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Metabolic and Immunological Biomarker Changes Following 12-Week Supplementation with a Processed Cirsium Brevicaule-Containing Powder: Two Independent Exploratory Single-Arm Studies

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1. Abstract

1.1. Background/Objectives

Preclinical studies have reported effects of Cirsium brevicaule on lipid metabolism and adipocyte differentiation, whereas human data on commercial multi-ingredient powders containing processed C. brevicaule remain limited. We reanalyzed two independent exploratory single-arm before-after studies in which a processed C. brevicaule-containing powder marketed under the same name was consumed for 12 weeks and evaluated changes in metabolic, vascular, and immune-related biomarkers.

1.2. Methods

Study 1 included 18 adults with type 2 diabetes, and Study 2 included nine untreated men aged ≥40 years with BMI ≥25 kg/m². The study product was supplied in 1.77-g sachets and consumed as two sachets in the morning and one in the evening (5.31 g product/day) for 12 weeks. The daily dose contained 2.5 g of a proprietary blended ingredient containing processed C. brevicaule; the exact amount and proportion of C. brevicaule within the blend were not disclosed by the manufacturer because they constitute proprietary formulation information. Study 1 included 11 outcomes and Study 2 included 30 outcomes. Benjamini-Hochberg false-discovery-rate (FDR) correction was applied within each study and analysis population. In Study 2, all nine participants were retained in the main analysis, and a post hoc sensitivity analysis was conducted in seven participants after excluding two with acute upper-respiratory symptoms and self-reported non-adherence at the final assessment. HOMA-IR was derived post hoc.

1.3. Results

In Study 1, HDL-C decreased from 51.17 ± 11.26 to 47.33 ± 9.78 mg/dL (mean change −3.83 mg/dL; 95% CI −6.15 to −1.52; p=0.0028; q=0.0306), and this was the only endpoint that remained significant after FDR correction. In the Study 2 main analysis, fasting insulin changed from 13.60 [9.28, 17.50] to 9.15 [6.03, 12.60] μIU/mL (p=0.0742), and NK-cell activity changed from 48.44 ± 14.60% to 51.89 ± 13.50% (p=0.2936); none of the 30 outcomes was significant after FDR correction.

In the seven-participant post hoc sensitivity analysis, fasting insulin (p=0.0469) and NK-cell activity (p=0.0230) changed nominally significantly, but neither remained significant after FDR correction. Post hoc HOMA-IR was likewise nonsignificant in the nine-participant analysis and nominally significant only in the seven-participant analysis.

1.4. Conclusions

Broad metabolic or immunological effects of 12-week supplementation with the processed C. brevicaule-containing powder were not established in the main analyses. The changes in fasting insulin, HOMA-IR, and NK-cell activity observed in the Study 2 post hoc sensitivity analysis should be regarded as hypothesis-generating signals concerning metabolic-immune responses. The HDL-C decrease observed in Study 1, safety, including the observed lipid and renal-function signals, and the reproducibility of the formulation-level findings should be evaluated in a prospectively registered, randomized, placebo-controlled trial.

2. Introduction

Cirsium brevicaule A. Gray is an Asteraceae plant native to the southwestern islands of Japan and has been used as a food. Cellular and animal studies of C. brevicaule have reported inhibition of adipocyte differentiation and lipid accumulation, including suppression of fatty acid synthase expression by leaf-derived monogalactosyldiacylglycerol and modulation of adipocyte differentiation by root-derived syringin [1–3]. Preclinical data on related Cirsium flavonoids and compounds have also been reported in relation to glucose metabolism, inflammation, JAK/STAT signaling, and immune responses [4–7]. However, because these studies differ in plant part, extract or compound, and animal model, they do not directly establish human effects of a multi-ingredient food containing processed C. brevicaule.

In clinical research on plant-derived foods, it is important to distinguish the effects of single compounds identified preclinically from the response to the formulation actually consumed. Even when the plant species is the same, bioavailability and biological effects may differ according to the plant part used, processing procedures, extraction conditions, coexisting ingredients, and dose. In commercial multi-ingredient foods, additive or interactive effects among ingredients are also possible; therefore, attributing a post-supplementation change to a specific botanical constituent requires additional compositional analysis or comparative trials. The processed C. brevicaule-containing powder examined here was likewise a multi-ingredient formulation. Accordingly, previous findings on C. brevicaule were used as biological background rather than as a basis for attributing the clinical results to that plant alone.

Insulin resistance is one of the central pathophysiological features of type 2 diabetes and obesity-related metabolic disorders. It is complexly related to hyperinsulinemia, hepatic glucose production, dyslipidemia, and ectopic fat accumulation, and insulin action is not uniform across organs [8–10]. Fasting insulin and the homeostasis model assessment of insulin resistance (HOMA-IR) are convenient surrogate indices used in large-scale and clinical studies [11,12]. Although these indices are practical surrogate measures that correlate with clamp-based measures in some settings, they do not directly measure individual-level insulin sensitivity and should be interpreted in light of their dependence on fasting glucose and insulin physiology [11–14]. Therefore, a decrease in fasting insulin cannot simply be treated as synonymous with improved insulin sensitivity.

Metabolic homeostasis and the immune system are not mutually independent. Natural killer (NK) cells are cytotoxic lymphocytes of the innate immune system that contribute to defense against infection and tumor immune surveillance, and their function is regulated by activating and inhibitory receptors, cytokines, and intracellular metabolic state [15–18]. NK-cell number, phenotype, and function change with aging and constitute part of immunosenescence [19]. NK-cell energy metabolism is also closely linked to effector function [20], and studies of obesity and adipose-tissue inflammation have shown that NK cells can participate in macrophage activation and insulin resistance [21,22]. Lipid accumulation in the obese environment can induce metabolic reprogramming of NK cells and impair antitumor function [23], whereas recovery of NK-cell metabolism and function has been reported after GLP-1 receptor agonist treatment in people with obesity [24], highlighting bidirectional links between metabolic state and NK-cell function.

For these reasons, observing metabolic indices and NK-cell activity under the same intervention has exploratory value. However, NK-cell activity is not a fixed individual trait, and short-term variation related to biological state and preanalytical conditions cannot be excluded. In the present studies, acute illness, sleep or stress, blood-collection timing, specimen transport, and assay conditions were not all fully standardized or documented. Furthermore, obesity-related changes in NK cells are not necessarily identical in peripheral blood and adipose tissue, and the tissue immune environment cannot be inferred from a single circulating functional assay. Thus, even if metabolic indices and NK-cell activity change concurrently, they should not immediately be reduced to a single common mechanism; reproducibility and mechanism must be evaluated stepwise.

Viewed from the perspective of aging biology, nutrient sensing, mitochondrial function, and chronic inflammation are major biological features of aging [25,26]. Forkhead box O3 (FOXO3) is a transcription factor downstream of insulin/IGF-1 signaling, and its association with human longevity has been reproduced in multiple populations [27–30]. Signaling from the insulin receptor through phosphoinositide 3-kinase (PI3K)-AKT regulates FOXO phosphorylation and intracellular localization [31–33], while SIRT1-dependent deacetylation and direct regulation by AMP-activated protein kinase (AMPK) also contribute to FOXO responses [34,35]. AMPK has been shown to connect NAD+ metabolism with SIRT1 activity [36], and NAD+-dependent sir-

tuins are closely related to mitochondrial biology, metabolism, stress responses, and age-related disease [37–41]. Nutritional and pharmacological approaches targeting FOXO3 have also been proposed [42], but none of these pathways was directly measured in the present studies. The key point is not to confuse discussion of longevity-associated genes or nutrient-sensing pathways with evidence of clinical efficacy. FOXO3, AMPK, NAD+, and sirtuins constitute candidate networks that could help organize possible explanations for the observed biomarker changes, but their activity cannot be inferred from short-term blood-test changes. Because these pathways connect insulin signaling, mitochondrial function, stress responses, and chronic inflammation [25–42], they nevertheless provide a useful theoretical framework for parallel evaluation of metabolic and immune outcomes and for selecting molecular markers to be measured directly in the next trial.

In the present study, we reanalyzed participant-level data from two independent single-arm before-after studies in which a multi-ingredient powder marketed under the same name and containing processed C. brevicaule was consumed for 12 weeks. Study 1 enrolled adults with type 2 diabetes, whereas Study 2 enrolled untreated men at metabolic risk; because the two populations differed substantially in background, it was not appropriate to combine them for an integrated efficacy assessment. The objective was to transparently quantify within-participant changes in metabolic, vascular, inflammatory, and immune-related biomarkers in each study, account for multiplicity, and identify human signals that should be retested in future controlled trials. In Study 2 in particular, the main analysis of all nine participants was clearly separated from the post hoc sensitivity analysis excluding two participants with acute symptoms and non-adherence, and the relationships among fasting insulin, HOMA-IR, and NK-cell activity were considered in a hypothesis-generating manner.

3. Materials and Methods

3.1. Study Design

This study was a reanalysis of two mutually independent exploratory single-arm before-after studies comparing measurements before and after 12 weeks of supplementation with a processed C. brevicaule-containing powder. Study 1 included 18 adults with type 2 diabetes, received ethics approval in 2024, and was conducted in 2025. Study 2 included nine untreated men aged ≥40 years and was conducted in 2026. There was no overlap in participants between the two studies. Because the studies differed in calendar year, participant background, and outcome set, data were not pooled across studies for the main analyses.

Both studies were exploratory and had no control group, randomization, or blinding. No formal a priori power calculation was performed. Neither study was prospectively registered in a public clinical-trial registry before initiation. Post hoc analyses are explicitly distinguished from the main analyses in this report.

3.2. Ethical Considerations

Both studies were conducted with approval from the Ethics Review Committee of the International Society for Geriatric and Longevity Clinical Medicine. The ethics approval identifiers were ISGN_N10052024 for Study 1 and ISGN_N10012026 for Study 2. Written informed consent for study participation was obtained from all participants. The Study 2 informed-consent document specified voluntary participation, the right to withdraw consent, protection of personal information, and anonymization when study results were published.

3.3. Study Product and Supplementation

The study product was ADIPON EX (HUNTKEY JAPAN Co., Ltd., Tokyo, Japan), a multi-ingredient powder containing processed C. brevicaule. Each sachet contained 1.77 g of product, and participants consumed two sachets in the morning and one in the evening, dissolved in water, for 12 weeks; the available study instructions described administration as under fasting conditions. Total product intake was therefore 5.31 g/day, of which the proprietary blended ingredient containing processed C. brevicaule accounted for 2.5 g/day. The exact proportion of C. brevicaule within the proprietary blend was not disclosed by the manufacturer as proprietary formulation information. Other listed ingredients were matcha powder, chlorella, Lumbricus powder, tricalcium phosphate, dried almond, and inulin. Information on the C. brevicaule plant part used, detailed processing conditions, individual quantities of the other ingredients, product lot numbers, marker compounds, and quality specifications was not available. Accordingly, observed changes were not attributed specifically to C. brevicaule.

In Study 2, the planned intervention period was 12 weeks (84 days ± 3 days). Participants received the study product at the initial examination and were instructed to begin intake the following day. They were instructed not to eat before examinations, whereas water and tea were permitted. The available records did not specify whether tea was always consumed without caloric additives; therefore, fasting status should be interpreted according to the study instructions rather than as a fully standardized zero-calorie fasting condition. The interval between the final study-product intake and blood collection could not be determined from the available records for each participant.

3.4. Study 1: Type 2 Diabetes Cohort

The Study 1 analysis included 18 adults with type 2 diabetes (8 men and 10 women), with a mean age of 68.3 ± 7.0 years (range, 50–79 years). Antidiabetic medications, with overlapping prescriptions, included metformin (n=13), DPP-4 inhibitors (n=9), SGLT2 inhibitors (n=11), alpha-glucosidase inhibitors (n=3), glinides (n=3), and an oral GLP-1 receptor agonist (n=1). No antidiabetic medication was newly initiated, discontinued, or dose-adjusted during the intervention period. Disease duration, details of concomitant medications other than antidiabetic drugs (including lipid-lowering agents), and quantitative records of diet and physical activity could not be determined from the

available analysis materials. Detailed laboratory assay methods and device-specific measurement procedures for ABI and CAVI were not sufficiently documented in the materials available for this reanalysis and were therefore not supplemented by inference.

The 11 outcomes were HbA1c, right and left ankle-brachial index (ABI), right and left cardio-ankle vascular index (CAVI), LDL cholesterol (LDL-C), HDL cholesterol (HDL-C), body weight, body mass index (BMI), body-fat percentage, and waist circumference. For each outcome, only participants with complete pre- and post-intervention values were analyzed, and missing values were not imputed.

3.5. Study 2: Untreated Men at Metabolic Risk

Study 2 included nine men aged ≥40 years with BMI ≥25 kg/m², no overt subjective symptoms, and no pharmacological treatment. Mean age at baseline was 50.9 ± 9.0 years (range, 41–68 years). One participant had a baseline fasting glucose concentration of 149 mg/dL and fasting insulin of 123 μIU/mL. This isolated abnormal fasting-glucose value was not treated as a new diagnosis of diabetes in this reanalysis because confirmatory testing and diagnostic records were unavailable in the materials used for analysis. Because there was no evidence of a data-entry error and the participant did not meet any prespecified exclusion criterion, this participant was retained in both the main and post hoc sensitivity analyses.

All nine participants completed pre- and post-intervention measurements. At the final assessment, P02 and P08 had transient cold/upper-respiratory symptoms and self-reported non-adherence to study-product intake instructions. The attending physician considered that the blood and immune-related measurements obtained that day might not be suitable for efficacy evaluation. Because this exclusion rule had not been formally prespecified in the analysis plan before the study, all nine participants were retained in the main analysis, and an analysis excluding only P02 and P08 was designated a post hoc restricted sensitivity analysis (n=7).

Study 2 evaluated 30 outcomes covering liver function, glucose metabolism, lipids, kidney function, inflammation, hematology and leukocyte differentials, fasting insulin, apolipoprotein A-I, adiponectin, remnant-like particle cholesterol, and NK-cell activity. Laboratory testing was commissioned to the external laboratory SRL, Inc. Detailed information on individual assay methods, reagents, and specimen transport time for NK-cell activity could not be consistently determined from the materials available for this reanalysis; therefore, these details were not supplemented by inference.

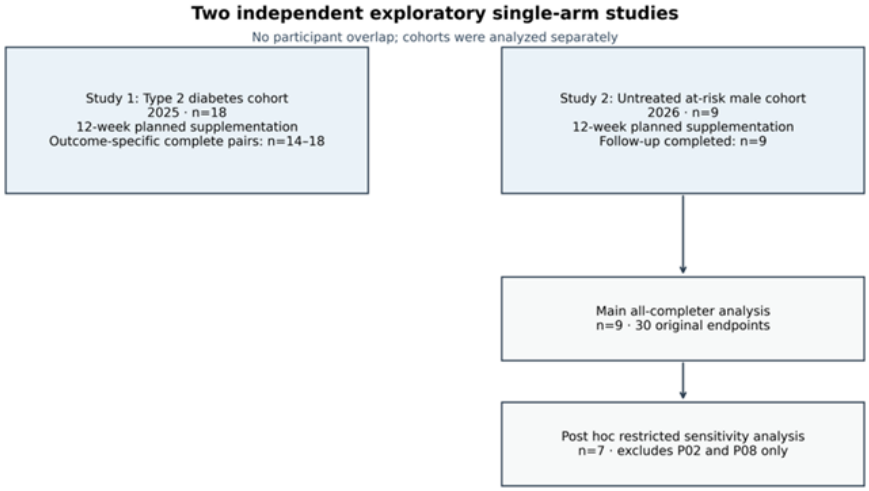

Figure 1: Study flow and analysis populations. The two studies were analyzed independently; no pooled efficacy analysis was performed.

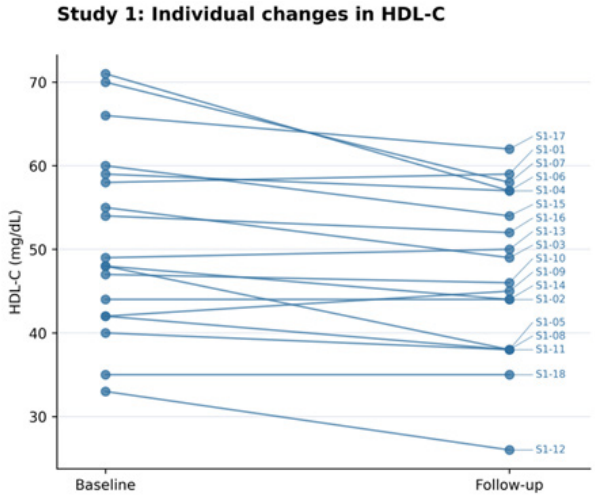

Figure 2: Individual Study 1 HDL-C values before and after 12 weeks. Lines connect paired observations; the thick line indicates the group mean.

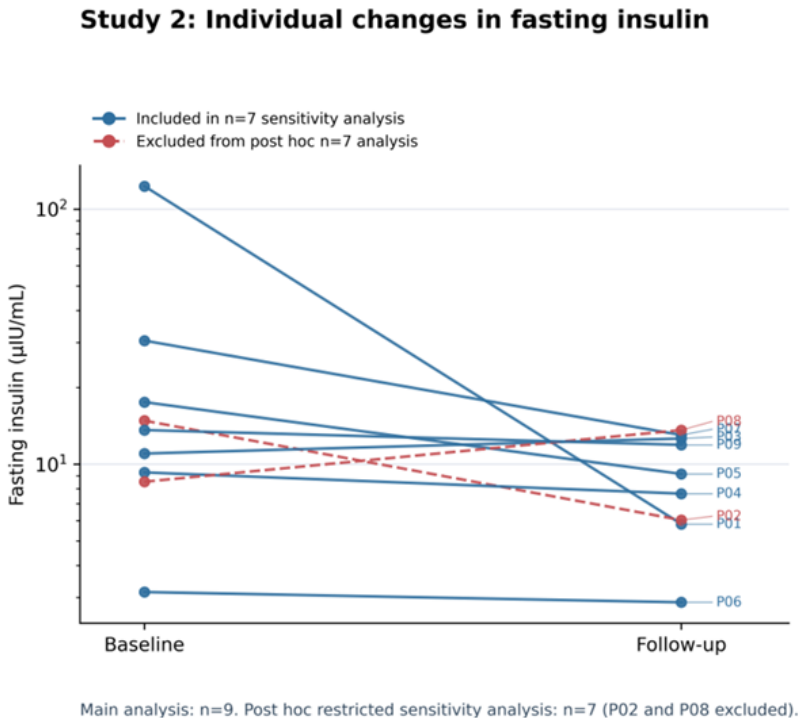

Figure 3A: Individual fasting-insulin values in the Study 2 main population (n=9). The P01 extreme baseline value was retained. The y-axis is shown on a logarithmic scale.

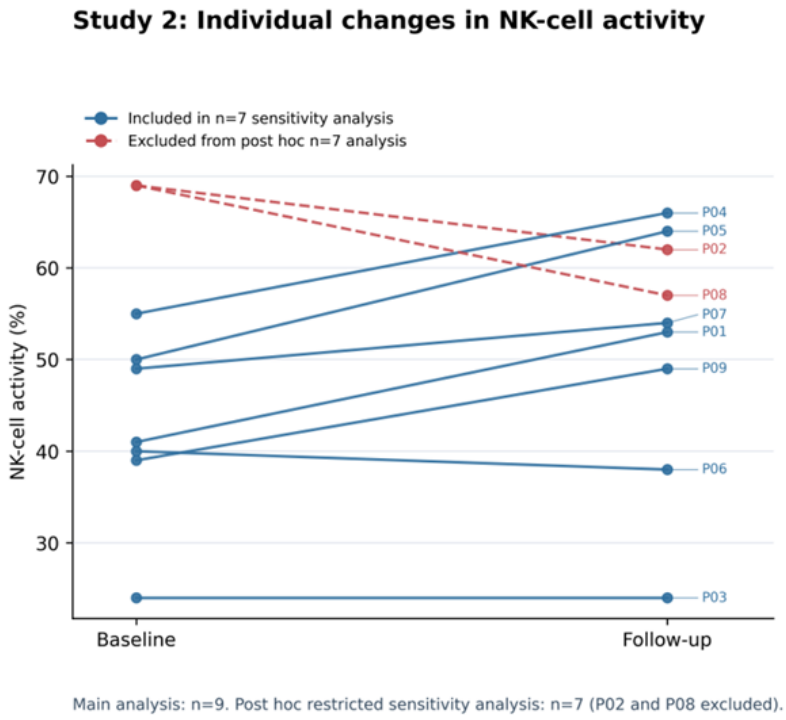

Figure 3B: Individual NK-cell activity values in the Study 2 main population (n=9).

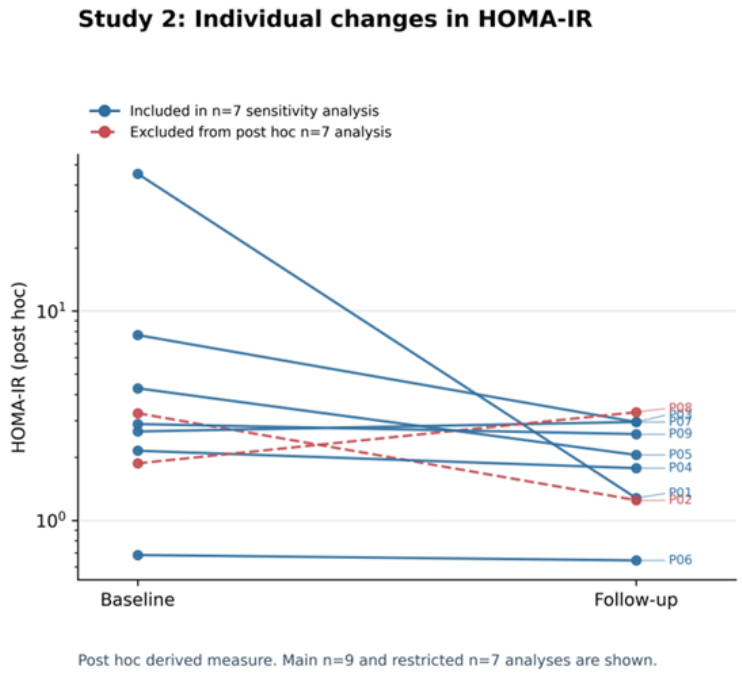

Figure 4: Individual post hoc HOMA-IR values before and after intervention in the Study 2 main population (n=9). The y-axis is shown on a logarithmic scale.

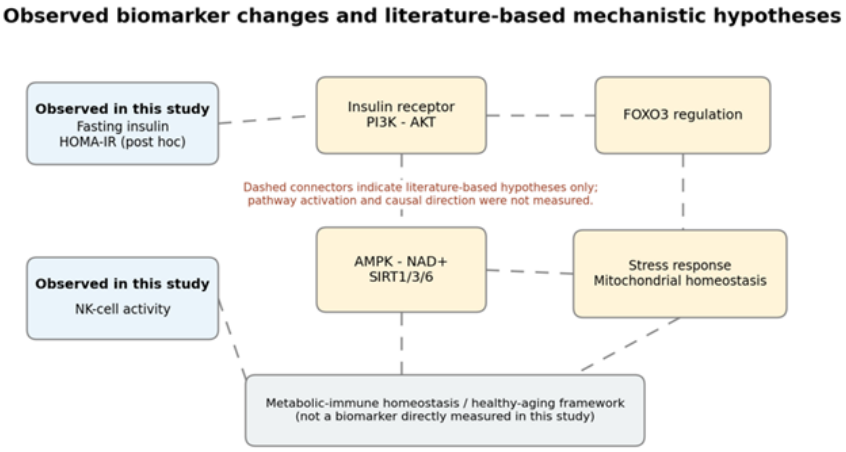

Figure 5: Biomarkers observed in the present study and literature-based mechanistic hypotheses. Dashed connectors indicate literature-based conceptual relationships only; they do not imply measured pathway activation or a demonstrated causal direction among PI3K-AKT, FOXO3, AMPK, NAD+, or sirtuins.

Table 1: Baseline characteristics and exposure. Values are mean ± SD (range) unless otherwise stated.

Characteristic	Study 1	Study 2
Population	Adults with type 2 diabetes	Untreated men at metabolic risk
Participants	18	9
Age, years	68.3 ± 7.0 (50–79)	50.9 ± 9.0 (41–68)
Sex	10 women / 8 men	9 men
BMI	27.81 ± 5.39 kg/m² (n=16)	Eligibility BMI ≥25 kg/m²; individual values unavailable
Exposure	ADIPON EX, 5.31 g/day for 12 weeks	ADIPON EX, 5.31 g/day for 12 weeks
Proprietary blend containing processed C. brevicaule	2.5 g/day; exact C. brevicaule content not disclosed	2.5 g/day; exact C. brevicaule content not disclosed

Table 2: Study 1 paired outcomes. Parametric rows show mean ± SD and mean paired change (95% CI); nonparametric rows show median [Q1, Q3] and the median of paired change scores. Effect is Hedges g_z or matched-pairs rank-biserial r. Change estimates were calculated from paired individual values and may differ slightly from differences between the displayed rounded baseline and Week 12 summaries.

Outcome (unit)	n	Baseline	Week 12	Change	Test	p	BH q	Effect
HbA1c (%)	18	7.25 ± 1.18	7.38 ± 1.41	+0.13 (-0.15, 0.41)	paired t	0.327	0.5941	g_z=0.23
ABI, right (ratio)	16	1.10 [0.98, 1.23]	1.11 [1.06, 1.14]	paired-change median +0.04	exact Wilcoxon	0.9446	0.9739	r=-0.03
ABI, left (ratio)	16	1.11 ± 0.15	1.07 ± 0.06	-0.04 (-0.13, 0.05)	paired t	0.3781	0.5941	g_z=-0.22
CAVI, right (unitless)	16	9.51 ± 1.29	9.45 ± 1.44	-0.06 (-0.51, 0.40)	paired t	0.797	0.9739	g_z=-0.06
LDL-C (mg/dL)	18	133.56 ± 25.59	128.94 ± 22.92	-4.61 (-15.26, 6.03)	paired t	0.3735	0.5941	g_z=-0.21
HDL-C (mg/dL)	18	51.17 ± 11.26	47.33 ± 9.78	-3.83 (-6.15, -1.52)	paired t	0.0028	0.0306	g_z=-0.79
Body weight (kg)	16	67.75 ± 12.67	67.11 ± 12.93	-0.64 (-1.52, 0.24)	paired t	0.1399	0.4821	g_z=-0.37
BMI (kg/m²)	16	25.95 [24.27, 30.73]	26.05 [24.43, 30.27]	paired-change median -0.40	exact Wilcoxon	0.096	0.4821	r=-0.48
Body fat percentage (%)	15	32.07 ± 9.00	31.48 ± 8.36	-0.59 (-1.48, 0.30)	paired t	0.1753	0.4821	g_z=-0.35
Waist circumference (cm)	14	93.07 ± 10.31	93.04 ± 11.46	-0.04 (-2.35, 2.28)	paired t	0.9739	0.9739	g_z=-0.01

Table 3: Selected Study 2 main-analysis outcomes (n=9). For space, selected outcomes are shown here; the complete 30-endpoint table is Supplementary Table S2. Parametric change values are mean paired changes (95% CI); nonparametric change values are medians of paired change scores. Change estimates were calculated from paired individual values and may differ slightly from differences between the displayed rounded baseline and Week 12 summaries.

Outcome (unit)	n	Baseline	Week 12	Change	Test	p	BH q	Effect
Glucose (mg/dL)	9	94.00 [89.00, 99.00]	91.00 [89.00, 94.00]	paired-change median -3.00	exact Wilcoxon	0.2656	0.6258	r=-0.47
HbA1c (%)	9	5.42 ± 0.27	5.41 ± 0.21	-0.01 (-0.16, 0.14)	paired t	0.8695	0.9524	g_z=-0.05
Glycated albumin (%)	9	14.00 [13.00, 15.00]	13.50 [12.80, 14.20]	paired-change median +0.00	exact Wilcoxon	0.8203	0.9524	r=-0.11
LDL-C (mg/dL)	9	128.56 ± 20.76	126.33 ± 27.65	-2.22 (-21.15, 16.70)	paired t	0.7934	0.9524	g_z=-0.08
Creatinine (mg/dL)	9	0.81 ± 0.10	0.85 ± 0.10	+0.05 (-0.01, 0.10)	paired t	0.1046	0.5022	g_z=0.55
eGFR (mL/min/1.73 m²)	9	81.26 ± 12.24	76.14 ± 10.30	-5.11 (-11.36, 1.14)	paired t	0.0959	0.5022	g_z=-0.57
CRP (mg/dL)	9	0.05 [0.05, 0.31]	0.05 [0.03, 0.40]	paired-change median -0.00	exact Wilcoxon	0.8203	0.9524	r=0.11
Apolipoprotein A-I (mg/dL)	9	157.11 ± 30.69	154.56 ± 32.25	-2.56 (-17.48, 12.36)	paired t	0.7032	0.9524	g_z=-0.12
Adiponectin (µg/mL)	9	7.13 ± 1.87	7.33 ± 1.64	+0.20 (-0.53, 0.93)	paired t	0.5447	0.9524	g_z=0.19
Fasting insulin (µIU/mL)	9	13.60 [9.28, 17.50]	9.15 [6.03, 12.60]	paired-change median -1.70	exact Wilcoxon	0.0742	0.5022	r=-0.69
NK-cell activity (%)	9	48.44 ± 14.60	51.89 ± 13.50	+3.44 (-3.62, 10.51)	paired t	0.2936	0.6258	g_z=0.34

3.6. Post Hoc HOMA-IR

In Study 2, HOMA-IR was calculated post hoc from fasting insulin and fasting glucose as fasting insulin (μIU/mL) × fasting glucose (mg/dL) / 405 [11,12]. HOMA-IR was not among the original 30 outcomes and was treated as a post hoc derived measure rather than an independent confirmatory endpoint; only nominal p values are reported.

3.7. Statistical Analysis

For continuous variables, the Shapiro-Wilk test was applied to the pre-post change score. When normality of the change score could be assumed, a two-sided paired t-test was used; otherwise, a two-sided Wilcoxon signed-rank test was used. Outcomes analyzed parametrically are described using mean ± standard deviation, mean change, 95% confidence interval, and paired-sample Hedges g_z. Hedges g_z was used as a bias-corrected standardized paired-change effect size. Nonparametric outcomes are described primarily using median [first quartile, third quartile], the median of paired change scores, and matched-pairs rank-biserial correlation (r). The nominal two-sided significance level was $p<0.05$.

To account for false-positive risk arising from exploratory evaluation of numerous outcomes, Benjamini-Hochberg FDR correction was applied independently by study and analysis population [43,44]. Specifically, the 11 Study 1 outcomes, the 30 outcomes in the nine-participant Study 2 main analysis, and the 30 outcomes in the seven-participant Study 2 post hoc sensitivity analysis were treated as separate multiplicity families. Post hoc HOMA-IR was not added to the original 30-outcome family and its nominal p value was interpreted as hypothesis-generating. No direct between-study statistical comparison or simple pooled analysis of Study 1 and Study 2 was performed. All statistical analyses were performed using IBM SPSS Statistics, version 30.0 (IBM Corp., Armonk, NY, USA).

4. Results

4.1. Participant Flow and Baseline Characteristics

All 18 Study 1 participants and all nine Study 2 participants contributed at least one paired outcome. Outcome-specific missingness in Study 1 produced n=14–18. Study 2 contained complete pairs for the 30 recorded outcomes. The main Study 2 analysis retained P02 and P08; only the explicitly labeled sensitivity analysis excluded them.

4.3. Study 2 Main Analysis (n=9)

Fasting insulin decreased from a median of 13.60 [9.28, 17.50] to 9.15 [6.03, 12.60] μIU/mL, but the exact Wilcoxon test did not reach nominal significance (p=0.0742; BH q=0.5022; rank-biserial r=−0.69). NK-cell activity changed from 48.44 ± 14.60% to 51.89 ± 13.50% (mean change +3.44 percentage points; 95% CI −3.62 to 10.51; p=0.2936; BH q=0.6258; Hedges g_z=0.34). None of the 30 Study 2 endpoints remained significant after false-discovery-rate correction.

4.4. Study 2 Sensitivity Analysis and Post Hoc HOMA-IR

After excluding P02 and P08 because of transient upper-respiratory symptoms and self-reported non-adherence at follow-up, fasting insulin decreased in seven participants (median 13.60 to 9.15 μIU/mL; exact p=0.0469; BH q=0.7031; rank-biserial r=−0.86), and NK-cell activity increased from 42.57 ± 10.15% to 49.71 ± 14.71% (mean change +7.14; 95% CI 1.38 to 12.90; p=0.0230; BH q=0.6898; Hedges g_z=1.00). These signals were nominal only and did not survive multiplicity correction. Because the exclusion was post hoc, selection bias cannot be excluded.

Post hoc HOMA-IR decreased from 2.89 [2.15, 4.28] to 2.06 [1.28, 2.95] in the nine-participant analysis (exact p=0.0742) and from 2.89 [2.41, 5.98] to 2.06 [1.53, 2.77] in the seven-participant sensitivity analysis (exact p=0.0469). These unadjusted values are exploratory and should not be treated as independent confirmation because HOMA-IR is algebraically derived from glucose and insulin.

4.5. Safety-Relevant Findings

No adverse events documented as related to the study product were identified in the available records. However, systematic safety surveillance using structured adverse-event questionnaires, severity grading, and standardized causality assessment could not be confirmed for either study. Accordingly, the absence of recorded product-related adverse events should not be interpreted as established safety. In Study 2, serum creatinine increased from 0.81 ± 0.10 to 0.85 ± 0.10 mg/dL (p=0.1046; q=0.5022) and laboratory-reported eGFR decreased from 81.26 ± 12.24 to 76.14 ± 10.30 mL/min/1.73 m² (p=0.0959; q=0.5022). Neither outcome showed statistically significant evidence of change, but these directions, together with the HDL-C decrease in Study 1, should be prospectively monitored in future studies.

5. Discussion

5.1. Principal Findings and Positioning of the Present Study

The present study reanalyzed participant-level data from two independent exploratory single-arm studies in which a processed Cirsium brevicaule-containing powder was consumed for 12 weeks, using the same statistical principles across the two studies. The most important point is that, rather than forcing all analytical results into a single conclusion of ‘effective’ or ‘ineffective,’ we separated the target populations and analysis populations. In Study 1, only the decrease in HDL-C remained after FDR correction, whereas no clear improvements were observed in HbA1c, LDL-C, anthropometric measures, or ABI/CAVI. In the main analysis of Study 2 (n=9), all 30 outcomes were nonsignificant after FDR correction, and neither fasting insulin nor NK-cell activity was statistically significant in the main analysis. Therefore, the present studies do not establish broad metabolic-improving or immune-enhancing effects of the processed C. brevicaule-containing powder.

On the other hand, in the post hoc sensitivity analysis of Study 2 excluding two participants in whom acute upper respiratory symptoms were documented and non-adherence to intake instructions was self-reported, decreases in fasting insulin and

post hoc HOMA-IR and an increase in NK-cell activity simultaneously showed nominal statistical significance. Fasting insulin and NK-cell activity did not remain significant after FDR correction, while post hoc HOMA-IR was evaluated using nominal p values only; chance findings therefore remain possible. Nevertheless, the simultaneous appearance of signals across biologically distinct metabolic and immune indices may be useful for narrowing the endpoints to be evaluated in the next-stage trial. The role of an exploratory study is not to anticipate confirmatory conclusions, but to clarify which signals should be retested and under what conditions. In this sense, the n=7 findings in the present study are appropriately positioned as hypothesis-generating human data. In interpreting the present study, we also do not equate 'absence of statistical significance' with 'absence of any effect.' In small samples, confidence intervals are wide and the precision of effect estimates is low; therefore, a nonsignificant result is not proof of a zero effect. Conversely, efficacy cannot be claimed merely because an unadjusted p value is below 0.05. Because 30 outcomes were explored in Study 2, the p values for insulin and NK-cell activity in the n=7 analysis must be evaluated in the context of multiple testing, and the failure of these findings to remain significant after FDR correction was explicitly considered alongside the main analysis. Accordingly, this manuscript evaluates the robustness of the signal by considering effect sizes, individual trajectories, and dependence on the analysis population, and focuses the discussion on whether the signals warrant retesting in a subsequent trial.

5.2. Fasting Insulin and HOMA-IR: What They Show and What They Do Not Show

Insulin resistance is a central pathophysiological process linking type 2 diabetes, obesity, dyslipidemia, and cardiovascular risk [8–10]. Fasting insulin and HOMA-IR are widely used surrogate indices [11–14], but they are not glucose-clamp measurements. In particular, HOMA-IR is a model reflecting the fasting equilibrium between hepatic glucose production and insulin secretion and is not a direct measure of whole-body insulin sensitivity, including peripheral skeletal muscle. Therefore, the decrease observed in the n=7 Study 2 analysis should not be stated as proof that insulin resistance improved, but rather as a change consistent with lower fasting insulin exposure and lower HOMA-IR.

Fasting insulin concentration is determined by both the amount of insulin secreted from pancreatic beta cells and insulin clearance, particularly hepatic clearance. Thus, when insulin concentration decreases at a similar glucose level, not only a reduction in the amount of insulin required but also changes in secretion dynamics or clearance remain possible explanations. Moreover, because HOMA-IR contains fasting insulin in its formula, in a dataset such as the present one, in which some participants showed large insulin changes, the change in HOMA-IR is not an independent second piece of evidence but largely a mathematical re-expression of the same physiological information. For this reason as well, insulin and HOMA-IR should not be counted as two independent efficacy endpoints.

More precise clinical characterization of insulin resistance would require a combination of dynamic indices derived from an oral glucose tolerance test (OGTT), assessment of secretion using C-peptide, estimation of insulin clearance, or a hyperinsulinaemic-euglycaemic clamp [13,14]. The present study was not designed to provide such mechanistic decomposition; therefore, the signal obtained here is valuable primarily because it indicates what should be measured in the next trial. In particular, it will be important to prospectively determine whether the decrease in fasting insulin is reproducible and, if so, how glucose homeostasis, C-peptide, body weight, liver function, and lipid metabolism change in parallel. The statistical plan should likewise move from the present design, in which numerous laboratory variables were explored together, to a design in which the primary endpoint and primary analysis population are fixed in advance. If a single primary endpoint is selected, sample size should be calculated using a clinically meaningful difference and its variance, with secondary and safety endpoints organized hierarchically. Even if several primary candidates are retained, the method for multiplicity control should be specified in the protocol and registry, and rules concerning exclusions, missing data, acute illness, and adherence should be decided before data lock. This would allow the distinction between the n=9 and n=7 analysis populations, which was necessarily post hoc in the present study, to be handled as a prespecified estimand in the next trial.

Furthermore, a decrease in fasting insulin has several possible physiological explanations. Improved insulin sensitivity that permits maintenance of the same glucose homeostasis with less insulin is one hypothesis, but changes in pancreatic beta-cell secretion and other determinants of fasting insulin also remain possible [11–14]. The present study did not measure C-peptide, perform OGTT or clamp testing, use continuous glucose monitoring, or assess indices of insulin clearance; therefore, changes in secretion, clearance, or short-term variation related to acute stress, sleep, diet, or pre-sampling behavior cannot be distinguished. The absence of clear improvement in HbA1c and glycated albumin is another reason not to infer long-term improvement in glucose metabolism from a single fasting-insulin change.

In P01, extreme baseline values were present for insulin (123 μIU/mL) and glucose (149 mg/dL), and the mean was strongly influenced by this single participant. For this reason, the present manuscript places emphasis on medians and individual trajectories when interpreting insulin and HOMA-IR. The decision not to exclude P01 solely because the value was an outlier is important for the transparency of the study. In a future study, repeated testing under identical conditions, with prespecified recording of fasting duration, sleep, exercise, acute infection, and alcohol intake on the preceding day, and, when necessary, use of averages from repeated measurements would be desirable to reduce within-participant variability.

5.3. The Insulin-PI3K-AKT-FOXO3 Axis as a Mechanistic Hypothesis

Any discussion linking lower insulin with FOXO3 must separate the observed clinical data from molecular mechanism. Stimulation of the insulin receptor regulates diverse metabolic responses through the PI3K-AKT pathway [31], and AKT-mediated phosphorylation of the FOXO family contributes to nuclear export and transcriptional suppression [32,33]. Thus, in theory, changes in the intensity of insulin/IGF signaling could alter the regulatory environment of FOXO. However, what was measured in the present study was circulating fasting insulin; phosphorylation of AKT or nuclear localization of FOXO3 in liver, skeletal muscle, adipose tissue, or other tissues was not measured. A decrease in circulating insulin therefore cannot be linearly translated into reduced tissue AKT signaling and then into FOXO3 activation.

FOXO3 is of particular interest as a biological background to the present study not only because of its position in molecular signaling pathways, but also because its association with human longevity has been relatively consistently reproduced. Associations between FOXO3 polymorphisms and longevity have been reported in multiple populations, including Japanese Americans, Germans, and southern Italians [27–29], and reviews and meta-analyses have positioned FOXO3 as a major candidate gene related to human longevity [30,45–47]. Genetic association, however, is entirely different from activation of a transcription factor by a short-term nutritional intervention. The present findings do not demonstrate activity of the FOXO3 pathway; they merely suggest a mechanistic bridge that should be tested in future research.

FOXO3 function varies according to tissue, cell type, nutritional state, oxidative stress, and growth-factor signaling; it is therefore not a simple axis on which more FOXO3 is always better. Different post-translational modifications, including AKT-dependent phosphorylation, SIRT1-dependent deacetylation, and AMPK-dependent phosphorylation, can influence FOXO3 target selection and intracellular localization [31–36]. Accordingly, if FOXO3 is evaluated in a future human intervention trial, total protein abundance alone would be insufficient; phosphorylation state, nuclear translocation, and expression of target genes should be interpreted together. Avoiding direct extrapolation from the serum insulin change observed here to such intracellular events is essential to maintain credibility in the mechanistic discussion.

To test this hypothesis in a subsequent trial, it will be necessary to prespecify and measure not only circulating insulin but also AKT/FOXO3 phosphorylation in peripheral blood cells or an appropriate surrogate tissue, FOXO3 target genes, and oxidative-stress-related markers. In addition, the processed C. brevicaule-containing powder is a multi-ingredient product, and the observed signals cannot be regarded as effects of C. brevicaule alone.

5.4. The AMPK-NAD+-Sirtuin Network and a Healthy-Aging Framework

FOXO3 also intersects with metabolic-stress responses mediated by AMPK and sirtuins. SIRT1 deacetylates FOXO3 and modulates stress-dependent transcriptional responses [34], while AMPK can directly phosphorylate FOXO3 [35]. AMPK is also linked to SIRT1 activity through NAD+ metabolism [36] and is positioned as a nutrient-sensing system integrating energy homeostasis, autophagy, mitochondrial adaptation, and inflammatory control [39,48]. NAD+ is a metabolic coenzyme that supports sirtuin activity, and age-related alterations in NAD+ metabolism and their relationship to mitochondrial function have been extensively investigated [37,40,49].

The AMPK-NAD+-sirtuin network is not a simple linear pathway that operates in one direction during energy deficiency. AMPK responds to cellular energy stress reflected in adenine-nucleotide balance, NAD+ metabolism differs across intracellular compartments, and individual sirtuins regulate different substrates in the nucleus, cytoplasm, and mitochondria [37-41,48-51]. Therefore, a change in a single peripheral-blood metabolic marker cannot be used to infer that AMPK activity increased, NAD+ increased, or sirtuins were activated. Nevertheless, because this network intersects with nutrient sensing, mitochondrial homeostasis, oxidative-stress responses, and inflammation, it provides a useful framework for organizing mechanistic candidates in next-stage studies that simultaneously track metabolic and immune biomarkers.

In particular, when discussing the concept of healthy aging, short-term biomarker changes must be clearly distinguished from lifespan and healthspan. Longevity-associated FOXO3 polymorphisms and research on NAD+/sirtuins provide a background in long-term aging biology [25-30,37-42], but they do not mean that a 12-week food intervention has demonstrated life extension or rejuvenation. The purpose of using a healthy-aging framework in the present manuscript is to place the observed metabolic and immune signals within aging-related nutrient-sensing biology and thereby make more concrete the molecular markers that should be measured in the next trial.

The mitochondrial sirtuin SIRT3 regulates the electron-transport chain, ATP production, and oxidative-stress responses [38,50], whereas SIRT6 participates in genomic stability, glucose and lipid metabolism, and inflammation [41]. Because mammalian sirtuins regulate multiple processes relevant to health span, they are considered an important network in aging research [51]. Pharmacological and nutritional possibilities for linking FOXO3 activation to healthy longevity have also been discussed [42].

However, none of AMPK, NAD+, SIRT1, SIRT3, SIRT6, or FOXO3 was measured in the present studies. Thus, conclusions such as the product activated AMPK, increased NAD+, or activated sirtuins and promoted rejuvenation are not supported by these data. What the present study provides is a framework for connecting the peripheral metabolic signals represented by fasting insulin/HOMA-IR to known nutrient-sensing networks

in order to design the next mechanistic study. Considering the intersection of deregulated nutrient sensing, mitochondrial dysfunction, and chronic inflammation among the hallmarks of aging [25,26], a study design that evaluates metabolic and immune indices in parallel is itself biologically reasonable.

5.5. NK-Cell Activity: Nutritional Intervention, Immunosenescence, and Immunometabolism

NK-cell activity is a functional index involved in defense against infection and tumour immune surveillance [15-18]. Its interpretation is context-dependent, and short-term variation related to acute illness and preanalytical conditions cannot be excluded in a small study such as the present one. Age-related changes in NK cells are not a simple decline but involve remodelling of subset composition, cytotoxic capacity, and cytokine production [19]. Because the present study did not measure NK-cell number, surface markers, cytokines, granzyme/perforin, or T-cell function, a change in a single NK-cell activity measure cannot be described as an improvement in overall immune function.

With regard to nutritional interventions and NK function, small intervention studies using beta-carotene, probiotics or fermented milk, beta-glucan, mushrooms, aged garlic, ginseng polysaccharide, Euglena, and related products have reported heterogeneous immune findings [52-63]. Increased NK-cell or closely related NK/NK-T outcomes were reported in several trials [52-56,58-60,62,63], whereas no significant NK-cell effect was found in other studies [57,61]. Meta-analyses of probiotic or HN019 interventions in older adults have suggested increases in NK-related outcomes, although substantial heterogeneity and the limited number of studies constrain the strength of inference [64,65]. Therefore, the p=0.0230 result obtained in the n=7 Study 2 analysis should not be treated as equivalent to an established effect of nutritional intervention.

Taken together, these prior studies indicate that NK-cell-related endpoints can respond to some nutritional interventions, but findings are heterogeneous and appear to depend on the intervention, participant background, baseline immune state, and assay conditions [52-65]. Several positive studies enrolled older adults or smokers [52-56,62], whereas other trials in healthy adults reported either positive or null NK-related findings [57-61,63]. Thus, baseline characteristics may influence the detectability of a response, but the direction and magnitude cannot be predicted reliably from the present literature. In Study 2, baseline NK-cell activity ranged widely from 24% to 69%, and with a sample of only nine participants, interindividual variability cannot be adequately averaged out. Individual trajectories were therefore shown so that this heterogeneity would not be concealed by mean values alone.

Furthermore, although the concurrent nominal changes in NK-cell activity and fasting insulin in the n=7 analysis excluding P02 and P08 are hypothesis-generating, there are no data showing that the two indices changed through the same mechanism. The inflammatory actions of adipose-tissue NK cells and the cytotoxic activity of peripheral-blood NK cells are not the same concept, and immune changes observed in adipose tissue in obesity cannot be directly transferred to the peripheral-blood functional assay used in the present study [21–24]. Therefore, future studies should measure NK-cell subsets, cytokines, and metabolic phenotypes in addition to NK-cell activity and incorporate their relationships with metabolic indices into a prespecified analysis plan.

At the same time, the intersection between NK cells and metabolic abnormalities is relevant to the present study. In obese adipose tissue, models have been reported in which NK cells promote pro-inflammatory macrophages and contribute to insulin resistance [21,22], while the obese environment impairs lipid metabolism and mTOR-dependent glycolysis in NK cells and reduces cytotoxic function [23]. Conversely, human data in people with obesity treated with a GLP-1 receptor agonist have suggested improvements in NK-cell metabolism and effector function independently of weight change [24]. These findings do not imply a simple causal relationship of higher NK activity equals metabolic improvement, but they support the existence of an immunometabolism network in which metabolic state and NK-cell function can influence one another.

P02 and P08 had acute symptoms at the final assessment in Study 2. Because acute infection can influence NK-related indices, there is biological rationale for excluding these participants in a sensitivity analysis; however, because the analysis rule was not prespecified, the n=7 results cannot be promoted to the status of the main analysis. A future protocol should prospectively record acute infection, fever, vaccination, and use of antibiotics or corticosteroids and should specify procedures for postponing blood collection or repeating testing when such conditions are present.

5.6. Study 1: Lipid, Glucose-Metabolic, and Vascular Indices in the Type 2 Diabetes Cohort

In the type 2 diabetes cohort, there was no clear improvement in HbA1c, and although LDL-C decreased numerically, there was no statistical evidence of change. The association between blood cholesterol and cardiovascular risk has been shown in large epidemiological studies, and long-term exposure to lower LDL-C is associated with lower coronary artery disease risk [66,67]. In contrast, the decrease in HDL-C was the only finding that remained after FDR correction, and low HDL-C has long been associated with coronary artery disease risk [68]. However, Mendelian-randomization studies and trials that pharmacologically increased HDL-C have shown that raising HDL-C concentration itself does not necessarily reduce cardiovascular events [69–72]. Therefore, it would be inappropriate either to directly link the mean -3.83 mg/dL decrease in HDL-C observed in the present study to adverse clinical outcomes or, conversely, to dismiss it on the grounds that HDL-C is not causal. In a future trial, it would be worthwhile to prespecify and follow a broader lipid profile including non-HDL-C, ApoB, triglyceride-rich lipoproteins, and, where feasible, direct HDL-related functional or particle-based measures. In addition, information on concomitant medications

other than antidiabetic drugs, including lipid-lowering therapy, was incomplete in the present study, and dietary fat intake and weight change during the 12-week period were not rigorously controlled. Therefore, the HDL-C decrease cannot be attributed to the product, but because it was the only finding that remained after FDR correction, confirming its reproducibility in a future study is important for safety assessment and overall benefit-risk interpretation.

Study 1 included a population resembling real-world clinical practice in that participants used antidiabetic medications, often in combinations including metformin, DPP-4 inhibitors, and SGLT2 inhibitors. No initiation, discontinuation, or dose change of antidiabetic medications occurred during the intervention; nevertheless, the medication background itself can influence glucose, lipid, and body-weight indices even under stable treatment. The clinical importance of glycemic management in diabetes has been established in studies such as UKPDS [73,74], but improvements in weight and metabolic indices achieved through lifestyle interventions do not invariably translate into improved cardiovascular outcomes [75]. Because the present study was small and lacked a control group, the absence of change in HbA1c cannot be interpreted either as confirmation of product inefficacy or as evidence that medication confounding masked an effect. In particular, SGLT2 inhibitors, DPP-4 inhibitors, metformin, and other therapies can exert different effects on glucose metabolism, body weight, lipids, and inflammation; therefore, in a heterogeneous group of 18 participants, it is difficult to distinguish small changes attributable to the intervention food. The absence of initiation, discontinuation, or dose change of antidiabetic medications during the intervention is important information, but it does not eliminate medication confounding. If future research again enrolls patients with diabetes, background therapy should either be stratified or eligibility should be restricted to a defined treatment regimen, and concomitant medications including lipid-lowering and antihypertensive agents should be collected prospectively.

No clear changes were observed in ABI or CAVI. CAVI has been proposed as an index of arterial stiffness designed to reduce dependence on blood pressure and has been associated with obesity, metabolic syndrome, and future cardiovascular events [76–78]. ABI is also used for peripheral arterial disease assessment and cardiovascular risk stratification [79]. However, considering the 12-week duration, only 16 complete pairs, and potential influences of rest before measurement, heart rate, blood pressure, and device conditions, the present study was not designed to detect small changes in vascular function. A future trial in which vascular function is a primary objective should standardize measurement conditions and use a longer follow-up period.

5.7. Safety-Related Findings and Kidney Function

Adverse events can occur with dietary supplements [80], underscoring the importance of systematic safety assessment in intervention studies. No adverse events documented as related to the study product were identified in the available records; however, because no record of systematic adverse-event collection was available, safety cannot be considered established. In particular, the decrease in HDL-C in Study 1 and the direction of change in renal-function indices in Study 2 should be prespecified as monitoring items in future studies even though their causal relationship to the intervention is unknown.

Mean eGFR in Study 2 changed from 81.26 to 76.14 mL/min/1.73 m² without a statistically significant difference. eGFR is a creatinine-based estimate influenced by factors such as muscle mass and hydration status. A creatinine-based equation specifically developed for Japanese adults has been reported [81], but the exact estimating equation used in the original laboratory report could not be confirmed from the available records. Associations between eGFR and clinical outcomes have been established in large cohort meta-analyses [82], but a mild short-term fluctuation in a small study such as this cannot be diagnosed as kidney injury. Future studies should standardize blood-collection conditions and include urinalysis and repeat testing when needed.

CRP also showed no clear change in the main analysis. CRP is an inflammatory marker associated with cardiovascular risk [83,84] but is affected by infection, obesity, exercise, trauma, and other factors. Inflammatory cytokines were not measured in the present study, and the results did not provide human evidence supporting the anti-inflammatory effects suggested by preclinical Cirsium-related studies [6,7].

5.8. Interpretation as a Multi-Ingredient Product

The study product was a multi-ingredient formulation providing 2.5 g/day of a proprietary blended ingredient containing processed C. brevicaule; the listed product ingredients also included matcha powder, chlorella, Lumbricus powder, tricalcium phosphate, dried almond, and inulin. The exact proportion of C. brevicaule within the proprietary blend was not disclosed by the manufacturer for proprietary reasons. Therefore, even if a specific biomarker change were reproduced, attributing that effect to C. brevicaule would require component-only groups, dose-response studies, or compositional analyses. It also could not be confirmed that the plant part or processing method of the ingredient used here was the same as that used in previous reports employing C. brevicaule leaves or roots [1–3].

Conversely, the fact that the product is multi-ingredient does not eliminate its value for clinical research. It can provide formulation-level evidence evaluating the response to a product intended for commercial or practical use. In that case, however, product lot, composition information, marker compounds, contaminants, stability, and manufacturing quality should be disclosed as far as possible, and formulation identity should be confirmed when studies span different years. In the present study, the total amount of the proprietary blend containing processed C. brevicaule was confirmed as 2.5 g/day, whereas publicly disclosable information on the internal blend ratio, exact amounts of the individual co-ingredients, product lot, and related specifications was limited, constraining reproducibility assessment. It would therefore

also be inappropriate to import the known effects of matcha, chlorella, inulin, or other individual ingredients to explain the observed results. Future studies evaluating clinical efficacy of the formulation as a whole should standardize the finished product as the analytical object; if the contribution of C. brevicaule alone is to be tested, a different design such as a component-removal group or comparison formulation will be necessary.

5.9. Strengths and Limitations

A strength of the present study is that two independent studies conducted at different times were not forcibly combined; instead, complete-pair analyses were reconstructed from participant-level pre/post data, with multiplicity explicitly adjusted within each study. In Study 2, the n=7 analysis, which yielded nominally significant findings, was not presented alone; the n=9 main analysis was shown first, and the n=7 analysis was positioned as a post hoc sensitivity analysis. The extreme baseline insulin value in P01 was not arbitrarily removed, and both individual trajectories and medians were presented. Results in potentially unfavorable directions, such as the HDL-C decrease and the direction of renal-function changes, were reported using the same criteria. Presenting the main analysis, sensitivity analysis, multiplicity correction, and individual trajectories together reduces the risk of emphasizing only statistically significant findings; this improves interpretability despite the small study size but does not overcome the limitations associated with limited statistical power. In exploratory research, showing effect direction, interindividual variation, confidence intervals, and dependence on the analysis population is more informative for designing a subsequent validation study than the p value alone. The present manuscript therefore neither replaces the main analysis with the nominally significant n=7 results nor discards those results entirely.

There are, however, many limitations. First, both studies were small single-arm studies without control groups, randomization, or blinding; natural variation, regression to the mean, seasonality, and behavioral changes cannot be separated from intervention effects. Second, there was no formal prespecified sample-size calculation, public trial registration, or detailed prespecified statistical analysis plan, and numerous outcomes were evaluated exploratorily. FDR correction was applied retrospectively, but this does not eliminate the design limitations. Third, although the n=7 analysis in Study 2 has medical rationale based on acute symptoms and non-adherence, it was post hoc and cannot completely exclude selection bias.

Fourth, potential confounders such as diet, physical activity, alcohol use, smoking, sleep, acute infection, vaccination, and concomitant supplements were not systematically quantified. In Study 2, participants were instructed not to eat before examinations, but water and tea were permitted; because the available records did not document whether beverage intake was uniformly noncaloric, fasting conditions may not have been fully standardized. In Study 1, antidiabetic therapy was not changed during the intervention, but other concomitant medications and duration of diabetes could not be sufficiently characterized. Fifth, adherence assessment relied mainly on self-report rather than quantitative returned-sachet counts or electronic records, so the actual intake rate could not be calculated. Sixth, Study 2 included men only, limiting generalizability to women.

Seventh, information on plant part, processing conditions, lot, detailed quantities of individual ingredients, and quality specifications was insufficient, and, within the 2.5 g/day proprietary blend containing processed C. brevicaule, the exact proportion of C. brevicaule itself was not disclosed by the manufacturer for proprietary reasons. Eighth, detailed assay or device-specific measurement information was incomplete for some outcomes, and transport and processing conditions for NK-cell specimens could not be fully traced. Ninth, systematic adverse-event surveillance was absent, limiting safety assessment. Tenth, mechanistic biomarkers such as FOXO3, AKT, AMPK, NAD+, sirtuins, mitochondrial function, and cytokines were not measured. Accordingly, the molecular-mechanistic discussion in this manuscript remains a literature-based hypothesis.

5.10. Recommendations for the Next Study

The next stage should be a prospectively registered, randomized, double-blind, placebo-controlled trial. The target population should be narrowed to a single clinical background, such as type 2 diabetes receiving pharmacological treatment or untreated obesity/insulin resistance, and one or a small number of primary endpoints should be prespecified. If the Study 2 signals are pursued, fasting insulin/HOMA-IR should be supplemented with OGTT-derived indices or, in a subset, clamp measurements, so that insulin sensitivity, secretion, and clearance can be distinguished to the extent possible.

If NK-cell activity is used as a primary or secondary endpoint, rules for postponing blood collection during acute infection, blood-collection time, transport time, and measurement conditions should be standardized, and NK-cell counts and subsets, granzyme/perforin, cytokines, and related measures should be combined. If the mechanistic hypotheses are to be tested, AKT/FOXO3-, AMPK-, NAD+-, and SIRT1/3/6-related biomarkers should be prespecified, and data directly linking changes in clinical indices with pathway activation should be obtained.

In addition, product lot, plant part, processing method, marker compounds, and quality control should be documented, and at minimum the composition information necessary to evaluate study reproducibility should be maintained in a form that investigators can verify while respecting proprietary restrictions. Adherence should be quantified using returned-sachet counts or intake diaries. Safety should be assessed through structured adverse-event collection, with lipids, kidney function, and liver function prespecified for monitoring. Such a design would allow a more rigorous determination of whether the hypothesis-generating signals observed here represent a reproducible formulation response or chance variation. Follow-up duration should also be selected according to the study objective. Even when short-term changes in NK-cell activity or fasting insulin are the main objective, repeated measurements across the 12-week period should

be considered; when the durability of changes in HbA1c, body composition, lipids, or vascular function is of interest, longer follow-up may be appropriate. For safety, pre-existing abnormalities should be distinguished from newly emerging post-intervention abnormalities, and the severity, causality, and outcomes of adverse events should be collected in a standardized manner. In particular, the HDL-C decrease and the direction of creatinine/eGFR changes observed here should be explicitly incorporated into the safety-monitoring plan of the next study.

6. Conclusions

Two independent exploratory single-arm studies of 12-week supplementation with a processed C. brevicaule-containing powder were reanalyzed. In Study 1, only the decrease in HDL-C remained significant after FDR correction; no clear improvement was observed in HbA1c, LDL-C, body-composition measures, ABI, or CAVI. In the nine-participant Study 2 main analysis, none of the 30 outcomes was significant after FDR correction, and fasting insulin and NK-cell activity were not significant in the main analysis. In a post hoc sensitivity analysis excluding two participants with acute symptoms and non-adherence, fasting insulin, post hoc HOMA-IR, and NK-cell activity showed nominal signals; fasting insulin and NK-cell activity did not remain significant after multiplicity correction, whereas HOMA-IR was evaluated using nominal p values only. These findings are hypothesis-generating rather than confirmatory. The HDL-C decrease, safety-related findings, limitations of attribution inherent to the multi-ingredient proprietary formulation, and the unmeasured FOXO3/AMPK/NAD+/sirtuin pathways support the need for a sufficiently powered, prospectively registered, randomized placebo-controlled trial to assess reproducibility and mechanism.

7. Author Contributions

S.K.: Conceptualization, Formal analysis, Visualization, Writing-original draft, Writing-review & editing. Y.M.: Investigation, Writing-review & editing. Y.F.: Supervision, Writing-review & editing. All authors reviewed the final manuscript and accepted responsibility for its content.

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9. Institutional Review Board Statement

Both studies were approved by the Ethics Review Committee of the International Society for Geriatric and Longevity Clinical Medicine (Study 1: ISGN_N10052024; Study 2: ISGN_N10012026).

10. Conflicts of Interest

Y.M. has a relationship with Med Pla Co., Ltd., which received the commissioned research support from HUNTKEY JAPAN Co., Ltd. for this study. The role of the funder is described in the Funding statement.

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