Supplementation of Spermidine at 40 mg/day has Minimal Effects on Circulating Polyamines: An Exploratory Double-blind Randomized Controlled Trial in Older Men

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List of Abbreviations: ADI, acceptable daily intake; AE, adverse events; ALP, alkaline phosphatase; ALT, alanine transaminase; AST, aspartate aminotransferase; BMI, body mass index; BUN, blood urea nitrogen; BW, body weight; CI, confidence interval; DBP, diastolic blood pressure; EMRL, Elmhurst Memorial Reference Laboratory; FDA, United States Food and Drug Administration; GLP, good laboratory practice; GRAS, Generally Recognized as Safe; HDL-C, high-density lipoprotein-cholesterol; HR, hazard ratio; hsCRP , high-sensitivity C-reactive protein; IFSH, Institute for Food Safety and Health; LDL-C, low-density lipoprotein-cholesterol; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; MCV, mean corpuscular volume; mITT, modified Intent-to-Treat; NHANES, National Health and Nutrition Examination Survey; NOAEL, no observed adverse effect level; NSAID, non-steroidal anti-inflammatory drugs; PP, per protocol; RBC, red blood cells; SBP systolic blood pressure; SD, standard deviation; hpSPD, high-purity spermidine-trihydrochloride supplement; TG, triglyceride; total-C, total cholesterol; WBC, white blood cells

Abstract

This study represents the first investigation into the safety of a novel, high-purity spermidine-trihydrochloride supplement (hpSPD) in humans. Spermidine, a natural compound found in various foods, has demonstrated potential health benefits in animal and epidemiological studies. However, evidence from clinical trials and safety evaluations of spermidine supplements is limited as pure spermidine for human administration has not been available. In this randomized, double-blind, within subject and placebo-controlled trial, 37 healthy men (50-70 years old, BMI 18.5-28 kg/m^2) were administered either hpSPD or a placebo. We hypothesized that 7-day and 28-day dosing of 40 mg/day of hsSPD would have minimal effects on safety, although metabolic and polyamine homeostasis has not previously been examined at this dosage level. Consistent with our hypothesis, 40 mg/day hpSPD did not result in any significant changes in clinical, lipids, chemistry, or hematological parameters compared to placebo. Compliance was high, and no study product-related adverse events were reported. Substantial changes in serum and urine polyamine concentrations were not observed following hpSPD supplementation, suggesting effective homeostatic control of full dose highly purified spermidine supplements with no evidence of adaptation of spermidine metabolism at 40 mg/day. These findings suggest that hpSPD at 40 mg/day for up to 28 days is safe and well-tolerated in healthy older men. The study is consistent with preclinical results and provides important evidence supporting the safety of high-purity spermidine supplementation, enabling further research with single molecule spermidine to investigate its potential biology for improving human health. This trial was registered at clinicaltrials.gov (NCT05459961).

Keywords: anti-aging, spermidine, autophagy, human dose, cardiovascular, natural, longevity, safety

1 Introduction

Spermidine, a naturally occurring polyamine found in cells, organs, and the circulating cardiovascular system, is integral to various physiological processes, including cell growth, proliferation, antioxidation, autophagy, apoptosis, and immune regulation [1-5]. Dietary intake of polyamines mainly comes from foods of plant origin, such as natto, beans, fruits, vegetables, potatoes, bread, and cereals [6]. The average daily intake of spermidine varies between countries, ranging from 4.8 to 17.0 mg/day [7].

The health effects of spermidine have been extensively investigated in animal models, with evidence from clinical epidemiology studies indicating improved mortality and morbidity outcomes in populations with higher polyamine or spermidine diets [8-15]. Notably, the Bruneck study, a 20-year cohort study of 829 adults aged 45-84 years, reported an inverse relationship between spermidine intake and all major causes of death, including cancer and vascular disease [14]. Adjusted all-cause mortality (deaths per 1000 person-years) decreased across tertiles of increasing spermidine intake from 40.5 [95% confidence interval (CI): 36.1, 44.7] to 23.7 (20.0, 27.0) and 15.1 (12.6, 17.8) [14]. Similar findings were observed in a prospective replication cohort (SAPHIR study) of 1770 healthy unrelated adults aged 39–67 years, indicating a 26% reduction in mortality for every 1-standard deviation (SD) increase in spermidine intake, after adjustments for potential confounders [14]. These findings were supported by an analysis of the 2003-2014 National Health and Nutrition Examination Survey (NHANES), including 23,894 adults (age $\geq$ 18 years), which showed a significantly lower risk of cardiovascular mortality (HR:0.68; 95% CI: 0.51,0.91) and all-cause mortality (0.70; 0.60, 0.82) for the highest quartile compared to the lowest quartile after adjustment for confounders [15].

Spermidine is of particular interest among the aging population due to aging-related increases in morbidity rates as well as evidence of declining spermidine concentrations with age in humans and other species [16-19]. It is currently unclear what happens to polyamines obtained from dietary sources (reviewed in [20]) but there is some evidence suggesting a rapid absorption of spermidine in human subjects [21]. Thus, spermidine supplementation is one strategy to increase spermidine intake. Thus far, commercially available spermidine supplements are limited to plant extracts such as wheat germ extracts with low concentrations of spermidine (<5%). Evidence from clinical intervention trials on the health benefits of spermidine are limited to studies using plant or algae extracts or bread containing spermidine dosed in a range of 1.2 mg/day to 15 mg/day [22-25]. Of these, only one reported on safety and this study provided a very low amount of mixed polyamines (~2 mg/day total polyamine) [22].

Chrysea Labs developed a novel high-purity (86%) spermidine-trihydrochloride supplement (hpSPD) using a patented biological process that sources spermidine through precision fermentation of a Generally Recognized as Safe (GRAS) yeast strain [26]. The hpSPD was shown to be safe as a dietary source of spermidine based on good laboratory practice (GLP)-compliant *in vitro* genotoxicity assays as well as GLP-compliant repeated dose oral toxicity studies in rats with a NOAEL of 728 mg/kg body weight (BW)/day [27]. The current study aimed to fulfill the gap in research on high-purity spermidine as well as provide further safety support for hpSPD by conducting a randomized, placebo-controlled, double-blind clinical trial in healthy, older men, dosed for up to a month at 40 mg/day. Safety assessments consisted of clinical chemistry, hematology, high-sensitivity C-reactive protein (hsCRP), lipid profile, body weight, blood pressure, serum and urinary polyamines, and adverse events (AEs) following 7

112 days and 28 days of hpSPD supplementation. We hypothesized that 7 days and 28 days of
113 supplementation with hpSPD would minimally affect these safety parameters.

114 ## 2 Methods and Materials

115 ### 2.1 Study design

116 The study was conducted in accordance with the Declaration of Helsinki, the United
117 States Food and Drug Administration Code of Federal Regulations, Title 21, and the
118 International Code of Harmonization (E6) Good Clinical Practice Guidelines after approval of
119 Protocol No. BIO-2203 and informed consent documents on 26 May 2022 by the Institutional
120 Review Board at Sterling IRB (Atlanta, GA). Signed informed consent and authorization for the
121 use of protected health information were provided by the participants before implementing any
122 protocol-specific procedures. The study was prospectively registered at ClinicalTrials.gov
123 (NCT05459961) and conducted between June and October 2022 at a single clinical research site
124 (Biofortis Research; Addison, IL, USA). CONSORT reporting guidelines were used [28].

125 This was a randomized, double-blind, placebo-controlled study with a 7-day two-period
126 crossover phase with a ≥ 2-week washout followed by an additional 21-days of supplementation
127 in the second crossover period for a total of 28 days of supplementation (Figure 1). Thus, this
128 study incorporated a 7-day crossover design with a 28-day parallel design. During each
129 supplementation phase (i.e., 7-day crossover or 28-day parallel), participants consumed one
130 capsule of hpSPD or a matching excipient placebo capsule daily. Before and after each
131 supplementation period, clinical chemistry, hematology, hsCRP, lipids, serum and urinary
132 polyamines, body weight, and blood pressure were measured. AEs were monitored throughout
133 each supplementation phase. Of note, the overall objective of this study was to examine the
134 safety of hpSPD as well as effect on metabolomics and transcriptomics in healthy, older men.

This report focuses on safety-related findings of the study; other measurements which include targeted and untargeted metabolomics will be reported in a future publication.

2.2 Study participants

Participants were healthy Caucasian men, 50-70 years old, with a BMI of 18.5-28 kg/m^2 who did not habitually consume foods high in spermidine (<30 mg/day spermidine determined using an abbreviated food frequency questionnaire (FFQ) at Visit 1). Additionally, participants were non-smokers, had no history of major illness or health conditions (e.g., cancer, cardiovascular disease) as assessed by the study physician, with no recent vaccination (within 30 days of Visit 1), antibiotic or steroid use (< 3 months within Visit 1), unstable (change within 6 months of Visit 1) use of United States Food and Drug Administration (FDA)-approved therapeutic medication, unstable intake of dietary supplements, herbal preparations, or complementary medicine (within 30 days of Visit 1), no chronic use (i.e., daily and regularly) of oral analgesics and non-steroidal anti-inflammatory drugs (NSAID; within 30 days of Visit 1), and no known allergies to any of the study product ingredients. At the screening visit (Visit 1), participants completed a medical history questionnaire in addition to the assessment of height, body weight, BMI, vital signs, current medication/supplement use, and review of inclusion/exclusion criteria to determine eligibility. Additionally, subjects completed an online abbreviated FFQ which was used to exclude individuals consuming a habitual diet high in spermidine. The eligibility criteria were selected to maximize homogeneity among participants to minimize metabolic and clinical variance within intervention groups. Additionally, participants were asked to maintain habitual exercise, diet, and medication/supplementation use during the study except for abstinence from alcohol. In addition, abstinence from structured, moderate-to-vigorous exercise for 24 hours and an overnight (≥8 hours) fast was specified before each visit.

2.3 Randomization

At Visit 2 (Day 0), eligible participants were randomized (1:1) to one of two product sequences, based on a statistician-generated allocation sequence using a permuted blocks algorithm in SAS PROC PLAN. The sequence was uploaded onto the electronic case report form platform (Medrio Inc., San Francisco, CA, USA). Once a participant qualified for the study, the randomization module was used to assign the study sequence which kept all personnel involved with the data collection, analysis, and interpretation blinded to the sequence assigned to participants.

2.4 Study products

The hpSPD and placebo capsules were prepared by Chrysea Labs. The hpSPD used in the study (Sprevive® lot C002) was produced using an engineered strain of *Saccharomyces cerevisiae* coupled with a novel purification methodology and consisted of 86% spermidine trihydrochloride with the remainder being ~13% sodium chloride and ~1% water [27]. Each hpSPD capsule provided 40 mg spermidine with mannitol, magnesium stearate, and silica as the excipients. The placebo capsule only contained the excipients. Both products were manufactured into opaque, white, size 1 capsules and were identical in appearance to ensure blinding of participants and all personnel involved with the data collection, analysis, and interpretation. A recent toxicology study in rats on the same hpSPD product indicated a no observed adverse effect level (NOAEL) of 12500 ppm, equivalent to 728 mg/kg BW/day for males and 829 mg/kg BW/day for females [27]. The acceptable daily intake (ADI) has been calculated to be 7.3 mg/kg BW/day for spermidine trihydrochloride (4.16 mg/kg BW/day for spermidine), giving a wide margin of safety dosing with 40 mg/day including accounting for estimated dietary intake of spermidine.

Study products were provided to participants as 10-capsule blister packs. During each supplementation period, participants were instructed to consume the study product once a day (1 capsule/day) with or without food. The first dose was consumed in the clinic and the time of consumption was recorded. Participants were instructed to consume all subsequent study products within ± 1 hour of the consumption time of their first dose and not to consume more than 1 capsule/day. During the 7-day crossover phase, participants were required to consume the placebo or hpSPD for 7 continuous days, starting on Day 0 (Visit 2) and crossing over to the next study product in their sequence starting on Day 21 (Visit 4) for the second test period (Figure 1). For the 28-day parallel phase, participants were required to stay on their assigned study product from the second test period of the 7-day crossover phase for an additional 21 days i.e., starting on Day 28 (Visit 5) through Day 49 (Visit 6) (Figure 1). Compliance was defined as the consumption of 100% of the scheduled intake. Compliance was assessed at the end of each supplementation period by the counting of returned unused products. Participants were also instructed to record time of consumption in a daily study product log to promote compliance. Overall, participants consumed the supplements between 6:00 am and 12:00 pm.

2.5 Spermidine FFQ

Habitual dietary spermidine intake was obtained at Visit 1 to assess eligibility. The spermidine dietary intake data were captured using an online, self-administered, point-and-click, photo-based FFQ designed to evaluate intake of foods, frequency of consumption, food choices, portion sizes, and habits over the past three months (VioScreen™, Viocare, Inc., Princeton, NJ) [29]. Originally developed through research funded by the National Institutes of Health (NIH) to be a complete, accurate and scientifically validated HIPAA compliant tool, the dietary analysis utilizes the food and nutrient information from the Nutrition Coordinating Center (NCC;

University of Minnesota, MN) which distributes and supports the Nutrition Data System for Research (NDSR). Specifically for the quantification of dietary spermidine, the spermidine content values for individual food items and groups were incorporated into the VioScreen food and nutrient database utilizing data from multiple publications [14, 30-38]. The data and outputs from this dietary recall FFQ were then analyzed to estimate the individual intake of spermidine. Conflicting food values were compared with other similar foods by a multidisciplinary group who made a decision on a value considered to represent the correct amount based on the weight of evidence.

2.6 Serum spermidine by ELISA

Fasting serum spermidine concentration was assessed at Visits 2, 3, 4, 5, and 6 (Days 0, 7, 21, 28, and 49) and analyzed by Abexxa ELISA (https://www.abbexa.com/spermidine-smd-elisa-kit; Abexxa Ltd, Cambridge, UK) according to manufacturer's instructions by the Institute for Food Safety and Health (IFSH) of the Illinois Institute of Technology (Bedford Park, IL). Blood was collected between 7:00 am and 10:30 am on the aforementioned visit days into serum separator tubes which were allowed to sit in an upright position at room temperature for approximately 30 to 60 minutes for clot formation. The tubes were then centrifuged for 10 minutes at 3000 rpm and separated into 1 mL aliquots. The samples were stored frozen at -80°C and batch shipped to IFSH at the conclusion of the trial.

2.7 Serum and urine polyamine by LC-MS

Fasting serum polyamine (diamopropane, *N*-acetyl putrescine, ornithine, putrescine, spermidine, and spermine) concentrations were analyzed by LC-MS at Visits 2, 3, 4, 5, and 6 (Days 0, 7, 21, 28, and 49) by Creative Proteomics (Shirley, NY). Blood was collected as described for ELISA and separated into 500 µL aliquots. Urine was collected for 24 hours and

analyzed by LC-MS for polyamine concentrations at Visits 2, 3, 4, 5, and 6 (Days 0, 7, 21, 28, and 49) by Creative Proteomics (Shirley, NY). Participants kept the urine collection containers refrigerated during the entire collection period. Study staff measured and recorded the total volume of the urine, thoroughly mixed the sample, and separated it into 2 mL aliquots. All serum and urine samples were stored frozen at -80°C and batch shipped to Creative Proteomics at the conclusion of the trial.

For the LC-MS analysis, standard solutions were prepared with reference standards of diamiopropane, *N*-acetyl putrescine, ornithine, putrescine, spermidine, and spermine in mimic urine. The standard solutions were diluted to a concentration range of 0.00005 to 100 μM. Thawed 20 μL of urine or plasma was mixed with 20 μL of an internal solution of 13C or 2H-labeled cadeverine, diaminopropane, putresine, and spermidine, and 100 μL of acetonitrile. The mixtures were vortexed for 1 minute, sonicated for 2 minutes and then centrifuged at 21,000 x g for 10 minutes. The supernatant of each sample or each of the calibration solutions (50 μL) was mixed with 50 μL of a benzoyl chloride solution and 50 μL of a pH buffer. The mixtures were allowed to react under a set of optimized conditions. Aliquots (5 μL) of the resultant solutions were injected into a C18 column (2.1x100 mm, 1.8 μm) to run LC-MRM/MS on an Agilent 1290 UHPLC system coupled to an Agilent 6495 QQQ mass spectrometer, which was operated in the positive-ion detection mode. The mobile phase for LC separation was 0.1% formic acid in water (A) and in acetonitrile (B) for binary-solvent gradient elution (20% to 80% B over 12 minutes), at 0.3 mL/minutes and 40 °C. Concentrations of the analytes were calculated by interpolating the constructed linear-regression calibration curves with internal standard calibration. All samples and standards were run in singlets. The concentrations of urinary polyamines were normalized to the 24-hour urine volume.

2.8 Body Weight and Vital Signs

Body weight was measured at all visits using a digital floor scale (Health-O-Meter Professional model 349KLX, Boca Raton, FL). Blood pressure was measured at all visits using an automated blood pressure device (Welch Allyn 300 Series, Skaneateles Falls, NY). Systolic and diastolic pressures were measured once after the participant had been sitting for at least 5 minutes using an appropriate sized cuff (bladder within the cuff encircled ≥ 80% of the arm).

2.9 hsCRP and plasma lipid profile

Blood samples for hsCRP and lipid profile assessments were collected following an overnight (≥ 8 hours) fast at Visits 2, 4, and 6 (Days 0, 21, and 49). Samples were collected in plasma separator tubes, centrifuged at 3000 rpm for 10 minutes within 2 hours of collection, and stored in the refrigerator. Samples were transported on ice to Elmhurst Memorial Reference Laboratory (EMRL; Elmhurst, IL) where it was stored refrigerated until analysis on the same day as sample collection.

2.10 Fasting blood chemistry profile and hematology

Blood samples for chemistry profile and hematology were collected following an overnight (≥ 8 hours) fast at all visits. For the blood chemistry profile, samples were collected in plasma separator tubes, centrifuged at 3000 rpm for 10 minutes within 2 hours of collection, and stored in the refrigerator. For hematology, samples were collected in EDTA tubes and stored in the refrigerator. Samples were transported on ice to Elmhurst Memorial Reference Laboratory (EMRL; Elmhurst, IL) and stored refrigerated until analysis on the same day as sample collection.

2.11 Adverse event assessment

AEs were defined as any untoward medical occurrence or undesirable clinical experience in a participant in a clinical trial, whether or not considered related to the study. This included any occurrence that is new in onset or aggravated in severity or frequency from the baseline condition, or abnormal results of diagnostic procedures (including laboratory test abnormalities where applicable). AEs were collected in-clinic at Visits 2 through 6 (Days 0 through 49) and categorized as either “not related”, “unlikely”, “possibly”, “probably”, or “definitely” related to the intervention [39]. The severity and causality of all AEs were determined by a study physician. Information describing the AE collected for assessment and documentation included the following: date of onset/resolution, severity, and causal relationship to study treatment or study conduct (as determined by the physician). All documented AEs were followed to resolution or until determined to be clinically insignificant by the study physician.

2.12 Statistical analysis

All statistical analyses were conducted using SAS for Windows (version 9.4; Cary, NC). No formal sample size calculation was performed for this exploratory study. A sample of 42 participants were randomized with a target of at least 30 participants completing in compliance. Primary analyses were completed for the modified Intent-to-Treat (mITT) sample set which included all participants who were randomized to the study and completed at least one post-baseline line assessment. Additionally, analyses were conducted for the per-protocol (PP) sample set such that data was excluded from the respective treatment period/time point for participants in violation of protocol requirements. If not explicitly specified, all reported results are for the mITT population.

Unless otherwise stated, tests of significance were two-tailed and performed at the 0.05 significance level. Outcomes collected at the start of each period, as well as the within-group change from the start to the end of the intervention period for safety outcomes, were compared with the Wilcoxon sign rank test. The Benjamini-Hochberg false discovery rate adjustment (q) was used to adjust for multiple testing of outcomes within each measurement grouping for the safety laboratory assessments and vital signs only where $q < 0.10$ were considered statistically significant. In the 7-day crossover phase of the study, the spermidine and polyamine outcomes were analyzed with a repeated measures mixed model where the period baseline was included in the response vector [40]. The model included fixed effect terms for test sequence, test period, time point, sequence, and the group by time point interaction. The participant nested within test sequence was included as a random effect with an adjustment for the repeated measures within a test period. In the 28-day parallel phase of the study, outcomes measured over time were analyzed with a repeated measures model including terms for the time point and group-by-week interaction with an adjustment to the residuals/errors for the repeated measures within participants. Extreme statistical outliers were identified by the 3*interquartile range rule and removed in sensitivity analyses.

3 Results

A total of 57 participants were screened. Although the goal was to randomize 40 participants, enrollment was stopped once 38 eligible participants were identified due to the slow rate of recruitment. The 38 participants were randomized (Figure 2) to one of two sequences: placebo/hpSPD (n = 18) or hpSPD/placebo (n = 20) in the 7-day crossover phase. One participant randomized to the hpSPD/placebo sequence was unwilling to comply with study procedures and withdrew consent after starting consumption of hpSPD, but before providing any

post-baseline data. All other participants completed the 7-day crossover phase. Therefore, the mITT for the 7-day crossover phase included 37 participants. One participant withdrew from the study while on hpSPD before completion of the 28-day parallel phase due to personal medical reasons not related to the study. Thus, the mITT for the 28-day parallel phase included 18 participants on hpSPD and 19 on placebo. The PP population excluded data from the treatment period/time point (i.e., set data to missing) of subjects who violated protocol requirements (i.e., under- or over-compliance, antibiotic use, NSAID use, and failure to replicate diet prior to each visit). This consisted of seven subjects from the first 7-day crossover period (before washout), seven subjects from the second 7-day crossover period (after washout), and 14 from Day 49 at the end of the 28-day parallel phase (Supplemental Table 1). Of these, four subjects were removed from all study time points. The mean compliance was 100.7% (standard deviation (SD) 2.8%) in the 7-day crossover phase and 99.5% (7.2%) in the 28-day parallel phase of the study.

Baseline characteristics are shown in Table 1. Average habitual spermidine intakes of our participant are consistent with global estimations [7].

3.1 7-day crossover phase

Serum polyamines during the 7-day crossover phase are shown in Table 2. No statistically significant interaction between the intervention group and time point was detected for serum spermidine concentrations measured by the ELISA. Similarly, no statistically significant interaction between the intervention group and time point was detected for the serum spermidine concentrations measured by the LC-MS. Additionally, no significant interactions were detected for diaminopropane, *N*-acetyl putresine, ornithine, putrescine, or spermine. However, in the PP population, a significant interaction was detected for spermine ($p = 0.024$) where a reduction in spermine was observed following hpSPD [pre: 0.024 (95% CI 0.019,

0.031), post: 0.021 (0.016, 0.027); p = 0.012] but not placebo [pre: 0.020 (0.016, 0.026), post: 0.021 (0.016, 0.027); p = 0.56].

Urine polyamines during the 7-day crossover phase are shown in Table 3. One data point in the urine diaminopropane results was deemed an influential outlier and was removed from subsequent analyses. No statistically significant interaction between the intervention group and time point was detected for diaminopropane, *N*-acetyl putrescine, ornithine, putrescine, or spermidine. A significant interaction was detected for spermine whereby spermine significantly decreased following placebo but increased following hpSPD. Exploring specific comparisons of interest, within-group comparisons revealed decreases in almost all urinary polyamines following placebo, but not following hpSPD. However, only the change from baseline for urinary spermidine and spermine was significantly different following placebo compared to hpSPD.

Body weight and blood pressure were unaffected by hpSPD or placebo (Supplemental Table 2). No significant difference between the start of the intervention periods (i.e., Days 0 and 21) was detected for hsCRP or plasma lipid profile (Supplemental Table 3). No clinically meaningful changes in blood chemistry (Supplemental Table 4) or hematology (Supplemental Table 5) were detected following hpSPD or placebo.

3.2 28-day parallel phase

There was no statistically significant interaction between the intervention group and time point for serum spermidine concentrations measured by ELISA or LC-MS (Table 4). Additionally, no statistically significant interaction between the intervention group and time point was detected for the other polyamines measured by LC-MS (i.e., diaminopropane, *N*-acetyl putrescine, ornithine, putrescine, or spermine; Figure 3; Supplemental Table 6). Similarly, there

was no statistically significant interaction between the intervention group and time point for the urine polyamines measured by LC-MS with the exception of spermine (Figure 4; Supplemental Table 7). However, within-group differences were not significant following placebo or hpSPD for urinary spermine or any other urinary polyamines.

Body weight and blood pressure was unaffected by hpSPD or placebo (Supplemental Table 2). No significant change after 28 days of supplementation (i.e., from Day 21 to Day 49) was detected between groups for hsCRP or any measures in the plasma lipid profile (Supplemental Table 8). No clinically meaningful changes in blood chemistry (Supplemental Table 4) or hematology (Supplemental Tables 5) were detected within each group.

3.3 Adverse events

In total, eight mild and five moderate AEs were reported by 10 participants during the intervention phases (Table 5). Four AEs (three mild and one moderate) occurred before the start of any product intake or during the washout phase. None of the AEs were judged by study physicians to be related to either study product. None of the participants withdrew from the study due to an AE. Only three AEs occurred while participants were supplementing with hpSPD and all three AEs resolved at follow-up.

3.4 ELISA vs. LC-MS spermidine

Acknowledging the heterogeneity and limitations in current methodologies for measuring polyamines from biological samples, we utilized two methods for measuring serum spermidine i.e., ELISA and LC-MS. Baseline (Day 0 and 21) serum spermidine measured by ELISA is consistent with a published study using the same ELISA method [17], indicating that circulating spermidine in our participants is as expected in a healthy adult cohort. In general, serum and urine spermidine measured by ELISA is higher than that measured by LC-MS. Bland-Altman

analysis of pooled data from all visits of the two methods revealed a positive trend whereby the difference between the methods tended to increase as the average increased (Supplemental Figure 1). Additionally, the two methods were not significantly correlated (Spearman r = 0.005, p = 0.94; Supplemental Figure 2).

4 Discussion

Presented are the safety outcomes of an exploratory clinical trial on a highly purified spermidine supplementation. At the conclusion of both the 7-day crossover intervention phase and the 28-day intervention phase, no significant differences were observed in body weight, blood pressure, hsCRP, lipids, clinical chemistry, and hematological parameters between participants who consumed hpSPD or placebo. Participant compliance was high, with no drop-outs and no study product-related adverse events. Additionally, supplementation with hpSPD marginally affected serum and urinary polyamines. In conclusion, these findings support the safety of the hpSPD and suggest minimal effect on polyamine metabolism and homeostasis.

Our observations following supplementation with high-purity spermidine complements that of previous studies using spermidine extracts. In a placebo-controlled, double-blind Phase II clinical trial using spermidine wheat germ extract supplements providing 1.2 mg/day spermidine, 0.6 mg/day spermine, and 0.2 mg/day putrescine, no differences in blood polyamine concentrations between controls and spermidine-supplemented older adults (60-80 years old) were reported at 3 months of follow-up [22]. In another clinical trial, the consumption of natto, a polyamine-rich food (37-71 μmol/day of polyamine mixture) for 26 weeks by healthy adults increased the mean spermine concentrations in whole blood but did not affect blood spermidine concentrations [24]. In a follow-up study by the same group, the doubling of dietary polyamine

through the consumption of a new form of natto (12.3-24.6 mg/day of spermidine and 3.6-7.2 mg/day of spermine) for a longer period (12 months) also increased spermine concentrations without changes in spermidine concentrations in healthy adults [25]. Finally, provision of 15 mg/day of spermidine in the form of a commercially available dietary supplement (wheat germ extract containing spermidine (~2%), chlorella algae powder, and linseed flour) for 5 days to healthy adults increased plasma spermine concentrations but not spermidine or putrescine concentrations [23].

A major difference between our study and that of others is the form of spermidine supplementation. To the best of our knowledge, our study is the first to administer a highly purified (86%) spermidine supplement, contrasting with prior human clinical trials that utilized polyamine extracts containing <5% spermidine [22-25]. Therefore, the disparity in circulating polyamines observed between our study and others may stem from the presence of additional non-spermidine compounds in the extracts used in those earlier human trials [22-25]. The mechanism underlying the selective increase in circulating spermine, but not spermidine, seen in previous studies remains unclear. Some authors hypothesized that this is a result of an almost complete rapid enterocytic and hepatic conversion of spermidine to spermine [23]. However, it is also plausible that the rise in circulating spermine reflects the increased intake of spermine from the polyamine-rich extracts. The clinical significance of non-spermidine polyamine intake on health is unclear. The Bruneck study found no associations between all-cause mortality and putrescine and arginine or methionine intakes, which are natural sources (i.e., metabolic precursors) of endogenous polyamine synthesis [14]. Meanwhile, the intake of spermine, a metabolite of spermidine, showed a weak inverse association for all-cause mortality [14]. This

knowledge gap supports the need for studies in highly-purified polyamines such as ours to better understand the individual and collective contribution of dietary polyamines to health.

Additionally, the hpSPD dose provided in our study is appreciably higher compared to previous human clinical trials [22-25]. The amount of spermidine provided corresponded to ~3 times the baseline dietary spermidine intake of our participants. A recent toxicology assessment of the same hpSPD supplement in Wistar rats indicates a NOAEL of 12500 ppm, equivalent to 728 mg/kg BW/day for males and 829 mg/kg BW/day for females [27]. The amount supplemented to our participants is ~1000-fold lower than the NOAEL. Despite the high amount, we observed minimal changes to serum and urine polyamines in our current human trial, suggesting effective homeostatic control with no evidence of adaptation of spermidine metabolism at 40 mg/day. The specific mechanisms involved in polyamine homeostasis following hpSPD supplementation is beyond the scope of this study, but it is likely complex and multifactorial [41, 42]. Regardless, our findings suggest the hpSPD supplement at 40 mg/day for up to 28 days is safe and well-tolerated. No AEs were related to the study product and compliance was high. Additionally, there were no statistically or clinically significant changes in body weight, blood pressure, hsCRP, lipids, clinical chemistry, or hematological parameters following hpSPD. Oral toxicity studies of this hpSPD product in rats also revealed no treatment-related effects on body weight, clinical chemistry, or hematology following 90 days of 100, 333, or 1000 mg/kg BW/day [27]. Only one other clinical trial assessed safety following spermidine supplementation and reported no study product-related AEs or changes in clinical chemistry and hematological parameters but this was following consumption of a mixture of polyamines providing a much lower amount of spermidine (i.e., 1.2 mg/day) [22].

Although hpSPD generally had little effect on serum or urinary polyamines, urinary spermine significantly increased following hpSPD and decreased following placebo during the 7-day crossover phase. These changes were also observed in the 28-day parallel phase, although within-group differences were no longer significant. Serum spermine concentrations also significantly decreased following 7-days of hsSPD supplementation in our PP population, but not the mITT population. These changes are likely temporary as they are absent or minimal following the longer 28-day supplementation phase. Additionally, there were significant within group decreases for spermidine, N-acetyl putrescine, and ornithine during the 7-day crossover phase following placebo dosing, but not following hpSPD dosing. The reason behind the decrease in urinary polyamines without changes in serum polyamines in the placebo group is unclear. A systematic error in LC-MS analysis is highly unlikely as the analysis was performed blinded and ordered by participant number. Dietary intakes of spermidine were only assessed at the start of the study and thus, it is unknown if there were any dietary changes that may have contributed to the observed pattern. Impact of dietary and lifestyle changes on measured outcomes were preemptively minimized by instructing all participants to maintain habitual diet and lifestyle throughout the study and by requiring all participants to replicate their 24-h diet prior to each visit. Further studies are needed to confirm these changes in serum and urinary polyamine and investigate their clinical significance.

The review of spermidine supplementation studies, including ours, should be performed while acknowledging the heterogeneity and limitations in current methodologies for measuring polyamines from biological samples [43]. Thus, although polyamines seem to be rapidly absorbed from the intestinal lumen [21], detecting changes in serum concentrations after oral spermidine supplementation is challenging. Previous studies have suggested that oral doses of up

to 15 mg/day of spermidine from plant extracts have minimal impact on serum concentrations of spermidine and other polyamines [22-25]. In our study, spermidine concentrations varied significantly within and between the two measurement methods used for serum and urinary spermidine (i.e., ELISA and LC-MS). Similar large inter-individual variations were noted in an earlier study with healthy adults using the same ELISA method [17, 19]. The weak correlation between methods suggests that the broad variation in circulating spermidine may be attributed to methodological differences rather than biological factors. Therefore, while significant impacts of hpSPD supplementation on serum and urinary spermidine concentrations can be ruled out, current analytical constraints may impede the detection of subtle changes. Inadequate methodology also poses challenges in using circulating polyamines as promising biomarkers for conditions such as periodontitis, chronic kidney disease, Alzheimer's disease, and cancer [44]. Thus, there is a pressing need to enhance current analytical capabilities for polyamine assessment in biological samples, which could be facilitated by the availability of safe and highly purified spermidine free from other interfering polyamines.

The eligibility criteria were designed to minimize metabolic and clinical variance. This included enrolling only male participants, restricting age, BMI, and dietary spermidine, and excluding significant medical issues and prescription drugs not widely used in this population. Additionally, care was taken to instruct participants to maintain a consistent habitual diet considering the presence of polyamines in various food sources. Other strengths include the use of a randomized, double-blind, placebo-controlled study design, high participant retention, and near-perfect compliance. The study is also the first of its kind to detail the safety of a high-purity spermidine supplement in healthy adults. Thus, caution should be taken when making direct comparisons to other studies that provide mixed-polyamine extracts of low purity. Finally, the

safety evaluation of the extract examined in this study is confined to the outlined conditions of use and additional safety assessments might be required should the conditions of use change.

In conclusion, consistent with the wide margin of safety with high-purity spermidine reported in the preclinical safety testing studies, the clinical and laboratory results and the lack of reported adverse events in this study demonstrate the safety of daily supplementation of 40 mg of high-purity spermidine supplement for up to 28 days in healthy older men. While significant impacts of hpSPD on serum and urinary spermidine concentrations can be ruled out, changes in intracellular concentrations were not examined. Additionally, current analytical constraints may impede the detection of more subtle changes in circulating polyamine and also pose challenges in using circulating polyamines as promising biomarkers. These findings will be foundational for the design of future clinical research studies aimed at understanding the safety, absorption and metabolism, and health effects of spermidine [45].

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7 Author Contributions

Patrick Keohane: Study design; Data interpretation; Methodology; Writing - review & editing. **Jeremy R. Everett:** Study design; Data interpretation; Methodology; Writing - review & editing. **Rui Pereira:** Study design; Data interpretation; Methodology; Writing - review & editing. **Chad M. Cook:** Funding acquisition; Methodology; Data interpretation; Writing -

review & editing. **Eunice Mah:** Data interpretation; Writing - original draft; Writing - review & editing. **Traci M. Blonquist:** Data interpretation; Methodology; Formal analysis; Writing - review & editing.

8 Author Declarations

Patrick Keohane and Rui Pereira are employees of Chrysea Labs. Jeremy R. Everett, Chad M. Cook, Traci M. Blonquist, and Eunice Mah received funding from Chrysea Labs.

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10 Figures and Legends to Figures

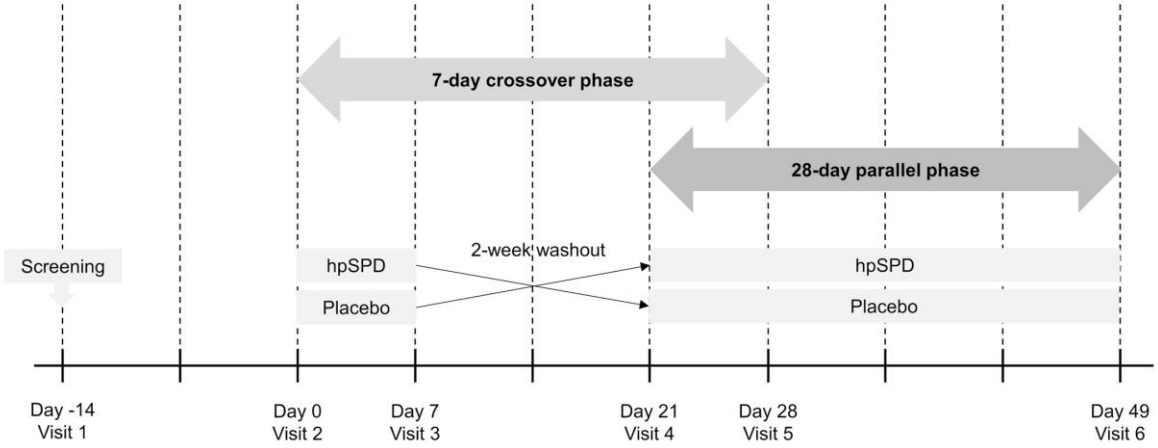

Figure 1. **Study design.** hpSPD = high-purity spermidine-trihydrochloride supplement

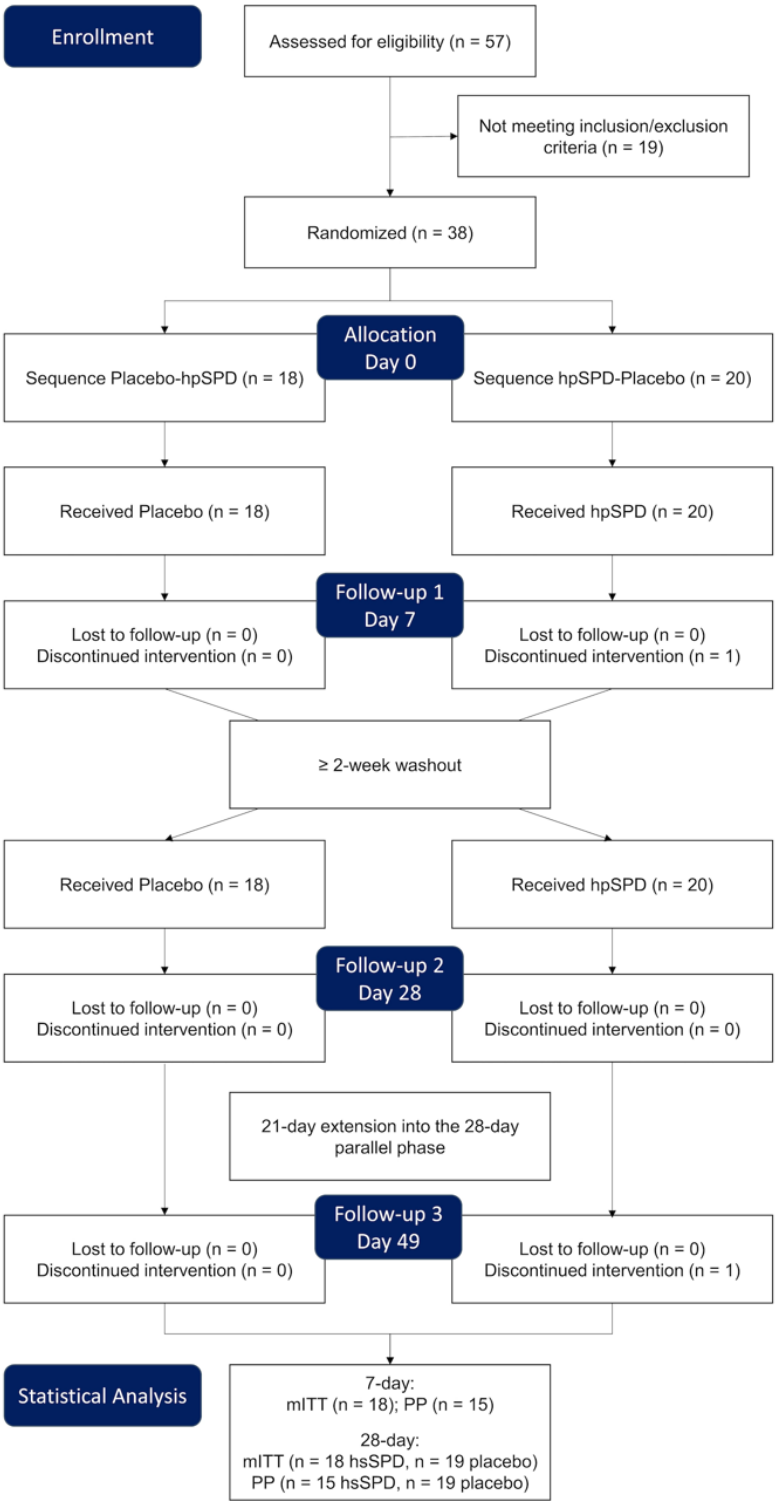

Figure 2. Participant flow diagram. mITT = modified intent-to-treat; n = sample size; PP, per protocol; hpSPD = high-purity spermidine-trihydrochloride supplement

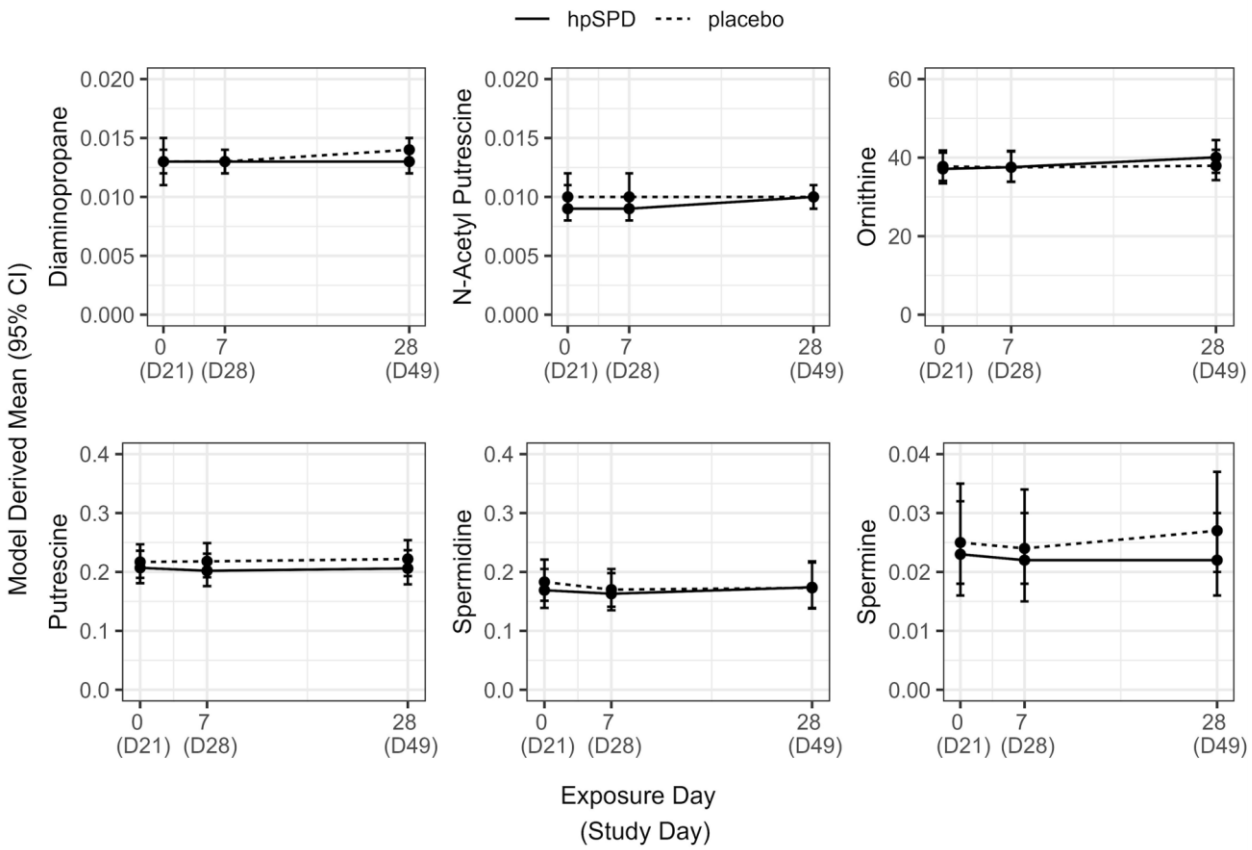

Figure 3. Serum polyamine concentrations (µM) of healthy male participants measured by LC-MS during the 28-day parallel phase. There was no statistically significant interaction between the intervention group (placebo or hpSPD) and time point as analyzed with a repeated measures mixed model. Measurements were log transformed prior to model fit and back transformed mean estimates are presented (95% confidence interval). CI = confidence interval; hpSPD = high-purity spermidine-trihydrochloride supplement; LC-MS = liquid chromatography mass spectrometry

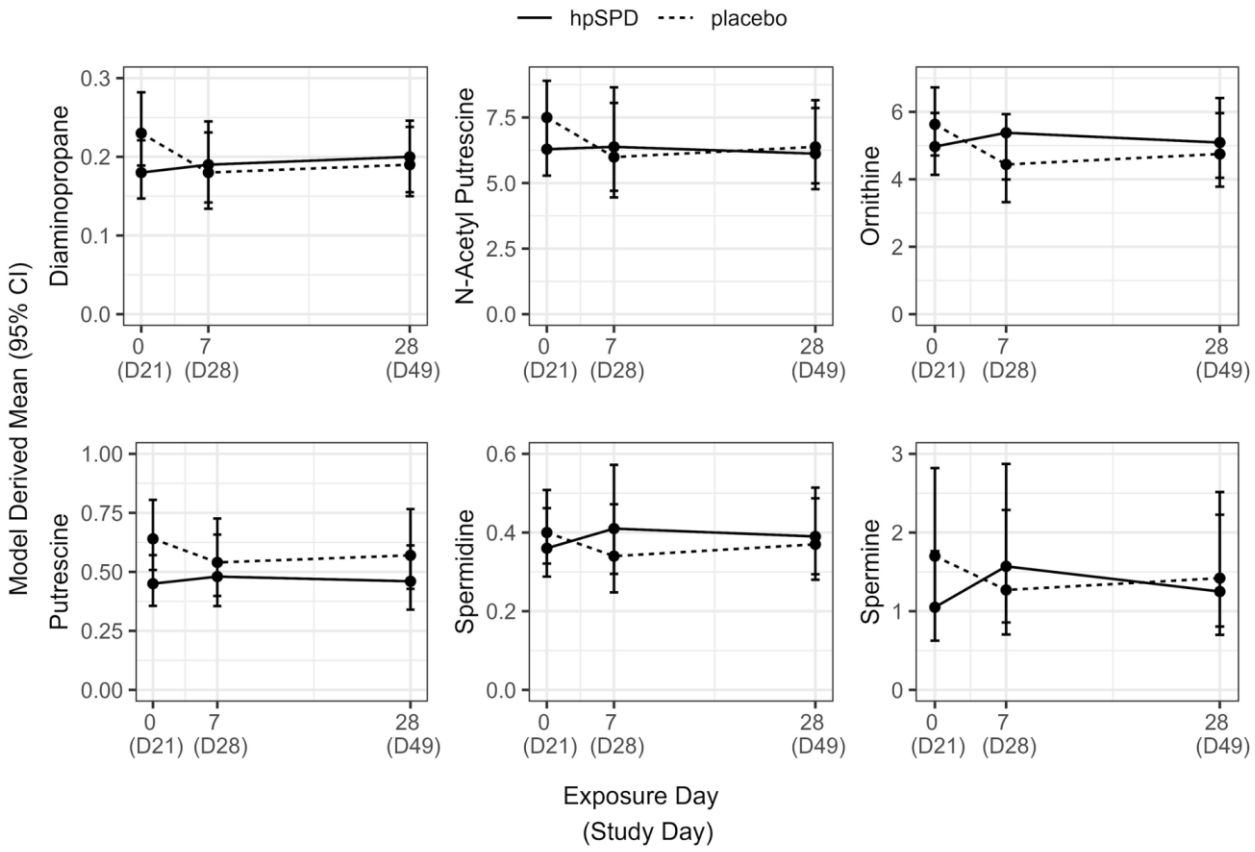

Figure 4. Urine polyamine concentrations (µM) of healthy male participants measured by LC-MS during the 28-day parallel phase. There was no statistically significant interaction between the intervention group (placebo or hpSPD) and time point as analyzed with a repeated measures mixed model. Measurements were log transformed prior to model fit and back transformed mean estimates are presented (95% confidence interval). CI = confidence interval; hpSPD = high-purity spermidine-trihydrochloride supplement; LC-MS = liquid chromatography mass spectrometry

11 Tables

Table 1. Subject characteristics of healthy male participants at the start of the study

Characteristics[1]	7-day crossover phase n = 37	28-day parallel phase hpSPD n = 18	28-day parallel phase Placebo n = 19
BMI (kg/m^2)	25.7 (21.4, 28.0)	25.6 (22.0, 28.0)	25.9 (21.4, 27.9)
Weight (kg)	83.2 (67.1, 109.4)	84.0 (73.8, 109.4)	82.2 (67.1, 96.9)
DBP (mm Hg)	77.0 (51.0, 96.0)	77.5 (51.0, 89.0)	75.0 (59.0, 96.0)
SBP (mm Hg)	122.0 (98.0, 142.0)	121.5 (98.0, 139.0)	122.0 (106.0, 142.0)
Spermidine intake (mg/d)	14.1 (5.0, 33.9)	10.5 (6.2, 27.3)	17.6 (5.0, 33.9)

Abbreviations: BMI, body mass index; DBP, diastolic blood pressure; SBP systolic blood pressure; hpSPD, high-purity spermidine-trihydrochloride supplement

Footnote:

[1] Values are median (minimum, maximum). Spermidine intake was obtained from food frequency questionnaire completed prior to Visit 3 (Day 7). All other characteristics were obtained at Visit 1.

683 Table 2. Model derived estimates and pairwise comparisons for the serum ELISA spermidine
684 and LC-MS polyamines of healthy male participants during the 7-day crossover phase

Polyamines[1]	Group	Pre[2]	Post[2]	P value within group	P value between groups[3]	P value int[4]
ELISA						
Spermidine (µM)	hpSPD	0.235 (0.183, 0.286)	0.217 (0.165, 0.269)	0.29	0.16	0.30
	Placebo	0.199 (0.147, 0.250)	0.214 (0.163, 0.266)	0.36		
LC-MS						
Spermidine (µM)	hpSPD	0.166 (0.144, 0.192)	0.160 (0.139, 0.185)	0.57	0.67	0.85
	Placebo	0.167 (0.144, 0.192)	0.167 (0.145, 0.193)	0.98		
Diaminopropane (µM)	hpSPD	0.013 (0.012, 0.014)	0.013 (0.012, 0.013)	0.34	0.88	0.25
	Placebo	0.013 (0.013, 0.014)	0.013 (0.012, 0.014)	0.46		
N-acetyl Putrescine (µM)	hpSPD	0.010 (0.009, 0.011)	0.010 (0.009, 0.010)	0.15	0.35	0.60
	Placebo	0.010 (0.009, 0.011)	0.010 (0.009, 0.011)	0.90		
Ornithine (µM)	hpSPD	37.869 (35.250, 40.683)	37.756 (35.14, 40.562)	0.88	0.94	0.81
	Placebo	38.224 (35.580, 41.064)	38.034 (35.40, 40.859)	0.80		
Putrescine (µM)	hpSPD	0.215 (0.194, 0.237)	0.211 (0.191, 0.233)	0.097	0.089	0.22
	Placebo	0.206 (0.186, 0.228)	0.215 (0.194, 0.237)	0.45		
Spermine (µM)	hpSPD	0.024	0.022	0.13	0.21	0.35

	(0.019, 0.030)	(0.017, 0.028)	
Placebo	0.022	0.022	0.81
	(0.017, 0.028)	(0.017, 0.028)	

Abbreviation: hpSPD, high-purity spermidine-trihydrochloride supplement; LC-MS, liquid chromatography mass spectrometry; ELISA, enzyme-linked immunosorbent assay

Footnotes:

[1] Outcomes were analyzed with a repeated measures mixed model. Measurements by LC-MS were log transformed prior to model fit and back transformed mean estimates are presented (95% confidence interval).

[2] Pre refers to Day 0 (before first dose) and Day 21 (after washout). Post refers to Day 7 (completion of first crossover period) and Day 28 (completion of second crossover period).

[3] Difference between groups in change from baseline.

[4] Interaction between the intervention group and time point.

Table 3. Model derived estimates and pairwise comparisons for the urine LC-MS polyamines of healthy male participants during the 7-day crossover phase

Polyamine[1]	Group	Pre[2]	Post[2]	P value within group	P value between groups[3]	P value int[4]
Spermidine (µmol)	hpSPD	0.38 (0.31, 0.46)	0.40 (0.33, 0.48)	0.55	0.038	0.11
	Placebo	0.42 (0.34, 0.51)	0.35 (0.29, 0.42)	0.020		
Diaminopropane (µmol)[5]	hpSPD	0.20 (0.17, 0.23)	0.20 (0.17, 0.24)	0.67	0.10	0.25
	Placebo	0.21 (0.18, 0.25)	0.18 (0.15, 0.22)	0.060		
N-acetyl Putrescine (µmol)	hpSPD	6.80 (5.74, 8.05)	6.60 (5.57, 7.82)	0.72	0.20	0.42
	Placebo	7.21 (6.09, 8.54)	6.03 (5.09, 7.14)	0.031		
Ornithine (µmol)	hpSPD	5.26 (4.52, 6.13)	5.27 (4.52, 6.15)	0.98	0.14	0.25
	Placebo	5.49 (4.71, 6.40)	4.63 (3.97, 5.39)	0.038		
Putrescine (µmol)	hpSPD	0.54 (0.45, 0.65)	0.57 (0.47, 0.68)	0.53	0.11	0.19
	Placebo	0.55 (0.46, 0.67)	0.48 (0.40, 0.58)	0.10		
Spermine (µmol)	hpSPD	1.19 (0.82, 1.73)	1.51 (1.04, 2.20)	0.049	0.002	0.010
	Placebo	1.58 (1.09, 2.30)	1.18 (0.81, 1.71)	0.016		

Abbreviation: hpSPD, high-purity spermidine-trihydrochloride supplement

Footnotes:

[1] Outcomes were analyzed with a repeated measures mixed model. Measurements were log transformed prior to model fit and back transformed mean estimates are presented (95% confidence interval).

[2] Pre refers to Day 0 (before first dose) and Day 21 (after washout). Post refers to Day 7 (completion of first crossover period) and Day 28 (completion of second crossover period).

[3] Difference between groups in change from baseline.

[4] Interaction between the intervention group and time point.

[5] One data point was outside the statistical extreme outlier limit and examination of the data point suggest that it is not likely biologically feasible.

Table 4. Model derived estimates and pairwise comparisons for the serum spermidine of healthy male participants measured by ELISA and LC-MS during the 28-day parallel phase

Polyamines[1]	Group	Pre[2]	Post[2]	P value within group	P value between groups[3]	P value int[4]
ELISA						
Spermidine (µM)	hpSPD	0.261 (0.187, 0.336)	0.258 (0.184, 0.332)	0.93	0.89	0.24
	Placebo	0.221 (0.148, 0.294)	0.225 (0.152, 0.298)	0.92		
Spermidine (µM)	hpSPD	0.169 (0.139, 0.205)	0.174 (0.139, 0.218)	0.75	0.55	0.92
	Placebo	0.183 (0.151, 0.221)	0.173 (0.138, 0.216)	0.6		

Abbreviation: hpSPD, high-purity spermidine-trihydrochloride supplement; LC-MS, liquid chromatography mass spectrometry; ELISA, enzyme-linked immunosorbent assay

Footnotes:

[1] Outcomes were analyzed with a repeated measures mixed model. Measurements by LC-MS were log transformed prior to model fit and back transformed mean estimates are presented (95% confidence interval).

[2] Pre refers to the start of the 28-day parallel phase (i.e., following a minimum 14-day washout prior to crossing over to the next study product in the randomization sequence at Day 21). Post refers to the end of the 28-day parallel phase (Day 49).

[3] Difference between groups in change from baseline.

[4] Interaction between the intervention group and time point.

Table 5. Adverse events of healthy male participants during the 7-day crossover phase, 7-day crossover washout period, and 28-day parallel phase

Adverse event	Severity	Phase[1]	hpSPD[3]	Placebo[3]
Squamous cancer cell	Mild	28-day parallel	1	0
Costochondritis	Moderate	28-day parallel	1	0
Exacerbation of deviated septum	Moderate	28-day parallel	0	1[4]
Headaches	Moderate	28-day parallel	0	1[4]
Hypertension	Mild	28-day parallel	0	1[4]
Nasal congestion	Moderate	28-day parallel	0	1
Blood glucose increased	Mild	7-day crossover	0	1
Abdominal pain	Mild	7-day crossover/28-day parallel[2]	0	1[4]
Diarrhea	Mild	7-day crossover/28-day parallel[2]	0	1[4]
Upper respiratory tract infection	Mild	28-day parallel	0	1[4]
Acne exacerbation	Mild	28-day parallel	0	1[4]
Intermittent headache	Moderate	7-day crossover/28-day parallel	1	0
Lightheadedness	Mild	7-day crossover	0	1
Plantar fasciitis	Mild	First washout	n/a	n/a[4]
Cellulitis	Mild	First washout	n/a	n/a[4]
Basal cell carcinoma	Mild	First washout	n/a	n/a[4]
Poison ivy	Mild	Before the start of any product intake	n/a	n/a[4]

Abbreviation: hpSPD, high-purity spermidine-trihydrochloride supplement

Footnotes:

[1] Only adverse events occurring during the intervention phases were listed. Four adverse events (three mild and one moderate) occurred before the start of any product intake or during the washout phase. None of the adverse events were judged to be related to either study products.

[2] 7-day crossover/28-day parallel phase refers to the period between Visits 4 and 5 whereby the two phases overlap.

[3] Values are number of participants.

[4] Four participants experienced multiple adverse events: Participant 021 had exacerbation of deviated septum, headache, and hypertension; Participant 027 had plantar fasciitis and cellulitis; Participant 044 had poison ivy, basal cell carcinoma, abdominal pain, and diarrhea; Participant 050 had upper respiratory tract infection and acne exacerbation.