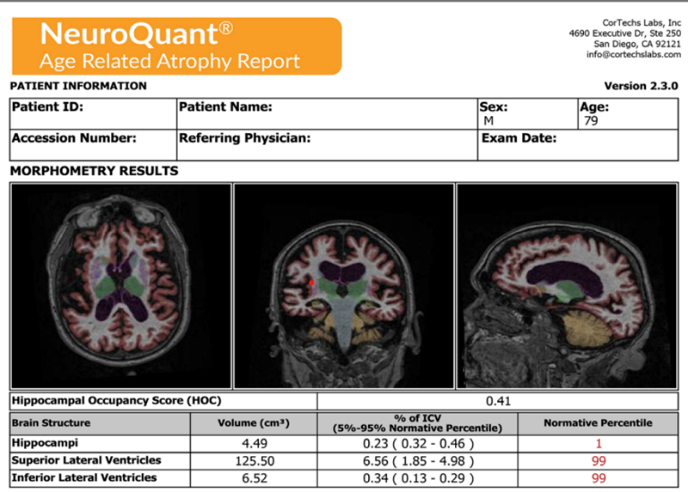
**Research Design and Methods**

For our research design, we will utilize qualitative research with complementary data collection. In addition to our previously obtained data (Table 1), we will conduct a follow-up trial to complete our dataset from previously performed trials.

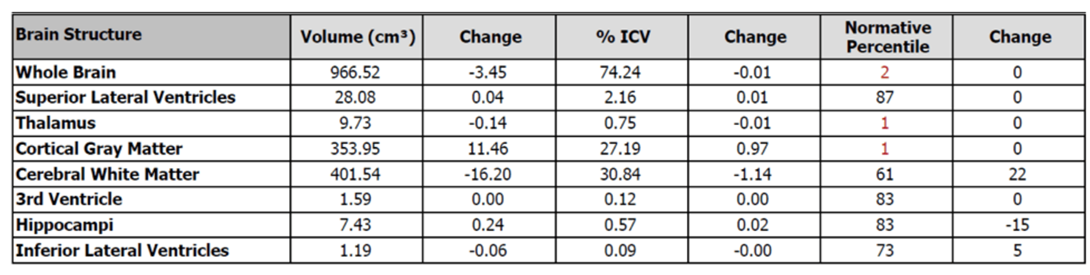
**MRI:** MRI technologists will review scans for quality at acquisition time and repeat as necessary for quality. All MRI scans will be performed after at least a 3-hour-long fast.

**Brain measurements:** MRI scans will be utilized for several brain-related measurements.

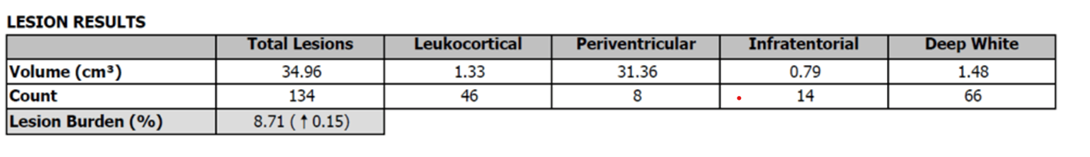
***Brain anatomy*:** We will acquire whole brain 3D imaging using the T1 sequence. To investigate regions of interest, we will further examine hippocampus and lateral-ventricle volumes by T1 3D T1-TFE sequence with 1.0 mm isometric voxels using an inversion pre-pulse acquired in the axial plane. The acquisitions will be made using a 3.0T Philips Ingenia.

***Brain volum******e:*** We will analyze brain images using the Food and Drug Administration cleared NeuroQuant®software for volumetric MRI processing. NeuroQuant® will provide data on 15 brain regions (left and right sides), totaling 30 measurements, which will be compared to volumes of normative references adjusted for age, sex, and intracranial volume (http://www.cortechs.net/products/neuroquant.php). The procedure will provide fully automated segmentations, including stripping the skull and mapping the brain with a Talairach atlas. Automatic segmentation includes image filtering, artifact correction, segmentation, error measurement, and report generation.A report of age-related atrophy will include hippocampal and lateral ventricle volume compared to age and gender norms, as measured in our previous publication [150].

***White matter and cerebrovascular outcomes*:** CMIs by DWI-DTI sequence. White matter lesion and CMI segmentations by DTI 3D FLAIR sequence. Lesions will be analyzed using **LesionQuant®** as described below:



An example of the lesion results section shows the total lesion volume (cm3) and counts in the anatomical regions. The lesion burden (8.71%) indicates the change in the ratio of the total lesion volume (34.96) to the white matter volume (401.54) as a percentage with an arrow indicating an increase (up) or decrease (down) as described below:



This personalized quantification detects lesions as regions brighter than the surrounding white or GM in a T2 FLAIR image ( ). We will analyze SWIp-Quantitative Susceptibility Mapping (QSM) for the detection of CMBs using the MATLAB software JHU/KKI\_QSM\_Toolbox\_v3.0. The software uses a graphical user interface (GUI) for QSM from MR magnitudes.

**Clinical, anthropometric, and BP parameters:**We will collect weight, height, waist circumference, and BP from all past participants. Additionally, we will utilize data from the “Ofe” and “Chameleo” databases for morbidity, mortality, habitual medicine regiment, and standard care routine checkups obtained by the primary care provider.

**MoCA Questionnaires:** MoCA is a quick scan for multiple brain areas and functions, with lower scores indicating more severe impairment. The tool is sensitive and useful in early and mild phases of progressive cognitive decline and is validated for conditions including MCI, AD, and Parkinson’s Disease dementia [99, 152]. All interviewers have undergone training and are certified to administer the test. MoCA scores range from 0 to 30, with 26 or higher being considered normal, with no cognitive issues apparent. MoCA accurately and quickly (~10 min) assesses short-term memory, visuospatial abilities, executive functions, attention, concentration, working memory, language, and time and place orientation. Additionally, we will use electronic questionnaires, as in our previous trials [ ], which contain a validated food-frequency questionnaire including 127 food items with three portion-size pictures and lifestyle, symptom, and medical questions. We will also use the 14-Item Mediterranean Diet Assessment Tool from the PREDIMED trial and VAS questionnaire for appetite.

**Table 1**: Available measurements from our four RCTs, ~2000 person-years of dietary interventions

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
|  | **DIRECT** NCT00160108 | **CASCADE** NCT00784433 | **CENTRAL** NCT01530724 | **DIRECT PLUS** NCT03020186 |
| Type of intervention | Low-fat vs. MED vs. Low-carb weight loss diets | Water vs. White wine vs. red wine; Based on MED diet | Low fat vs. MED low carb weight loss diets +/- PA | PA vs. MED vs. green MED Weight loss diets |
| Duration | 24m + 4 years follow-up | 24m | 18m | 18m |
| Number of participants | 322, obesity | 224, type 2 diabetes | 278, abdominal obesity | 294, abdominal obesity |
| Adherence rate | 85% | 87% | 86% | 89% |
| **Outcome Measurers available** | | | | |
| Adiposity, Clinical | 0, 6, 24m | 0, 6, 24m | 0, 6, 18m | 0, 6,1 8m |
| Lipid, glycemic, inflammatory, hepatic, endocrine, nutritional profiling biomarkers | 0, 6, 24m | 0, 6, 24m | 0, 6,1 8m | 0, 6, 18m |
| sub-metabolome profiles (omics) | 0, 6, 24m | 0, 6, 24m | 0, 6, 18m | 0, 6, 18m |
| fat depot re-distributions (MRI derived) |  |  | 0, 6, 18m | 0, 18m |
| Epigenetics |  |  | 0, 18m | 0,18m |
| Adherence measures: nutrition, exercise, lifestyle | 0, 6, 24m | 0, 6, 24m | 0, 6, 18m | 0, 6, 18m |
| Atherosclerosis (US derived) | 0, 24m | 0, 24m |  |  |
| Brain volume (MRI derived) and cognitive function |  |  |  | 0, 18m |
| Gut Microbiome metagenomics and metabolomics |  |  |  | 0, 6, 18m |

**Biological sample collection:** We will collect various samples, including blood, feces, urine, hair, nails, and saliva. We will obtain measurements followed by a 12-h fasting state, and blood, fecal, urine, and saliva samples will be stored at -80oC with a backup system.

**Measurements of biomarkers and metabolites:** We will collect the brain-related biomarkers BDNF and p-tau proteins 181 217 from plasma. For genetic and epigenetic sampling, we will extract DNA from blood samples following a standard protocol. Sample integrity will be controlled using gel-electrophoresis and double-stranded DNA concentration. We will use genotyping or sequencing techniques to investigate targeted genes such as *APOE ε4 and PSEN1/2* and untargeted genes. For metabolites, we will measure traditional lipids (LDL-C, HDL-C, triglycerides, total cholesterol), glycemic control (FPG, insulin), and adipokines (adiponectin, FGF21, RBP4, chemerin, progranulin, bone morphogenetic protein 2, RBP4, omentin, asprosin). We will also measure HbA1c, liver enzymes (Alkaline Phosphatase, Alanine Aminotransferase, Aspartate Aminotransferase), bilirubin, CRP, vaspin, MCP-1, IL-1beta, IL-6, IL-17, and TNFalpha. We will include the plasma hormone-like molecules GIP, GLP-1, ghrelin, PYY, FGF-21, and RBP4.

**Multi-omics analyses:** We will include lipidomics to measure fatty acid and triglyceride composition and amino acids for the metabolome. We will also assess DNA methylation and microRNAs as epigenetic markers.

**Microbiota deep sequencing:** We will use taxonomic and functional profiling of microbial communities from shotgun metagenomes and meta-transcriptomes to detect differences in microbial compositions.

**Statistical analyses:** We will use ML/AI models to determine individual retrospective predictors of brain age based on deviations from chronological age. We will develop classifiers for short and long-term outcomes using machine-learning methods for feature selection, such as LASSO, Boosting, Network analyses, or our previously published methods [153]. We will perform extensive data pre-processing and quality control using analysis pipelines developed for the different multi-omics techniques. The pipelines may provide an extensive annotation of analyzed features, facilitating functional interpretations. Cross-validation techniques will assess the performance of predictors. Analyses for genome-wide associations of genotypes, eQTL-, mQTL, and phenotypes, as well as causal inference modeling, may utilize automated high-throughput and quality-assured genetic analysis. We will consider molecular and clinical parameters as potential predictors. Beyond association analyses, we will identify molecular factors showing causal effects on our interventional trials’ short- and long-term outcomes. The factors will be identified by:

1. Time series analyses using univariate (generalized) linear mixed models with appropriate link functions to determine molecular parameters associated with the outcomes.

2. Longitudinal correlations using bivariate mixed models to identify parameters inter-related in time series.

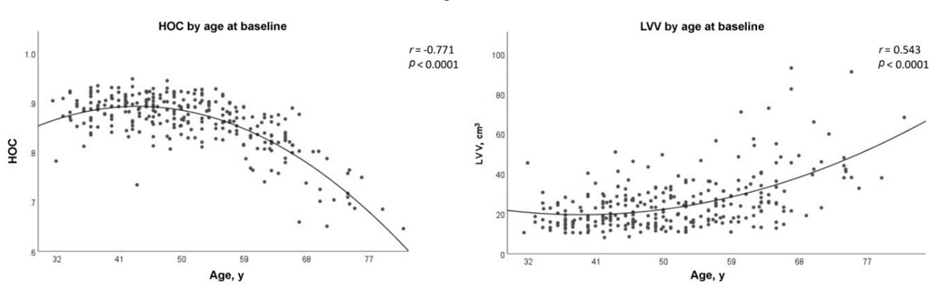
3. Analyzing latent curve models with structured residuals to identify within-patient lagged relationships between interventions, molecular parameters, and outcomes, which surpasses simple association analyses. This structural equation modeling will be performed for all pairs of molecular and outcome parameters found in steps 1 and 2. The resulting cross-lagged relationships can be evidence of causality.

We will present continuous variables as means ± standard deviations (SD), with dependent variables analyzed for normal distribution using the Kolmogorov–Smirnov test. Subgroup differences will be tested by T-test, analysis of variance (ANOVA), Mann-Whitney, Kruskal-Wallis, or Chi-square tests, depending on the number of groups and distribution of dependent variables. Kendall Tau correlation was used to examine the p-of-trend, and Benjamini-Hochberg's false discovery rate correction will be applied for multiple comparative analyses. The latest observations will be subjected to intention-to-treat analysis for missing values.

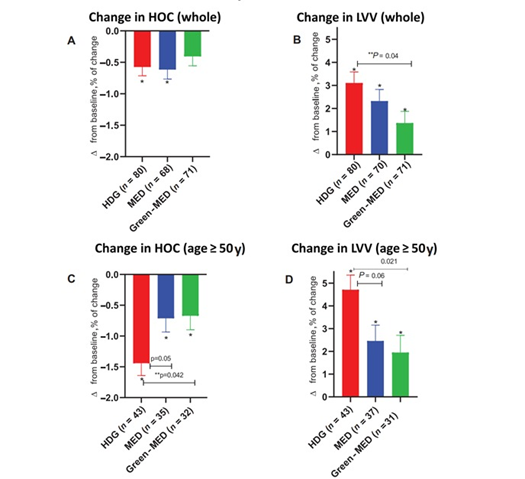
For ***Specific Aim I***, a cross-sectional or case-control study will be performed. The dependent variable is biological brain age and brain volume, and lesion quantification (assessed by neuro/lesion quant software) compared to expected chronological age. Independent variables will be biomarkers such as multi-omics, hormones, clinical, lifestyle, gender, anthropometrics, etc. We will perform a cross-sectional, and case-control analysis of the FIT cohort by multivariate linear regression or machine learning models lasso or boosting. For associations between independent variables and biological brain age acceleration, we will use standardized residual volumes or lesions accounting for chronological age, which reflect differences between brain and chronological aging. For ***Specific Aim II***, the dependent variables will be brain volume, lesion, CMI, and CMB quantifications, with MoCA score at the follow-up timepoint. The independent variables will be biomarker changes, such as insulin resistance, weight, WC, VAT, etc., based on previously measured baseline (pre-intervention) and post-intervention (after 18/24 months) data points. Linear regression (LM) or linear mixed-effects modeling will predict the dependent variables at the follow-up timepoint. For ***Specific Aim III***, the dependent variable (brain volumes quantified NeuroQuant®) will be the trajectory of the brain state over three time points (baseline, post-intervention, follow-up). The independent variables will be profiles, such as changes in blood biomarkers, gender, responders/non-responders to dietary intervention, etc., characterized by the clustering algorithm K means. We will analyze variance with ANOVA to explore the distinction between dynamic brain patterns and present our statistical analyses using SPSS software (version 26.0) and R (version 3.6.0). Our statistical significance will be p<0.05, two-sided, and graphing will use GraphPad Prism 7.

**Preliminary Results**

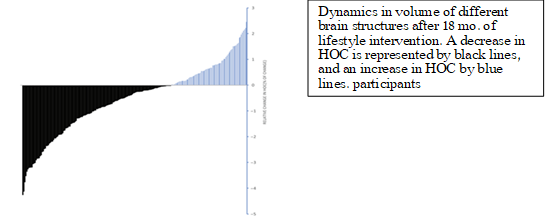
Recently, we reported in the DIRECT PLUS trial that lower HOC and higher lateral ventricle volume (LVV) at baseline are strongly correlated nonlinearly with age, with the most prominent threshold for changes in the slopes of HOC decline and LVV expansion at age 50 years [ ] (Figure 3).

**Figure 3.** A change in slope is significant at age 50 y. Baseline results from the DIRECT-PLUS trial show brain MRI-derived HOC and LVV across age (n = 284). Data were quantified and segmented in a fully automated manner using NeuroQuant®.

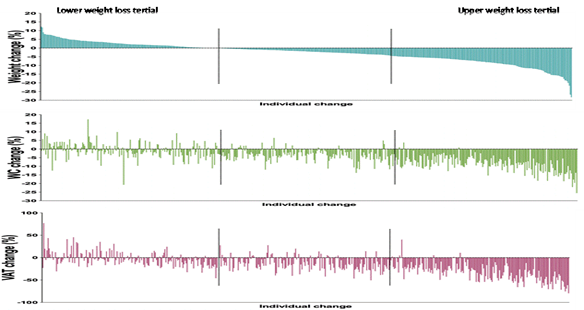
We presented ground-breaking findings that the MED diet, rich in polyphenols, has a beneficial effect on anatomical brain atrophy evolution. Among participants ≥ 50 years old, the MED and Green-MED diet groups demonstrated ~50% less of the neurodegeneration markers HOC decline and LVV expansion than the control group (Figure 4).

**Figure 4.** The effect of green MED diet on brain atrophy. The effect of 18-month dietary interventions on MRI-derived brain volume structures. A. Participants in the Green-MED group did not significantly change HOC after 18 months of intervention. However, no significant difference in change in HOC (from baseline) between groups of the entire study population. The Healthy Dietary Guidelines (HDG) and the MED group participants significantly decreased HOC. B. Compared with the HDG group, the Green-MED group had attenuated LVV expansion across the entire study population. C. and D. Among participants ≥ 50 y of age, the MED and Green-MED diet groups demonstrated less HOC decline and LVV expansion than the HDG group.

In addition, among participants ≥ 50 years old, a lower decrease in HOC is associated with lower HOMA-IR, weight loss, and TG. After adjusting for age, sex, weight change, and TG changes, *a beneficial change in HOMA-IR was independently associated with a beneficial change in HOC*. We found an association of weight, BMI, and WC losses as well as HOMA- IR and BP decline with favorable changes in LVV. Following our novel association of HOC% and biomarkers, such as insulin resistance, lifestyle intervention, etc., the reproducibility of the GM volume and its association with our various biomarkers in the FIT is scientifically important.

We found that attenuation of HOC decline was significantly associated with biomarkers such as weight loss, improved insulin sensitivity, and lower TG levels. Other biomarkers that attenuated brain atrophy were weight, BMI, and WC loss, as well as HOMA- IR and BP declines, which present favorable changes of LVV. In addition, individual response to the 18-month lifestyle intervention was pronounced in the HOC score change (Figure 5). **Figure 5.** Individual response of change in HOC (% change). Data from the DIRECT PLUS trial. Dynamics in the volume of different brain structures after 18 months of lifestyle intervention. Decreased HOC (black lines) and increased HOC (blue lines) are shown.

The findings may strengthen the assumption of individual response to lifestyle interventions. In unpublished results (Figure 6), an integrative outlook of the CENTRAL and the DIRECT PLUS trials sorted by tertials of weight change results in distinct 18-month changes in weight, WC, and VAT.

**Figure 6.** Individual response to dietary interventions (unpublished data from the CENTRAL and DIRECT PLUS trials). Waist circumference (WC) and VAT individual data are sorted by the extent of weight loss. Although some participants did not achieve successful weight loss “upper weight-loss tertia”), they still improved their health status, reflected by reductions in WC and VAT “lower weight-loss tertia”).

***The distinction in the individual responses to lifestyle interventions emphasizes the need for more personalized interpretation.*** ***To meet this challenge in our FIT study, we propose to utilize integrated metadata from our four RCTs and follow-up trials to explore how individual responses induced by lifestyle intervention can predict brain health.***

**Feasibility**

We started re-inviting participants from our four previous RCTs in the past four months. Currently, 42% of the participants have agreed to register for follow-up measurements.

**Power Calculation**

Our power calculation is based on HOC mean and LVV measurements at baseline in the DIRECT PLUS trial. The time between the DIRECT PLUS and the FIT trials will be five years. Differences between the mean and SD of 51 years (mean of DIRECT trial) and 56 years were -0.009% (SD ±0.03 (51 years) and ±0.0259 (56 years)) and +3.1 cm3 (SD ±8.797 (51years) and ±10.676 (56years)) for HOC and LVV, respectively. Based on the retention rate of 85-90% in our previous RCTs, we estimate a retention rate of 60% to 70% (between 600 to 700 participants) for the FIT project. The power calculation is described in the table below for several sample sizes:

|  |  |  |
| --- | --- | --- |
| *Sample size* | *HOC%\_time\_0* | *LVV\_time\_0* |
| *400* | *89.31%* | *88.52%* |
| *500* | *94.77%* | *94.25%* |
| *600* | *97.54%* | *97.23%* |
| *700* | *98.88%* | *98.7%* |

**Ethical Considerations**

DIRECT, CASCADE, CENTRAL, and DIRECT PLUS were conducted according to the Declaration of Helsinki. The protocols were approved by the Medical Ethics Board and Institutional Review Board at Soroka University Medical Centre, Be’err Sheva, Israel (approval numbers: DIRECT: 3809; CASCADE: 4781; CENTRAL: 5008; DIRECT PLUS: 0280-16-SOR). No participants received any financial compensation. We have received ethical approval for the FIT trial (0373-21-SOR). All participants will sign an informed consent. The personal details of the participants will be confidential and secured.

**Existing Resources**

**PI: Iris Shai** is a Professor of Nutrition and Epidemiology at Ben Gurion University (BGU) in nutrition and chronic diseases, Adjunct Prof., Dept. of Nutrition, Harvard Univ., and Honorary Prof., Dept. of Medicine, Univ. of Leipzig. Shai is chair of the Cathedra of Epidemiology, named by Dr. Herman Kessel, and a member of the Israeli Government Health Ministry Committee for Healthy Nutrition Regulations and the Israeli Cardiology Association committee for nutritional guidelines for preventive cardiology. Her focuses are obesity, diabetes, and cardiometabolic risk while performing long-term, large-scale, compressive dietary RCTs in standards of drug trials and long-term cohorts. Prof. Shai was PI of the two-year Dietary Intervention Randomized Controlled Trial (DIRECT) and four-year follow-up Dietary Intervention-Randomized Controlled Trial (DIRECT) Study, the two-year CArdiovasCulAr Diabetes & Ethanol (CASCADE) trial, the 18-month Diet and Body Composition (CENTRAL) trial, and the 18-month DIRECT PLUS trial.

**BGU of Negev** is an institute where the clinical trials DIRECT, CASCADE, CENTRAL, and DIRECT PLUS was done. BGU includes a well-established platform and the necessary infrastructure for long-term clinical trials, including follow-up studies. BGU includes laboratories, -80°C freezers, imaging, metabolic units, MRI services (three Tesla Philips Ingenia), image analysis software (MNOVA, PHILIPS, MATLAB software developed specifically for our research projects), database management and interpretation services, and epidemiological and statistical backup. Our proposal will benefit from an outstanding scientific environment, a unique design for well-controlled, unique, 24/18-month dietary intervention trials. We will provide comprehensive traditional and novel biomarkers for times 0, 6, and 18/24 months for glycemic control, lipids, inflammation, etc., already measured and analyzed. Beyond these data, we will perform follow-up measurements to enrich and deepen our datasets for the suggested prediction models.

**PI: Ilan Shelef, Soroka Medical Center, Israel,** is director of the imaging department and member of the BGU Faculty of Health Sciences. Prof. Shelef is an editorial member and reviewer of several internationally reputed journals and has authored many research articles/books related to medicine. Prof. Shelef is an expert in neuroimaging and collaborated with Prof. Shai's group on the CENTRAL and the DIRECT PLUS trials.

**Collaborator: Markus Scholz, Leipzig Univ, Germany**; Prof. Scholz leads a Genetical Statistics and Systems-Biology group at the Institute for Medical Informatics, Statistics, and Epidemiology experienced in molecular data analyses, including quality control, classifier construction by machine-learning approaches, and cross-omics. Their expertise includes causal inference by Mendelian Randomization Network analyses or structural equation modeling of multivariate time series and will support all aspects of data analysis.

**Collaborator: Galia Avidan, Ben Gurion Univ, Israel;** Prof. Avidan is an expert in cognitive neuroscience. She studies the bidirectional physiological and anatomical interactions between the gastric system and the brain. She is also interested in the implications of such interactions to gastrointestinal disorders, obesity, eating disorders, and high-level cognition by using a multidisciplinary combination of functional and structural brain imaging, behavioral, and physiology. Prof. Avidan employs a computational perspective to study connectivity patterns in the human brain associated with specific cognitive abilities and individual differences. Prof. Avidan collaborated with Prof. Shai in the DIRECT PLUS trials.

***Collaborative work:*** The database will be managed and analyzed using the server of the Faculty of Health Science at BGU and bioinformatic services at Leipzig University. HSPH will support the analysis of long-term follow-ups. BGU’s computer facility consists of servers and PCs networked for primary data management and analysis for international analyses. All trial data will be saved on BGU-secured servers. The software includes SPSS, RStudio, and Stata, with extensive bioinformatics software. Office space for the proposal exists in the PI’s Dept. of Epidemiology. The institutional support, physical resources, and intellectual and collaborative environment will ensure success.

**Expected Results**

We anticipate that retrospective predictors and drivers for attenuated/ accelerated/ appropriate biological brain age compared to the chronological age could be assessed via the individual response induced by past lifestyle interventions. Additionally, integrating our four RCTs and the FIT trial will explore novel prevention treatments and attenuation of age-related brain deterioration.

***Potential pitfalls****:* Lost follow-up is a potential pitfall. However, based on our experience, and the recruitment of participants from the same workplace, we will be able to contact most of them and obtain any updated contact information. The design limits our ability to determine causality and to directly explore the legacy effect of the lifestyle intervention itself for all our aims. However, we will explore the individual response induced by this intervention based on four intensive and unique RCTs with vast and various data. Previous imaging measurements of brain volume structure are available only for one out of four interventional randomized clinical trials. To face this limitation, we intend to compare the brain volume measurements to the age and gender norms of the general population. Furthermore, there is a difference in the follow-up duration among our previous RCTs. Thus, analysis adjusted for the distinct duration is necessary to prevent this bias. Additionally, selection bias may occur since all participants were recruited from their workplace, and the health of employed people is generally better than that of the unemployed population. However, approximately half of them have already retired. Moreover, we have a relatively low number of women participating, but the integrated sample size extends the absolute number of women compared to our previous RCTs.

***The strengths of this proposal:*** the proposal will provide a rich dataset based on clinical intervention and long-term follow-ups. Some of these measurements were obtained using state-of-the-art tools (e.g., MRI) at multiple time points. We will combine results from different trials but with similar inclusion criteria, and our study population was recruited from the same workplace/geographical area. Finally, our human resources – our well-trained and experienced multidisciplinary team will work together towards achieving our aims.

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