1. SUMMARY OF THE PHASE 2 PROTOCOL

Specific Aims [Protocol Section 2]: The overall aim of CALERIE Phase 2 is to test the hypothesis that two years of sustained caloric restriction (CR), involving a reduction in energy intake to 75% of baseline (25% CR), in healthy men and women aged 25 to 45, will result in the same adaptive changes that occur in rodents subjected to CR. Particular emphasis on the adaptive responses thought to be involved in slowing the aging process and protecting against age-related disease processes. Primary outcomes include core body temperature and resting metabolic rate. Secondary outcomes include triiodothyronine and catecholamines (as potential mediators of the predicted metabolic adaptation), and plasma concentrations of TNF-α (because inflammation is one of the adaptive responses suggested as a mediator of the salutary effects of CR on the aging process in rodents). An important secondary aim is to identify potential adverse effects of CR in humans. A number of exploratory aims will be assessed to evaluate the effect of CR on body composition, serum hormones, plasma growth factor concentrations, serum lipid and lipoprotein levels, skeletal muscle, adipose tissue and psychological factors. Full details are provided in Protocol Section 2. Consistency between the two sexes and across levels of body composition will be explored. In addition, biological samples will be stored in a biosample repository for future analysis.

Basic Study Design [Protocol Section 5]: The study will be conducted as a multi-center, parallel-group, randomized, controlled trial (RCT). A sample of 250 participants will be enrolled, and assigned to either the CR intervention or an *ab libitum* (AL) control group. A 2:1 allocation ratio in favor of the CR intervention will be applied in order to maximize the number of subjects receiving the intervention of greater scientific interest. Participants in both treatment arms will be followed over a period of 24 months. A comprehensive set of evaluations will be performed prior to initiating the intervention, with follow-up evaluations at Months 1, 3, 6, 9, 12, 18 and 24 after randomization. It is expected that 10% of study subjects will drop-out in each of the two follow-up years, so that a sample of approximately 200 subjects is expected to complete the study.

Study Population and Eligibility Criteria [Protocol Section 6]: Participants must be between 25 and 45 years of age (inclusive), and body mass index must be greater than or equal to 22.0 and less than 28.0 kg/m^2. Otherwise, healthy individuals from both genders and all races are eligible to participate. Volunteers will be ineligible if there are significant medical conditions (e.g., history or clinical manifestation of cardiovascular disease, diabetes, cholelithiasis or cancer); abnormal laboratory markers (e.g., elevated potassium levels, hemoglobin or hematocrit below the lower limit of normal); psychiatric or behavioral problems (e.g., eating disorders or a history of drug and alcohol abuse); concomitant medications (e.g., steroids). Never-smokers of tobacco products or ex-smokers who quit completely at least 12 months ago are eligible. Breast-feeding or pregnant women (or those intending to become pregnant before the scheduled end of the intervention) and individuals performing any kind of heavy physical activity will be excluded. Volunteers will be screened out if they are unwilling or unable to adhere to the rigors of the CR intervention or the evaluation schedule over the entire two-year period.

Treatment Interventions [Protocol Section 7]: The active intervention will target a sustained 25% restriction in calorie intake vis-à-vis *ad libitum* energy intake measured by doubly labeled water (DLW) at baseline. There will be no gradual ramping of CR, and the 25% energy reduction goal will be maintained for the entire 24 months. Control participants will be advised to continue their current diets on an *ad libitum* basis. The CR

intervention will be implemented by a multi-disciplinary team including dietitians, psychologists, and physicians. No specific diet composition will be mandated. Rather, the CR intervention will employ an algorithmic approach that combines specific nutritional and behavioral guidance so that each participant can maintain the prescribed level of calorie restriction. The approach will be tailored to the needs of the individual participant, with specific nutritional and behavioral strategies selected from an intervention "toolbox." Examples include increasing dietary fiber, modifying recipes to decrease energy density, adding novel foods to relieve boredom, strategies to avoid impulse eating, obtaining desired foods through home delivery or take-home meals, strategies for limiting caloric intake in public settings like restaurants, parties and work, and so on. Selections from the toolbox will be based on the participant's success in achieving adherence, and on problems arising at that point in time. This algorithmic approach will also provide for continuous feedback and communication between individual participants and interventionists. The behavioral component will include group sessions and individual counseling, and during these sessions, the interventionist will provide specific and individualized dietary information to help the participant adhere to the CR regimen and meet his/her calorie target.

No specific level of physical activity will be required or recommended. However, all participants will be advised of current recommendations from the Surgeon General (Centers for Disease Control) for minimum levels of dedicated physical activity. A complete daily vitamin and mineral supplement will be provided to intervention and control participants to ensure that they meet the current recommendations for these nutrients.

Recruitment and Screening [Protocol Sections 8, 9, 11.1 and 23]: Participants will be recruited at the three CALERIE clinical centers using procedures that were successful in the Phase 1 studies. Recruitment will be continuous, and generally include media advertising, direct mail, health promotion events, databases, and referral sources. An effort will be made to recruit an ethnically diverse group based on the demographics of the three clinical sites.

An initial telephone screening will record the volunteer's contact information as well as age, height, weight, and basic eligibility information. Volunteers who are clearly ineligible will be screened out at this point. Then, a staged screening process will be undertaken over a series of 3-4 visits. Exclusion criteria outlined above will be evaluated. Volunteers will meet with the study psychologist or a trained member of the behavioral team to assess any barriers to participation. A 14-day food record will be collected to assess the volunteer's ability to adhere and complete a food record continuously over a two-week period.

Randomization and Blinding [Protocol Section 10]: CALERIE participants will be assigned to intervention using a random process. A telephone-based, interactive voice-response system (IVRS) will be applied. Randomization will be stratified by sex and BMI within each clinical center, and within each stratum, subjects will be allocated in a 2:1 ratio in favor of the 25% CR intervention. A permuted block randomization technique will be applied so that the desired allocation ratio is maintained at periodic intervals throughout the recruitment process. Given the nature of CR intervention and control conditions, it is not possible to blind study participants or CALERIE staff members to the treatment assignments. Nevertheless, within the resources available to this study, intervention staff will not be engaged in evaluating participants, and evaluation staff will be blinded to the treatment assignments.

Outcome Determinations [Protocol Section 12]: A detailed series of evaluations will be performed on participants in both treatment arms at baseline and at periodic intervals during the study. They include the following: measures of energy metabolism, cardiovascular risk factors, glucose tolerance and insulin, immune function, endocrine response, qualify of life (QoL), psychological and cognitive functioning, physical activity measures, body height and weight, body composition, bone turnover, and nutrient intake. Biological material including blood, urine, muscle biopsy and abdominal fat biopsy will be collected and stored in a biosample repository for future analyses. A process is described for extending the protocol to incorporate advanced clinical endpoints as the opportunity arises. Complete details are provided in Protocol Section 12.

Schedule of Evaluations [Protocol Sections 11 and 23]: Evaluations will be performed with participants in both treatment arms at baseline and at periodic intervals during the study. Follow-up visits will be performed at Months 1, 3, 6, 9, 12, 18 and 24 following the start of the assigned intervention. The baseline visits and follow-up visits at Months 12 and 24 are the most elaborate and a complete set of evaluations will be performed. A smaller set of evaluations will be performed at Months 6 and 18, while an abbreviated follow-up will be performed at Months 1, 3, and 9. The schedule of evaluations in summarized in Protocol Section 23 and provides complete details.

Adherence Calculations [Protocol Section 14]: Adherence measures will be used both as an outcome to determine the degree of CR actually achieved, and to inform decisions about modifications to a participant's intervention program and selecting toolbox options. Adherence will be characterized as the percentage of CR achieved, $\%CR_P = 100\ [1 - (EI_P/EI_{AL(t)})]$, where EI_P represents average daily energy intake over the period of interest, and $EI_{AL(t)}$ represents *ad libitum* long-term average daily energy intake before the start of the intervention. *Ad libitum* EI will be characterized by the average of two consecutive measures of energy expenditure performed at baseline using DLW methods as described in Protocol Section 13.

We will use two versions of the intake/balance method, based on the relationship, EI = EE + ΔES, where EE is average daily energy expenditure during the period of interest and ΔES is the change in body energy stores during the period of interest. The "long-term" version will consider EE over the interval between any two time points by taking the average of the EE estimates across the time points. For intervals spanning more than two DLW measures, the average of the estimates for each interval, weighted by the duration of the interval, will be applied. ΔES will be estimated by calculating the change in energy stores (measured by DEXA) from the beginning to the end of the interval. ΔES will be calculated using standard coefficients for changes in fat mass and in fat-free mass. "Short-term" calculations will be performed at 6 months only. EE will be estimated by 14-day DLW measures; ΔES will be estimated by 14-day changes in FM and FFM, measured by DEXA at the beginning and end of the DLW measurement period.

Quality Control Procedures: [Protocol Section 15]: A detailed quality assurance plan will be developed to safeguard the scientific integrity of the study. A comprehensive Manual of Procedures will be developed, initial and ongoing training and certification of each staff member will be conducted, and each clinical site will have a Study Manger with substantial experience in clinical research operations and management of the multi-center clinical trials. S/He will supervise and oversee day-to-day operations of the clinical site, ensure adherence to the Good Clinical Practice guidelines, study protocol, Manual of Procedures, study timeline and budget. Prior to study start, the Coordinating Center

(CC), reading centers and laboratories will develop appropriate monitoring plans for their study functional areas with the guidance and oversight by the Quality Control Committee.

Formal reliability studies will be conducted for two of the central facilities, i.e., the DLW lab and the central biochemistry laboratory. Duplicate urine sample sets from the same subject at the same protocol time point will be collected and forwarded to the DLW lab for analysis. The specimens will be labeled in such a way that facility personnel are blinded to which participant (and treatment arm) the specimens correspond, and whether it is the original or duplicate specimen. Similar procedures will be applied for blood samples sent to the biochemistry lab with the exception that the duplicate samples will be drawn from the blood stored in the repository. Statistical analyses will be performed to quantify the reliability of the procedures, and whether there is a significant difference between the test and retest specimens.

Participant Safety and Adverse Events [Protocol Section 16]: Protection of subjects from risks related to the study is of paramount concern to investigators and institutions participating in CALERIE. An adverse event (AE) is defined as any unfavorable and unintended sign (including an abnormal laboratory finding), symptom, or disease temporally associated with the intervention irrespective of whether it is considered related to the intervention. Participants will be given a diary to record signs, symptoms and adverse events occurring between clinic visits. An expedited reporting protocol will be followed for reporting serious adverse events (SAEs) to the Coordinating Center, the NIA Program Official and the Data and Safety Monitoring Board (DSMB).

Clinical laboratory tests include hematology, serum chemistry and urinalysis will be performed at screening, baseline and at Months 1, 3, 6, 9, 12 and 24. A serum pregnancy test for women at screening only; otherwise, a urine pregnancy test will be performed immediately prior to any DXA evaluation. A heightened surveillance protocol for elevations in potassium and the incidence anemia will be applied. On-going surveillance for mental / behavioral health conditions as well as bone mineral density will be performed. CR participants will be removed from the intervention, either temporarily or permanently, if abnormal values develop. Periodic reports summarizing participant safety will be presented to the CALERIE Steering Committee and the DSMB. Remedial action, including additions and changes to the protocol, will be taken as appropriate.

Statistical Considerations [Protocol Section 17]: The study size is limited by the feasibility of finding eligible subjects, enrolling them into the study, and maintaining their commitment to this extensive intervention over a period of 24 months. Enrolling 250 subjects over a period of 20-24 months is thought to be feasible with the resources available. Based on the experience in the Phase 1 studies, a drop-out rate of around 10% per year is expected, so that a sample of approximately 200 subjects is expected to complete the study. Power calculations indicate that based on this sample size, clinically meaningful differences can be detected for the most important outcome variables.

This is the first detailed investigation of effects of CR over an extended period of time in humans. Many of these comparisons are exploratory in nature, and will need to be confirmed by follow-up studies. Type-II error, i.e., failing to detect a significant effect when it exists, is important to this study. Thus, all tests of significance for between-group comparisons will be performed at the $\alpha = .05$ level of significance. The primary analysis strategy is under the Intention-to-Treat principles. CALERIE is also interested in mechanistic questions concerning the effect of CR, and to address these issues, the Marginal Structural Model (MSM) of Robins and colleagues will be applied. All major

outcomes are observed repeatedly at well-defined time points over participant follow-up, so that statistical methods for longitudinal and repeated measures analysis will be applied. Subgroup analyses will be tested by evaluating the treatment by subgroup interaction. Withdrawal from the intervention, drop-out from the study, cross-over to the alternate intervention or death (if any) will be analyzed using the standard techniques for survival data.

Data Management [Protocol Section 18]: All data arising from the study will be forwarded to the Coordinating Center. A database will be created on the DCRI computer network specifically for this study. Paper Case Report Forms (CRFs) will be designed to capture all the information required for reports and analyses. Staff at the clinical sites will record the data mandated by the study on the CRFs, and a copy of the CRF will forwarded to the CC. Double data entry by two different operators at two separate occasions will be performed. As well, a variety of supplementary material and procedures will be conducted with study participants, including blood and urine samples, DXA evaluations and dietary recall. The resulting files and/or materials will be forwarded directly to central laboratories and reading centers for processing and interpretation. At periodic intervals, electronic data files containing the results of these determinations will be forwarded to the CC using a secure FTP server and merged into the master database. A series of validation checks will be conducted on the database. They will search for impossible and implausible values as well as logical inconsistencies across the different data fields. A variety of progress reports will be prepared during the course of a trial reviewed with the appropriate CALERIE working groups.

Participant Rights and Confidentiality [Protocol Sections 19 and 25]: All participant data will be kept strictly confidential, and no subject-identifying information will be released to anyone outside the project. Each participant will be assigned an anonymous study ID, which will be used on all study forms. Any study forms and paper records which do contain participant information (e.g., address lists, phone lists) will be kept at the CALERIE clinical site in secured, locked areas. At the Coordinating Center, only authorized personnel will have access to the data files containing study data. Participants will not be identified by name in any reports or publications, nor will the data presented in such a way that the identity of individual participants can be inferred.

Before initiating this study, the protocol, site-specific informed consent form (including HIPAA Authorization) recruitment materials, and other relevant information will be reviewed by a properly constituted Institutional Review Board (IRB) at each participating clinical site. A copy of the IRB approval notification and approved informed consent and HIPAA Authorization Form will be collected by the CC study monitor prior to site initiation and archived at the Coordinating Center. All CALERIE participants will provide written informed consent to participate in the study before any study-related procedures are initiated. The consent will describe the study's aims and objectives, procedures and activities to be undertaken in the study, as well as a summary the potential risks and benefits of participating. Two consents will be undertaken. First, because there is an extended screening phase to determine eligibility and many study procedures are performed during this process, the first consent will occur during the first screening visit. The second informed consent will occur after eligibility has been confirmed and the participant is ready begin the baseline evaluations.

Study Administration [Protocol Section 20]: The administrative and funding mechanism for this research is an NIH cooperative agreement (U01). The Steering Committee is the main governing body, and is composed of the Principal Investigators of the clinical

centers and the Coordinating Center as well as the NIA Project Scientist. Each member has one vote and all decisions are determined by majority vote. In addition to the Steering Committee, a number of subcommittees will be formed to provide broad direction to the study. Oversight is provided by a Data and Safety Monitoring Board. The DSMB will approve the protocol before the study is initiated; monitor recruitment, retention and adherence; evaluate data completeness and data quality; and, ensure that participant safety is addressed adequately. It reports directly to the Director of NIA, and makes recommendations on all study activities including terminating the study for safety or operational reasons.