Official Title: Modeling the Epidemiologic Transition: Energy Expenditure, Obesity and Diabetes

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A. SPECIFIC AIMS

Populations all over the world are experiencing rapid increases in the prevalence of obesity and diabetes [1-5]. To date, the public health response to the emerging obesity epidemic has been almost totally ineffective. As a first line of response, professional bodies and government organizations have issued prevention guidelines, all of which include recommendations on levels of activity energy expenditure (AEE) [6-10]. However, even if fully implemented, it is not clear that the current recommendations on AEE would impact the trend in age-related weight gain. *In fact, there is virtually no direct evidence that can be brought to bear on the question of whether the obesity epidemic has resulted primarily or even partially from society-wide declines in habitual physical activity (PA).*

To date, epidemiologic research on AEE has relied on very crude measurement tools—primarily questionnaires. These self-reported data capture only a small fraction of the total variance in activity and are potentially confounded when they focus on leisure-time PA. By contrast, direct measurement with doubly labeled water (DLW) provides a precise, unbiased estimate of all forms of non-resting energy expenditure. DLW has also been used to validate the new generation of accelerometers which are efficient measurement tools for larger studies.

In this application we request support to apply these objective measurement tools to examine the "ecology of AEE". The study will take place in 5 countries, spanning the range of the activity-obesity spectrum, and combine both within-population person-level and between-population ecological analyses. First, we will examine whether an individual's amount or pattern of AEE is related to adiposity in a diverse sample of 2,500 (i.e., 500 subjects/site x 5 sites). Second, we will test the ecological hypothesis that a decline in levels of AEE is an important cause of the rapid increases in obesity that are currently taking place in many societies. As an exploratory aim we will examine the role of selected adipocytokines and the appetite hormone ghrelin.

Accordingly, we propose to test the following Hypotheses:

1) AEE is negatively related to percent body fat in the 5 study populations at baseline, independent of dietary intake.

2) AEE is negatively related to change in body weight during follow-up, independent of dietary intake.

3) Population mean levels of AEE are negatively related to population mean levels of obesity/relative weight.

4) Adipocytokines (e.g., adiponectin and leptin) and hormones (e.g., ghrelin) influence weight regulation and insulin sensitivity and interact with AEE.

5) Heavy metals, such as arsenic, mercury, lead and cadmium, are positively associated with obesity and markers of diabetes and CVD risk.

6) Human gut and oral microbiome and plasma metabolites are associated with obesity through short chain fatty acids, a byproduct of gut fermentation of complex carbohydrates and is mediated by lifestyle and geographic location.

To test these hypotheses we will complete the following Specific Aims:

1) Recruit 500 participants, ages 25-45, from each of 5 populations (total N = 2,500); measure AEE with accelerometry; collect 24-hour dietary recalls; assess fasting glucose/insulin, ghrelin, and adipocytokines (adiponectin, leptin).

2) Measure AEE using DLW in a random subset of 75 participants from each of the 5 populations at baseline (total n = 375).

3) Repeat AEE measurement with accelerometry on all participants at 24 months; repeat weight at 12 and 24 months and body composition at 24 months.

4) Examine the relationship between AEE and body composition within and among populations at baseline and at follow-up; assess inter-relations of AEE and diet, glucose, insulin, ghrelin and adipocytokines.

5) Measure serum 25(OH)D and intact parathyroid hormone (iPTH) levels in 500 adults from the 5 METS sites (total N=2,500) at baseline and determine associations with latitude, diet, physical activity, adiposity, blood pressure (BP), and biochemical risk factors for CVD (eg, insulin, glucose, adiponectin, leptin, cholesterol).
6) Examine the association between baseline serum 25(OH)D and iPTH levels and changes in body composition and blood pressure over 6.5 years of follow-up.
7) Repeat measurement of serum 25(OH)D and iPTH at third follow-up examination (approximately 3.5 years after baseline) and measure BMD using DXA in the available cohort.
8) Identify a metabolite profile that predicts progression to IGT/T2D in diverse black populations spanning the epidemiologic transition.
9) We hypothesize that the plasma metabolite profile at baseline (2009) will be significantly associated with changes in diabetic status over the same 6-year period (2009 to 2015), i.e. normal glucose tolerance (NGT) to IGT and/or T2D.
   Examine whether this metabolite profile is common between sites, and characterize the lifestyle components, including diet, and physical activity, which may account for any country differences.
10) We hypothesize that, in addition to a baseline metabolite profile, lifestyle factors (improved diet quality, and greater physical activity) that vary according to epidemiologic transition are also associated with development of IGT/T2D over a 6-year period.
11) To evaluate the independent and combined effects of metals (As, Cd, Pb, Hg) on body composition and markers of CM risk at baseline in 2,500 individuals living in 5 diverse countries. Hypothesis 1: Baseline metals concentrations are positively associated with a) elevated adiposity / obesity and b) markers of CM risk (central obesity, impaired fasting glucose (IFG), insulin resistance (IR), hyperlipidemia, high blood pressure), adjusting for diet, physical activity, and other potentially important confounding variables.
12) To evaluate the independent and combined effects of metals (As, Cd, Hg, Pb) on incident obesity, IGT, IR and hypertension after 8 years of follow-up in the full cohort. Hypothesis 2: Exposure to metals is associated with increased risk of new-onset a) obesity, b) impaired glucose tolerance, c) insulin resistance, and d) hypertension in individuals who were healthy at baseline, adjusting for diet, physical activity, and other potentially important confounding variables.
13) To explore the independent and combined effects of metals (As, Cd, Hg, Pb) on adipose-derived bioactive molecules/hormones as intermediate markers of diabetes risk.
14) Hypothesis 3: Exposure to metals is associated with lower adiponectin/leptin ratio, adjusting for diet and physical activity and other potentially important confounding variables, and modifies the effects of adipose-derived hormones on body weight regulation, fat composition, and insulin sensitivity.
15) Examine the associations between obesity, plasma metabolites and gut/oral microbiome.
16) As a proof of concept examine the contributions of the oral/gut microbiome to the development of obesity and type 2 diabetes in sterile mice, using samples identified from in specific aim 15.

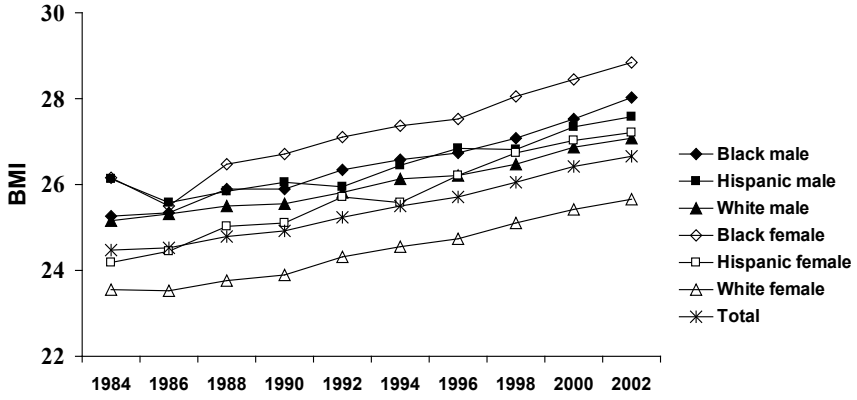
B. BACKGROUND AND RATIONALE

B.1 Physical Activity and Risk of Obesity

The energy budget is defined by the direct relationship between energy intake and expenditure. Obesity can only result from an excess of calories consumed over calories expended. It is often assumed, therefore, that a similarly straightforward, simple relationship exists between variation in AEE within the range of normal for a population and the risk of weight gain. The second conclusion which follows from this line of reasoning is that population-level weight gains taking place have resulted—at least in part—from declining AEE, and small increases in AEE for individuals will prevent weight gain. In fact, neither of these conclusions is justified by theory alone or supported by evidence. Increases in expenditure would normally be accompanied by increases in intake, while excess intake can be stimulated independently by high caloric density of food, changes in food availability, and eating patterns. As pointed out repeatedly by investigators in this field [17-20], the regulation of energy stores and body composition must be seen as a complex, dynamic process influenced by the interplay of factors that modify both intake and expenditure. An individual ultimately uses both biological stimuli, via the hormones that control satiety, and social cues to achieve energy balance [21-24]. Whether variation in patterns of activity observed in modern, free-living populations plays a key role in this process is unknown. Since recommendations to increase AEE to reduce weight gain are at the core of current public health policy this issue deserves careful study.

Large shifts in chronic disease have been associated with recognizable shifts in lifestyle—for example cigarette smoking and lung cancer, and animal fat intake and heart disease [25, 26]. "Macro-level" or ecological relationships are, of course, subject to confounding. However, these relationships must be present for an etiologic hypothesis to be correct, and their absence is a serious weakness for a hypothesis. While lifestyle trends that increase risk of obesity are obviously taking place, it is difficult to identify clear temporal associations. As clearly demonstrated in the trends from the CDC's Behavioral Risk Factor Surveillance System (BRFSS), the US experienced an abrupt upward deviation of the trend for BMI in the mid-1980s, resulting in the current "obesity epidemic" [27] (Figure 1). However, there was no temporally related break in activity patterns or eating habits [27]. In the absence of direct evidence most observers have defaulted to a "common sense" explanation that people are "eating more and exercising less". Unfortunately, this formulation is both trivial conceptually and unsubstantiated empirically. A more sober assessment was offered recently by a well-known obesity expert: *"Clearly some kind of change in energy balance has occurred, but we have surprisingly little ability to explain exactly why this has happened. Why was BMI relatively stable and why did it begin to increase fairly suddenly? Despite the intense attention paid to obesity and numerous hypotheses that have been put forward, we still lack explanations supported by data, and we lack understanding of the mechanisms driving these changes" [28].* **The central purpose of our proposed project is to test whether change in AEE can be identified as a contributory mechanism to the population-wide weight gain, and, if so, to quantify its importance.**

Figure 1. BMI Trend in US Population



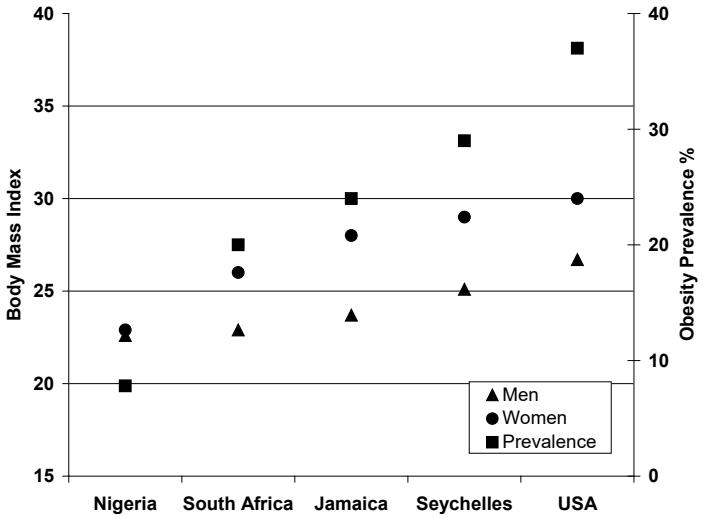
B.2 Activity Energy Expenditure and Risk of Future Weight Gain

Under experimental conditions, increases in activity *with control of energy intake* will obviously lead to weight loss. However, more relevant to population prevention efforts is the question of whether variation in normal activity mitigates the tendency to gain weight. Despite the view that this relationship is self-evident, the data are not convincing. By far, the largest number of studies has employed questionnaires. In most analyses, categories of sedentary activities (e.g., hours of TV watching) or leisure time sports are used as the

exposure measures. In two specialized cohorts, i.e., Nurses and Health Professionals, sub-sets of significant relationships were observed (e.g., TV watching and obesity, increase in vigorous PA and reduction in waist size) [29, 30]. In other prospective surveys, change in PA was an important predictor of weight change, explaining 4.4% of change in body weight in a UK cohort [31] and predicting weight change in both black and white adults over 10 years of follow-up in CARDIA [32]. Occupational activity was predictive of weight change in China [31]. An older review of 16 prospective, observational studies examining the role of PA in the prevention of weight gain among white males found inconsistent results: 3 studies reported an inverse association between baseline PA and weight change, 2 showed a positive association, 4 reported an inverse association between follow-up PA and weight change, and 7 had no association [33]. In addition, other recent large community studies have shown no association [34, 35]. Questionnaires are both insensitive and potentially biased instruments. A RECENT COMPREHENSIVE REVIEW OF VALIDATION STUDIES FOUND THAT ON AVERAGE THE CORRELATION BETWEEN AEE BY QUESTIONNAIRE AND DLW WAS ~ 0.2 (Neilson et al). The challenge in this field is clearly to move toward objective measurement tools.

DLW has been used in 4 observational and longitudinal studies with a minimum follow-up of 12 months in adults (cf. Appendix A, Table 3) [36-39]. Only 3 of these studies—including two of our own—examined weight gain in a population sample [36, 38, 39]. While all three studies found an association between AEE and body composition at baseline, variation in AEE did not predict future weight change. These results are similar to our larger pilot data on women in Chicago and Nigeria (cf. Preliminary Studies). Some interventional studies, focusing on the risk of regaining weight after dieting, have suggested that higher AEE is preventive [40, 41]. Accelerometers, either alone or in combination with heart rate monitors, have so far received limited attention in population studies of this question. While several studies have quantified activity, none have yet reported longitudinal results. A varied literature also exists among children using these methods, with similarly equivocal results (cf. Appendix A, Table 4 for summary). To our knowledge, at the present time there are no data using objective measurements that permit the "macro-level" or ecological hypothesis of AEE versus obesity to be tested.

Figure 2. Mean BMI in 5 Countries of African Origin, 25-45 year olds



The question framed in this section is at the heart of our proposal, and therefore we want to be certain it is stated with complete clarity. For sake of emphasis, we repeat the key point. Whether or not variation in AEE by itself influences body fat stores is a fundamental biological and public health question. Under normal circumstances increased activity stimulates increased energy intake which allows the organism to remain in balance. At the present time the "accepted wisdom" states that AEE added to a daily life routine of humans will reduce the risk of obesity. In fact, the available data do not support that "optimistic" view. For example, in a recent well controlled clinical trial involving 468 overweight or obese women, graded increases in exercise led to highly consistent increases in fitness, but did not result in participant weight loss [42]. This result is consistent with the general finding in the literature on this question which shows that increased AEE without intentional calorie restriction does not reduce weight [43-45].

Further, it is repeatedly stated that the environment of modern society is "obesogenic" in part because of the greatly reduced need for physical exertion [46-48]. Once again, while it seems "self evident" that industrialized societies require less AEE across a range of domains, the empirical data on this question lead to the opposite conclusion. As summarized in a recent review of the world's literature, "*The seemingly obvious conclusion that energy expenditure is systematically higher in Third World countries is not supported by the evidence… (Based on a review of the data) we conclude, therefore, that there are no systematic differences in the level of habitual activity between developed and developing countries"* [49].

While AEE has many indisputable health benefits, its role in the regulation of body weight requires careful additional study.

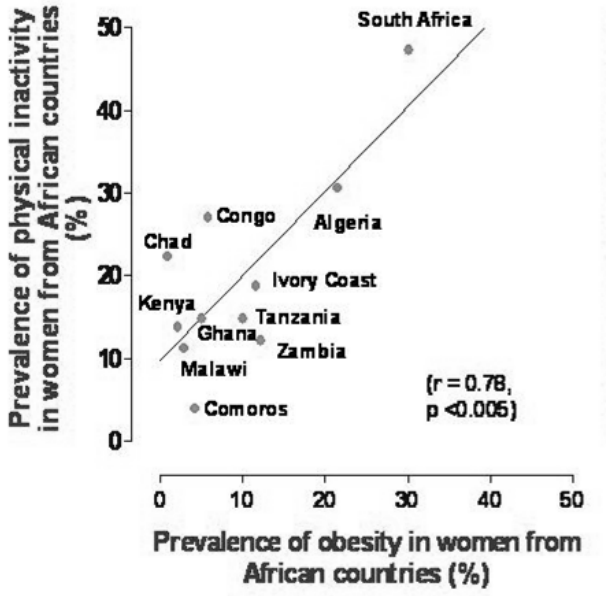
B.3 Population Trends in Weight

Long-term trends in weight have been tracked in only a few countries. Somewhat surprisingly, what has emerged, as noted above, is a long period of stability until the last two decades of the 20th century when an abrupt upturn took place. In the US, this inflection point is sharply defined at 1985 (cf, Figure 1) [27]. In Norway, for example, the upturn occurred around 1990 [50]. Many developing countries are also experiencing even more rapid increases in relative weight, which in turn have led to an abrupt up-turn in the prevalence of CV risk factors and diabetes. Trend data analyses of longitudinal cohorts in Nigeria, Jamaica, and the US demonstrate that while they all gained weight during follow-up, the rate of change varied markedly with the steepest slope in Jamaica (0.4 kg/y in Nigeria vs. 1.4 kg/y in Jamaica) [51]. The 5 countries selected for our study span the full range of the obesity spectrum (Figure 2). The rapidity of the obesity trends has been particularly striking in developing countries. We would therefore expect that the risk relationships would be most clearly delineated in that setting.

B.4 Trends in Physical Activity – The Critical Unmeasured Variable

While in the US we have very good data on obesity prevalence beginning in the 1960's, there are no comparable population-level trends in AEE or total EE [31]. Trends in occupation and transportation clearly suggest declining activity, however, this trend has been going on for over three-quarters of a century in industrialized countries while the upturn in obesity is much more recent [31]. Data from a recent WHO project on a subset of African countries demonstrate an emerging relationship between questionnaire-based levels of inactivity and obesity (Figure 3); while intriguing, these results are driven primarily by one country. Questionnaires, as the sole direct source of information, are clearly limited. For example, data from the US suggest that leisure-time AEE has *increased* consistently since 1989 while the prevalence of obesity has almost doubled [52]. Belatedly, we have come to recognize that surveillance for AEE will be crucial to unravel the causes of this epidemic, and it must be conducted using objective measures.

Figure 3. Physical Inactivity and Obesity in the AFRO Region



A recent comprehensive review by a panel of experts advising the National Heart, Lung, and Blood Institute (NHLBI) highlighted the importance of this missing piece of information needed to solve the puzzle of the rise in obesity. First, they noted that while reduced activity and increased consumption have obviously fueled the current epidemic, *"the decrease in energy expenditure over the last decade and its contribution to the obesity epidemic have not been quantified"* [53]. The second missing data item, in many ways the complement of the first, will also be addressed by this study: *"Insufficient data are available to determine the amount of physical activity needed for the primary prevention of weight gain*" [53]. An estimate of this "permissive minimum," which we view as being analogous to the "recommended daily allowance" of nutrients, is needed to guide public health policy and recommendations. We highlight these issues because of our near-universal experience that many in the epidemiology community have come to view DLW as a novelty or specialty tool rather than a robust measurement technique that could be more widely used in population research. We are proposing, to the contrary, that an important new set of questions can be addressed with systematic use of these tools. Given the dramatic decrease in cost for DLW, this is a particularly propitious moment to pursue this neglected agenda.

B.5 Assessment of Energy Balance

The premise of this project rests on the assertion that epidemiologic research has not yet applied sufficiently rigorous methods in studies of PA. Questionnaires are both limited in scope—because they focus on sub-domains, such as leisure activity—and biased, because a sedentary lifestyle has taken on increasingly negative social connotations and will influence responses [54, 55]. The only technique that measures total EE directly in free living people relies on stable isotopes, i.e., doubly labeled water (DLW). Total EE is the sum of resting EE and voluntary PA. The 3rd component, the thermic effect of food, is only about 10% of EE in adults and thus numerically minor. Subtracting resting EE and a fixed estimate of the thermic effect of food yields EE in PA. Resting EE can be measured using a metabolic cart or estimated from lean body mass (cf. Methods for details). In contemporary populations, variation in time spent standing or "fidgeting" may therefore be a major contributor to non-resting EE, and it cannot be measured with questionnaires [56]. Recent data from *Health ABC*, a NIH-sponsored study of the elderly that was one of the first to use DLW in an epidemiologic setting, demonstrates the value added by this technique. In that cohort, questionnaires captured much less than half of the variation in AEE [55].

The *Health ABC* study also demonstrates the potential value of objective measurement of AEE as an exposure variable. Among the 302 community-dwelling elderly individuals, the hazard ratio for all-cause survival was 0.3 in the top vs. bottom tertiles of EE [55]; this large survival advantage was not captured by the questionnaire, demonstrating the enormous gain in information observed when objective, precise measures are applied.

Considerable effort has now been invested in making better accelerometers and validating them against DLW. In preparation for this grant we conducted a comprehensive review of the published literature (see Appendix A, Table 1). The current generation of triaxial accelerometers is now sensitive, robust and easy to use. Based on 4 separate studies (total n = 91), the weighted correlation between kcal/day from the accelerometer and DLW was 0.62 (see Appendix A, Table 2) [57-60]. On-going methodological work (cf D.4.1.1) should further improve precision and make it feasible to move this research forward rapidly.

Data on energy intake (EI), and as well as macronutrient composition (eg, % of calories from fat, carbohydrate and protein) are also important for assessing energy balance. Measurement of intake in free-living people can be difficult, however [61-67]. Food frequency questionnaires (FFQ) are known to have important limitations, both in estimation of total calories and composition [68]. Furthermore, no single FFQ would be appropriate for our heterogeneous populations. Multiple twenty-four hour recalls are therefore the best option for international comparisons, even though they are labor-intensive [69-72]. Energy adjustment will also improve estimation of composition [68, 73]. Likewise, intake of manufactured foods, soda, etc, which are measured with less error may be key aspects of an obesogenic environment, especially in the context of rapidly modernizing societies.

B.6 Rationale for the Proposed Study Design

In our experience, studying AEE to gain insight into the pathogenesis of obesity remains controversial. We therefore describe explicitly the logic of the hypothesis testing we propose. As noted, energy balance can be described in a simple equation with two variables, viz, in a weight stable individual energy intake = energy expenditure (EI = EE). How energy stores are actually regulated, and correspondingly dysregulated in obesity, is much more complicated, however. For most individuals, obesity results from the gradual accumulation of excess energy stores over many years. In the US, average weight gain is ~1.5–2.0 lbs/year, which is equivalent to an excess of ~30 kcal/day [74]. This can result from excess intake, reduced expenditure, or a combination of the two. The rationale for this project is based on the assumption that persons with low levels of AEE are, on average, at increased risk of accumulating excess energy stored as fat. About 70% of total EE is required for resting metabolism and there is relatively little variation in resting metabolic rate/kg among people; AEE accounts for most of the between-person variation. Alternatively of course, since weight gain represents the final balance of EI and EE, consistent over-consumption could also be a co-factor or the determining factor. Assuming both are operative, persons in the quadrant of "low AEE", "high EI" would be at the highest risk. EI is difficult to measure in free-living people. The hypothesis which can be rigorously tested, because precise, objective measures are available, is whether baseline PA is related to future change in

weight. Since EE and EI are not perfectly balanced (if they were there would be no change in weight over time), EE will have a separate, independent influence on change in energy stores. Given that AEE is a highly stable trait (cf. Section C), persistence of low AEE will lead to future increase in weight, and this can occur in combination with, but independent of, excess EI.

We acknowledge that the energy balance models presented here are simplified to allow valid hypothesis testing. We submit, however, that the basic model is no different from established risk factor mechanisms. Thus, a diet high in saturated fat and cholesterol leads to a gradual increase in serum LDL over a lifetime, independent of other modifying factors and without the assumption that diet composition continues to change over time. The same logic applies to the impact of a high salt diet on blood pressure. In our framework low AEE is therefore being characterized as a classic chronic disease lifestyle risk factor. ***AEE should be detectable as a risk factor when measured with precision in an adequately powered sample.***

B.7 Rationale for Cross-Population or "Ecological" Studies

We have now missed the historical moment when obesity risk factors changed in the US. By sampling from global populations across the range from low to high risk, we can compress the time dimension and model this transition with standard epidemiologic methods. In the past, this "population as the unit of analysis" design has made crucial contributions to research in common disease. The key role of nutrition as the underlying cause of the CHD epidemic was first recognized by ecological comparisons [75]. The "Seven Countries Study" subsequently demonstrated the relationship of individual nutrients to atherosclerosis [75-78]. "Intersalt" employed a similar design to study mineral intake and blood pressure [79, 80], and our recently completed "International Collaborative Study on Hypertension in Blacks" (ICHSHIB) used population comparisons to examine a broad range of risk factors and physiologic processes in hypertension (cf C.2.3) [81]. Similar studies have suggested etiologic factors for breast, colon and stomach cancer. The study proposed here will extend this methodology into the area of obesity for the first time. The advantages and disadvantages of this design are outlined in general terms in the section below and in more detail under Methods (Section D).

Descriptive epidemiology is usually the only available tool that can be used to examine etiologic processes in chronic disease. For many "common source" diseases, however, methodological problems undermine its effectiveness. Within a homogeneous society some degree of exposure may be universal, and the relevant quantitative variation can be hard to characterize. This is often complicated by large *within-person* relative to *between-person* variation in the exposures which makes person-level analyses imprecise. The large contrasts obtained by comparing social groups as the unit of analysis and the marked shifts that occur among migrants can help focus attention on the key exposures. For example, in ICSHIB low correlations were noted between BMI and Na with BP at the individual level (.01-.15); however, these rose to 0.85 when populations were the unit of analysis. As with all study designs, however, there are trade-offs in cross-cultural studies. For example, a concern sometimes raised is the "ecologic fallacy," whereby group-level relationships are falsely inferred to be causal at the individual level [82]. We are well aware of this potential threat and address it further in the analytic section of the Methods. With consistency at both levels and control for confounding, however, these relationships can be inferred with confidence. Likewise, once a strong hypothesis has been established, larger within-population studies can be justified.

B.8 Adipocytokines, Appetite Hormones and Regulation of Body Fat Stores.

Once considered metabolically inert, adipose tissue has now been shown to secrete multiple cytokine hormones [83-85]. In addition, other components of the system that regulates body fat stores have been identified [21, 86]. Despite initial enthusiasm, from this long list—which includes, among others, leptin, adiponectin, ghrelin, resistin, visfatin, omentin, NPY3, serum amyloid A, TNF-α, RBP4, IL-6, IL-10—only for the first 3 is there reasonable evidence of a physiologic function in regulating fat mass or influencing EE [22, 87, 88]. Leptin accurately reflects fat mass, and, while not an "adipostat", appears to function at the lower range to prevent depletion of calorie reserves [89]. Adiponectin is a potent insulin sensitizing agent, and in prospective studies—including our own—it predicts onset of glucose intolerance [90, 91]. Surprisingly, adiponectin is not higher in women than men despite higher body fat, and it varies cross-culturally with BMI [92], suggesting a more complex role that has not yet been defined. Ghrelin, which is secreted by the stomach, clearly

contributes to appetite control but is more complex to study in observational cohorts [93, 94]. Although data in the literature are inconsistent as to whether or not these hormones, in concert with insulin, play a role in the regulation of energy balance [95-103], four recent studies suggest associations with EE independent of body composition for all three, adiponectin, leptin and ghrelin [104-107], and we will measure them in all subjects. Additional inter-relations to EE from our pilot data are described in section C below.

B.9 Summary

Although the "obesity epidemic" has received enormous attention in recent years, the mechanisms through which the energy budget is being perturbed are still not well understood. In particular, the role of declining AEE as a causal mechanism in population-wide increases in relative weight deserves much greater attention. Despite widespread perception that variation in normal activity levels (as opposed to voluntary increases in high-intensity activity, as typical of trials) is correlated with change in weight, the data do not support this conclusion. Research in this vital area has been handicapped by lack of access to precise objective methods of measurement. A new generation of epidemiologic research will be required before we can base causal inferences or public health recommendations on something more than assumptions. As with other chronic CVD, international comparisons could be particularly informative.

The test of our study hypothesis will be "two-sided". That is to say, the presence or absence of an association can be given a clear interpretation. Of course, given the body of evidence that increased AEE has health advantages, absence of an association would not be taken to suggest that AEE is of no value. Rather, the correct interpretation would be that declining AEE is unlikely to be the driving force behind the rise in obesity, nor will modest increases in AEE be adequate to reverse the trends.

C. PRELIMINARY STUDIES

C.1 Experience in International Collaborative Research

C.1.1 Experience of the PI

The applicant is a nutrition epidemiologist with a primary interest in AEE and obesity. Her doctoral thesis involved the development of improved laboratory methods for the analysis of stable isotopes to measure AEE [108-111]. At Loyola she has been involved in large-scale cross-cultural studies comparing CV risk status, nutrition and AEE in populations of African descent in Nigeria, Jamaica and Chicago [112-120]. She is currently PI on a grant to define AEE and obesity risk in women in rural Nigeria and Chicago; the preliminary data from Nigeria-Chicago comparisons provide part of the framework for this study. This project represents the largest cross-cultural application of DLW to population samples. As part of a related project, she has collected resting EE data on 2,500 individuals in Nigeria, Jamaica, and the US—the largest such sample ever studied [114, 121].

In Chicago she has supervised studies enrolling more than 2,000 African-American and Mexican-American participants over the last 6 years. She recruited and supervised bilingual staff and established effective working relationships with communities and local institutions. The PI is also working as a consultant with the Tropical Medicine Research Institute of the University of the West Indies in Kingston, Jamaica, on an International Atomic Energy Agency-sponsored project [118]. The project is designed to assess EE and intake in both rural and urban older adults using the DLW method and their relationship to parameters of glucose tolerance in populations at elevated risk of diabetes.

C.1.2 Experience within the Department

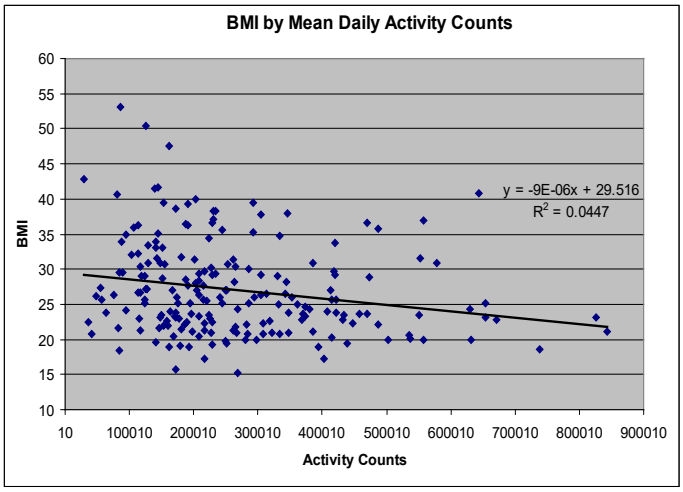
The applicants have extensive experience in collaborative research. In 1992, the Department initiated the largest international study of hypertension yet to be conducted, involving 12,000 participants in 8 countries of African origin [112, 122-124]. This research project has produced comparable population prevalence estimates, new insights into physiologic pathways, estimates of disease burden from prospective cohorts, and numerous genetic analyses [125-128]. The study has been continuously funded by the NIH for 15 years. The PI has participated actively in all aspects of this collaborative program and has extensive field experience in

Africa and the Caribbean. The Department of Preventive Medicine has additional collaborative research in Mexico, Cuba and Puerto Rico.

C.2 Previous Research on Physical Activity, DLW, Body Composition and Weight Change

C.2.1 Physical Activity by Accelerometer

Figure 4



Accelerometers have been used by the applicants to measure AEE in all of the study sites; in addition, our consultants, Drs. Ekelund and Brage, have published extensively on the methodology and validation of accelerometry [129-134]. In preparation for this application, preliminary data have been collected in all 5 populations using the proposed AEE protocol (Table 1). In each of the field sites, men and women were recruited and wore the accelerometer (Actical, Respironics/Mini-Mitter, Bend, OR) for a minimum of 7 consecutive days.

Our experience with the accelerometers was overwhelmingly positive at each of the sites. Acceptance of the instruments by the participants was uniformly high, there were no instances of instrument failure with accompanying loss of data, no reports of excessive participant burden, and virtually no missing data due to non-compliance by participants (cf. D4.1.1 for further details). Over the course of 3 months we enrolled 198 adults. As can be observed in Table 1, there is a range of mean activity EE measured by the accelerometers across the sites with the highest levels recorded among Jamaican and South African males and the lowest among women from Nigeria and the Seychelles.

Table 1. Participant Characteristics by Site – mean (SD)

	Jamaica	Maywood	Nigeria	Seychelles	S. Africa
n (M/F)	41 (18/23)	36 (16/20)	38 (20/18)	45 (22/23)	38 (16/22)
Age (yrs)	37.9 (8.6)	38.3 ± 10.1	38.9 ± 13.2	35.2 ± 5.7	27.2 ± 5.0
BMI					
males	23.6 (3.5)	27.3 ± 6.5	22.5 ± 2.9	26.6 ± 3.3	21.8 ± 2.0
females	30.6 (5.3)	33.7 ± 9.4	26.2 ± 6.4	26.9 ± 6.4	29.8 ± 7.3
Activity Counts					
males (cts/d)	364040 (158584)	313988 ± 217868	241191 ± 103401	275078 ± 130813	379480 ± 159859
females	209277 (94637)	280929 ± 175078	181312 ± 105667	170670 ± 76242	293548 ± 182584
AEE (kcal/d)					
males	1050 (306)	1090 ± 387	688 ± 253	902 ± 254	911 ± 329
females	884 (306)	1090 ± 566	608 ± 230	617 ± 292	1000 ± 514
AEE/kg (kcal/kg/d)					
males	15.06 (3.76)	13.25 ± 5.71	10.45 ± 3.83	11.45 ± 3.08	14.37 ± 5.35
females	11.13 (3.75)	12.42 ± 5.66	9.14 ± 3.88	8.73 ± 3.16	13.29 ± 6.06
AEE adj. for wt					
males (kcal/d)	1094 (271)	985 ± 426	761 ± 262	866 ± 239	1011 ± 319
females	838 (287)	970 ± 532	660 ± 220	667 ± 227	999 ± 467
Pop. Mean BMI*					
males	23.7 (4.5)	26.7 (5.5)	22.6 (4.5)	25.1 (4.4)	22.9 (6.0)
females	28.0 (6.4)	29.8 (8.1)	22.9 (6.0)	27.6 (6.3)	27.1 (9.1)

* Population mean BMI for 25-45 year olds based on representative sampling

The absolute activity EE levels are supported by data on the % of time spent in sedentary vs. moderate activity, with Jamaican and South African males, who were primarily laborers, spending less time in sedentary activity than Nigerian or Seychelles women, as recorded by the accelerometer (Table 2). There was an inverse association between activity counts and BMI (Figure 4) indicating a cross-sectional relationship between adiposity and PA. We acknowledge that heterogeneity exists by gender, but caution against interpretation of sub-groups given the small sample size.

Due to varying degrees of literacy amongst our populations, we did not require participants to record activity in a log while wearing the accelerometer. We did, however, have participants in Nigeria and the Seychelles complete the Global Physical Activity Questionnaire (GPAQ) via interviewer. The purpose of the GPAQ is to obtain internationally comparable data on self-reported, health-related AEE done in the past 7 days. The correlation for activity EE (kcal/d) between the GPAQ and the accelerometer, r = 0.51 ($p<0.002$), did not differ significantly between Nigeria and Seychelles. In both sites the GPAQ overestimated AEE by as much as 145 kcal/d. The GPAQ or other comparable questionnaire used in conjunction with accelerometry can provide useful information on types and patterns of PA.

Table 2. Average Percent of Awake Time Spent in Varying Levels of Physical Activity by Site

	Jamaica	Maywood	Nigeria	Seychelles	S. Africa
			(Mean ± SD)		
Sedentary Activity (%)	43.3 ± 11.3	46.6 ± 15.6	57.7 ± 13.6	53.0 ± 11.3	40.9 ± 20.7
males	39.7 ± 8.9	46.3 ± 14.4	56.8 ± 14.4	49.6 ± 9.5	41.5 ± 18.4
females	46.0 ± 12.4	46.8 ± 16.8	58.7 ± 13.1	56.3 ± 12.1	40.4 ± 22.7
Light Activity (%)	31.9 ± 7.4	27.9 ± 6.1	22.3 ± 6.3	28.8 ± 6.0	33.6 ± 11.2
males	30.3 ± 8.9	27.7 ± 5.6	21.7 ± 7.2	29.9 ± 5.7	32.2 ± 9.5
females	33.1 ± 5.8	28.0 ± 6.6	23.0 ± 5.4	27.7 ± 6.2	34.5 ± 12.4
Moderate Activity (%)	24.7 ± 9.9	25.2 ± 12.7	19.9 ± 8.4	17.9 ± 6.2	25.1 ± 11.5
males	29.6 ± 9.0	25.5 ± 11.9	21.4 ± 8.4	20.0 ± 4.8	25.4 ± 11.9
females	20.9 ± 9.0	24.9 ± 13.5	18.3 ± 8.4	15.9 ± 6.7	24.9 ± 11.5
Vigorous Activity (%)	0.13 ± 0.42	0.40 ± 0.78	0.05 ± 0.13	0.30 ± 0.65	0.47 ± 0.82
males	0.28 ± 0.60	0.58 ± 1.09	0.08 ± 0.18	0.52 ± 0.85	0.84 ± 0.90
females	0.01 ± 0.04	0.25 ± 0.37	0.01 ± 0.02	0.09 ± 0.23	0.20 ± 0.65

C.2.2 Doubly Labeled Water Studies

C.2.2.1 DLW & Accelerometer in South Africa

In South Africa, DLW was used to assess free-living total daily EE in a small sample of adults (n = 27). Of this total, 18 also wore the Actical accelerometer. The characteristics of the participants wearing the accelerometer and undergoing DLW did not differ from those listed in Table 1 for South Africa. The total daily EE measured using DLW was 2600 ± 655 kcal/d and using the accelerometer was 2775 ± 990 kcal/d; the correlation was r=0.6. This pilot study was conducted in Khayelitsha, the community in which we propose to carry out this larger study. The correlation between total EE measured using DLW and the accelerometer was comparable to our weighted correlation calculated from published literature (Appendix A, Table 2), giving us confidence that the accelerometer is measuring the same domain in the field setting that it is in more tightly controlled laboratory settings.

Table 3. Participant Characteristics – Loyola-Ibadan Women's Study - mean (SD)

	Nigeria (n = 150)	US (n = 180)
Age (y)	32.9 (11.4)	33.9 (10.8)
Weight (kg)	58.1 (12.5)	83.6 (21.6)
BMI	23.0 (5.1)	30.9 (7.7)
Fat-free mass (kg)	40.4 (5.3)	48.9 (8.6)
Fat mass (kg)	17.9 (8.8)	34.8 (15.4)
% Body Fat	29.3 (8.6)	39.8 (9.0)
Total EE (kcal/d)	2262 (398)	2442 (462)
Resting EE (kcal/d)	1284 (157)	1400 (212)
PAL (TEE/REE)	1.77 (0.27)	1.75 (0.24)
AEE/kg (kcal/kg/d)	13.5 (6.0) *	10.0 (3.8)
Adj. AEE (kcal/d)	810 (311)	765 (299)

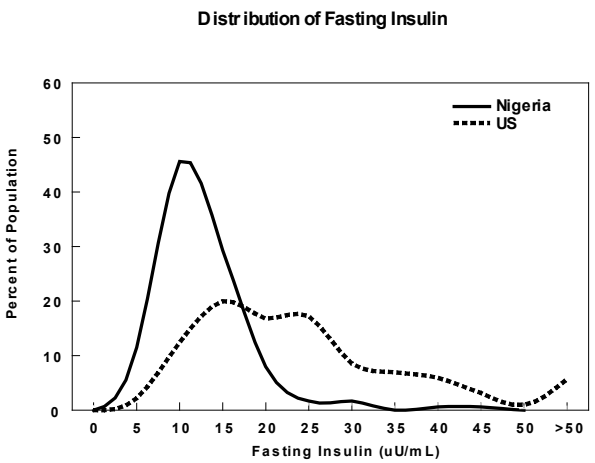
* $p<0.05$

C.2.2.2 Physical Activity and Weight Change

The Loyola-Ibadan Women's Study was initiated in 2000 to study the relationship between resting EE, AEE, and weight change in women from two contrasting social settings. In both metropolitan Chicago and rural Nigeria, women were enrolled in a longitudinal survey in which EE was measured at baseline using DLW, and weight and body composition were measured for 3 consecutive years (Table 3) [135]. Mean change in weight during the follow-up interval was 0.73 and 0.76 kg/y for the Nigerian and US women, respectively. Contrary to our hypothesis, no association was observed between activity EE at baseline and weight change in either site [136]. This result underscores our prior assertion that variation in AEE in free-living populations has not been convincingly shown to predict risk of obesity. Unfortunately, the sample size in this cohort is somewhat small, and we were limited to two sites.

C.2.3 Energy Expenditure, Body Composition and Markers of Chronic Disease

Our international collaborative study on hypertension has led to new insights on the relationship between EE, body composition and markers of chronic disease. In our initial survey, we noted a robust correlation between resting EE and BP. This observation was subsequently confirmed in a total sample of 1,567 individuals from independent samples in Nigeria, Jamaica and the US [121]. Resting EE is in turn strongly correlated with weight and BMI, since adiposity leads to an obligatory increase in muscle mass required for movement. Multivariate analysis subsequently confirmed, however, that the impact of resting EE on BP is independent of BMI and weight. In fact, no measures of adiposity remain significant after accounting for resting EE [121]. This observation suggests that at least one important mechanism by which obesity leads to increases in BP is through the correlated increase in muscle mass and resting EE. To further test this hypothesis, we recently completed a person-level meta-analysis of body composition and BP in 18,072 individuals from the populations we propose to include in this study [137]. Persons at the upper end of the BMI distribution have higher percent body fat, thus a unit increase in BMI leads to a smaller increase in lean mass than a comparable increase in BMI among the lean. Under the assumption that it is the increase in lean mass that influences BP, the slope of the BMI-BP relationship should be steeper among the lean. In meta-regression analysis of 13 population samples, we, in fact, observed a strong negative relationship between mean BMI and slope of the BMI-BP correlation for both SBP and DBP ($p < 0.001$). This relationship had never been detected in studies of individual populations and demonstrates the value of studies that sample from a broad range of social contexts. Using this international comparative design, we have also detected novel relationships between BMI and physiologic variables (e.g., leptin and angiotensinogen) as well as impacts of environmental factors on the heritability of common traits (e.g., height, BP, and BMI) [137]. We have also reported on the relationship between PA, measured using DLW, and blood pressure; in a small sample of Nigerian, Jamaican, and African-American adults, activity EE, independent of body size or composition, was found to be inversely associated with both SBP and DBP [48].



The Loyola-Ibadan's Women's Study allowed us to examine the relationship between components of the energy budget and hormones and cytokines associated with obesity and glucose homeostasis. Along with measurements of total, resting and activity EE, fasting blood samples were obtained from the women in both rural Nigeria and suburban Maywood. Among the significant associations recorded was an inverse association between activity EE and fasting insulin after controlling for body size and composition among the Maywood women ($r=-0.24$, $p<0.05$), but none among the Nigerian women. This lack of association among the Nigerian women is likely due to the relatively modest variability in insulin levels compared to the Maywood cohort (10±6 vs 24±19 μU/mL). As can be observed in Figure 5, the distribution of insulin concentrations is much narrower among the Nigerian than the US women. These data illustrate the obvious importance of AEE in maintenance

of glucose homeostasis. Interestingly, in regression models resting EE was a significant determinant of both leptin ($p<0.005$) and adiponectin ($p<0.05$) concentrations in both cohorts of women after controlling for body composition and age. In addition, resting EE was inversely associated with ghrelin in the Nigerian cohort ($p<0.01$). These observations require confirmation. It has been hypothesized that high adiponectin may be protective of the increased risk of obesity that theoretically accompanies low resting EE [106], however, our group demonstrated recently that there was no association between low resting EE and subsequent weight gain[138]. This preliminary data does suggest a strong link between hormones and the energy budget and deserve further investigation in larger samples.

C.2.4 Dietary Intake Across the 5 Sites

Dietary intake data have been collected in each of our 5 study sites. In the US and Jamaica our group has significant experience in the collection and analysis of 24-hour recall and FFQ data with analysis completed using the Nutrient Data System for Research (NDSR) [139-141]. In Nigeria, until very recently a nutrient database specific to Nigerian foods was not available and thus dietary analysis was limited to foods and food patterns; a site-specific database is now available and nutrient analyses will be conducted on previously collected 24-hour recalls [142]. In the Seychelles, our co-investigator has been working with dietitians at the Ministry of Health to create and validate a nutrient database specific to Seychelles using the UK system, WISP (personal communication, S. Romain & P. Bovet) [143, 144]. In South Africa, our new co-investigator Dr. Steyn, has extensive experience with dietary intake measurement at the national level [145] and developed a system with colleagues at the MRC to improve estimation of portion sizes, the Dietary Assessment and Education Kit (DAEK); there is a South Africa-specific nutrient database available.

In Table 4, the most frequently consumed foods, assessed by repeated, multiple pass 24-hour dietary recalls, are presented for each of the 5 study sites.

Table 4. Most Frequently Reported Foods and Energy Intake by Site – 24-hr Dietary Recall Data

	Jamaica (n = 270)	**Maywood (n = 197)**	**Nigeria (n = 125)**	**Seychelles (n = 243)**	**South Africa (n = 563)**
Instrument	24-hr recall	24-hr recall	24-hr recall	24-hr recall	24-hr recall
Most frequent	Brown bread	Soft drinks	White bread	Rice	Sugar
#2	Cheese	Chicken	Pepper stew	Sugar	Maize porridge
#3	Rice	Potatoes	Cow peas	Whole milk	Bread
#4	Beans	White bread	Vegs-greens	White bread	Potatoes
#5	Chicken	Beef	Beef	Tea	Milk
Energy intake (kcal/d)	2130	2520	2475*	2150	1860
Fat (% kcal)	31%	41 %	26%*	26%	26%

* estimated from sub-sample of recalls

It is of interest to note – illustrating the rapid environmental change developing countries are undergoing - that in rural Nigeria 8 years ago white bread accounted for only 2.3% of the foods, compared to 24.9% in 2007; cassava, the most frequently reported food in 1999, was not in the top 5 in 2007 (unpublished observation, A. Luke). In Seychelles, there was a 17% decrease in the consumption of fresh fish and concomitant increase in dairy and red meat intake between 1993 and 2003 (personal communication, P. Bovet).

D. EXPERIMENTAL DESIGN AND METHODS

D.1 Project Overview

D.1.1 Participant Recruitment and Baseline Exam

Population-based samples, ages 25-45, will be drawn from communities in 5 countries: Nigeria, Seychelles, South Africa, Jamaica and the US; 500 per site, 2500 total. Based on our overall experience with

longitudinal studies, we experienced 2% annual attrition in Nigeria and 10% in the US site, we anticipate no more than 20% attrition by the 24-month examination, resulting in a final 'N' of at least 400 per site (Table 5).

D.1.2 Hypotheses to be Tested

1) PA is negatively related to percent body fat in the study populations at baseline, independent of dietary intake.
2) PA is negatively related to change in body weight during follow-up, independent of dietary intake.
3) Mean levels of AEE are negatively related to mean levels of obesity/relative weight among the populations.
4) Adipocytokines and hormones influence weight regulation and insulin sensitivity and interact with AEE.

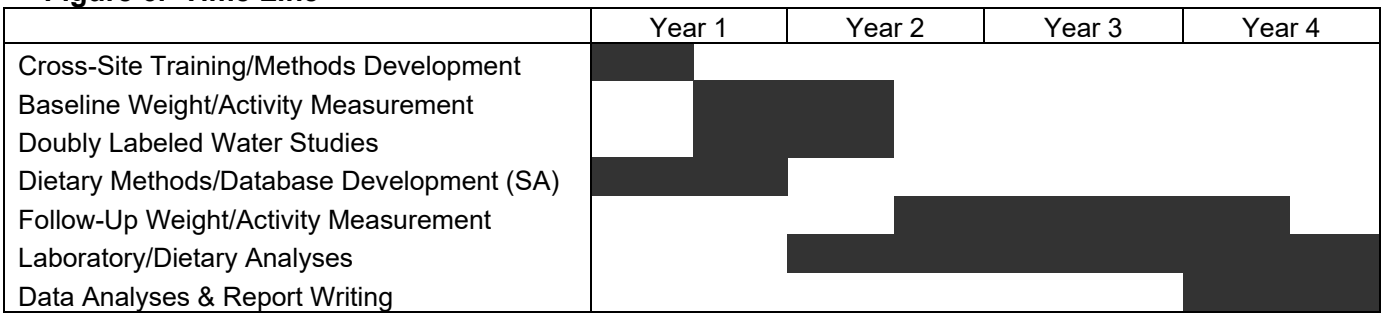
Table 5. Proposed Study Measures

	Baseline (N)	12-Mo Follow-Up (N)	24-Mo Follow-Up (N)	Use of Data
Physical Activity				
Accelerometer	2500		2500	Exposure
DLW	375			Concordance, exposure
Questionnaire	2500			Exposures
Body Composition				
BIA	2500		2500	Outcome: baseline & change
Isotope Dilution	500		500	Calibrate BIA change data
Dietary Intake	2500			Covariate
Anthropometrics	2500	2500	2500	Outcome: baseline & change
Blood pressure	2500			Outcome
Adipocytokines/Hormones	2500			Outcome

D.2 Study Organization and Time Line

The Department of Preventive Medicine in Chicago will serve as the coordinating center. During the first 6 months, a detailed protocol will be finalized, and staff from all sites will come to Chicago for a group training session. Staff at each site will be responsible for translating and back translating all patient materials, including consent forms and questionnaires, into the local language. These materials will be piloted on 10 people at each site in the first 6 months. The DLW protocol and concurrent accelerometer baseline protocol for the 75 selected individuals/site will be conducted between month 6 and 18 in all sites. The baseline accelerometer protocol for the additional 425 participants/site will be carried out over the course of a year beginning at 6 months. The follow-up examinations will begin at 1.5 years, for a 2.0-year period. As discussed previously, these will involve measuring weight for all 500 participants/site at 12 and 24 months of follow-up with accelerometer and body composition measurements at 24 months of follow-up. Laboratory analyses of the DLW samples will commence at approximately year 1. Deuterium analyses for body composition assessments will be conducted in years 1 and 4. Fasting hormone and glucose samples will be collected at the baseline examination and stored at -80C; the samples will be analyzed in year 4. Primary data analysis and report writing will be conducted in year 4. Figure 6 shows a graphical representation of the previously discussed timeline. A detailed timeline can be found in Appendix B.

Figure 6. Time Line



Communication between investigators will be maintained by monthly conference calls coordinated by the Loyola group. The project coordinator and/or PI will travel to each site annually for site visits with staff and co-investigators.

D.3 Clinic Overview

D.3.1 Clinic Examinations and Data Acquisition

Each of the sites has been conducting community-based CV research and has community-centered clinics from which the present study will be based (see below). For participants enrolled in the DLW/deuterium protocol, the initial clinic examination will last approximately 4 hours. They will return to the clinic, or be visited by a staff member at their home, on day 5 (± 1 day) to provide a midpoint urine sample and to ensure the accelerometer is comfortable and being worn properly. They will be asked to return to the clinic on day 9 (± 1 day) to provide a final urine sample and have the accelerometer removed. For the participants enrolled in the accelerometer protocol, the clinic examination will last approximately 1 hour. A staff member will check on each participant on day 2 to ensure that the accelerometer is comfortable and being worn properly, and the participant will return on day 9 to return the accelerometer and have a final body weight measurement taken.

All anthropometric measurements, BP, and blood collection will be made at the initial clinic examination, with participants fasted from the evening prior. Demographic data and medical history, including age, gender, season, birth place, occupation, education, tobacco use, alcohol use and pregnancy history, will also be collected at this time. At 12 and 24 months after the baseline examination, all participants will be recontacted for follow-up clinic examinations.

D.3.2 Sampling Procedures

In each of the 5 sites, 75 individuals aged 25-45 years will be recruited for the DLW protocol and 425 will be recruited for the accelerometer protocol, with equal numbers of men and women. We will exclude individuals with obvious chronic or infectious diseases (including active malaria), pregnant or lactating women, and HIV positive individuals (see D.3.3 for HIV testing procedures). Any individual that has a condition preventing normal AEE will also be excluded from our study. Population-based surveys have been carried out in each of the sites; the sampling procedures used previously will be employed in the present study. These study sites were chosen because mean BMIs and prevalence of obesity span the healthy physiological range.

Jamaica – Spanish Town is an urban area 25 km from the center of Kingston. Once the capital of colonial Jamaica, Spanish Town is an established community with a population of 92,000 in 1991; about 45,000 individuals are between the ages of 25 and 74. A community-based study of hypertension and diabetes (n=3500) has been completed [123]. Districts were randomly sampled; beginning from a fixed point in each district (e.g., the north-west corner), door-to-door screening took place. Examinations will be carried out in the local health clinic and specimens taken immediately to the Univ. of the West Indies for processing.

US – Maywood is an African-American working class community adjacent to the western border of Chicago, Illinois. Approximately 3,500 participants have been examined in our clinic since 1991 for both observational and interventional studies [123]. Participation rates for observational components of these studies have been 60% or greater. Project staff are residents of the community and conduct all recruitment and home visits. We have now contacted individuals on approximately two thirds of the blocks in Maywood and are well known to the community. The Department clinic is on campus, within walking distance of most neighborhoods and is well known to local residents. We will also recruit from the adjoining west side of Chicago.

Nigeria – Developed in 1965 as a teaching site for the University College Hospital (UCH), University of Ibadan, Igbo-Ora is a rural community with a population of 50,000 in the Yoruba-speaking region of southwest Nigeria. All permanent houses have been mapped and assigned numbers by public health workers. Most residents are subsistence farmers or petty traders. Loyola has been engaged in collaborative research with the UCH Dept of Social & preventive Medicine since 1992 and maintains a permanent facility in Igbo-Ora. Ten-thousand adults are being followed in a prospective cohort study on hypertension, and 3,000 family members have been enrolled in genetic studies [123, 124, 126, 127]. Prior studies on EE and obesity have been carried out in this community.

Seychelles – The Republic of Seychelles is an archipelago with 81,000 inhabitants located approximately 1,500 km east of Kenya in the Indian Ocean, and approximately 2,000 km north of the island of Mauritius. Population-based surveys in 1989 and 2004 have shown increasing prevalence of several cardiovascular risk factors in the adult population [146, 147]. The sampling method will be a sex- and age-stratified random sample generated from the national census.

South Africa – Khayelitsha, the 3rd largest township in South Africa, is adjacent to the city of Cape Town. The population is about 500,000 people with 80 percent of the residents living in shacks and 40 percent unemployed. There are no hospitals in Khayelitsha, but there are three community health clinics. Investigators in Cape Town have recently been funded to conduct a population-based 5-year prospective study on AEE and diabetes in 1000 members of this urban community. A sex- and age-stratified random sample will be generated from the national census.

D.3.3 HIV Testing in South Africa

The prevalence of HIV in the rural regions of Nigeria in which this study will be conducted is less than 2% [148], and less than 1% in the Seychelles [149]; however, the prevalence is as high as 12% in the Western Cape, South Africa [150]. The hyper-metabolic state produced by HIV infection can significantly impact body weight well before other signs of infection appear. In order to eliminate the impact of HIV infection, we will work with the community health clinics in South Africa in which study enrollment will take place. All participants will be tested for HIV and undergo pre- and post-test counseling both before study enrollment at baseline and again at follow-up (cf. E.2.2 Protection Against Risk). The officials at LifeLine Clinic in Khayelitsha, CapeTown, have agreed to counsel, test, and treat all participants in the screening process of this study. All the above options are free of charge in South Africa as a result of both national and international efforts. HIV-positive individuals will be excluded from our study.

D.4 Measurement Procedures

D.4.1 Activity Energy Expenditure

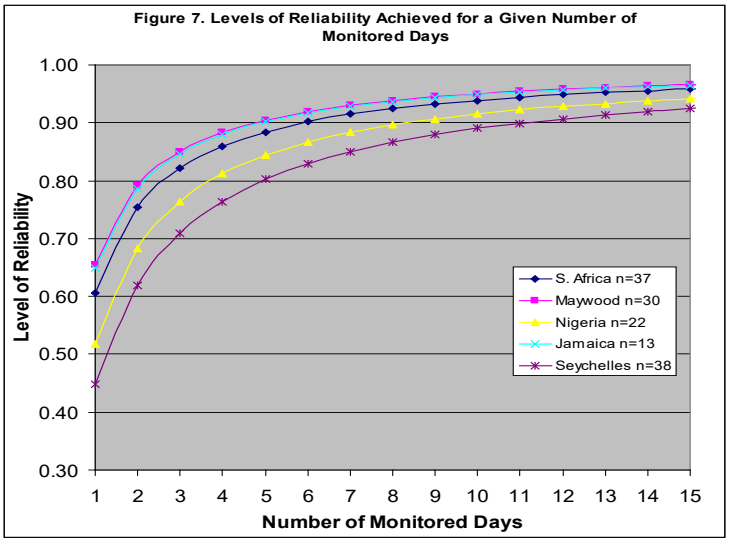
Total daily EE is comprised of three components: resting EE, AEE, and thermic effect of food. In this study, we are most interested in AEE and its relationship to weight change and insulin sensitivity. AEE will be measured by two methods: accelerometers and DLW. All participants in each site will wear an activity monitor for 6-9 days, while a subset will undergo the DLW procedure as well. Our previous experience with measurement of EE indicates that both resting EE and AEE are very stable traits, with within-person correlations of 0.9 and 0.8 at intervals of 1-2 years, respectively [135]. For those participants in the DLW

subset, resting EE will be measured by indirect calorimetry, and thermic effect of food will be estimated as 10% of total daily EE. All methods are described below.

D.4.1.1 Physical Activity by Accelerometer

PA will be measured using the Actical activity monitor (Respironics/Mini-Mitter, Bend, OR) with all participants. Previous studies have shown that accelerometer-based activity monitors can discriminate differing intensities of activity [151-156], making it possible to adequately characterize each of the study communities with regard to overall intensity of PA. We will record the data as activity counts/min and kcal/d. The activity monitor records the intensity, duration and frequency of physical motion through the use of an accelerometer which produces a variable electrical current based on the combination of the amplitude and frequency of motion. Accelerometers are omnidirectional motion sensors that count the vertical and horizontal acceleration of the user. This information is stored within the instrument as activity counts per epoch, or specified subunit of time, e.g., per minute. As the intensity of the activity increases, so does the number of activity counts per epoch. The Actical monitor is lightweight, waterproof and made to be worn on the wrist, waist or ankle. Consistent placement of the activity monitor ensures comparability of data. For this study, the monitor will be worn at the waist. There are concerns about the ability of the accelerometer to discriminate between some activities with differing energy expenditures, eg, stair climbing versus level walking. As described below, our consultants are developing methods to improve accuracy of these instruments.

There is controversy over the number of days of recording needed to adequately capture habitual activity levels; the suggested range is from 4 to 9 days [152]. We used the Spearman-Brown prophecy formula with calculated, site-specific intraclass correlation coefficients [157] to estimate the level of reliability achieved for a given number of days of monitoring in each site (Figure 7) [152]. Most studies use 0.8 as an acceptable level of reliability [152, 158-161]. For our data, all sites achieve this level with five days of monitoring (ie, excluding the first and last partial days of monitoring which are dropped from analysis), however the Seychelles are on the borderline. Given the practicality of six days of monitoring (i.e., participants will receive the monitor and return the monitor on the same day of the week), we have chosen to monitor all participants for six days (i.e., a total of 8 after taking the two partial days into consideration). With six days of monitoring, the expected level of reliability will be 0.83-0.92.



The Actical activity monitor will be initialized for each participant on the morning of the initial clinic examination, using the on-site computer. The participant's ID, epoch period (1 minute), start date and time, and mode will be entered into the main program. Use of the activity monitor will be explained to the participant: the participant will be asked to wear the activity monitor at all times over the next 8 days, including during sleep; the only time the monitor should be removed is while bathing, showering, or swimming. The activity monitor will be placed on the participant on a belt at the waist, positioned just behind the left hip bone. The participant will be trained in proper handling of the accelerometer, including removal and placement of the monitor on the belt as well as placement of the belt on the body, in order to ensure accurate measurements. If the participant has problems sleeping with the monitor, they will be encouraged to remove it during sleep hours. An additional belt will be provided in case it gets wet and needs to be swapped out. Staff members will contact participants within 24 hours after the initiation of the monitoring session to ensure that there is no discomfort with the monitors. In addition, the monitor will be checked at the midpoint home or clinic visit to ensure proper placement and recording. On day 8, each participant will return to the clinic, and the staff member will verify end date and time and download the data. Data will be presented as activity counts per minute for the whole day and during hours the participant is awake, as well as kcal/day or METs/wk in AEE as calculated using the accompanying software [153, 155, 156, 162, 163].

Investigators at each site have significant experience with accelerometer-based activity monitors and with the validation of monitors using the DLW method. Staff will be collectively trained on the use and handling of the Actical. In addition, we will validate the Actical activity monitor for estimation of energy expenditure in AEE at each site through the comparison with DLW results. Validation of this instrument will help in future studies for the quantification of AEE at significantly lower cost than available with DLW.

Over the years, we have had extensive experience with several models of accelerometers, activity monitors, and heart rate monitoring devices. Missing data is a well-documented problem with activity monitors in general and can result from instrument failure or non-compliance of the participant, i.e., the monitor is simply removed for part of the measurement period. In our experience, all cases of truncated data collection (which occurred in less than 2% of participants) had at least 4 days of data collected over the 7-9 day measurement period, providing at least some data for analysis. Methods of handling missing data are further discussed in the analytical section.

The consultants on this project, Drs. Brage and Ekelund, are currently working on advanced methods of analysis for data generated from accelerometers. They have demonstrated that converting the output from accelerometry to units of acceleration (in m/s^2) from counts provides substantial additional information by capturing speed of motion [130]. After adjustment for built-in frequency filtering by the specific accelerometer model, acceleration is linearly associated with activity counts, as well as with EE. Brage and Ekelund are also developing methods using Hidden Markov Models and other mathematical approaches to improve the accuracy of accelerometer data, particularly with regard to recognition of patterns of activity that allow further differentiation among activities. The DLW data from this project will therefore help improve the accuracy of accelerometery as a tool in survey research, while improving the precision of the data collected in our project.

D.4.1.2 Physical Activity by Questionnaire

All participants will have AEE assessed by questionnaire, in their own language, using the Global Physical Activity Questionnaire version 2 (GPAQ) [164]. The GPAQ was developed by the World Health Organization (WHO) as part of the WHO STEPwise approach to chronic disease risk-factor surveillance (STEPS manual available online at http://www.who.int/chp/steps/manual/en/) [165] to produce reliable and valid estimates of physical activity for use in culturally diverse populations in developing countries. Approximately 50 developing countries are currently using the GPAQ for physical activity data collection. The main outcome variables are: a categorical variable of total physical activity (high, moderate and low) and a continuous variable of total physical activity within each domain of work, transport and leisure.

D.4.2 Body Composition

D.4.2.2 Bioelectrical Impedance Analysis

Body composition will also be assessed in all participants using BIA. BIA measures the impedance to the flow of an applied mild alternating current by body tissues. The measured impedance of body tissues can be used to calculate total body water, from which fat-free mass and fat mass can be calculated [170]. BIA is a relatively inexpensive method to estimate body composition in populations.

The participants will be placed in the supine position with limbs abducted. Current-supplying electrodes will be placed on the dorsal surfaces of the right hand and foot at the metacarpals and metatarsals, respectively. Detection electrodes will be placed at the pisiform prominence of the right wrist and the anterior surface of the true ankle joint [170]. The single-frequency instrument (BIA Quantum, RJL Systems, Clinton Township, MI) will be attached to electrodes and generate an excitation current of 800 μA at 50kHz.

The investigators on this application have extensive experience in the application of BIA in field situations and have developed equations for use in Nigerian, Jamaican and African-American adult and child populations [112, 119]. Unless evidence from the ID analyses, described above, indicates a need for the development of separate equations for the other African sites, we propose to use the pre-existing validated BIA equations. The ID data will be used to calibrate BIA for change in body composition within individuals over the 2-year follow-up.

D.4.2.3 *Dual X-ray Absorptiometry (DXA)*

Participants will undergo a bone mineral density measurement using dual x-ray absorptiometry (DXA). The bone scan takes approximately 30 minutes and will require the participant to remove all metal jewelry and piercings. To ensure women are not unknowingly pregnant, they will be asked the date of their last period, given a pregnancy test and will be excluded if they are pregnant. Individuals with pacemakers will be excluded.

D.4.3 Anthropometrics

At the initial clinic visit, height, weight, waist and hip circumferences, and blood pressure will be measured. Weight of the participant will be measured without shoes and dressed in light clothing to the nearest 0.1 kg using a standard balance (Health-o-meter, Bridgeview, IL). Height will be measured using a stadiometer without shoes and head held in the Frankfort plane to the nearest 0.1 cm. Waist circumference will be measured to the nearest 0.1 cm at the umbilicus. Hip circumference will be measured to the nearest 0.1 cm at the point of maximum extension of the buttocks. For both waist and hip circumferences, repeat measures will be taken. If two measurements differ by more than 0.5 cm, a third measurement will be taken.

D.4.4 Dietary Intake

Dietary intake is not the primary focus of this application, however, we recognize that estimates of EI and diet composition data may improve the analysis of energy balance. We are cognizant of the challenges involved in comparing dietary intake across multiple populations [69-72, 171]. Using both INTER-MAP and EPIC as models, we will attempt to collect and measure dietary intake, with appropriate quality control measures, in these 5 populations at varying stages of the epidemiologic transition. We regard this as an additional contribution to the long-term effort to collect international comparative diet data for epidemiologic studies, and one that will continue to be refined.

The dietary component of this project will be directed by Dr. Steyn at the Medical Research Council (MRC) of South Africa. Dr. Steyn has extensive experience in assessing the diets of multiple countries in sub-Saharan Africa [145, 172-175], and has developed a system for portion size estimation in collaboration with a MRC colleague which has been utilized in South Africa and Kenya (DAEK, [176]). Quality control measures will include centralized training for all interviewers led by Dr. Steyn (cf. section D.5); training will include discussion and documentation of site-specific prompts and portion sizes for the dietary interviews, decision on standardized coding of the recalls, and methods for the audiotape recording of the interviews. We will use the five indicators suggested by INTERMAP to assess performance quality: evaluation of audiotapes, number of foods coded, coding error rates, duration of the recall and total energy intake (kcal or kJ) per recall [171]. Dr. Steyn and/or a post-doctoral fellow will travel to each during pilot training to insure proper implementation of the protocol. The Nutrient Data System for Research (NDSR; U. Minnesota, Minneapolis, MN) will be modified and utilized for nutrient analysis. The first year will be spent setting up the NDSR to accommodate those foods/recipes local to each site using the currently available site-specific nutrient databases and the previously collected 24-hour recall data (cf. Table 4). In the 3 African countries, this will not be an excessive number of foods – eg, in Nigeria only 75 different foods/food mixtures mentioned in over 200 collected recalls.

In brief, each participant will complete at least two 24-hour recalls using the multiple pass method, one at the initial baseline examination and the second when the activity monitor is collected. The DLW subset of participants will have 3 dietary recalls - the third recall conducted at the midpoint urine sample collection. With these multiple recalls, usual intake distributions of nutrients can be estimated (cf. Section D.7.1.3; [177-179]. At each site, the 24-hour recalls will be collected by trained interviewers using pen and paper; an audiotape recording will also be made of the recall interviews and used to augment the written record where necessary, as well as for quality control. The 24-hour recalls will be scanned and sent via e-mail to the MRC in South Africa where data entry and analysis will take place. Endpoints of interest will be total EI and macronutrient composition (ie, % kcals from fat, carbohydrate and protein), as well as indicators of intake of processed foods (ie, # of sweetened beverages, pre-packaged foods and restaurant fast foods per day) and of intake of fruits, vegetables and site-specific staples (cf. Table 4). While we recognize the importance of micronutrients for

overall health and well-being of individuals, many nutrient databases are lacking sufficient data on local food micronutrient content.

D.4.4 Blood Pressure

Blood pressure will be measured using the protocol and training procedures developed for our ongoing international hypertension studies. Blood pressure will be measured using the Omron Automatic Digital Blood Pressure Monitor (model HEM-747Ic, Omron Healthcare, Bannockburn, IL, USA). With the antecubital fossa at heart level, three pulse and blood pressure readings will be taken.

D.4.5 Biochemistry assays

Participants will be asked to fast from the evening prior to the baseline clinic examination. One 10-mL lavender-top (adipocytokines, ghrelin and insulin) and one 5-mL grey-top vacutainer (glucose) will be drawn for fasting blood samples. The blood samples will be processed and plasma separated within two hours of collection and stored at -20C (in the field) and -80C in the laboratory. Plasma will be aliquotted into 6 cryovials. Fasting plasma glucose will be measured using the glucose oxidase method at each site at the time of collection using the instrument available in each collaborator's laboratory. Two vials will be shipped or carried with the PI, along with the DLW samples, to Loyola for the assays. Insulin, total ghrelin, leptin and adiponectin will be measured using RIA kits (Linco Research, Inc., St. Charles, MO). In addition, a small plasma aliquot will be shipped to the Swedish Metabolomics Unit for metabolite analysis from all 2,500 participants. The analysis will focus on known, as well as novel metabolites linked to obesity and type two diabetes mellitus. Again, samples from all sites will be batched together, and blinded replicates will be included. Our experience with the insulin and leptin RIA indicates an intra-assay CV of <8%, ghrelin is <6% and adiponectin is ~9%. The 3 remaining cryovials will remain in the laboratory of origin in case of shipping mishap or laboratory error.

a. Participants will have a blood sample drawn to assess vitamin D status.

b. Participants will provide a stool sample.

c. Participants will provide a sputum sample.

D.4.6 Mouse model

Following the identification of obesity-related fecal/oral microbiome in the METS participant samples, and as a proof of research concept, fecal/oral microbiome samples, collected in D.4.5, will be transplanted in sterile mice, who will be evaluated for the development of adiposity and type 2 diabetes. All the mice work will be preformed at Division of Endocrinology, Metabolism and Molecular Medicine, Northwestern University, under the supervision of Dr Brian Layden. Specifically, male mice will have their gut microbiota ablated by oral gavage of ampicillin over a 2 week period. After the final oral antibiotic dose, mice will have the fecal transfer, once a week for 4 weeks. At the end of each week, mice will receive an intraperitoneal glucose tolerance test, in addition to adiposity measurements. Mice will be sacrificed after 4 weeks for tissue collection. This proof of concept study has been approved by the Northwestern University Institutional Animal Care and Use Committee

D.4.7 Skin Pigmentation

Participants will have their skin pigmentation measured by a handheld DSM II ColorMeter at the inner upper right and left arms.

D.5 Centralized Training of Staff and Certification

In the first 6 months, after protocols and procedures have been approved by the investigators, a centralized training session for all staff will be held in Chicago. Over the course of one week, all staff will be

trained by previously certified project coordinators on procedures to be used for the study. All methods have been employed in previous studies at the coordinating institution, Loyola University. The project coordinator from Loyola will be responsible for the training and certification on the DLW and accelerometer methods, and anthropometric, body composition and blood pressure measurements. Dr. Steyn and her coordinator will be responsible for training and certification on the dietary intake methods.

D.6 International Transport of Samples

International shipment of biological samples has become complicated by security precautions. Based on prior experience, it has been decided that samples to the US, i.e., DLW and plasma samples, will be carried by investigators as they travel to/from the annual investigator meeting if there is evidence of problems with shipping, e.g., from Jamaica. After appropriate aliquotting, the samples will be packed on dry ice in insulated shipping containers and checked as baggage. In the case of Nigeria and South Africa, no such problems have arisen to date; therefore, samples will be shipped directly on dry ice by an express shipping company. For DLW samples transported to Loyola, they will subsequently be re-packed on dry ice and shipped overnight to the University of Wisconsin in Madison, Stable Isotope Core Laboratory. Duplicates of all samples will be stored in the laboratories of origin.

D.7 Statistical Considerations

Statistical analyses will be supervised by Dr. Durazo. He will generate reports on accrual, missing data items, and alerts (if any). With the assistance of the PI and the programmer, he will undertake the analyses outlined below. A data coordinating center (DCC) will be established to manage and administer data transfers between sites. Data quality control and assurance processes will be put in place by the DCC.

D.7.1 Statistical Analysis

In the initial stage of the analysis, a complete set of descriptive tables will be produced by gender within site. The distributional properties of each variable will be examined and appropriate re-scaling undertaken, if necessary. All outliers (i.e. > 3 SD's) will be identified and either verified as correct or set to missing. When possible, internal validity will be assessed by the use of bivariate scatter plots of variables known to be correlated (e.g., resting EE vs. BMI or weight vs. BP). These plots provide a useful search procedure for unanticipated outliers and/or non-linear relationships.

As in any other longitudinal study, some measurements may be unavailable in a subset of the study participants. A brief description of methods used to handle non-ignorable missing data are presented at the end of this section (cf. D.7.4)

We describe below the analytic procedures and the power calculation, in relation to the following 4 primary hypotheses as stated in the Aims:

1) AEE is negatively related to % body fat at baseline, independent of dietary intake.
2) AEE is negatively related to change in body weight during follow-up, independent of dietary intake.
3) Mean levels of AEE are negatively related to mean levels of obesity/relative weight among the populations.
4) Adipocytokines and hormones influence weight regulation and insulin sensitivity and interact with AEE.

D.7.1.1 Weight and Body Composition are Related to AEE at Baseline

D.7.1.1.1 AEE, Weight and Body Composition

Two measures of AEE will be available. At baseline, we will have 500 participants/site with data from the accelerometer, inclusive of those who have both DLW and accelerometer (total N, all sites = 2500; 6 days

of monitoring). We will have data generated by DLW for 75 participants/site (total N, all sites = 375). The structure of the analysis will be similar with both exposure measures.

We anticipate that data for all the primary outcomes will not conform entirely to a normal Gaussian distribution. Using BMI, PAL and AEE data collected in previous studies in Maywood (n = 567) and Nigeria (n = 735), we examined the probability distributions. A formal test statistic was performed that combines kurtosis and skewness [180] as implemented in STATA with p-values less than 0.001, suggesting deviations from normality. Kernel density estimates of the BMI, AEE, and PAL distributions are depicted in Figure 7. After applying the ladder of power approach suggested by Tukey [181], we found that BMI, AEE and PAL for both sites appear to deviate from normality. Although no transformation is preferred after application of the ladder of powers method, two potential transformations are suggested by this approach, namely log X and X^{-1}. Tukey's approach followed by formal testing will be carried out once data on all subjects in the study has been collected and a decision about data transformations has been made. Despite the lack of normality exhibited and corroborated by the statistical tests performed, a visual exploration of Figure 7 suggests only slight deviations from normality, mostly due to well-known skewness of these distributions. Thus, we anticipate the statistical methods that make use of normally distributed data will be appropriate to analyze these data. Of course, the degree of appropriateness of these methods will depend on their robustness to deviations from normality. If it is necessary, appropriate transformations will be carried out before statistical tests are performed.

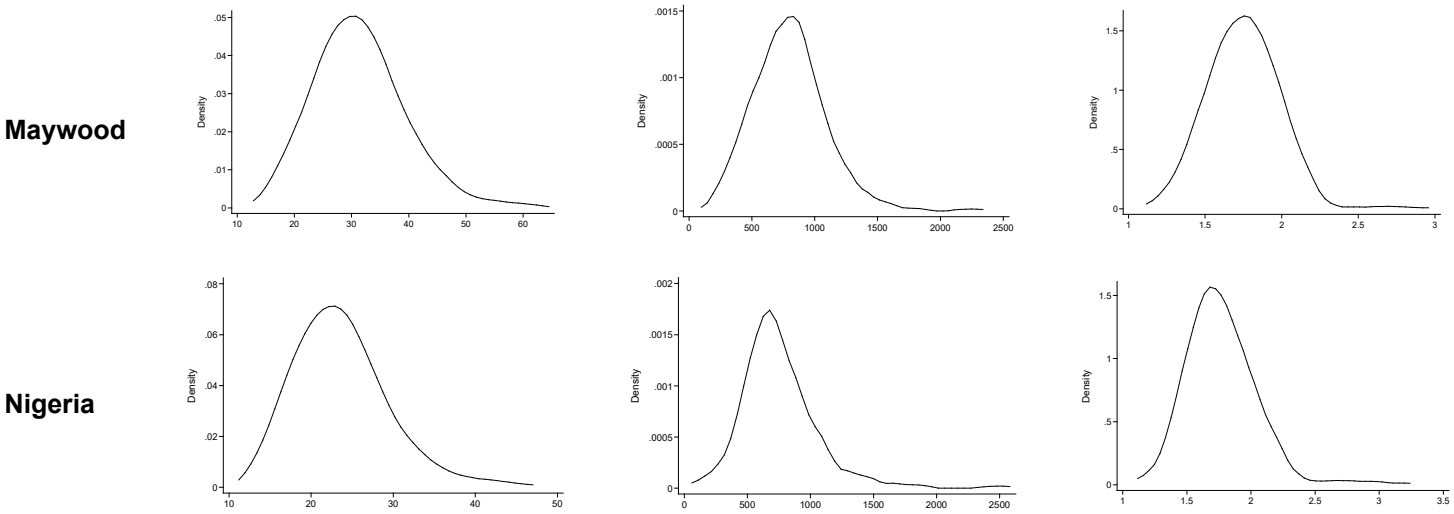


Figure 7. Distribution estimates of main analysis variables BMI, AEE, and PAL in two sites: Maywood (US) and Nigeria.

Following transformation of the appropriate exposure and outcome variables, Fisher's Z-transformation test will be applied in order to test hypotheses involving correlations. The resulting parametric test will be more powerful than other distribution-free approaches. The results will be corroborated by applying Spearman rank correlations and another parametric test based on the concordance coefficient introduced by Lin [182] and later extended by Chinchilli and colleagues to include repeated measures [183]. Lin's concordance correlation allows the comparison of the correlations accounting for possible differences in the mean values of the two variables under consideration. Furthermore, approximate permutation tests [184, 185] will be implemented using each of the three statistics: Pearson, Spearman and Lin's concordance, to compare agreement of the association between AEE and BMI across the three groups under investigation.

Correlation coefficients will be estimated using the Pearson correlation and confidence intervals constructed after applying the Fisher's Z-transformation. Furthermore, computer intensive statistical methods, such as bootstrapping, will be used to construct confidence intervals for both parametric and nonparametric approaches. Comparisons of these correlations will be carried out using this statistic for 1 and 2 samples [186], as well as approximate permutation tests [184, 185]. In addition, multivariate regression analysis will be fit to adjust for potential confounders. Confounders will include age, gender, and any other significant

predictors defined by the univariate analyses. Potential non-linear functions will be explored to see if they fit the data better. Sub-group analyses will be undertaken, with participants in each site divided by gender.

We have no evidence to suggest that the relationship between AEE and BMI is non-linear in the parameters. However, we will perform all our analyses using multivariate linear regression methods allowing for non-linearity of the covariates. Standard statistical methods based on the F-statistic and R^2 will be used to decide the best fitting model to our data. It is anticipated that there will be more than one "best model" obtained. We will choose one based on two approaches. First, subject matter considerations will eliminate some models. Second, a computer intensive approach based on bootstrap sampling will be implemented in a fashion similar to that introduced by Sauerbrai and Schumaker [187] to aid automated variable selection.

Repeat accelerometer measures will be made in all individuals at the 2 year exam (with estimated 20% attrition, N=2000). We will use these data to assess tracking of AEE levels and identify potential cross-site variation in seasonal effects, etc. A second measure will offer important information about potential between-site heterogeneity that might help explain anomalous findings (i.e., weak or absent relationships at a particular site). If appropriate, we could also use this data in a "regression-dilution" type of analysis that corrects for the underestimation of exposure-outcome effects from "noise" in individual exposure levels.)

D.7.1.1.2 AEE, Adipocytokines and Diabetes

We will have measures of fasting glucose and insulin, adiponectin, ghrelin and leptin on this unique sample of 5 populations of African origin. The following additional variables will be collected: age, gender, place of birth, prior medical history, occupation, education, height, weight, activity by questionnaire, pregnancy history, and season of exam.

In the analysis phase we will first compare the descriptive characteristics in each population (e.g., mean adipocytokine/hormone values and prevalence of hyperglycemia). We will explore the univariate correlation structure for the continuous variables listed above. We will then construct multivariable regression models to assess the potential independent role of AEE as a predictor of risk factor status independent of body composition. Risk factor status will be examined as continuous and categorical outcomes. For instance, how is insulin level affected by AEE after accounting for body composition?

An important focus of these analyses will be looking for potential heterogeneity of risk relationships across sites. Three main aspects are of interest, namely whether the AEE-risk factor (e.g. insulin level) relationship is present, or of the same magnitude, in each sample and whether there exists co-variation with the mean level of AEE; testing for interaction will be undertaken where appropriate. Precisely,

Relative effect of AEE on risk factor: We will examine the magnitude of the association using 1) regression coefficients; 2) standardized regression coefficients. This approach allows within and between site comparisons. It is recognized that cross-site comparisons could be problematic when using standardized regression coefficients since the standard deviation of the variables involved may differ across sites. In this case, standardized regression coefficients will be obtained using pooled estimates of the mean and standard deviation.

Co-variation of the association with mean level of AEE: Multilevel models will be applied, in which the site specific variable, mean AEE, will be used to estimate the effect of the average AEE on the regression coefficient.

Interactions between AEE and site: Multiplicative interaction terms will be included in regression models to assess how much site modifies the association between AEE and the risk factor. We are fully aware of the lack of statistical power to test this type of hypotheses and thus results will be exploratory in nature. Thus, one might anticipate that anthropometric and AEE measures are stronger in low risk population samples than in the US. In addition, we will test whether the slope of the exposure-outcome relationships are influenced by the mean BMI of the population using multilevel regression models.

Questionnaire data on AEE will be summarized using methods previously developed [188]. Mean activity levels and prevalence of inactivity will be assessed in relation to objective measures of EE and the relevant outcome variables. As a sub-analysis we will describe types of activities reported to assess overall population patterns.

D.7.1.2 AEE and Weight Change

The study design contemplates 3 weight determinations over time: one at baseline, a second after 12 months, and a final assessment 2 years after entering the study. To better understand the associations between AEE and body composition, the analysis plan will be carried out in incremental steps corresponding to the degree of sophistication of the statistical methods used.

1) Descriptive: Weight change is computed for each subject as the subject's last weight minus baseline weight. The correlation between weight change and AEE will be computed.
2) Regression of weight change (as computed above) on AEE will be performed, adjusting for potential confounders of the association.
3) AEE will be dichotomized at the median value, resulting in high and low AEE groups. Weight change will be estimated for each individual as the slope of the regression of weight on time (0, 12, and 24 months). Average weight change across AEE groups will be compared. That is, individual regression lines of weight on time will be obtained for each individual, and the average regression among "Low PA" will be compared to the average regression among "High PA" subjects.
4) Multilevel analysis (i.e., mixed effect models), which generalizes the previous methods, will be used in our final analysis. This approach will allow for the use of all the available information (i.e., there will be no need to categorize AEE values), thus improving the statistical power [189]. Furthermore, this method allows one to account for the within-subject correlation of the weight determinations and to account (adjust for) subject-specific covariates that may confound the associations. In addition, the dependence of weight change on AEE values can be specifically modeled by the use of random coefficients. Specifically,

$$
\begin{aligned}
&Level\ 1: \quad W_{ij} = \beta_{0i} + \beta_{1i} T_{ij} + \varepsilon_{ij} \\
&Level\ 2: \quad \beta_{0i} = \gamma_0 + \gamma_1 AEE_i + \Omega X_i + \xi_{0i} \\
&\qquad\qquad \beta_{1i} = \alpha_0 + \alpha_1 AEE_i + \Psi Z_i + \xi_{1i} \\
&\varepsilon_{ij} \approx N(0, \sigma^2);\ \xi_{oi} \approx N(0, \delta_0^2);\ \xi_{1i} \approx N(0, \delta_1^2);\ corr(\xi_{0i}, \xi_{1i}) = \delta_{01} \\
&corr(\varepsilon_{ij}, \xi_{0i}) = corr(\varepsilon_{ij}, \xi_{1i}) = 0 \\
&i = 1,...n; \quad j = 0,1,3
\end{aligned}
$$

Where $T_{i1} = 0$, $T_{i2}=12$, and $T_{i3}=24$ correspond to the three times at which weight will be measured for the i-th subject; X_i, and Z_i are potential confounders; and Ω and Ψ are model parameters associated to the confounders. Confounders will include demographic variables and EI variables (e.g. total intake, percent calories from fat, etc). This approach corresponds to a multivariate nutrient density method suggested in the literature [73]. The effect of type of food most frequently consumed (e.g. vegetables, industrially processed foods, etc) will be accounted for by including dummy variables in our multivariate regression models. Note that the parameter β_{1i}, in the Level-1 equation, represents the weight change of the i-th subject. Thus, the Level-2 equation for this parameter models the dependence of weight change on AEE after adjusting for other covariates. A similar interpretation for the intercept parameter, β_{0i}, holds. In both instances site-specific factors could be included.

The multilevel approach can be extended to accommodate time-varying covariates, which we will apply to investigate the effect of changing AEE on weight change. In addition, the effect of AEE in the i-th subject (ΔAEE_i) on weight change will be assessed by replacing AEE_i in the Level 2 step of the multi-level analysis above by (ΔAEE_i).

Finally, one additional co-variate capturing exposure information will be created which will define the pattern of AEE. After examining the within-person distribution over the monitored days (eg, within-person SD) we will use both continuous and categorical transformations to characterize "consistent vs variable" patterns. Our pilot data suggest substantially more consistent patterns in Nigeria vs. the US and this may impact on the AEE-weight relationship.

D.7.1.3 Comparison of Mean AEE Levels Among Sites

The 2 sample t-test will be used to compare means of the primary continuous variables, e.g., BMI, PA, weight, and height. Relevant co-variates will be identified from the within-site analyses and added using multivariable models [186, 190]. We recognize that some modifying factors within sites may not have the same significance across sites, and we will be very careful how variables are chosen for these analyses. Finally, we will test whether the regression slopes of the PA-BMI relationship varies significantly among any pair of sites (i.e., test for interaction).

The association between mean levels of AEE and mean levels of obesity/relative weight will be assessed using regression analysis, following a meta-regression type of approach. That is, mean AEE values will be regressed on mean obesity/relative weight values, weighting by the inverse of the squared standard error of the mean AEE values [191]. This is a widely-used approach in meta-analysis [192]. It is recognized that EI is an important potential confounder of the associations at the individual level. We will estimate the median (and other quantiles of the distribution) usual intake of nutrients (e.g. fat, percent fat) using methods developed by the ISU research group [179, 193] and include them in the meta-regression models.

D.7.2 Power Analysis

A statistical justification for the selected sample size is provided for each aim of the study according to the suggested analytical plan. Sample size estimation for each of these hypotheses will be obtained, and the maximum number required to test them with sufficient power (80% or higher) will be selected.

1) PA is negatively related to percent body fat and CV risk factors in the study populations at baseline.

It has been previously estimated (cf. Preliminary Studies) that the correlation between PA, measured with DLW, and body composition is between -0.6 and -0.5. A correlation of -0.33 or smaller (larger in absolute value) will be detectable with 83% power and maximum probability of type I error alpha = 5% using a one-sided Fisher's Z test and 75 subjects per site [194].

2) PA is negatively related to change in body fat composition.

a) *Physical Activity Measured by Accelerometer:*

PA, measured by the accelerometer, is associated with changes in body composition. For design purposes we will use the approach described above in the analysis section (see D.7.1.2. method 3). That is, two AEE groups will be formed by using the median AEE as the cut point. A method developed by Frison and Pocock [195] will be applied to compare mean changes between groups in a longitudinal analysis, adjusting for baseline values. In this method the following assumptions are made: the correlation between any 2 weight determinations is ~ 0.60 and the minimum acceptable difference between the means is 0.25 standard deviations (i.e., effect size equals 0.25) [186]. There will be one baseline measure of weight and two follow-up measurements. An analysis of covariance approach will be used to adjust for baseline values. Thus, to detect the pre-specified minimum difference with 90% statistical power we will need to accrue 460 subjects per AEE group (230 in the Low and 230 in the High group). We will accrue a total of 500 individuals per site in anticipation of a conservative total attrition rate of ~ 20%.

b) *Physical Activity Measured by Doubly Labeled Water:*

The correlation between AEE and change in body composition is anticipated to be weaker (i.e., smaller in absolute value) than that observed between AEE and body composition at baseline. Data from previous studies (cf. Section C: Preliminary Studies) suggest that the association between AEE and body composition remains constant across studies.

Although the study is not powered to test for differences in the association across sites (i.e., effect modification), we will undertake this task to ensure that large differences among the correlation coefficients do not exist. In addition, sites in which the correlation coefficient is deemed to be similar will be combined, and then comparisons of the correlations will be performed. For the sake of illustration, assume that 2 sites (n = 150 individuals) are compared to other 2 sites (n = 150 individuals). Thus, a two-sample, one-sided test of the correlations, assuming -0.4 versus -0.65, will yield over 91% power, whereas the power will be over 80% for comparison of -0.45 and -0.65, and between -0.35 and -0.6 the power is greater than 85%. Combining the data samples from all 5 sites and assuming constant

association will demonstrate a correlation between AEE and change in body composition of -0.15 or smaller (larger in absolute value) with over 83% power.

3) Mean levels of AEE are negatively related to mean levels of obesity/relative weight among the populations.

As previously demonstrated, sample sizes within sites will allow us to rule out correlations larger than 0.33 (absolute value), even when AEE is determined by DLW. Furthermore, we have estimated (cf. Preliminary Studies) that the within-site correlation between AEE and body composition of individual data could be as large as 0.6. We anticipate that the correlation between mean AEE levels and mean body composition will exceed 0.85. This estimate is justified in the following manner. A Fisher's Z-transformation 5%-significance test will have over 55% power to detect this correlation with 5 values corresponding to the 5 sites of the study. However, after dividing our samples by gender there will be 10 observations (2 X 5 countries), resulting in over 95% statistical power. Assuming an effective sample size of 7, to account for within-country correlations, the statistical power is 80%. Note that additional power will be gained when weighted linear regression methods are applied to estimate the correlation (slope) between AEE and body composition using meta-regression. Given the strong a prior hypothesis we can also compare mean AEE in the groups with the highest and lowest rates of obesity to test the ecological association.

In this instance, we acknowledge that we are forced to make the assumption that important relationships will be inherently strong statistically. This assumption is justified since the unit of the analysis will be the average for the entire group in each site, and therefore estimated with precision. For example, in our study of 11,000 individuals in 8 populations we demonstrated a correlation of 0.85 between mean BMI and prevalence of hypertension [123]. To enhance our ability to find these relationships, we have selected populations from across the full range of BMI (e.g., from 21-30). We are also fully aware of the possibility of "ecological confounding" in these analyses. Thus, while a strong correlation could be observed, the causal process could be entirely different from, but simply co-linear with, the AEE relationship. We will guard against this outcome by testing the individual-level relationships first, as discussed previously.

D.7.3 Data Management

The data coordinating center (DCC) will be established in Chicago, where the study statistician and statistical programmers are housed. Dr. Durazo has significant experience supervising DCCs. He has maintained large data repositories on international collaborative studies at Loyola for over 10 years. He also was the director of the DCC for the Chicago Initiative to Raise Asthma Health Equity for which he was in charge of maintaining a database of asthma screening on over 50,000 children from the Chicago Public Schools. Data entry will be the responsibility of each site coordinator. Data will be sent to the DCC along with copies of the paper forms used to collect them for storage, editing, cleaning, and preparation for publication. Data quality and assurance processes will be implemented and kept in place for the duration of the study. All data files will be stripped from personal identifiers to ensure participant confidentiality. Data backups will be performed regularly following the guidelines of the Department of Preventive Medicine IT.

D.7.4 Missing Data

Briefly, missing data will be handled statistically by using mixed effects models, which will (1) allow use of all data even if some time points are missing, (2) accommodate subject measurements that were not obtained at the precise time, (3) use growth curve models, which is a special case of mixed effects models, which again permits use of the full data set despite missing values and (4) allow for correlations of variables within a participant. The mixed models approach assumes that the data is missing at random (MAR) and, as such, will not bias the analysis. However, if it is determined that the data may not be randomly missing and thus non-ignorable, then two well accepted approaches to handle this situation will be implemented, namely selection models [196, 197] and pattern mixture models [198-201]. ***Selection models*** comprise to steps. In the first, a predictive model for drop-out (missingness) is developed as a function of variables measured prior to dropout (e.g. baseline information). Thus, the probability (propensity) of missingness is estimated for each participant.

Then, in the second step, the propensity score is entered into the regression model as a covariate to adjust for the effect of drop-outs. We will also apply mixed-effect selection models to account for non-ignorable missing data [200]. In ***pattern-mixture models*** we first group the data according to a missing pattern. For instance, in a study with 3 time point determinations there will be total of 8 (2^3) missing data patterns (e.g. OOO, OOM, OMM, OMO, where O stands for observed and M for missing). Then, a mixed-effects model is fit to the data with the same covariates as the original mixed-effects model, in addition to a grouping variable representing the missing data patterns to account for drop-out. Formally, dummy variables are created to represent each of the data patterns. The final model includes all the covariates, the dummy variables and interactions of these dummy variables with other variables in the model. This approach allows determination of i) the degree of dependence of the outcome variable on the missing data patterns; ii) the extent of the influence of the missing data patterns on the association between other variables and outcome (effect modification). Overall estimates of the association between a given variable and outcome can be estimated by averaging over all missing data patterns.

D.8 Limitations

This study is based on the premise that objective measurement of EE will be required to fully understand the causes of the on-going secular trends in obesity. Given prior research on the role of specific sedentary activities, it is reasonable to test the hypothesis that AEE will vary across the social settings we have chosen to study. The value of our study will be in quantifying the overall magnitude of this difference and its within-site relationship. We recognize that it is possible that increases in energy intake—rather than expenditure—could be the determining, or even sole factor, in weight gain and diabetes risk; demonstrating the absence of a PA-obesity relationship will directly support this alternative hypothesis. We will capture data on EI from questionnaires as well, although we recognize its limitations.

On the other hand, the net positive energy balance required to cause gradual weight gain can be quite subtle (in the range of 20-40 kcal/d for adults). Given that this net excess is likely to be due to a combination of both decreased AEE and increased caloric intake, the contrast in population means for AEE could be hard to define. As in all such etiologic studies, therefore, we run the risk of a false negative due to low power. This result, however, would not be uninformative since it would be important to know that large mean shifts in AEE were not driving this epidemic. At the same time, a wide distribution of AEE levels can be anticipated among participants within each site, and we anticipate finding important relationships in the individual-level analyses. The entire success of the project, therefore, does not rest on the group comparisons.

The focus of this study is on the net outcome of a complex regulatory system in natural populations. The class of effects that include the interaction of socially-embedded behavior, food patterns, cultural norms, required and voluntary PA, etc., can only be examined in observational studies. Controlled experiments are therefore not useful to address this question. Long-term prospective studies have clear advantages over cross-sectional and short-term studies; however, they are also difficult to conduct and do not fit within the NIH funding framework. In most instances where epidemiologic research has tried to define risk for chronic disease, cross-sectional studies have effectively summarized the cumulative effect of prior exposures and have consistently identified true relationships among CVD risk factors (e.g., BMI vs. BP, glucose, and cholesterol). Clearly, studies of much longer duration are ultimately necessary to support causal inferences and will be needed in the future.

This project involves recruitment in 5 countries, collection of a complex set of data, and follow-up for two years. The methods must be rigorously standardized to ensure comparability. We recognize the challenge posed by these aspects of the study. We have many years of experience in cross-cultural research and have implemented standardized data collection protocols for similar complex surveys. Our study of the African diaspora was both the largest international collaborative study ever conducted on hypertension and the only one that has yielded standardized cross-cultural prevalence estimates [81, 202]. We regard project described in this application as a major research opportunity which could have long-term significance. It will be the sole research focus of the PI who has invested more than 6 years bringing this project to maturity. We are therefore committed to a maximal effort at study management and are convinced that we can obtain complete high quality data from all the study sites.

HUMAN SUBJECTS

E.1 Risks to the Subjects

This study will involve the collection of medical information, activity data, plasma and urine samples from 2,500 human participants, ages 25-45 years, from communities in 5 countries. The participants will be recruited from residential neighborhoods in Igbo-Ora, Nigeria; Cape Town, South Africa; Seychelles; Spanish Town, Jamaica; and Maywood, IL, USA. Participants will be recruited for this study as the primary aim is to determine whether or not differences in energy expenditure are responsible for the differences in relative weight, change in relative weight over time, and diabetes risk between these countries.

E.1.1 Sources of Materials

Information, activity monitoring data, and plasma samples will be collected from all participants (n = 2,500); urine samples will be collected on a subset participating in the DLW component of the study (n = 375). Demographic, anthropometric, PA, environmental, and lifestyle data will be collected on all participants. They will be assigned an identification number upon enrollment into the study. Linkage of identification number with name, address, birth date or any other identifying information will be kept by the data manager at the respective institutions in a separate, password-protected data file. Only the data managers and study investigators will have access to the linkage file. AEE data, anthropometrics, and specimens will be collected for this study by specifically trained field staff at each of the institutions. Demographic, health history, and questionnaire data will be collected by a one-on-one interview. The participants will be provided with an extensive explanation of the intended study in his/her native language. The data will be coded, cleaned and double entered by trained staff at Loyola.

E.1.2 Potential Risks

The potential risk to the research participants includes hematoma at the site of venipuncture and possible disturbed sleep due to the presence of the activity monitor. The second risk can be alleviated by removal of the monitor during the night. There are no risks associated with the DLW or the questionnaires proposed for this study. There are no alternate treatments or procedures offered. Participation is strictly voluntary and participants may choose to leave the study at any time during the protocol.

E.2 Adequacy of Protection Against Risks

E.2.1 Recruitment and Informed Consent

Recruitment in all sites will be conducted through health professionals in the respective communities. The procedure has been agreed upon by the respective institutions. The institutional review procedures at Loyola will be initiated once this application has been submitted to NIH for review. At that time, an informed consent document will be created and translated/back-translated into each local language. Our investigators and staff are trained to explain the study in detail to all participants, review the informed consent documents, and answer any questions that may arise. There is no aspect of the study that will be withheld from any participant.

E.2.2 Protection Against Risk

The study protocol itself has minimal risk to the participant or family. All phlebotomy staff have been trained and certified, so risk of bleeding and hematoma trauma will be minimized.

Confidentiality will be protected in several ways. The participant will be assigned a unique identification number upon enrollment into the study. This ID will be used on all subsequent materials, including laboratory samples, activity monitoring data and questionnaires. The electronic file linking the name and ID number will be accessed only by the data manager in a separate passworded file at the respective study sites and available only to study investigators.

Appropriate referral will be made for any detected conditions, including elevated fasting serum glucose or blood pressure. The study, however, will not assume responsibility for treatment or provision of drugs.

Due to the relatively high prevalence of HIV in many regions of sub-Saharan Africa, we will undergo screening in Khayelitsha (South Africa). All participants will be tested for HIV and undergo pre- and post-test counseling both before study enrollment at baseline and again at follow-up. The LifeLine Khayelitsha Clinic, CapeTown, will counsel and test all individuals recruited for the study and treat all those found to be HIV-positive. This is policy in South Africa, and we have received confirmation from the officials at the LifeLine Khayelitsha Clinic that all those recruited for the study will be provided for. All counseling, testing as well as treatment for HIV/AIDS is free of charge in South Africa as a result of both national and international efforts. HIV-positive individuals will be excluded from our study, as mentioned previously, because of its effects on metabolism and body weight.

E.3 Potential Benefits of the Proposed Research to the Subjects and Others

Participants will be asked to join this study for its potential scientific and public health benefits. All individuals in the study may benefit directly from the screening activities, e.g., blood pressure, BMI, and fasting glucose. The potential benefits to others include the knowledge of AEE requirements for prevention of excess weight gain. The research risks are reasonable in relation to the anticipated benefits because the possibility of a hematoma, the only real risk to a participant in this study, is no greater than that from a trip to a doctor's appointment. Yet, we have the chance to increase the body of knowledge which will inform national and international recommendations on AEE guidelines.

E.4 Importance of the Knowledge to be Gained

Obesity is an increasing problem worldwide and carries a large risk for diabetes and CVD. We lack an adequate understanding of the underlying determinants of this problem. This study will provide an answer to one facet of the puzzle and will allow us to define a minimum threshold of AEE above which excess weight might be minimized. In addition, there are few objectively obtained data on AEE expenditure. National and international recommendations have been made based on relatively sparse information. The data collected from this study will greatly enhance the extant literature on this topic. Again, the risks of this study are very minor relative to these potential gains.

E.5 Data and Safety Monitoring Plan – N/A

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