HHS Public Access

Author manuscript

J Nutr Biochem. Author manuscript; available in PMC 2016 August 01.

Published in final edited form as:

J Nutr Biochem. 2015 August ; 26(8): 876–882. doi:10.1016/j.jnutbio.2015.03.012.

Effect of six-month caloric restriction on Cu bound to Ceruloplasmin in adult overweight subjects

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Abstract

In a randomized clinical trial of calorie restriction (CR), we demonstrated that important cardiovascular disease (CVD) biomarkers were favorably influenced by CR alone and in conjunction with physical exercise. The aim of this study was to examine the effects of CR with or without exercise on Copper bound to Ceruloplasmin (CuCp), a well-known biomarker for CVD, in overweight men and women enrolled in the CALERIE phase 1 study. Forty-six individuals were randomized to one of four groups for 6 months: control: healthy weight maintenance; CR: 25% CR from baseline energy requirements; CR + exercise: 12.5% CR and 12.5% through aerobic exercise; low calorie diet: low calorie diet until 15% reduction in body weight followed by weight maintenance diet. CuCp was determined in fasting blood samples by an HPLC-ICP-MS methodology and compared with changes in body composition and markers of CVD.

After 6 months, CR combined with exercise induced a decrease in plasma concentration of CuCp. CuCp was inversely correlated with insulin sensitivity (*Si*) at baseline and after 6 months of intervention. A cluster analysis showed that the percent change of weight after 6 months of intervention was the most important variable that could discriminate the intervention groups. The percent change of CuCp was the only other variable selected by the analysis.

Decreased CuCp in overweight subjects by CR combined with exercise suggest a positive effect of this intervention on metabolic health. Further studies to explain the relationship between weight loss and CuCp and its relevance for cardiovascular health are needed.

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Declaration of interest

There is no conflict of interest

Keywords

caloric restriction (CR); Copper bound to ceruloplasmin (CuCp); insulin sensitivity (Si); physical exercise (EX)

1. Introduction

It has long been known that caloric restriction (CR) increases average and maximum lifespan in many animals in laboratory studies, but the physiological and molecular mechanisms and their implications for humans are still unclear [1]. Studies in primates, although contradictory regarding maximum lifespan extension, have shown that CR can delay the onset of age-associated pathologies including cardiovascular diseases (CVD) [2,3], a major cause of death in humans [4,5]. CR trials in humans have also shown that many biomarkers related to major risk factors for CVD, i.e. subclinical atherosclerosis [6] and type 2 diabetes [7], can be favorably influenced by CR, thus suggesting that delaying the onset and progression of subclinical atherosclerosis and diabetes may be among the potential mechanisms by which CR promotes health-span. In addition, it is well established that overweight and obesity individuals have increased CVD mortality [8,9] and weight loss in these individuals improves CVD risk factors with a reduction in coronary heart disease (CHD) event rates [10,11]. In addition, it has been also shown that physical exercise in overweight subjects reduces the risk for atherosclerosis, acute cardiovascular events, stroke and type 2 diabetes mellitus (T2DM) [12,13].

The serum copper (Cu) and Cu binding protein Ceruloplasmin [CuCp] are well-established biomarkers for conditions predisposing to CVD [12]. Among these conditions, obesity [14], atherosclerosis [15,16] and T2DM [17] are the most relevant. Ceruloplasmin was also found increased in subjects with metabolic syndrome (insulin resistance syndrome) suggesting the assessment of this biomarker for metabolic stresses [18]. In a multiple regression analysis, Cp level was independently associated with age, fasting glucose, triglyceride, HDL-cholesterol, and Urinary Albumin Excretion Rate [18].

Copper, is one of the essential trace elements of our body [19]. It has pivotal roles as cofactor for a multitude of enzymes, including ceruloplasmin [20]. It is also needed both in humans and animals in important pathways, such as growth and development [21]. All foods contribute to the copper consumed by humans [22–24]. The most abundant dietary sources include seafood (especially shellfish), organ meats (e.g., liver), whole grains, legumes (e.g., beans and lentils) and chocolate. The major storage of Cu is in the liver, primarily bound to Metallothioneins (MT) [25]. The adult human dietary recommendation for copper (estimated safe and adequate dietary intake) is suggested between 1.5 and 3.0 mg Cu/day [26]. Inadequate nutritional intake of Cu could affect plasma Cu levels and with severe consequences for a multitude of body functions. Many of the pathologies due to copper deficiency may be traced to metabolic defects involving various copper-containing enzymes [27]. It has been shown in fact how an altered Cu intake might affect Cp enzyme function leading to an impaired Cp ferroxidase activity [28]. Moreover, a subclinical Cu deficiency might lead to neutropenia, skeletal demineralization and anemia [27]. On the other hand, at high concentrations, Cu is known to produce oxidative damage to biological systems,

including peroxidation of lipids or other macromolecules [29]. Another interesting feature of Cu physiology is represented by its different amount in blood between women and men. This gender difference discovered many years ago [30–34] has been confirmed recently by several groups [35–37]. Previous studies addressed the increased female plasma Cu to the use of copper containing supplements [38] and to the menstrual cycle [39]. However, others have found that even after excluding women on oral contraceptives, female serum copper remained higher than those observed in males [37].

Ceruloplasmin, is an abundant plasma protein that contains seven Cu atoms per molecule and accounts for 95% of the total circulating Cu in healthy adults [40, 41]. The physiological function(s) of Cp is uncertain. The fact that it is an acute-phase reactant protein with bactericidal activity [42] suggests that Cp may participate in inflammatory responses to foreign agents. Moreover, Cp antioxidant activity also blocks protein [43] and DNA damage [44] and may protect cells against free radical–initiated cell injury and lysis [45]. On the contrary, several studies have shown Cp pro-oxidant activity in vivo [46–48]. Although the assessment of CuCp may be considered a useful tool to monitor health status and therapy response to interventions, to our knowledge CuCp has yet to be measured in a randomized clinical trial of weight loss in overweight individuals with risk factors for CVD.

Using data and samples from a landmark study of CR in overweight individuals [49], we hypothesized that six months of CR and CR plus structural exercise would improve CuCp by decreasing its value with respect to baseline. Concerning the physiological plasma Cu difference between men and women, we expect different results between the two genders. Moreover, since Cu level, might be affected by various confounding factors in women, estrogen concentration will be also taken in consideration. Finally, since individuals received different diets along the trial, nutritional intake of Cu will be also considered in the analysis.

The objectives of this study were therefore a) to examine the effects of CR with or without exercise on CuCp in adult overweight participants of the CALERIE study (Comprehensive Assessment of Long term Effects of Reducing Intake of Energy) phase 1 trial after 6 months (M6) of intervention and b) to determine eventual correlations between CuCp and other parameters measured in the trial.

2. Material and Methods

The CALERIE study was approved by the Pennington Biomedical Research Center IRB and the Data Safety Monitoring Board of CALERIE. All participants provided written informed consent for the trial, collection of samples and subsequent analysis. Details related to the CALERIE can be found online (ClinicalTrials.gov Identifier: NCT00099151) and in the manuscript by Heilbronn et al. 2006 [49].

2.1. Study design

In brief, forty-eight healthy, non-smoking male (25–50y) and female (25–45y), overweight participants ($25 \leq BMI < 30$) were recruited to participate in a 6-month intervention [49]. Participants were excluded if they had a history of CVD, elevated blood pressure (>160/90

mmHg), high fasting blood glucose (>126 mg/dL), chronic medications (except oral contraceptives), smoking, regular exercise (more than twice a week), abnormal thyroid function or abnormal ECG. Results herein report in 46 subjects who completed the study.

Participants were randomized into one of four groups for 6 months: a) control (CTRL) = healthy weight maintenance based on an American Heart Association; b) CR = 25% caloric restriction from baseline energy requirements; c) CR+EX = 12.5% caloric restriction and 12.5% increase in energy expenditure through structured aerobic exercise; d) LCD = low calorie diet (890 kcal/d) to achieve a 15% reduction in body mass followed by weight maintenance. The group assignment was stratified to ensure equal distributions of sex and BMI in the four groups. Comprehensive physiological and psychological testing was conducted over a 5-day stay on the inpatient unit at baseline (BL), after 3 months (M3) and after 6 months (M6). Whenever possible, study personnel collecting study data and samples were blinded to the treatment assignment of the subjects. Included in this analysis are data derived from DXA and abdominal CT scans, fasting blood chemistries and intravenous glucose tolerance test (IVGTT).

2.2. Baseline period

The baseline period was conducted over 5 wk to carefully establish individual Energy requirements and thereafter to perform baseline testing. Subjects had normal fasting plasma glucose concentration and normal glucose tolerance [50]. Average baseline levels for all groups were within normal ranges for blood lipids (Total-C, HDL-C, LDL-C, TG) and blood pressure (SBP and DBP) [6].

The energy intake required for weight maintenance during baseline and the subsequent energy deficit necessary to achieve the desired caloric restriction during the intervention were calculated from total daily energy expenditure assessed during two 14-d periods by doubly labeled water and from a 14-d period when participants consumed all meals prepared by the metabolic kitchen with adjustments for weight maintenance [49].

During the last week of baseline, participants were admitted to the inpatient unit for 5-d during which body composition and metabolic assessments were conducted. The same inpatient stay was repeated at M3 and M6. The composition of the study menus were designed using Moore's Extended Nutrient Database (MENu 2000, PBRC, Baton Rouge, LA) and ProNutra 3.0 (Viocare, Princeton, NJ) [49]. Cu content was determined for all the meals and menus used throughout the trial.

2.3. CuCp determination

Plasma samples obtained in the fasted state while participants were consuming a controlled diet were used in this analysis. The analytical method used for the CuCp determination has been reported in details in a previous manuscript [51]. In brief, analysis of Cu bound to Cp was performed by strong micro anion exchange (SAX) high-performance liquid chromatography (HPLC) coupled with inductively coupled plasma mass spectrometry (ICP-MS). The HPLC system used for the separation of plasma proteins consisted of a Biocompatible Dionex Ultimate 3000 Titanium (Dionex, USA) equipped with a WPS-3000TBPL capillary autosampler (Dionex, USA) fitted with a 1uL loop. Monolithic

anion exchange micro columns [Dionex ProSwift SAX1-S, 1×50 mm id] were employed in the tandem HPLC system for the separation of proteins. The ICP-MS system used for detection and quantification of trace elements was a Thermo X II Series (Thermo Fischer, USA). All speciation measurement were made with a low volume Cinnabar Cyclonic spray chamber (Glass Expansion, Melbourne, AU) working at room temperature, a single piece quartz torch (1.5 mm i.d. injector) together with Xs interface cones and MicroMist U-Series (0.2 – 0.6mL/min) Nebulizer (Glass Expansion, Melbourne, AU). The interface between the HPLC system and the ICP-MS for automated run and acquisition of chromatogram was performed with an external trigger card included in a LC coupling kit (Thermo Fischer, USA). The system was proven to provide a satisfactory measure Cu bound to Cp even in EDTA plasma samples [51].

2.4. Statistical Analysis

SPSS Version 15.0 was used for analysis. Statistical tests were based on pairwise comparison of estimating marginal means with $p < 0.05$. The Generalized Linear Model (GLM) was applied to analyze the effect of sex, time and treatment on the changes of CuCp after 3 (M3) and 6 (M6) months with respect to baseline. Estrogens levels were considered in the analysis as covariate in order to avoid analytical bias due to both the use of oral contraceptives and the menstrual cycle. Concerning the correlation analysis, parametric test was performed for all groups. All parameters included in the analysis were expressed by "Delta %" (percentage of change between M6 and M3 from baseline values). To establish the best variable, which percent change can be used to characterize the interventions, a decision tree analysis was performed by "Chi-squared Automatic Interaction Detector" (CHAID) algorithm. The variables considered in the decision tree analysis included sex, and the changes (% at M6) of CuCp, Weight; BMI; FM, FFM, and percent fat (from DXA); insulin sensitivity (*Si*) from IVGTT; TATMass, VATMass and SATmass (from CT) and, Chol, LDL, HDL, Trig (from fasting blood samples). To determine the best split at any node, the CHAID algorithm choose the predictor variable with the smallest adjusted p-value, i.e., the predictor variable that will yield the most significant split; if the smallest (Bonferroni) adjusted p-value for any predictor is greater than some alpha-to-split value, then no further splits will be performed, and the respective node is a terminal node. The process repeats recursively until one of the stopping rules is triggered. In growing the tree it was used the following stopping rules: minimum terminal parental node size of 10 cases, minimum terminal child nodes size of 5 cases and alpha = 0.05 for splitting nodes. The convergence criteria for the CHAID were: Epsilon = 0.001 and 100 as the maximum number of iterations before stopping the process.

3. Results

3.1. Baseline data

CuCp levels at baseline were higher in females than in males ($p<0.009$) (Table 1). There were no differences among 4 groups. Weight at baseline, was higher in females than in males ($p<0.001$) Table 1. Baseline values of metabolic parameters including BMI, fat mass (FM), fat-free mass (FFM), percent fat, insulin sensitivity (Si), total adipose tissue mass (TATMass), visceral adipose tissue mass (VATMass), subcutaneous abdominal adipose

tissue mass (SATmass), total Cholesterol (Chol), low density lipoprotein (LDL), digh density lipoprotein (HDL), triglycerides (Trig) were previously reported to be unchanged among the intervention groups at baseline [6,49,52]. At baseline, there was only one significant correlation between all the variables above cited included in the analysis. CuCp was negatively correlated with *Si* in females ($R^2 = 0.262$; $p<0.01$).

3.2. Food composition analysis

All the menus, independently from the calories, presented similar Cu content: Cu = 1.66 ± 0.14 mg. (mean ± SD). Moreover the Cu content ratio between all the menus remained consistent from the beginning to the end of the study.

3.3. Test of model effects

The analysis showed statistically significant results for sex ($p = 0.001$) and time (baseline, M3 and M6) ($p = 0.001$) whereas no statistically significance was found for estrogen levels (considered as covariate) ($p = 0.384$) or treatment ($p = 0.527$). However the interaction between sex, time and treatments (estrogens considered as covariate) showed a statistically significant result ($p = 0.046$).

3.4. Effect of treatments on female CuCp

All the results are reported in Table 2. In CR+EX group, after 3 months, the levels of CuCp were significantly decreased from baseline ($p<0.05$). After 6 months, the levels of CuCp were significantly decreased from baseline ($p<0.05$) in both LCD and CR+EX groups. After 6 months in CR+EX group, the CuCp change (%) was significantly different from those one of CTRL ($p<0.05$) and CR ($p<0.01$) groups. After 6 months in LCD group, the CuCp change (%) was significantly different from those one of CTRL ($p<0.01$) and CR ($p<0.01$) groups.

3.5. Effect of treatments on male CuCp

All the results are reported in Table 3. In CR+EX group, after 6 months, the levels of CuCp were significantly decreased from baseline ($p<0.05$) and from those ones at month 3 ($p<0.01$). After 6 months, in CR+EX group, CuCp changes (%) from baseline were significantly different from those one of CR ($p<0.05$) group.

3.6. Effects of treatments on female Weight and BMI

All the results are reported in Table 2. After 3 months, both weight and BMI were statistically significantly decreased in CR, CR+EX and LCD groups with respect to baseline values ($p<0.01$). These differences remained significant after 6 months, too. Moreover, CR and CR+EX groups showed statistically significant decreased values with respect to those ones after 3 months. After 3 months CR, CR+EX and LCD groups showed statistically significant changes of weight and BMI (in %) different to that one of CTRL group ($p<0.01$). Moreover, LCD and CR+EX group showed statistically significant changes of weight and BMI in % different to that one of CR group ($p<0.01$). Finally, LCD group showed statistically significant changes of weight and BMI in % different to that one of CR+EX group ($p<0.01$). After 6 months the differences were the same with the only exception that the weight and BMI changes in % between CR+EX and CR groups were anymore different.

3.7. Effects of treatments on male Weight and BMI

All the results are reported in Table 3. After 3 months, both weight and BMI were statistically significantly decreased in CR, CR+EX and LCD groups with respect to baseline values ($p<0.01$). These differences remained significant after 6 months, too. Moreover, CR and CR+EX groups showed statistically significant decreased values with respect to those ones after 3 months. After 3 months CR, CR+EX and LCD groups showed statistically significant changes of weight and BMI (in %) different to that one of CTRL group ($p<0.01$). Finally, LCD group showed statistically significant changes of weight and BMI in % different to that ones of both CR ($p<0.01$) and CR+EX groups ($p<0.01$). After 6 months CR, CR+EX and LCD groups showed statistically significant changes of weight and BMI (in %) different to that one of CTRL group ($p<0.01$).

3.8. Correlations analysis of CuCp with metabolic parameters

In the correlation analysis were included all the variables above cited (see *Baseline data*). Here we report only those ones statistically significant. After 3 months there were no significant correlations between changes (%) of CuCp and changes (%) of other metabolic parameters. However, after 6 months the change (%) in CuCp values from baseline was positively correlated with the changes (%) in weight, BMI ($p=0.03$; $R^2 = 0.100$), FM ($p=0.04$; $R^2 = 0.086$) and negatively correlated with the change in *Si* ($p<0.01$; $R^2 = 0.215$). Even if at baseline CuCp was not correlated with *Si* in males, when we included all the measures at BL, M3 and M6, CuCp resulted negatively correlated with Si both in females ($p < 0.001$; $R^2 = 0.180$) and in males ($p < 0.01$; $R^2 = 0.120$). The correlations remained significant also excluding data at M3 (data not shown). CuCp was positively correlated with TRIG in females ($p<0.05$; $R^2 = 0.075$) and in males with FM ($p<0.05$; $R^2 = 0.104$), DXA %fat ($p<0.01$; $R^2 = 0.186$), TATMass ($p<0.01$; $R^2 = 0.187$), VATMass ($p<0.01$; $R^2 = 0.121$) and SATMass ($p<0.05$; $R^2 = 0.074$).

3.9. Classification tree of the experimental groups by CHAID algorithm

In this analysis, we identified the variables that play important roles in the characterization of the metabolic changes with the intervention. The variables included in the analysis were: BMI = body mass index, FM = fat mass, FFM, fat free mass, % fat = fat percent by DXA, Si = insulin sensitivity, TATMass = total adipose tissue mass, VATMass= visceral adipose tissue mass, SATmass = subcutaneous abdominal adipose tissue mass, chol = total cholesterol, LDL= low density lipoprotein, HDL= high density lipoprotein, Trig = triglycerides.

The change in weight after 6 months was the most important discriminating variable. This first-level split ($p<0.001$) produced the three initial branches of the classification tree: a) weight <= −8.708% (CTRL=0%, CR=32,1%, CR+EX=28,6%, LCD=39,3%); b) −8.708%< weight< −4.084% (CTRL=22,2%, CR=33,3%, CR+EX=44,4%, LCD=0%); c) weight > −4.084% (CTRL=100%) (Fig. 1).

Within the subjects who displayed the greatest weight loss (weight <= −8.708%), the change (%) of CuCp after 6 was identified as unique discriminating variable. In particular, if the

change in CuCp after 6 months was lower than −4.7%, the LCD group was the best characterized with a 61.5% of cases (Fig. 1).

5. Discussion

The aim of the current study was to compare the effects of 6 months of calorie restriction and calorie restriction plus structured exercise in overweight men and women on plasma concentration of CuCp, a known risk factor for CVD [53]. Taking into account that most of plasma Cu is bound to Cp [51,54], our data showed a mean Cu value both for men and for women was comparable to those published previously [36,37,54]. Moreover, plasma Cu gender difference has been confirmed. The method we used to determinate CuCp was optimized [51] in order to avoid a little Cu overestimation. In order to obtain CuCp results not affected by confounding factors such as the use of oral contraceptives [38] and the menstrual cycle [39] serum estrogen levels were considered in the statistical analysis as a covariate. Indeed, both the use of oral contraceptives and the menstrual cycle induce an alteration of estrogens [55]. However, no statistical significance of estrogen was found in the *"test of model effects"* (see Results 3.3), suggesting that neither the use of oral contraceptives or hormonal fluctuations during the menstrual cycle induced changes on CuCp. Importantly we found that the Cu level was similar (Cu = 1.66 ± 0.14 mg.) in all the meals independently from the treatment both at the beginning and after 6 months, we are confident that effects observed in our analysis were due to the dietary groups and changes in metabolic markers and not to changes in dietary Cu. In regard to the intervention, 6 months of dietary restriction (−12.5% of caloric intake) combined with exercise (−12.5% of calorie by energy expenditure) (CR+EX group) induced a significant decrease from baseline in plasma concentrations of CuCp by approximately 25%. When caloric intake was reduced in order to achieve 15% weight loss by low calorie diet (LCD group), the percent decrease of CuCp with respect to baseline was different from the control group. Conversely, CuCp remained unchanged when caloric intake was reduced by 25% (CR group). This latter result was a little unexpected, considering that changes in body composition and abdominal fat distribution after 6 months were similar in the CR and CR+EX groups [49,6]. However, these results appeared less surprising after seeing that a metabolic biomarker, insulin sensitivity (*Si*), displayed similar changes to those shown for CuCp, with significant improvements only in CR+EX and LCD groups [50]. In agreement with this observation, *Si* was found correlated with CuCp at baseline and with the changes of CuCp at month 6. Considering that an increased Cp was associated with metabolic syndromes, such as insulin resistance [18], the correlation between CuCp and *Si* confirms a link between copper homeostasis and metabolic changes. In this context, the observed changes reinforce the concept that calorie restriction plus structural exercise might be effective on promoting health.

Another hypothesis could be that 6 months of CR in humans (a relatively short time compared to the treatment performed in animal models) were not enough to induce changes of CuCp and that the improvements observed by others in CR [6,49,52] occurred independently from the pathways that modulate CuCp. Interestingly, increment in GH and IGF-1 levels in CR+EX and in LCD groups, but not in the CR one, have been also previously observed [56]. These results were partially explained with the larger reduction in

body weight and body fatness in the LCD group and the involvement of the somatotropic axis induced by the addiction of exercise to CR. However, why CR intervention did modulate neither GH nor IGF-1, remained unclear.

Although the mechanisms involved in CuCp regulation are still not completely understood, a possible involvement of IGF-1 and GH in this regulation is likely to occur. By the way, physical exercise has been shown to increase GH secretion [57–59] and to decrease serum Cp [60].

An additional intriguing hypothesis might consider improvements of *Si* in CR+EX and LCD groups as a consequence of the reduction of phenomena related to the accumulation of senescent cells [61,62]. In this context, a variety of evidence suggests that cellular senescence is involved in obesity [63] and development of type 2 diabetes [64]. Moreover, in the vessel wall, Cu bound primarily to Cp can contribute to local redox-dependent reactions [65–67] that might be involved in promoting a senescent phenotype. Even if this mechanism has never been evaluated in CR trials, it might be possible to preliminary estimate this phenomenon by assessing the most important circulating micro-RNAs related to cellular senescence [68,69].

In this study, a cluster tree analysis was also performed in order to characterize the intervention groups. Interestingly only two parameters (among all the metabolic variables included in this study) were identified by CHAID algorithm in order to provide the best classification of clusters. The percent change of weight after 6 months of intervention was the first whereas the percent change of CuCp was the second one. In particular, CuCp was able to discriminate better LCD and CR+EX groups in those individuals who had the highest changes of body weight (see Fig. 1). This analysis confirms CuCp as an important and independent parameter related to metabolic health and suggests that its assessment may be considered a useful tool in order to monitor response to interventions. Further studies should be addressed to evaluate other potential trace elements, such as Zn and the ratio between Cu and Zn as biomarkers for nutritional interventions.

In conclusion, reduced levels of CuCp in overweight subjects by LCD and CR combined with exercise suggest a positive effect of these interventions on metabolic health. This assumption is in line with the pivotal role played by the physical exercise in maintaining good performances of many body homeostatic mechanisms in overweight condition, in which high CuCp levels represent a risk factor for CVD [14,70]. Further studies in a larger CR trial would be useful to confirm these data and investigate the mechanism that could explain the relationship between weight loss and CuCp and its relevance for cardiovascular health.

Acknowledgments

Funding

This work was supported by grants from the National Institutes of Health: U01AG20478 and P30DK072476.

This paper was supported by INRCA and CALERIE Team.

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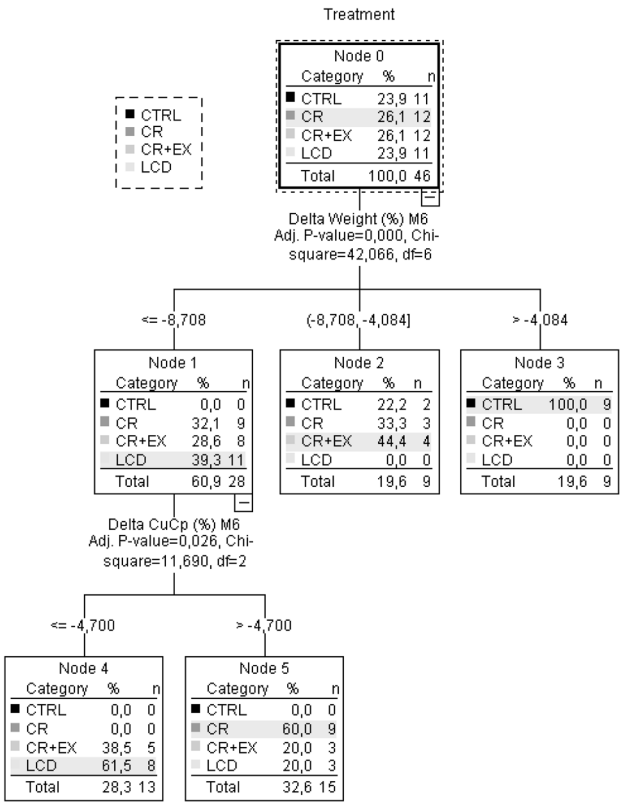

Figure 1.

Classification tree by CHAID algorithm of the 4 experimental groups (CTRL= control group, CR = calorie restriction group, CR+EX = calorie restriction plus exercise group, LCD = low calorie diet group). To establish the best variable which percent change can be used to characterize the interventions, a decision tree analysis was performed by “Chi-squared Automatic Interaction Detector” (CHAID) algorithm. The variables considered in the decision tree analysis included sex, and the changes (% at M6) of CuCp, Weight; BMI; FM, FFM, and percent fat (from DXA); insulin sensitivity (Si) from IVGTT; TATMass, VATMass and SATmass (from CT) and, Chol, LDL, HDL, Trig (from fasting blood samples). To determine the best split at any node the CHAID algorithm choose the predictor variable with the smallest adjusted p-value, i.e., the predictor variable that will yield the most significant split. In growing the tree it was used the following stopping rules: minimum terminal parental node size of 10 cases, minimum terminal child nodes size of 5 cases and alpha = 0.05 for splitting nodes. The convergence criteria for the CHAID were: Epsilon = 0.001 and 100 as the maximum number of iterations before stopping the process.

Table 1

CuCp, Weight and BMI at baseline in male and female subjects enrolled in CALERIE

Group (n. males; n. females)	CuCp (ppb) mean ± SD		Weight (Kg) mean ± SD		BMI	
	Males	Females	Males	Females	Males	Females
CTRL (5; 6)	1054 ± 172	1238 ± 290	89.4 ± 9.4	76.4 ± 4.2	27.9 ± 2.6	27.6 ± 1.8
CR (6; 6)	880 ± 182	1205 ± 360	88.8 ± 8.2	73.5 ± 8.9	28.5 ± 1.1	27.3 ± 1.4
CR+EX (5; 7)	1013 ± 284	1376 ± 577	89.2 ± 11.5	76.9 ± 7.3	27.1 ± 1.4	27.9 ± 1.9
LCD (4; 7)	900 ± 185	1113 ± 382	88.6 ± 11.2	76.8 ± 8.1	28.1 ± 1.7	27.6 ± 2.1
Overall (20; 26)	960 ± 207	1233 ± 409 *	89.0 ± 9.1	76.0 ± 7.0 **	27.9 ± 1.7	27.6 ± 1.7

Absolute CuCp data are expressed in part per billion (ppb) as Mean ± SD. CTRL (n=11): healthy weight maintenance; CR (n=12): 25% caloric restriction from baseline energy requirements; CR + exercise (n=12): 12.5% caloric restriction and 12.5% energy expenditure through aerobic exercise; d) low calorie diet = achieve a 15% reduction in body mass

* Statistically different from male (p= 0.009);

** Statistically different from male (p< 0.001)

J Nutr Biochem. Author manuscript; available in PMC 2016 August 01.

Table 2

Effect of treatments on CuCp, weight and BMI and their changes (%) from baseline in females.

	CuCp (ppb)		Δ CuCp %		Weight (Kg)		Δ Weight %		BMI, kg/m^2		Δ BMI %	
	M3	M6	M3	M6	M3	M6	M3	M6	M3	M6	M3	M6
	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD
CTRL (n=5)	1248 ± 278	1215 ± 259	1.6 ± 14.3	−1.0 ± 8.2	75.7 ± 5.6	75.3 ± 6.6	−1.0 ± 3.3	−1.6 ± 4.5	27.4 ± 2.5	27.3 ± 2.8	−1.0 ± 3.3	−1.6 ± 4.5
CR (n=6)	1255 ± 356	1232 ± 254	4.6 ± 6.9	4.5 ± 10.5	68.2 ± 8.3**	65.9 ± 8.6$^{**\#}$	−7.3 ± 1.3 *b*	−10.5 ± 2.0 *b*	25.3 ± 1.4**	24.4 ± 1.2$^{**\#}$	−7.3 ± 1.3 *b*	−10.5 ± 2.0 *b*
CR+EX (n=7)	1016 ± 294*	935 ± 306*	−11.5 ± 28.1	−23.8 ± 28.0 *a,x*	72.4 ± 6.9**	69.1 ± 7.6$^{**\#}$	−5.8 ± 0.6 *b,y*	−10.3 ± 2.5 *b*	26.3 ± 1.8**	25.1 ± 2.1$^{**\#}$	−5.8 ± 0.6 *b,y*	−10.3 ± 2.5 *b*
LCD (n=7)	1021 ± 326	851 ± 319*	−5.6 ± 20.2	−45.0 ± 41.7 *b,x*	66.1 ± 7.4**	65.6 ± 7.9**	−14.1 ± 1.6 *b,y,j*	−14.8 ± 1.5 *b,y,j*	23.7 ± 2.1**	23.5 ± 2.1**	−14.1 ± 1.6 *b,y,j*	−14.8 ± 1.5*b,y,j*

Absolute CuCp data are expressed in part per billion (ppb) as Mean ± SD. CTRL (n=11): healthy weight maintenance; CR (n=12): 25% caloric restriction from baseline energy requirements; CR + exercise (n=12): 12.5% caloric restriction and 12.5% energy expenditure through aerobic exercise; d) low calorie diet = achieve a 15% reduction in body mass. Statistically different from baseline:

*p<0.05;

**p<0.01.

Statistically different from the same parameter at M3:

$^{\#}$p<0.01.

Statistically different from CTRL:

a p<0.05;

b p<0.01.

Statistically different from CR:

x p<0.05;

y p<0.01.

Statistically different from CR+EX:

j p<0.01.

Table 3

Effect of treatments on CuCp, weight and BMI and their changes (%) from baseline in males.

	CuCp (ppb)		Δ CuCp %		Weight (Kg)		Δ Weight %		BMI, kg/m^2		Δ BMI %	
	M3	M6	M3	M6	M3	M6	M3	M6	M3	M6	M3	M6
	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD
CTRL (n=5)	960 ± 134	931 ± 58	−7.4 ± 18.3	−10.0 ± 13.9	88.6 ± 9.2	88.9 ± 8.2	−0.9 ± 1.9	−0.5 ± 2.1	27.7 ± 2.6	27.7 ± 2.2	−0.9 ± 1.9	−0.5 ± 2.1
CR (n=6)	851 ± 220	882 ± 181	−4.0 ± 8.5	0.5 ± 6.9	82.1 ± 8.0**	79.7 ± 8.4$^{**\#}$	−7.6 ± 1.9 *b*	−10.3 ± 4.4 *b*	26.3 ± 1.2**	25.5 ± 1.6$^{**\#}$	−7.6 ± 1.9 *b*	−10.3 ± 4.4 *b*
CR+EX (n=5)	892 ± 344	719 ± 300$^{*\#}$	−8.9 ± 30.9	−26.5 ± 29.4 *x*	84.1 ± 10.9**	80.2 ± 8.7$^{**\#}$	−5.8 ± 1.7 *b*	−9.9 ± 3.8 *b*	25.6 ± 1.4**	24.4 ± 0.9$^{**\#}$	−5.8 ± 1.7 *b*	−9.9 ± 3.8 *b*
LCD (n=4)	867 ± 126	800 ± 289	0.5 ± 31.6	−8.1 ± 34.6	76.9 ± 9.3**	77.6 ± 9.4**	−13.2 ± 2.4 *b,y,j*	−12.4 ± 3.2 *b*	24.4 ± 1.6**	24.6 ± 1.7**	−13.2 ± 2.4 *b,y,j*	−12.4 ± 3.2 *b*

Absolute CuCp data are expressed in part per billion (ppb) as Mean ± SD. CTRL (n=11): healthy weight maintenance; CR (n=12): 25% caloric restriction from baseline energy requirements; CR + exercise (n=12): 12.5% caloric restriction and 12.5% energy expenditure through aerobic exercise; d) low calorie diet = achieve a 15% reduction in body mass. Statistically different from baseline:

*p<0.05;

**p<0.01.

Statistically different from the same parameter at M3:

$^{\#}$p<0.01.

Statistically different from CTRL:

^{b}p<0.01.

Statistically different from CR:

^{x}p<0.05;

^{y}p<0.01.

Statistically different from CR+EX:

^{j}p<0.01.