# Effects of calorie-restricted diet on health state and intestinal flora in Hashimoto's thyroiditis patients: Study protocol for a randomized controlled trial

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**Running title:** Protocol of CR diet improves Hashimoto disease

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## ABSTRACT

**Background and Objectives:** Hashimoto's thyroiditis (HT) is an autoimmune disease, characterized by abnormal elevation in thyroid peroxidase antibody and/or thyroglobulin antibody. In recent decades, HT disease has become more and more widespread. Patients always report multiple symptoms, even though their thyroid hormone levels are kept in normal ranges. However, no treatment exists to effectively reduce the levels of thyroid antibodies. Our study aims to determine whether calorie-restricted diet is helpful in improving health of HT patients. **Methods and Study Design:** This is a 3-month randomized controlled trial. HT patients will be randomized into a calorie-restricted (CR) group or a calorie-unrestricted control group. All the participants will be instructed to consume a diet that includes a combination of 45-55% calories from carbohydrates, 20-30% from fats, and 15-25% from proteins, according to current Chinese Dietary Guidelines. Participants in CR group need to limit their calories intake equal to their basal energy expenditure, which means that their daily caloric intake will be limited by about 20-30%. **Results:** The study population is planned to be 66 HT patients aged 18 to 65 years. The primary outcome is change of thyroid antibody levels from baseline. Secondary outcomes include the changes of non-hypothyroid symptoms scores, thyroid function indexes, morphology of thyroid, T lymphocyte subpopulations, inflammatory biomarkers and lipids from baseline to 12 weeks. **Conclusions:** This trial will have implications for nutrition treatment policy in regard to thyroid antibodies control, immune dysfunction and related non-hypothyroid symptoms improvement among HT patients.


**Key Words: Hashimoto's thyroiditis, calorie restriction, thyroid antibody, non-hypothyroid symptoms, gut microbiota**

## INTRODUCTION

Hashimoto's thyroiditis (HT) is an autoimmune disease characterized by elevated levels of serum thyroglobulin antibody (TG-Ab) and/or thyroid peroxidase antibody (TPO-Ab), which resulting in damage of thyroid gland.[1] In recent years, HT has become one of the most prevalent autoimmune disease, with a morbidity of 0.03-0.15%.[2,3] Women are 7-10 times more susceptible than men to the disease.[4] HT patients are more prone to hypothyroidism, thyroid nodules and thyroid cancer than healthy controls.[5,6] Despite medical management with thyroid hormone replacement, patients with HT always report multiple persistent extra-thyroidal symptoms, such as fatigue, forgetfulness, muscle and joint tenderness, dry skin, rash

and poor sleep quality.[7,8] Our previous study has shown that serum TPO-Ab and TG-Ab levels were both inversely associated with health-related life quality of HT patients, and positively correlated with pro-inflammatory factors of tumor necrosis factor (TNF)-α and interferon (IFN)-γ, as well as severity of abdominal distension, diarrhea, chilliness, forgetfulness and fatigue (unpublished data). However, to date there is no therapeutic option for HT patients to improve immune dysfunction by reducing TG-Ab or TPO-Ab levels.[9] Therefore, there is an urgent need to find effective intervention method to decrease thyroid antibodies and improve immune dysfunction and multiple symptoms of HT patients.

Calorie restriction (CR) is one of the most studied intervention method in recent years, which reduces daily energy intake by approximately 20-30% of daily recommended allowance, but no malnutrition occurs.[10-12] CR has shown positive effect on extending lifespan, promoting weight loss, improving metabolic and neurodegenerative diseases.[13-15] In addition, recent studies also suggested that CR has favorable ameliorative effects on modifying immune dysfunctions. Data from animal studies have shown that CR diet could maintain production of thymic lymphocyte, increase diversity of T cell receptors, and reverse the damaged immune system.[16] In human studies, CR can significantly reduce the levels of pro-inflammatory cytokines in serum, slow down aging of thymus, increase CD4+ and CD8+ T cell production and the content of T cell receptor excision rings (sjTRECs).[16-18] Furthermore, CR diet has a potent anti-inflammatory effect on the human body. Previous evidences have shown that CR can increase the levels of hormones that inhibit inflammation such as cortisol, adiponectin and gastrin. In addition, CR can also reduce the expression of inflammatory cytokines such as TNF-α, interleukin (IL)-6, IL-10, IL-12 and IFN -γ.[19-21]

In the present study, a 3-month randomized controlled trial was conducted to firstly investigate whether CR diet could decrease TPO-Ab and TG-Ab levels of HT patients. Secondly, this study also aimed to determine the influence of CR on thyroid function, immune dysfunction, inflammation, morphology of thyroid, gut microbiota, and severity of non-hypothyroid symptoms among HT patients with normal thyroid hormones.

***Hypothesis and outcomes***

We hypothesize that CR diet has favorable ameliorative effects on HT disease. The primary outcome is the between-group difference of the changes in thyroid antibody levels (TPO-Ab and TG-Ab) from baseline to the end of the intervention. The secondary outcomes include the effect of CR diet on thyroid function indexes (thyroid stimulating hormone (TSH), free triiodothyronine (FT3), free thyroxine (FT4)), morphology of thyroid, T lymphocyte

subpopulations (contents of CD3+, CD4+ and CD8+ T lymphocytes), immune biomarkers (immunoglobulin (Ig)-G, IgA, IgM, complement (C) 3, C4), inflammatory biomarkers (TNF-α, IFN-γ, interleukin), liver function indicators (alanine aminotransferase (ALT), aspartate aminotransferase (AST), total protein (TP), albumin (ALB), globulin (GLOB)), sex hormones (follicle stimulating hormone, luteinizing hormone, estradiol, progesterone, testosterone, prolactin), lipids profiles (total cholesterol (TC), triglycerides (TG), low density lipoprotein cholesterol (LDL-C), high density lipoprotein cholesterol (HDL-C)), high sensitivity C-reactive protein (hs-CRP), cortisol, and self-reported symptoms related to HT.

## MATERIALS AND METHODS

### *Ethics approval and clinical registry*

This study has been approved by Ethics Committee of Zhejiang Chinese Medical University (No. 20220607-2), and has been registered in the Chinese Clinical Trial Registry as ChiCTR2200060968.

### *Design summary*

This is a 3-month, randomized controlled trial. The trial is to be conducted at the Second Affiliated Hospital of Zhejiang Chinese Medical University, Hangzhou, China. The protocol consists of an enrollment period and an intervention period (Figure 1). The intervention will last 3 months during which time all participants will be requested to eat according to the recipes provided by the research team and to send photos/records of their daily food to researchers via WeChat as feedback.

### *Target population and inclusion/exclusion criteria*

The target population of this study is the diagnosed HT patients living in Hangzhou. Participants are planned to be recruited through community announcements, internet posters, fliers, and advertisements on social media platforms (WeChat, QQ and Forum). The power calculation will be based on a between group difference in TPO-Ab changes of 100 KU/L with a SD of 150 KU/L, referring to a previous study and considered to be clinically meaningful. We required at least 52 individuals to complete the study to achieve a power of 80% for a two-side test with significant level of 0.05. Considering a loss-to-follow-up rate of 20%, sample size is raised to 66 patients. To be eligible in the trial, subjects need to fulfil all inclusion criteria and none of the exclusion criteria as in Table 1.

***Population screening, informed consent and randomization***

People who respond to recruitment will be interviewed through the internet/telephone by brief questions on major eligibility issues. Appropriated individuals from the pre-screen contact will be arranged for the following two formal screening visits. At the first visit, research team aims to determine the conformity of each participant in non-clinical test indicators for both inclusion and exclusion criteria. In addition, their previous attempt to lose weight or dietary restriction, as well as their motivation for this trial will be surveyed through a one-to-one conversation, to assess the participants' willingness and ability to adhere to the protocol. Participants who pass the first round of screening will undergo the second screening visit, when we will request a 12-hour fasting blood sample for testing the clinical indicators.

Participants who meet the screening criteria will be invited to attend an online conference. At the meeting, the chair of this study will introduce the research content, research procedures, precautions, possible benefits and risks to each potential participant in detail, and allow them three days to fully consider whether to participate. Furthermore, the rights and interests of the potential participants will also be indicated. For example, participation in this study will be completely voluntary, and the participants can terminate it at any time during the intervention. Of course, the project chair will also emphasize the expectation that the participants would better to follow up to the end of the study once they have decided to join the study after consideration. After completing the above steps, participants will be required to sign an informed consent and are formally enrolled.

Randomization will be block-stratified by age, sex and thyroid antibody levels of the participants to avoid potential difference at baseline, using a computer-generated random number list prepared by a data analysis manager with no clinical involvement in this trial. Volunteers are numbered after enrollment in the project, and this random number will remain the same throughout the trial. Sample collectors, laboratory testing personnel, and data analysis personnel are unclear about the group allocation, and only identify them by random number code. Dietitians will be aware of the group assignment of each participant, but they will not take part in the rest of the trial, including laboratory measurement and data analysis. Because of the obvious difference in the menus provided, blinding participants is not feasible, though they will not be informed of the group allocation and will be managed with two different WeChat groups according to their group allocation.

***Implementation of dietary intervention***

The intervention will last for 3 months, during which all the participants will be instructed to consume a diet that includes a combination of 45-55% calories from carbohydrates, 20-30% from fats, and 15-25% from proteins. The dietary calorie distribution was similar to the recommendation of macronutrient intake in current Chinese Dietary Guidelines. Only the participants in CR group will be required to limit their calories intake equal to their basal energy expenditure, also called basal metabolic rate, which will be measured by body composition analyzer at baseline. It means that daily caloric intake in CR group will be limited by about 20-30%, as the basal energy expenditure accounts for approximately 70-80% of the daily calorie requirement for the human body.

During the intervention period, all the participants will receive a seven-day meal plan and several sample menus weekly via WeChat. A sample of meal plan is showed in Table 2. Participants will be encouraged to weigh and record foods to ensure they accurately reported their food intake, and they will be required to note and take photo of the food they eat and send them to the dietitian through WeChat every day. Using each participant's note and photograph of the food, two researchers will assess the macronutrients and energy intake of each participant every day on the basis of the nutrient content shown on the Chinese Food Composition Table via Nutrition Calculator version 11.0 (Medical College, Qingdao University, Shandong, China). Two trained dietitians and two researchers will conduct a summary meeting weekly, to discuss the problems of the participants' diet and provide them reasonable suggestions as feedback. Each participant will receive a follow-up telephone call weekly and meet with the dietitian every two weeks to aid their adherence to the project and to ensure their adequate nutrient intake. In addition, all participants will be encouraged to maintain their original exercise habits throughout the intervention period.

***The ways to promote compliance and adherence assessment***

To increase compliance to the diets, we will introduce several initiatives as follows: A). Nutritional consultation: All the participants will have an individual nutritional consultation with our clinical dietitian at the beginning of the trial. On the one hand, it will help the dietitian to understand the participants' eating habits and dietary preferences, and therefore develop sound and easy-to-implement recipes for them exclusively. On the other hand, it will provide more detailed nutrition knowledge for the participants, helping them understand the dietary guidelines well. B). Meal plan and sample menus: During the intervention period, all the participants will receive a seven-day meal plan and sample menus via WeChat weekly.

Furthermore, we also list multiple alternative food, which can be replaced with the specified food on the menu with equal energy, according to personal preference. C). Detailed record: We encourage the participants to weigh and record foods to ensure they accurately control their caloric intake, and they need to take photo of the food they eat and send to the dietitian through WeChat every day. D). Regular follow-ups: The participants will receive follow-up telephone calls weekly and meet with the dietitian every two weeks. The communication between the participant and the dietitian will be mainly focus on the compliance to the choice of food sources and adequate calorie intake, everyday situations that challenge their compliance to the diet, as well as their struggles and problems. E). Group chats: Our research team will share related information that the patients may be interested in about three times a week in our WeChat group. The information will be showed by pictures stories or PPT slides. The topic may be: (1) how to vent emotions properly; (2) the tips for a happy life; (3) how to improve insomnia; (4) good ways to reduce stress; (5) how to choose food healthily when dining out; (6) how to read nutrition labels and ingredient lists; (7) the reason for the limited food; (8) the tips for precise control of oil and sugar intake; (9) recommendation of dietary therapy tea soup and flower tea that strengthen the spleen and stomach, and so on. It will promote communications among patients in the WeChat group, and enhance their participation sense in this trial, with the aim to increase compliance.

Adherence to the dietary intervention will be evaluated from two perspectives. On the one hand, the follow-up research personnel will assess it basing on the daily records and pictures send by each participant, which will account for 65% of the final results of compliance assessment. On the other hand, participants' self-report assessments will be collected in the form of questionnaires, which will contribute 35% of the final compliance assessment results. Of note, if the participant continues to deviate from the study protocol or can't take photos on time to record the diet intake more than three times a week, she or he will be withdrawn from the trial. In addition, absence from the study visit, occurrence of serious adverse events, and withdrawal of informed consent are all considered as withdrawal from the study.

***Data collection and methods***

**Tools**

*The following clinical information will be collected*:

(1) General demographic information: including marital status, occupation, degree, physical activity, family history of diseases (Hashimoto's thyroiditis, hyperthyroidism,

Graves' disease, etc.), medication (whether the participant is taking thyroid hormone substitution and the dosage), use of dietary supplements and so on;

(2) Hashimoto's thyroiditis symptom questionnaire: to assess the severity of multiple symptoms related to HT disease;

(3) The MOS 36-item short form health survey: to assess the health-related quality of life;

(4) Magnetic resonance imaging (MRI): to determine the morphology of thyroid;

(5) Anthropometric measures: basal metabolic rate, weight, BMI, body fat mass, fat free mass, body fat percentage, visceral fat area.

*For blood sample, we will evaluate markers of*:

(1) Thyroid antibodies: TG-Ab and TPO-Ab;

(2) Immune biomarkers: T lymphocyte subpopulations (contents of CD3+, CD4+ and CD8+ T lymphocytes, CD4+/CD8+), IgG, IgM, IgA, C3, C4;

(3) Inflammatory factors: TNF-α, IFN-γ, IL-2, IL-4, IL-6, IL-10, IL-17A, hs-CRP;

(4) Metabolic indicators: TC, TG, LDL-C, HDL-C, glucose, ALT, AST, TP, ALB, GLOB;

(5) Hormonal indicators: TSH, FT3, FT4, cortisol, follicle stimulating hormone, luteinizing hormone, estradiol, progesterone, testosterone and prolactin.

*For fecal samples, we will conduct the following analyses*:

(1) Gut microbiota (taxonomic and functional analysis)

(2) Targeted metabolomics of short-chain fatty acids and bile acids.

D. For urine samples, we will evaluate iodine level and untargeted metabolomics.

**Thyroid MRI analysis**

We will use a 1.5T MRI system equipped with an 8-channel coil which is specific for head and neck (Siemens, Germany) to evaluate the morphological changes of thyroid. Participants will be advised to breathe slowly and steadily, and not to swallow or speak during the scanning process to reduce motion artifacts.

T1-weighted images (T1WI) scan and T2WI scan will be conducted with the parameters showing in Table 3. Apparent diffusion coefficient (ADC) value will be obtained as follows: Performing a diffusion-weighted imaging scan, the instrument will generate an ADC map. When a radiologist plots an area of interest along the edge of the thyroid parenchyma, the tool will automatically display the ADC values within the area. The measurement was conducted

for three times at the same area as close as possible by two radiologists. The final ADC value of each participant will be the average of the three measurements of two radiologists.

**Anthropometric measurement**

All anthropometric measurement will be conducted by body composition analyzer (DONGHUAYUAN MEDICAL, BC-210) after an overnight fast. Before the measurement, patients need to empty fecal and urine, and rest quietly for about 15 min. Then, they will be required to remove their jackets, metal accessories and shoes, just wear light clothes. Anthropometric measurements will be taken at week 0, week 6 and week 12.

***Sampling***

Participants will be asked to provide blood, urine and fecal samples at each visit.

**Biochemical measurement**

After an overnight 12-h fasting, blood will be drawn from the antecubital vein between 7:00 and 9:00 am to avoid influence of circadian fluctuations. The blood samples will be centrifuged with 3,000 rpm at 4°C for 10 min to obtain serum. The serum samples will be stored at -80°C until use. Biochemical measurements will be taken at week 0, week 6, and week 12.

**Gut microbiota analysis**

Each participant will be instructed to collect the fecal sample (at least bigger than a broad bean) after an overnight fasting to avoid the influence of diet. Participants will be asked to store the sample at a low temperature and send it to us within 24 hours of collection. Upon receiving it, the sample will be immediately quick-frozen with liquid nitrogen, and then stored at -80℃ until use.

DNA was extracted from the stool using the QIAamp Power Fecal DNA Kit following the instruction manual. The extracted DNA will be quantified by Nano Drop spectrophotometer, and the quality of the DNA extraction will be detected by 1.2% Agarose Gel Electrophoresis. It will be amplified using primers flanking the V3-V4 hypervariable region of the 16S rRNA gene for PCR amplification, and the primers sequences are 338F (5'-ACTCCTACGGGGAGGCAGCA-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3'). Sequencing library preparation and sequencing will be performed using Illumina MiSeg

PE150 sequencing platform (Illumina, USA). Sequencing library construction will be performed using Illumina TruSeq Nano DNA LT Library Prep Kit.

The raw sequencing data will be processed using the pipeline tools QIIME and PEAR. According to the quality of the sequences, preliminary screening will be carried out, and the problematic samples will be re-tested. Sequences will be clustered into operational taxonomic units (OTUs) at a similarity level of 97% use Uparse algorithm of Vsearch software, and the chimeric sequences will be removed during the clustering process to obtain OUT representative sequences, which will be compared with the database for species annotation and taxonomic analysis.

***Data management and analysis plan***

**Data analysis principles**

*Primary outcome*

The primary outcome is the change of thyroid antibody (TPO-Ab and TG-Ab) from baseline to 12 weeks.

*Secondary outcomes*

Secondary outcomes are the between-group differences in non-hypothyroid symptoms scores, immune biomarkers (including contents of CD3+, CD4+ and CD8+ T lymphocyte, ratio of CD4+/CD8+, IgG, IgM, IgA, C3, C4), inflammation factors (including TNF-α, IFN-γ, IL-2, IL-4, IL-6, IL-10, IL-17A, hs-CRP), metabolic indicators (including TC, TG, LDL-C, HDL-C, glucose, ALT, AST, TP, ALB, GLOB), hormonal indicators (including TSH, FT3, FT4, FSH, cortisol, luteinizing hormone, estradiol, progesterone, testosterone and prolactin). Meanwhile, gut microbiota and targeted metabolomics of short-chain fatty acids and bile acids in the fecal, as well as the level of urine iodine and untargeted metabolomics in urine samples will also be determined.

**Monitoring data collection**

All team members will be trained according to what they're responsible for in the project, to ensure accurate execution of procedures and data collection. Standard procedures for data collection will be conducted as mentioned above. After each visit, at least two team members will check the quality and correctness of the data collection. The supervisor will review the checklist to evaluate the quality of data collection.

**Statistical analysis**

Baseline demographic variables, thyroid antibody levels, anthropometric measures, thyroid function indexes, immune biomarkers, inflammatory factors, liver function indicators, sex hormones, lipid metabolism factors, self-reported symptoms, dietary characteristics and physical activity level will be presented using descriptive statistics.

Main data will be analyzed according to the intention-to-treat principle. Exploratory data analysis and Shapiro-Wilk tests will be conducted to assess the normality of the data distribution. Continuous variables will be described as means ± standard deviation (SD) when they are normal distributed, and as median and inter quartile range (IQR) if not. Between-group difference at baseline and in the change from baseline to the middle or end of the intervention will be analyzed with unpaired t test when data are normal distribution, while with Mann-Whitney U tests for abnormal distribution data. For categorical variables, data will be expressed as counts and percentages. Between-group comparisons of categorical variables will be determined with the chi-square test. Correlations will be evaluated by Spearman rank correlation analysis. Correction for multiple testing will be performed based on the false discovery rate or Bonferroni correction. In all above analyses, the level of statistically significant will be set as a two-sided *p* value less than 0.05.

Alpha diversity of gut microbiota will be estimated using Chao1 index (richness), Shannon index (diversity), Simpson index (diversity), and Pielou's evenness index (evenness). Comparison within the groups (week 0 and week 12) and between the groups at the end of the study will apply the non-parametric Wilcoxon signed-rank test or Mann-Whitney (or Kruskal-Wallis) test. We will measure beta-diversity using means of the Bray-Curtis dissimilarity metric and the weighted UniFrac distance metric. The relative abundance of flora at the phylum and genus levels will be analyzed using both multidimensional and unidimensional statistics. Furthermore, we will use partial least squares discriminant analysis (PLS-DA), orthogonal partial least squares-discriminant analysis (OPLS-DA) and linear discriminant analysis effect size (LEfSe) to screen metabolites or species with significant differences and perform metabolic pathway analysis. Changes in each phylum and genus in the groups before and after the intervention will be analyzed using the paired Wilcoxon rank-sum test, supplemented by the Benjamini-Hochberg method for false discovery rate (FDR) correction for multiple comparisons. For phylum or genus with significant changes before and after the intervention, the Mann-Whitney U rank-sum test will be used for two-way comparisons between groups, with Bonferroni correction. Correlations between significantly different

genus and the main outcomes (TPO-Ab, TG-Ab) will be measured by Spearman rank correlation analysis.

***Project Management***

**Data and sample management**

Each participant's test results (baseline and follow-up period) should be carefully recorded and included in a file. Storage of the data should be done in triplicate (one for cloud databases, one for work computers, and one for paper) to prevent data loss. In order to protect the privacy of the participants, the staff who have access to the data should sign a confidentiality agreement, and unauthorized staff will not be allowed to access and use the data. The sample supervision personnel will be responsible for: 1) ensuring that the storage conditions of various samples are complied with the rules; 2) managing the use of the samples; 3) assuring that the remaining sample are harmless.

**Study schedule**

This study will include four visits, which can be seen in Table 4:

(1) Visit 0: All potential patients will be screened to confirm whether they meet the recruitment criteria and to assess their potential adherence to the trial protocol. In addition, informed consent will be obtained from each participant during the period.

(2) Visit 1: After screening visit, eligible patients will be asked to complete body composition analysis, questionnaires and biological samples collection (blood, urine, and fecal), as well as to undergo thyroid MRI.

(3) Visit 2: After 6 weeks from baseline visit, patients will visit again to complete body composition analysis, questionnaires and biological samples collection.

(4) Visit 3: Visit 3 takes place after 12 weeks from baseline visit. The procedure will be the same as visit 1.

**DISCUSSION**

HT is a T-cell mediated autoimmunity disease of uncertain cause. It is characterized by abnormally elevated TPO-Ab and/or TG-Ab in serum. The disease process finally leads to hypothyroidism. Current treatments are only available for patients progressed to hypothyroidism, by supplementing with levothyroxine to keep hormone levels within normal ranges. However, despite patients' euthyroid status after adequate levothyroxine supplementation, patients often report a wide range of symptoms that seriously decrease their

life quality, such as profound fatigue, poor sleep quality, forgetfulness, muscle and joint tenderness. These symptoms are therefore thought to be associated with autoimmune disease rather than with hypothyroidism.

The pathogenesis of HT is complex, involving imbalance of immune response, increase of intestinal permeability and intestinal flora imbalance. Previous studies have demonstrated that several increased pro-inflammatory factors in HT patients, such as IL-4, IL-6, IL-12, IL-17A and TNF-α, could to some extent aggravate the immune disorder by increasing the expression of human leukocyte antigen.[22-24] In addition, it has been reported that dysbiosis of intestinal flora and increased intestinal permeability (leaky gut) contribute to HT development.[25] A decrease in beneficial bacteria, such as *Bifidobacteria*, *Lactobacillus*, and *Prevotella*, while an increase in harmful bacteria, such as *Parasutterella*, were observed in patients with HT disease.[26]

CR diet has been extensively studied and has shown many potential benefits for health, such as improving cardiovascular disease, enhancing stress resistance, and strengthening immune function.[13-15] The mechanisms may be associated with its function of modulating the gut microbiota and its metabolite profile in the host.[27] CR diet has been shown to establish a gut microbiota dominated by probiotic *Lactobacillus* and *Akkermansia*.[28] It has been suggested that CR could remodel the composition of gut microbiota, increase the abundance of lactate-producing bacteria, inhibit several harmful bacteria that promote inflammation, such as *Desulfovibrionaceae*, *Streptococcaceae*, and TM7, and hence modify numerous metabolites of gut microbiota, such as short-chain fatty acids, bile acids and vitamins.[29-31]

The present study aims to investigate whether CR diet is effective for decreasing the levels of TPO-Ab and TG-Ab in HT patients, as well as to evaluate the effects of CR on non-hypothyroid symptoms, inflammatory biomarkers, immune dysfunction, hormone levels, serum lipids, intestinal flora and metabolites in Hashimoto's thyroiditis. To the best of our knowledge, the present randomized controlled trial study protocol is the first to evaluate the effects of CR on health and gut microbiota of HT patients. It will provide reliable evidence for clinicians to use the CR diet as a possible therapeutic strategy for HT patients.

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## CONFLICT OF INTEREST AND FUNDING DISCLOSURE

All authors declare that they have no conflicts of interest.

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## REFERENCES

1. Klubo-Gwiezdzinska J, Wartofsky L. Hashimoto thyroiditis: an evidence-based guide to etiology, diagnosis and treatment. Pol Arch Intern Med. 2022;132:16222. doi:10.20452/pamw.16222.
2. Caturegli P, De Remigis A, Rose NR. Hashimoto thyroiditis: clinical and diagnostic criteria. Autoimmun Rev. 2014;13:391-7. doi:10.1016/j.autrev.2014.01.007
3. Ralli M, Angeletti D, Fiore M, D'Aguanno V, Lambiase A, Artico M, et al. Hashimoto's thyroiditis: An update on pathogenic mechanisms, diagnostic protocols, therapeutic strategies, and potential malignant transformation. Autoimmun Rev. 2020;19:102649. doi:10.1016/j.autrev.2020.102649.
4. Ragusa F, Fallahi P, Elia G, Gonnella D, Paparo SR, Giusti C, et al. Hashimotos' thyroiditis: Epidemiology, pathogenesis, clinic and therapy. Best Pract Res Clin Endocrinol Metab. 2019;33:101367. doi:10.1016/j.beem.2019.101367.
5. Orgiazzi J. Thyroid autoimmunity. Presse Med. 2012;41:e611-e625. doi:10.1016/j.lpm.2012.10.002.
6. Xu S, Huang H, Qian J, Liu Y, Huang Y, Wang X, et al. Prevalence of Hashimoto Thyroiditis in Adults With Papillary Thyroid Cancer and Its Association With Cancer Recurrence and Outcomes. JAMA Netw Open. 2021;4:e2118526. doi:10.1001/jamanetworkopen.2021.18526.
7. Hu X, Chen Y, Shen Y, Tian R, Sheng Y, Que H. Global prevalence and epidemiological trends of Hashimoto's thyroiditis in adults: A systematic review and meta-analysis. Front Public Health. 2022;10:1020709. doi:10.3389/fpubh.2022.1020709.
8. Ott J, Promberger R, Kober F, Neuhold N, Tea M, Huber JC, et al. Hashimoto's thyroiditis affects symptom load and quality of life unrelated to hypothyroidism: a prospective case-control study in women undergoing thyroidectomy for benign goiter. Thyroid. 2011; 21:161-167. doi:10.1089/thy.2010.0191.
9. Samuels MH, Kolobova I, Antosik M, Niederhausen M, Purnell JQ, Schuff KG. Thyroid Function Variation in the Normal Range, Energy Expenditure, and Body Composition in L-T4-Treated Subjects. J Clin Endocrinol Metab. 2017;102: 2533-2542. doi:10.1210/jc.2017-00224.
10. Palma C, La Rocca C, Gigantino V, Aquino G, Piccaro G, Di Silvestre D, et al. Caloric Restriction Promotes Immunometabolic Reprogramming Leading to Protection from Tuberculosis. Cell Metab. 2021;33:300-318.e12. doi:10.1016/j.cmet.2020.12.016.

11. Ravussin E, Redman LM, Rochon J, Das SK, Fontana L, Kraus WE, et al. A 2-Year Randomized Controlled Trial of Human Caloric Restriction: Feasibility and Effects on Predictors of Health Span and Longevity. J Gerontol A Biol Sci Med Sci. 2015;70: 1097-1104. doi:10.1093/gerona/glv057.
12. Heilbronn LK, de Jonge L, Frisard MI, DeLany JP, Larson-Meyer DE, Rood J, et al. Effect of 6-month calorie restriction on biomarkers of longevity, metabolic adaptation, and oxidative stress in overweight individuals: a randomized controlled trial. JAMA. 2006;295:1539-48. doi:10.1001/jama.295.13.1539.
13. Johnson ML, Distelmaier K, Lanza IR, Irving BA, Robinson MM, Konopka AR, et al. Mechanism by Which Caloric Restriction Improves Insulin Sensitivity in Sedentary Obese Adults. Diabetes. 2016;65: 74-84. doi:10.2337/db15-0675.
14. Colman RJ, Beasley TM, Kemnitz JW, Johnson SC, Weindruch R, Anderson RM. Caloric restriction reduces age-related and all-cause mortality in rhesus monkeys. Nat Commun. 2014;5:3557. doi:10.1038/ncomms4557.
15. Stekovic S, Hofer SJ, Tripolt N, Aon MA, Royer P, Pein L, et al. Alternate Day Fasting Improves Physiological and Molecular Markers of Aging in Healthy, Non-obese Humans. Cell Metab. 2019; 30 :462-476.e6. doi:10.1016/j.cmet.2019.07.016.
16. Spadaro O, Youm Y, Shchukina I, Ryu S, Sidorov S, Ravussin A, et al. Caloric restriction in humans reveals immunometabolic regulators of health span. Science. 2022; 375:671-677. doi:10.1126/science.abg7292.
17. Meydani SN, Das SK, Pieper CF, Lewis MR, Klein S, Dixit VD, et al. Long-term moderate calorie restriction inhibits inflammation without impairing cell-mediated immunity: a randomized controlled trial in non-obese humans. Aging (Albany NY). 2016;8: 1416-1431. doi:10.18632/aging.100994.
18. Yang H, Youm Y-H, Dixit VD. Inhibition of thymic adipogenesis by caloric restriction is coupled with reduction in age-related thymic involution. J Immunol. 2009;183: 3040-3052. doi:10.4049/jimmunol.0900562.
19. Guo Y, Luo S, Ye Y, Yin S, Fan J, Xia M. Intermittent Fasting Improves Cardiometabolic Risk Factors and Alters Gut Microbiota in Metabolic Syndrome Patients. J Clin Endocrinol Metab. 2021;106: 64-79. doi:10.1210/clinem/dgaa644.
20. Fontana L, Villareal DT, Das SK, Smith SR, Meydani SN, Pittas AG, et al. Effects of 2-year calorie restriction on circulating levels of IGF-1, IGF-binding proteins and cortisol in nonobese men and women: a randomized clinical trial. Aging Cell. 2016;15: 22-27. doi:10.1111/acel.12400.
21. Mihaylova MM, Chaix A, Delibegovic M, Ramsey JJ, Bass J, Melkani G, et al. When a calorie is not just a calorie: Diet quality and timing as mediators of metabolism and healthy aging. Cell Metab. 2023;35: 1114-1131. doi: 10.1016/j.cmet.2023.06.008.
22. Wiersinga WM. Clinical Relevance of Environmental Factors in the Pathogenesis of Autoimmune Thyroid Disease. Endocrinol Metab (Seoul). 2016;31: 213-222. doi:10.3803/EnM.2016.31.2.213.
23. Rodríguez-Muñoz A, Vitales-Noyola M, Ramos-Levi A, Serrano-Somavilla A, González-Amaro R, Marazuela M. Levels of regulatory T cells CD69(+)NKG2D(+)IL-10(+) are increased in patients with autoimmune thyroid disorders. Endocrine. 2016;51: 478-489. doi:10.1007/s12020-015-0662-2.

24. Castoro C, Le Moli R, Arpi ML, Tavarelli M, Sapuppo G, Frittitta L, et al. Association of autoimmune thyroid diseases, chronic atrophic gastritis and gastric carcinoid: experience from a single institution. J Endocrinol Invest. 2016;39: 779-784. doi:10.1007/s40618-016-0445-5.
25. Fröhlich E, Wahl R. Microbiota and Thyroid Interaction in Health and Disease. Trends Endocrinol Metab. 2019;30: 479-490. doi: 10.1016/j.tem.2019.05.008.
26. Virili C, Fallahi P, Antonelli A, Benvenga S, Centanni M. Gut microbiota and Hashimoto's thyroiditis. Rev Endocr Metab Disord. 2018;19: 293-300. doi:10.1007/s11154-018-9467-y.
27. Sbierski-Kind J, Grenkowitz S, Schlickeiser S, Sandforth A, Friedrich M, Kunkel D, et al. Effects of caloric restriction on the gut microbiome are linked with immune senescence. Microbiome. 2022;10: 57. doi:10.1186/s40168-022-01249-4.
28. Fabbiano S, Suárez-Zamorano N, Chevalier C, Lazarević V, Kieser S, Rigo D, et al. Functional Gut Microbiota Remodeling Contributes to the Caloric Restriction-Induced Metabolic Improvements. Cell Metab. 2018;28 :907-921.e7. doi:10.1016/j.cmet.2018.08.005.
29. Li G, Xie C, Lu S, Nichols RG, Tian Y, Li L, et al. Intermittent Fasting Promotes White Adipose Browning and Decreases Obesity by Shaping the Gut Microbiota. Cell Metab. 2017;26 :672-685.e4. doi:10.1016/j.cmet.2017.08.019.
30. Zheng X, Wang S, Jia W. Calorie restriction and its impact on gut microbial composition and global metabolism. Front Med. 2018;12: 634-644. doi:10.1007/s11684-018-0670-8.
31. Fan Y, Qian H, Zhang M, Tao C, Li Z, Yan W, et al. Caloric restriction remodels the hepatic chromatin landscape and bile acid metabolism by modulating the gut microbiota. Genome Biol. 2023; 24: 98. doi:10.1186/s13059-023-02938-5.

**Table 1.** Major inclusion and exclusion criteria

| |
|---|
| Major inclusion criteria: |
| • Long-term residence in Hangzhou for work, study or other reasons<br>• Aged between 18-65 years<br>• Previously diagnosed as HT by an endocrinologist according to the Guidelines for the Diagnosis and Treatment of Thyroid Diseases in China<br>• Had a baseline TPO-Ab level above 27 KU/L or TG-Ab level above 12 KU/L (three times above the critical values).<br>• Agreed to follow the study protocol |
| Major exclusion criteria: |
| • Have a history of thyroid trauma or surgical treatment<br>• With other autoimmune diseases, such as systemic lupus erythematosus or rheumatoid arthritis<br>• With serious chronic diseases, such as cancer, heart disease, hepatic or renal dysfunction<br>• Taking antibiotics within one month for any reason<br>• Pregnant or lactating women<br>• Participation in another clinical trial within three months<br>• Do not sign the informed consent |

**Table 2.** Simplified version of a meal plan sample send to the participants

| Meal and Diet | Food | Exchangeable food |
|---|---|---|
| Breakfast | | |
| tomato and egg noodles | buckwheat noodles (70 g) | buns, rolls, cakes, noodles, breads |
| | hen egg (60 g) | eggs |
| | tomato (100 g) | zucchini, cucumber, loofah |
| Additional meal | | |
| milk | milk (250 mL) | milk products |
| Lunch | | |
| mixed grain rice | rice (50 g) | rice |
| | barley (25 g) | sorghum, oat, barley, millet, buckwheat |
| meat with mushroom | lean pork (40 g) | lean pork, beef, lamb |
| | mushroom (40 g) | fresh mushrooms |
| shrimp with celery | shrimp (60 g) | shrimp, crab, shell |
| | celery (50 g) | rape, spinach, fennel, amaranth |
| vegetable tofu soup | tofu (50 g) | tofu product |
| | baby cabbage (60 g) | cabbage, cauliflower, bamboo shoots |
| oil | rapeseed oil (10 g) | olive oil, soybean oil, peanut oil |
| Additional meal | | |
| fruit | grapefruit (200 g) | tangerine, orange, lemon |
| Dinner | | |
| mixed grain rice | black rice (50 g) | sorghum, oat, barley, millet, buckwheat |
| | brown rice (25 g) | sorghum, oat, barley, millet, buckwheat |
| carrot with pea | carrot (50 g) | white radish, red radish |
| | pea (40 g) | cowpea, lentil, sword bean |
| steamed perch | perch (100 g) | carp, silver carp, pomfret |
| oil | soybean oil (10 g) | olive oil, rapeseed oil, peanut oil |
| Additional meal | | |
| nut | walnut (20 g) | almond, hazelnut, pistachio, cashew |

**Table 3.** Scanning parameters of thyroid MRI measurement

| Variables | TR | TE | EF | FOV | ST | SG | SL |
|---|---|---|---|---|---|---|---|
| T1WI | 524ms | 11ms | 2 | 220mm × 220mm | 4mm | 1.2mm | 20 |
| T2WI | 2410ms | 75ms | 2 | 220mm × 220mm | 4mm | 1.2mm | 20 |

MRI, magnetic resonance imaging; T1WI, T1-weighted images; T2WI, T2-weighted images; TR, time of repetition; TE, time of echo; EF, excitation frequency; FOV, field of view; ST, slice thickness; SG, slice gap; SL, scanning layers.

**Table 4.** Schedule of study procedure

| | Screening period | Intervention period | | |
|---|---|---|---|---|
| List | Visit 0<br>Week -1 | Visit 1<br>Week 0 | Visit 2<br>Week 6 | Visit 3<br>Week 12 |
| Informed consent | √ | | | |
| Online conference | √ | | | |
| Questionnaire of dietary information | | √ | | √ |
| Questionnaire of medication | | √ | | |
| Questionnaire of physical activity | | √ | | |
| Questionnaire of general demographic information | | √ | | √ |
| Questionnaire of Hashimoto's Thyroiditis Symptom | | √ | | √ |
| The MOS 36-item short from health survey | | √ | | √ |
| Dietary counseling | | | Daily | |
| Take photos to record the food and give feedback | | | Daily | |
| Thyroid morphology test (MRI) | | √ | | √ |
| Body composition analysis | | √ | √ | √ |
| 12-h fasting blood sample | | √ | √ | √ |
| Urine sample | | √ | √ | √ |
| Fecal sample | | √ | √ | √ |

MRI, magnetic resonance imaging; T1WI, T1-weighted images; T2WI, T2-weighted images; TR, time of repetition; TE, time of echo; EF, excitation frequency; FOV, field of view; ST, slice thickness; SG, slice gap; SL, scanning layers.

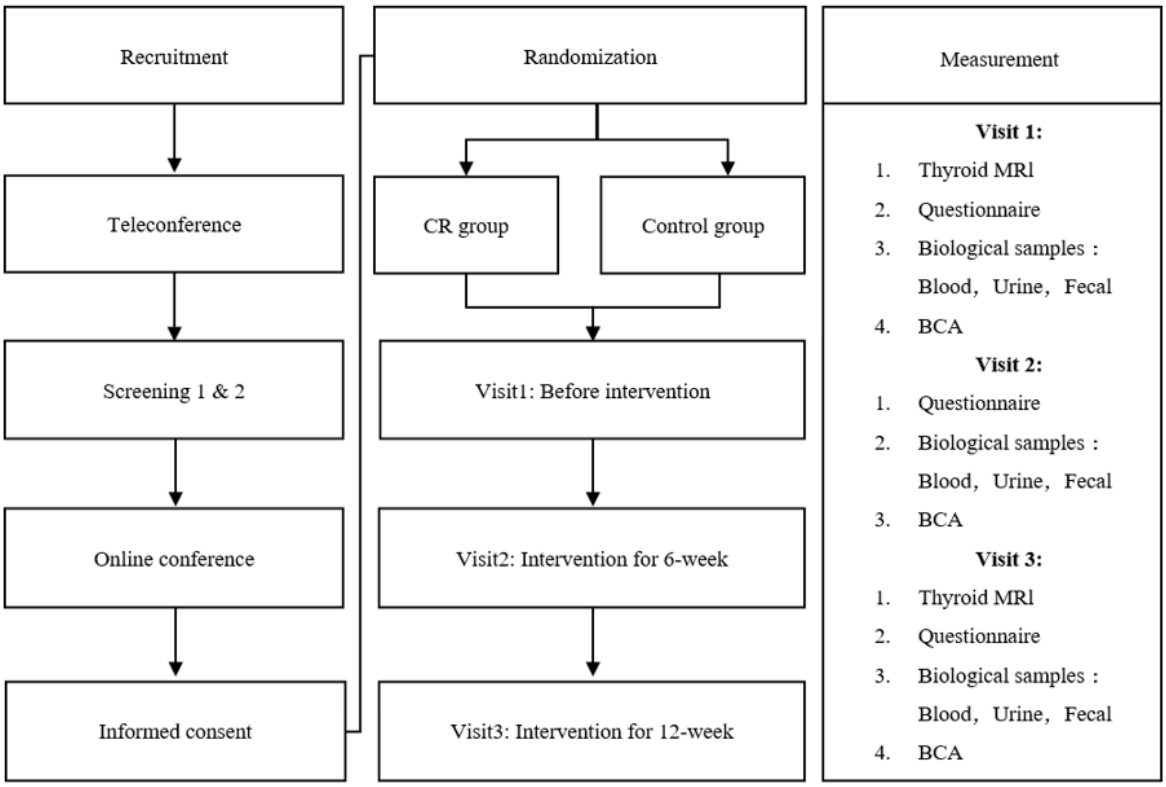


**Figure 1.** Study protocol flow diagram. MRI, magnetic resonance imaging; BCA, body composition analysis