Changes in the Urinary Proteome of Healthy Subjects After One Month of Continuous Nutritional Supplement Intake

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Abstract

Composite nutrient supplementation is a common health management strategy. No study has yet investigated the overall effects of composite nutrient supplements on healthy individuals from the perspective of urinary proteomics. This study conducted a comparative analysis of urinary proteomes from healthy individuals before supplementation and after 2 and 4 weeks of composite nutrient supplementation, finding that the urinary proteome exhibited more significant changes after 2 weeks of supplementation. The differentially expressed proteins and their enriched pathways may be related to nutrient supplementation. For example, erythropoietin receptor (Erythropoietin receptor) (after two weeks of composite nutrient supplementation, 4 out of 9 individuals showed a de novo appearance, with the average fold change (FC) for the 9 individuals reaching 450). The results of this study provide novel insights into the effects of composite nutrient supplements on health from the urinary proteomics perspective, which may help optimize usage guidelines and recommendations for composite nutrient supplements and facilitate the development of personalized nutrient supplementation strategies for different populations.

Full Text

Preamble

Changes in Urinary Proteome in Healthy Individuals Following Multi-Vitamin/Mineral Supplementation

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Abstract

Multivitamin/mineral supplements (MVM) are widely used across many populations. However, no studies have investigated the holistic effects of complex nutrient supplementation on healthy individuals from the perspective of the urinary proteome. This study compared and analyzed the urinary proteomes of healthy individuals before supplementation and after 2 and 4 weeks of compound nutrient supplementation. We found that the urinary proteome changed more significantly after 2 weeks of supplementation, with differential proteins and their enriched pathways potentially associated with nutrient supplementation. Notably, erythropoietin receptor exhibited a striking average fold change (FC) of 449.5 after 2 weeks of supplementation, with 4 out of 9 individuals showing a de novo appearance. These findings provide novel insights into the health effects of complex nutrient supplements from a urinary proteome perspective, which may help optimize usage guidelines and recommendations and facilitate the development of personalized supplementation strategies for different populations.

Keywords: Urine; Proteome; Compound nutrients; Multivitamins/minerals

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Introduction

Micronutrients (MNs), comprising trace elements and vitamins, are essential for human metabolism. In modern society, growing health consciousness has made multi-vitamin and mineral (MVM) supplementation a common health management practice. Complex nutrient supplements provide a broad spectrum of nutrients, supplying trace elements and vitamins that may be lacking in the diet to meet diverse nutritional needs. As awareness of health maintenance increases, more individuals use these supplements to promote overall well-being through comprehensive nutritional support. However, despite widespread use for health improvement, many questions remain regarding their specific physiological effects.

Previous research on complex nutrient supplements has several limitations. Early studies predominantly focused on animal experiments, while human research mainly examined single vitamin or mineral supplementation effects in disease populations or special groups such as the elderly, pregnant women, and athletes. Few studies have investigated complex nutrient supplementation interventions in healthy general populations. Some research indicates that complex nutrient supplements can provide essential trace elements and vitamins, potentially strengthening immune function, improving cardiovascular health, and enhancing antioxidant capacity. Multiple studies demonstrate

that vitamins and minerals interact synergistically in various physiological processes, including immune regulation, energy metabolism, and cellular repair. The effects of complex nutrients on nutritional status, antioxidant capacity, exercise performance, and subjective stress and mood have been extensively investigated. While these studies provide evidence for potential benefits, they often require large cohorts or extended experimental durations. We propose that urinary proteomics offers a novel and sensitive approach to nutrition research, capable of detecting subtle physiological changes following short-term nutrient supplementation.

Unlike blood plasma, urine is not subject to homeostatic mechanisms, allowing it to accumulate early changes in physiological status and reflect systemic alterations with greater sensitivity, making it an ideal source for next-generation biomarkers. Urinary proteins contain rich information that can reveal subtle changes across different organ systems. Our laboratory previously demonstrated that the urinary proteome can systematically and comprehensively reflect the effects of magnesium L-threonate intake, showing potential for clinical nutrition research and practice. To date, no study has examined the impact of complex nutrient supplementation on the urinary proteome of healthy adults.

This study aims to investigate changes in the urinary proteome following complex nutrient supplementation in healthy individuals and to elucidate the potential health implications of these changes. Our findings will help reveal how complex nutrient supplements affect human metabolism, physiological function, and potential disease risk, providing novel insights for understanding their health effects and developing personalized nutritional intervention strategies. This research promises to offer new scientific evidence for improving quality of life in healthy populations and provide valuable implications for chronic disease prevention and management.

2. Materials and Methods

2.1.1 Experimental Consumables

1.5 ml/2 ml centrifuge tubes (Axygen, USA), 50 ml/15 ml centrifuge tubes (Corning, USA), 96-well cell culture plates (Corning, USA), 10 kDa filters (Pall, USA), Oasis HLB solid-phase extraction columns (Waters, USA), 1 ml/200 l/20 l pipette tips (Axygen, USA), BCA assay kit (Thermo Fisher Scientific, USA), high-pH reversed-phase peptide fractionation kit (Thermo Fisher Scientific, USA), iRT (indexed retention time, BioGnosis, UK).

2.1.2 Experimental Instruments

Refrigerated high-speed centrifuge (Thermo Fisher Scientific, USA), vacuum concentrator (Thermo Fisher Scientific, USA), DK-S22 electric constant-temperature water bath (Shanghai Jinghong Experimental Equipment Co., Ltd.), full-wavelength multifunctional microplate reader (BMG Labtech,

Germany), vortex mixer (Thermo Fisher Scientific, USA), TS100 thermomixer (Hangzhou Ruicheng Instrument Co., Ltd.), electronic balance (METTLER TOLEDO, Switzerland), -80°C ultra-low temperature freezer (Thermo Fisher Scientific, USA), EASY-nLC1200 ultra-high performance liquid chromatography (Thermo Fisher Scientific, USA), Orbitrap Fusion Lumos Tribrid mass spectrometer (Thermo Fisher Scientific, USA).

2.1.3 Experimental Reagents

The nutritional supplement used was 21 Jinweita Multi-Vitamin and Mineral Tablets (Hangzhou Minsheng Health Pharmaceutical Co., Ltd.). Additional reagents included Trypsin Gold (Promega, USA), dithiothreitol (DTT, Sigma, Germany), iodoacetamide (IAA, Sigma, Germany), ammonium bicarbonate (NH_4HCO_3, Sigma, Germany), urea (Sigma, Germany), purified water (Wahaha, China), MS-grade methanol (Thermo Fisher Scientific, USA), MS-grade acetonitrile (Thermo Fisher Scientific, USA), MS-grade pure water (Thermo Fisher Scientific, USA), Tris-Base (Promega, USA), and thiourea (Sigma, Germany).

2.1.4 Analysis Software

Proteome Discoverer (Version 2.1, Thermo Fisher Scientific, USA), Spectronaut Pulsar (Biognosys, UK), Ingenuity Pathway Analysis (Qiagen, Germany), R Studio (Version 1.2.5001), Xftp 7, Xshell 7.

2.2.1 Study Design and Subject Selection

Eleven healthy adult volunteers aged 22–27 years (5 males, 6 females) were enrolled based on health examination results, with exclusion of cardiac, pulmonary, hepatic, or splenic disorders. Participants were instructed to maintain consistent dietary and lifestyle habits throughout the intervention period and to avoid nutritional supplements or dietary supplements for one week prior to and during the study. Each volunteer consumed two compound nutrient tablets daily for four weeks.

The supplement used was 21 Jinweita Multi-Vitamin and Mineral Tablets. Each tablet contained: L-lysine hydrochloride 12.5 mg, vitamin A 2500 IU, vitamin D_2 200 IU, vitamin E 5 mg, vitamin B_1 2.5 mg, vitamin B_2 2.5 mg, vitamin B_6 0.25 mg, vitamin B_{12} 0.5 g, vitamin C 25 mg, niacinamide 7.5 mg, calcium pantothenate 2.5 mg, choline bitartrate 25 mg, inositol 25 mg, iron 5 mg, iodine 0.05 mg, copper 0.5 mg, manganese 0.5 mg, zinc 0.25 mg, calcium hydrogen phosphate 279 mg, magnesium 0.5 mg, and potassium 5 mg.

[Figure 1: see original paper] Research methodology and technical workflow

2.2.2 Urine Sample Collection

Midstream morning urine samples were collected from 11 healthy volunteers before supplementation (W0, samples 301–311), after 2 weeks (W2, samples 401–411), and after 4 weeks (W4, samples 501–511), avoiding menstrual periods in female participants. Samples were stored at -80°C until analysis.

2.2.3 Urine Sample Processing

Two milliliters of thawed urine were centrifuged at 12,000×g for 30 minutes at 4°C to remove cellular debris. The supernatant was treated with 40 l of 1 M dithiothreitol (DTT, Sigma) to a final concentration of 20 mM, mixed, and incubated at 37°C for 60 minutes in a metal bath. After cooling to room temperature, 100 l of iodoacetamide (IAA, Sigma) stock solution was added to reach the working concentration, followed by 45 minutes of dark incubation at room temperature. Proteins were precipitated by mixing the reaction mixture with three volumes of ice-cold absolute ethanol and storing at -20°C for 24 hours. Precipitated proteins were pelleted by centrifugation at 10,000×g for 30 minutes at 4°C, dried, and resuspended in 200 l of 20 mM Tris solution. Protein concentration was determined by the Bradford method. Using the filter-aided sample preparation (FASP) method, urinary protein extracts were loaded onto 10 kDa ultrafiltration tubes (Pall, Port Washington, NY, USA), washed three times with 20 mM Tris solution, and resuspended in 30 mM Tris solution. Trypsin (Trypsin Gold, Mass Spec Grade, Promega, Fitchburg, WI, USA) was added at a 1:50 enzyme-to-protein ratio for digestion at 37°C for 16 hours. The resulting filtrate containing peptide mixtures was desalted using Oasis HLB solid-phase extraction columns, vacuum-dried, and stored at -80°C. Dried peptides were resuspended in 30 l of 0.1% formic acid, and peptide concentration was determined using a BCA assay kit and diluted to 0.5 g/ l. Four microliters from each sample were pooled to create a mix sample.

2.2.4 LC-MS/MS Analysis

All samples were spiked with iRT standard solution (diluted 1:100) at a 20:1 sample-to-iRT ratio for retention time alignment. Data-independent acquisition (DIA) was performed with three technical replicates per sample, and a mix sample was inserted every 10 runs as a quality control. One microgram of peptides was separated using an EASY-nLC1200 system (90-minute gradient: mobile phase A, 0.1% formic acid; mobile phase B, 80% acetonitrile) and analyzed by an Orbitrap Fusion Lumos Tribrid mass spectrometer to generate raw files.

2.2.5 Data Processing and Analysis

Raw DIA files were analyzed using Spectronaut software. High-confidence proteins were defined by peptide q-value < 0.01. Protein quantification was performed using peak area integration of all fragment ions from secondary peptides,

followed by automatic normalization. Proteins with two or more unique peptides were retained, missing values were imputed as 0, and differential proteins were identified through comparative analysis.

Unsupervised hierarchical clustering analysis (HCA), principal component analysis (PCA), and OPLS-DA were performed using the Wukong platform (https://omicsolution.org/wkomics/main/). Functional enrichment analysis of differential proteins was conducted using the DAVID database (https://david.ncifcrf.gov/) to identify biological processes, cellular localization, and molecular functions. Differential proteins and related pathways were searched in PubMed (https://pubmed.ncbi.nlm.nih.gov/). Protein-protein interaction networks were analyzed using STRING (https://cn.string-db.org/).

3. Results

3.1 Protein Identification Results

Following urinary protein extraction, enzymatic digestion, and LC-MS/MS analysis, an average of 3,077 proteins (protein and peptide FDR $< 1\%$) were identified per sample (including mix samples), with a standard deviation of 628. Specifically, the average number of identified proteins was 3,026 $\pm$ 663 before supplementation (W0, samples 301-311, n = 11), 2,890 $\pm$ 434 after 2 weeks (W2, samples 401-404, 406-410, n = 9), and 3,058 $\pm$ 657 after 4 weeks (W4, samples 501-511, n = 11). The mix samples yielded an average of 3,745 $\pm$ 337 proteins, indicating good data reproducibility.

3.2 Changes After 2 Weeks of Supplementation

3.2.1 Differential Protein Analysis After imputing missing values as 0, a two-tailed paired t-test comparison between pre-supplementation (W0) and 2-week (W2) samples identified 228 differential proteins ($P < 0.05$, $FC > 1.5$ or < 0.67). Among these, 14 proteins showed fold changes greater than 10, and 24 showed changes greater than 5. Due to space limitations, only proteins with $FC > 5$ or < 0.2 are listed in the table.

Notably, erythropoietin receptor exhibited a fold change of 449.5. Erythropoietin promotes the proliferation and differentiation of hematopoietic progenitor cells, and both erythropoietin and its receptor exert neurotrophic and neuroprotective effects in humans. Research has shown that iron and complex micronutrients significantly increase hemoglobin, hematocrit, and serum iron levels in mice. In our cohort, 4 out of 9 individuals showed de novo appearance of erythropoietin receptor (from 0 to 14,635, 11,851, 11,181, and 5,930 according to Spectronaut analysis). Four proteins showed complete disappearance (FC = 0), including 26S proteasome non-ATPase regulatory subunit 8, diphosphomevalonate decarboxylase, protocadherin gamma-A2, and 2-amino-3-carboxymuconate-6-semialdehyde decarboxylase.

Significantly differential proteins between pre-supplementation (W0) and 2-week

(W2) groups (FC > 5 or < 0.2, P < 0.05)

3.2.2 Functional Annotation of Differential Proteins Gene Ontology (GO) analysis of the 228 differential proteins (P < 0.05, FC > 1.5 or < 0.67) using DAVID identified 74 biological processes (BP) (P < 0.05), including regulation of cell shape, ubiquitin-dependent protein catabolic process via multivesicular body sorting pathway, protein transport, Rac protein signal transduction, viral budding via host ESCRT complex, multivesicular body assembly, adenylate cyclase-modulating G-protein coupled receptor signaling pathway, positive regulation of cell migration, signal transduction, and positive regulation of cell proliferation. The top 40 BPs by p-value are presented.

Biological processes (BP) enriched in differential proteins between W0 and W2 groups (top 40 by p-value)

GO analysis also enriched 24 molecular functions (MF) (P < 0.05), including protein binding, cadherin binding, GTP binding, GTPase activity, G-protein beta/gamma-subunit complex binding, guanyl nucleotide binding, cytoskeletal protein binding, G-protein coupled receptor binding, GDP binding, ATPase binding, calcium-dependent protein binding, actin binding, ubiquitin binding, protein kinase activator activity, proteoglycan binding, cAMP response element binding, calcium ion binding, transmembrane signaling receptor activity, protein kinase A binding, ATPase activator activity, collagen binding, cell adhesion molecule binding, natriuretic peptide receptor activity, and heparin binding.

Molecular functions (MF) enriched in differential proteins between W0 and W2 groups (P < 0.05)

Additionally, 49 KEGG pathways were enriched (P < 0.05), including endocytosis, long-term depression, cholinergic synapse, regulation of actin cytoskeleton, growth hormone synthesis/secretion/action, sphingolipid signaling pathway, human cytomegalovirus infection, dopaminergic synapse, alcoholism, relaxin signaling pathway, cGMP-PKG signaling pathway, melanogenesis, estrogen signaling pathway, Apelin signaling pathway, parathyroid hormone synthesis/secretion/action, axon guidance, Parkinson' s disease, PI3K-Akt signaling pathway, prion disease, chemokine signaling pathway, serotonergic synapse, pathogenic Escherichia coli infection, gap junction, HIV-1 infection, tight junction, circadian entrainment, renin secretion, retrograde endocannabinoid signaling, phagosome, adrenergic signaling in cardiomyocytes, oxytocin signaling pathway, Cushing syndrome, leukocyte transendothelial migration, vasopressin-regulated water reabsorption, MAPK signaling pathway, Rap1 signaling pathway, platelet activation, cocaine addiction, pathways of neurodegeneration-multiple diseases, chemical carcinogenesis-reactive oxygen species, cAMP signaling pathway, vascular smooth muscle contraction, tuberculosis, human papillomavirus infection, adherens junction, GnRH signaling pathway, thermogenesis, Ras signaling pathway, and aldosterone synthesis and secretion.

Notably, KEGG pathways related to cognition and mood were identified, including long-term depression, dopaminergic synapse, axon guidance, Parkinson' s disease, serotonergic synapse, and retrograde endocannabinoid signaling. Studies have shown that nutrients such as omega-3 fatty acids, antioxidants (vitamin C and zinc), B vitamins (B12 and folate), and magnesium can protect against mitochondrial and lipid oxidative damage in neuronal circuits associated with cognitive and emotional behaviors. Ten differential proteins were enriched in the cholinergic synapse pathway, consistent with the choline bitartrate content (50 mg/day) in the supplement. Several KEGG pathways were also related to immune system function.

KEGG pathways enriched in differential proteins between W0 and W2 groups ($P < 0.05$)

3.3 Changes After 4 Weeks of Supplementation

3.3.1 Differential Protein Analysis Comparison between pre-supplementation (W0) and 4-week (W4) samples identified 79 differential proteins ($P < 0.05$, $FC > 1.5$ or < 0.67). Five proteins showed fold changes greater than 5: ADAMTS3 (a disintegrin and metalloproteinase with thrombospondin motifs 3), guanidinoacetate N-methyltransferase, fibrinogen alpha chain, vacuolar protein sorting-associated protein 26A, and RNA-binding protein 14.

Significantly differential proteins between pre-supplementation (W0) and 4-week (W4) groups ($FC > 5$ or < 0.2, $P < 0.05$)

3.3.2 Functional Annotation of Differential Proteins GO analysis of differential proteins ($P < 0.05$, $FC > 1.5$ or < 0.67) enriched 20 biological processes ($P < 0.05$), including protein deglycosylation, epithelial cell differentiation, protein secretion, intermediate filament organization, epidermis development, intracellular protein transport, negative regulation of extrinsic apoptotic signaling pathway, ER-to-Golgi vesicle-mediated transport, positive regulation of epidermis development, centriole assembly, endocytic recycling, negative regulation of centriole replication, cell-cell adhesion, endocytosis, mannose metabolic process, protein processing, positive regulation of intracellular signal transduction, retrograde transport from endosome to Golgi, negative regulation of axon regeneration, and keratinization.

Biological processes (BP) enriched in differential proteins between W0 and W4 groups

Eight molecular functions were enriched ($P < 0.05$), including structural constituent of epidermis, GDP binding, cytokine binding, GTP binding, calcium ion binding, protein binding, alpha-mannosidase activity, and GTPase activity.

Molecular functions (MF) enriched in differential proteins between W0 and W4 groups

Differential proteins were enriched in two KEGG pathways: six proteins in endocytosis and three proteins in amino acid biosynthesis.

Discussion

Our results demonstrate that one month of complex nutrient supplementation induces measurable changes in the urinary proteome of healthy individuals. The most striking finding was the dramatic increase in erythropoietin receptor after 2 weeks (average FC = 449.5), with 4 out of 9 individuals showing de novo appearance (from 0 to 14,635, 11,851, 11,181, and 5,930). However, substantial inter-individual variability was observed, likely due to several factors. First, differences in baseline dietary habits and nutrient reserves may create heterogeneous responses, as individuals require different types and doses of nutrients. Second, the relatively low supplement dose and short supplementation period may produce modest effects that vary across individuals.

Personalized supplementation strategies tailored to specific populations are crucial to maximize benefits and minimize risks. The urinary proteome shows promise for providing clues to guide personalized nutrient supplementation. Notably, changes were more pronounced after 2 weeks than after 4 weeks, possibly reflecting decreased compliance over time or suggesting that nutrient effects are more acute initially, with diminishing impact over longer periods.

This study provides novel insights into the health effects of complex nutrient supplements from a urinary proteome perspective, which may help optimize usage guidelines. Our personalized analytical approach also offers a reference for related research fields. Future studies should measure micronutrient levels in body fluids and physiological parameters to better understand how complex nutrient supplementation modulates metabolism, physiological function, and health status, providing a foundation for developing innovative nutritional interventions and functional foods.

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Note: Figure translations are in progress. See original paper for figures.