Research article

Blood-Based Proteome Profiles among Healthy and Obese Men with Carotenoid-Rich Jelly Consumption

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ABSTRACT

Proteomics has clarified the link between obesity and the development of cardiovascular diseases, which involves a combination of genetic predisposition and environmental factors. This randomized, placebo-controlled study investigated the effects of carotenoid in carrot jelly on protein profiles and the clinical and biochemical markers of metabolic syndrome in adult men with obesity. Thirty participants were divided into three groups: healthy (n = 10), obese-treatment (n = 10), and obese-placebo (n = 10). Daily consumption of 200 mL carrot jelly for 3 months in the treatment group led to significant decreases in low-density lipoprotein-cholesterol, fasting plasma glucose and plasma malondialdehyde, and a significant increase in plasma β-carotene ($p < 0.05$). After 3 months of supplementation, protein interactions in the treatment group indicated important pathways involving Wnt regulation and cell surface-receptor signaling pathways for the upregulated proteins, as well as regulation of cytokine production and cell proliferation pathways for the downregulated proteins. The proteomic patterns clarified the molecular mechanisms underlying obesity-induced metabolic disorders and the potential benefits of carotenoid consumption. Such studies may critically improve serious nutrition-related health issues.

Keywords: obesity; carotenoid; serum proteome; plasma β-carotene; molecular pathways

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บทความวิจัย

รูปแบบของโปรตีนในเลือดของกลุ่มคนเพศชายสุขภาพดีและกลุ่มภาวะอ้วนที่บริโภคเจลลี่ ที่มีสารแคโรทีนอยด์สูง

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บทคัดย่อ

โปรตีโอมิกส์ ช่วยอธิบายความสัมพันธ์ระหว่างภาวะอ้วนและการเกิดโรคหัวใจและหลอดเลือดซึ่งเกี่ยวข้องกับปัจจัยร่วมจากพันธุกรรมและสิ่งแวดล้อม งานวิจัยนี้เป็นแบบสุ่มที่มีกลุ่มศึกษาและกลุ่มหลอก เพื่อศึกษาผลของการบริโภคเจลลี่ที่มีสารแคโรทีนอยด์สูงต่อรูปแบบของโปรตีนในเลือด ตัวชี้วัดทางคลินิกและทางชีวเคมีในกลุ่มเพศชายที่มีภาวะอ้วน โดยมีอาสาสมัครจำนวน 30 คนแบ่งเป็น 3 กลุ่มๆละ 10 คน ได้แก่ กลุ่มคนสุขภาพดี กลุ่มศึกษาคือคนอ้วนที่ได้รับเจลลี่ที่มีสารแคโรทีนอยด์สูง ขนาด 200 มิลลิลิตรวันละ 1 ครั้งเป็นเวลา 3 เดือน และกลุ่มคนอ้วนที่เป็นกลุ่มหลอก ผลการศึกษาพบว่ากลุ่มศึกษามีระดับไขมัน (LDL-C) น้ำตาลและ malondialdehyde ในเลือดลดลง แต่มีระดับระดับเบต้าแคโรทีนในเลือดเพิ่มขึ้นอย่างมีนัยสำคัญทางสถิติ ($p<0.05$) การติดตามผลที่ 3 เดือนพบโปรตีนที่เพิ่มสูงขึ้นในกลุ่มศึกษามีความสัมพันธ์กับ Wnt regulation และ cell surface-receptor signaling pathways ส่วนกลุ่มของโปรตีนที่ลดลงพบความสัมพันธ์กับ regulation of cytokine production และ cell proliferation pathways ทั้งนี้รูปแบบโปรตีนในเลือดที่พบสามารถอธิบายกลไกระดับโมเลกุล ที่เป็นสาเหตุของการเกิดความผิดปกติที่มีภาวะอ้วนเป็นสาเหตุและแสดงถึงประโยชน์ของการบริโภคอาหารที่มีแคโรทีนอยด์สูง ซึ่งงานวิจัยในลักษณะดังกล่าวมีความสำคัญอย่างยิ่งในการช่วยลดผลกระทบต่อสุขภาพที่มีสาเหตุหลักมาจากภาวะโภชนาการ

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Introduction

Obesity is an important cause of morbidity associated with dyslipidemia, type 2 diabetes (T2DM), hypertension, cardiovascular disease (CVD), and cancer[1]. According to a 2016 World Health Organization report, the prevalence of obesity, defined as a body mass index (BMI) ≥ 30 kg/m^2 in adults 18 years and older, was 13% globally and 10% for Thailand[2]. Furthermore, mean total cholesterol (TC, age-standardized estimate) among Thais (193.35 mg/dL) was slightly higher than the global figure (174 mg/dL)[2]. Of great concern is that the prevalence of obesity increased concurrently with abnormalities in lipid metabolism, with approximately 60%-70% of obese patients exhibiting dyslipidemia[3]. Genetic factors, excessive energy intake, sedentary lifestyle, and environmental factors all contribute to the increased accumulation of body fat and the adverse metabolic consequences of dyslipidemia characterized by hypertriglyceridemia and low levels of high-density lipoprotein-cholesterol (HDL-C). Along with excessive adipocyte hypertrophy and hyperplasia, these changes are associated with the generation of reactive oxygen species (ROS), oxidative stress, and expression or secretion of various proinflammatory and anti-inflammatory adipocytokines[4]. Nutritional status is a crucial modifiable lifestyle factor for individuals interested in health-promotion strategies. Among the micronutrients with bioactive properties, carotenoids are a class of effective phytonutrients with varying chemical structures, and include α-carotene, β-carotene, β-cryptoxanthin, lutein, zeaxanthin, and lycopene[5]. Their important roles in molecular functions include the following: (1) anti-inflammatory potential via inhibition of the nuclear factor (NF)-ΚB/Janus kinase 2/signal transducer and activator of transcription 3 and c-Jun NH2-terminal kinase (JNK)/p38-mitogen-activated protein kinase (MAPK) signaling pathways[6]; (2) antioxidant capacity as efficient scavengers of ROS and other free radicals[7]; (3) modulation of gene transcription of nuclear peroxisome proliferator-activated receptors (PPARs) in hepatic lipid homeostasis[8]; (4) anti-diabetic effects through increased insulin sensitivity and decreased insulin resistance (IR); and (5) protective antioxidant effects on pancreatic β cells[9]. Several previous studies focused on the effect of a provitamin A carotenoid, β-carotene, in obesity-induced metabolic diseases. A systematic review and meta-analysis showed an inverse association between serum total carotenoids and metabolic syndrome, with a pooled odds ratio of 0.66 (95% confidence interval [CI]: 0.56–0.78)[10]. A double-blind randomized placebo-controlled crossover clinical trial determined the effect of 6 weeks of β-carotene-fortified synbiotic food consumption in patients with T2DM. These patients showed significant decreases in levels of insulin, homeostasis model assessment of IR, triglyceride (TG), and very-low-density lipoprotein-cholesterol, and the TC/HDL-cholesterol ratio compared to those receiving control food[11]. Such data support the essential role of carotenoids in downstream signaling of oxidative stress, IR, adipokine concentrations, and abdominal adiposity[12,13]. However, another study investigated the association between β-carotene supplementation and increased CVD mortality

(relative risk: 1.12; 95% CI: 1.04–1.19)[14]. Mass spectrometry-based proteomic studies can provide insight into the mechanisms of biological processes associated with obesity and associated comorbidities. A recent study using the human blood plasma proteome from an obese population (N = 4600) discovered alterations associated with lipid metabolism and inflammatory pathways that have an impact on clinically significant adiposity pathways[15]. A study among Nepalese school-aged children reported associations between plasma protein biomarkers and plasma concentrations of carotenoids with functions in lipid and vitamin A transport, antioxidation, and anti-inflammatory processes[16]. To better understand the biological basis of the relationship between diet and disease, the underlying mechanisms related to carotenoid supplementation and metabolic effects in people with obesity should be further investigated. Consequently, we adopted a serum proteomics approach to identify the molecular pathways indicative of the biological functions/dysfunctions related to consumption of carotenoid-rich jelly among a group of participants with obesity. The findings from this study provide novel insights for future research on the mechanism underlying the preventive role of carotenoids on obesity-induced comorbidities and suggest candidate biomarkers.

Materials and Methods

Study design, setting, and participants

This randomized placebo-controlled study was conducted among eligible patients ranging in age from 25 to 40 years old with obesity (BMI ≥ 30 kg/m^2). Changes in biochemical and proteomic profiles through the effect of carotenoid-rich jelly consumption versus a placebo were investigated. Exclusion criteria included individuals with a medical history involving cardiovascular, hepatic, renal, thyroid, autoimmune or chronic inflammatory abnormalities, cancer, or drug or alcohol abuse. Randomization was conducted using computer-generated random numbers to assign each participant to one of two groups: a treatment group (n = 10), in which participants received one cup (200 mL) of carrot jelly containing β-carotene (10 mg) once daily for 3 months; and a placebo group (n = 10), in which participants received a similar-looking jelly without carrots/β-carotene. A third group, the healthy group (n = 10), comprised male participants aged 25-40 years with a BMI between 18.5–22.9 kg/m^2 and no metabolic syndrome or other chronic diseases. This group was used as a reference for the proteomic analysis.

Data collection, blood sampling, and biochemical measurements

Venous blood was obtained from all participants after overnight fasting for 10-12 h, both at baseline and at 3-month follow-up. The following biochemical parameters were measured using automated methods (Cobas-Mira, Roche, Italy): glycosylated hemoglobin (HbA1C), TG, TC, HDL-C, and LDL-C. Levels of the inflammation marker high-sensitivity C-reactive protein (hsCRP) were analyzed using the immunoturbidimetric method (Abbott Alinity ci-series, USA). Plasma malondialdehyde (MDA) concentration was measured by high-performance liquid

chromatography (HPLC) using the isocratic method on an Agilent 1200 HPLC system (USA) with a commercial MDA kit (Immundiagnostik AG, Germany) as previously described[17]. Plasma vitamin A (retinol) levels were determined by reversed-phase HPLC. A commercially available kit of ready-to-use reagents (Chromsystems Instruments and Chemicals GmbH, Germany) was used for sample preparation following the manufacturer's protocol[18]. Analysis of plasma β-carotene concentration was determined by isocratic HPLC system-UV/VIS detection, and samples were prepared using a commercially available ready-to-use reagents kit (Chromsystems Instruments and Chemicals GmbH, Germany) as previously described[19].

Carotenoid-rich carrot jelly and nutritive values

The 1-cup (200 mL) servings of jelly were prepared at the Institute of Nutrition, Mahidol University, using a standard protocol. The carotenoid-rich carrot jelly contained carrot juice from carrot extractions, gelatin, sorbitol, sucralose, orange flavoring, and orange food coloring power, while the placebo jelly contained an orange food-coloring powder, water, and a clouding agent. We determined the nutritive values following the standard method of the Association of Official Analytical Collaboration International[20]. The levels of soluble and insoluble fiber in the carotenoid-rich carrot jelly were 0.5 ± 0.07 g and 0.8 ± 0.08 g, respectively, while those in the placebo jelly were both <0.05 g. β-carotene content in the carotenoid-rich carrot jelly was 10.23 ± 2.15 mg/200 mL, as determined by HPLC using a previously described method[21].

2.4 Proteomic analysis

Protein samples from the 10 participants in each group were pooled and the protein concentration was measured using Bradford assay[22]. All samples were subjected to in-solution digestion followed by trypsin digestion[23]. Protein quantification and identification were carried out using an Ultimate3000 Nano/Capillary LC System (Thermo Scientific, UK) coupled to a hybrid quadrupole Q-Tof impact IITM (Bruker Daltonics). Briefly, peptides were enriched on a 300-um inner diameter (i.d.) × 5-mm μ-Precolumn, separated on a 75-μm i.d. × 15-cm C18 Pepmap100 (5 μm, 100 A) (Thermo Scientific, UK), and packed with an Acclaim PepMap RSLC C18 (2 μm, 100 A), nanoViper (Thermo Scientific, UK). Using this method, we prepared two solvents (A and B) to supply the analytical column. Solvent A contained 0.1% formic acid in water and solvent B contained 0.1% formic acid in 80% acetonitrile. The peptides were eluted at a constant flow rate of 0.30 μL/min for 30 min using a gradient of 5%-55% solvent B. Using the Captive Spray, electrospray ionization was performed at 1.6 kV. A range of 150-2200 m/z was used to obtain positive-ion mode mass spectrometry (MS) and tandem MS/MS (Software Compass 1.9, Bruker Daltonics). Each sample was analyzed by liquid chromatography-MS in triplicate. For bioinformatics and data analysis, we used various software. MaxQuant 1.6.1.12 was applied to quantify protein in samples using the Andromeda search engine to correlate MS/MS spectra to the Uniprot human database. The following parameters were used for data processing: a maximum of two missed cleavage sites; mass tolerance of 20 parts

per million for the main search; trypsin as the digestion enzyme; carbamidomethylation of cysteine as a fixed modification; and the oxidation of methionine and acetylation of the protein N-terminus as variable modifications. For protein identification, peptides with a minimum of seven amino acids and at least one unique peptide were needed. Only identified proteins with at least two peptides and at least one distinctive peptide were used for further data analysis. Next, all differentially expressed proteins (DEPs) were analyzed for intersections among the different sample groups using Venny 2.1 (https://bioinfogp.cnb.csic.es /tools/venny/). The cutoff for DEPs was a log2 fold change ratio >1.5. Gene ontology annotations, including biological processes and molecular functions, were created using Panther (http://www.pantherdb.org). The identified proteins were simultaneously submitted to the Search Tool for Interactions of Chemicals (STITCH, http://stitch.embl.de) to better understand the cellular functions and interactions between proteins and small molecules.

Ethical considerations

This study was approved by the Committee on Human Rights Related to Research Involving Human Subjects, Faculty of Medicine Ramathibodi Hospital, Mahidol University (protocol number: COA.MURA2021/760). Prior to their participation, all participants signed consent forms outlining the objective, procedure, advantages, and possible risks of the study.

Statistical analysis

Statistical analyses were performed using the SPSS for Windows software package, v. 25 (IBM Corp., Armonk, NY, USA). Categorical data are reported as percentages and continuous data as means ± standard deviation. Comparisons of categorical variables and continuous data between different groups were analyzed by Pearson's chi-square test and one-way analysis of variance (ANOVA) with Tukey's honest significant difference post-hoc test. For differences within groups at baseline and 3-month follow-up, all variables were analyzed by dependent t-tests. In all analysis, the significance cutoff was 0.05.

Results

Baseline characteristics and changes in clinical and biochemical parameters at baseline and 3-month follow-up

The men in the healthy, treatment, and placebo groups had average ages of 42.00 ± 4.73, 40.00 ± 2.86, and 39.30 ± 5.61 years, respectively **(Table 1).** There were baseline differences in waist circumference, body fat, fat mass, and systolic (SBP) and diastolic blood pressure (DBP) between the healthy group and the treatment and placebo groups ($p < 0.05$). Additionally, the participants with obesity in both the treatment and placebo groups displayed significantly higher levels of fasting plasma glucose, HbA1C, TC, LDL-C, and TG compared to the healthy group (all $p < 0.05$). The markers of inflammation and lipid peroxidation, hsCRP, and MDA, also showed statistically significant differences between the healthy group

Table 1. Clinical and biochemical parameters among three study groups at baseline and 3-month follow-up.

Biochemical parameters	Healthy group (N=10)	Treatment group (N=10)	Placebo group (N=10)
Age, years	42.00 ±4.73	40.00 ±2.86	39.30± 5.61
Waist circumference, cm	85.49± 8.36	98.96± 7.97 *	107.32± 7.81*
BMI, kg/m^2	21.56± 1.38	32.97± 4.58*	32.62± 3.18 *
Body fat, %	19.25± 4.08	31.16± 4.42*	30.70± 4.12 *
Body fat mass, kg	13.27± 4.03	31.68± 9.67*	28.95± 6.23 *
Fasting plasma glucose, mg/dL			
Baseline	90.56 ±9.78	94.10 ±5.75 *	91.33 ±5.59*
3-month follow-up	86.11 ±6.54	88.20 ±8.17 #	92.34 ±5.91
HbA1C, %			
Baseline	5.30 ±0.53	5.71 ±0.48 *	5.66 ±0.18*
3-month follow-up	5.25 ±0.56	5.64 ±0.41	5.67 ±0.27
Total cholesterol, mg/dL			
Baseline	187.24 ±16.32	209.30 ±45.08*	230.44 ±37.80*
3-month follow-up	189.47 ±15.34	207.90 ±39.62	238.22 ±34.79
HDL-cholesterol, mg/dL			
Baseline	47.30 ±9.92	38.70 ±7.05	44.33 ±9.28
3-month follow-up	51.60 ±9.47	42.10 ±7.52#	45.11 ±9.22
LDL-cholesterol, mg/dL			
Baseline	122.67±7.23	147.80 ±32.86*	162.11 ±44.39*
3-month follow-up	131.59 ±16.36	131.40 ±37.40#	165.67 ±41.90
Triglyceride, mg/dL			
Baseline	129.44 ±46.88	160.78 ±55.47*	162.56 ±54.96*
3-month follow-up	127.11±51.62	140.19 ±67.32#	178.23 ±96.27
hsCRP, mg/L#			
Baseline	0.84 ±0.38	2.89 ±1.81*	2.92 ±1.28*
3-month follow-up	1.09 ±0.78	2.32 ±0.91#	3.24 ±1.98
Plasma β-carotene, ng/mL#			
Baseline	184.23 ±1.89	72.44 ±2.31*	69.18 ±1.38*
3-month follow-up	172.61 ±46.55	284.56 ±1.65#	73.88 ±0.98
Plasma vitamin A, mg/L			
Baseline	0.56 ±0.13	0.60 ±0.11	0.56± 0.18
3-month follow-up	0.54± 0.10	0.71 ±0.19	0.58±0.24
Plasma MDA, µmol/L			
Baseline	2.78 ±0.70	4.03 ±0.23*	3.93 ±0.28*
3-month follow-up	2.21 ±0.36	2.23± 0.12#	4.15 ±0.39
SBP, mmHg			
Baseline	122.58±7.03	149.50 ±5.26*	146.11 ±6.35*
3-month follow-up	124.99 6.35	132.47±13.04#	144.36 ±9.43
DBP, mmHg			
Baseline	62.80 ±18.68	92.36 ±9.25*	91.67 ±11.58*
3-month follow-up	77.70± 11.58	82.19±7.92#	94.23 ±11.11

* Significant difference from healthy group at p<0.05.; # Significant difference from baseline at p<0.05.

and the obesity groups, whereas plasma β-carotene levels were inversely related ($p < 0.05$). After 3 months of jelly supplementation, returned supplement cup counts indicated that compliance was > 80% in both groups, and the same biochemical parameters were re-evaluated in the treatment and placebo groups only. There was a significant increase in plasma β-carotene levels from baseline to 3-month follow-up in the treatment group (72.44 ± 2.31 vs. 284.56 ± 1.65 ng/mL, $p < 0.05$), reflecting a 292.38% change. We also observed decreasing trends in fasting plasma glucose, TC, LDL-C, TG, hsCRP, and MDA in the treatment group compared to the placebo group (all $p < 0.05$). Additionally, the treatment group exhibited decreases in SBP (149.50 ± 5.26 vs. 132.47 ± 13.04 mmHg) and DBP (92.36 ± 9.25 vs. 82.19 ± 7.92 mmHg) from baseline to 3-month follow-up. No significant differences in other parameters were observed between the groups.

Comparative proteomic analysis in the three groups

Proteomic profiles were compared between the healthy, obese-placebo, and obese-treatment groups. Serum from each group was pooled to lower biological variability and three replicates were analyzed. All proteins were identified at a 1% false discovery rate. As shown in the Venn diagram in **Figure 1A**, 1310, 1085, and 1977 unique proteins were identified in the healthy, treatment, and placebo groups, respectively, of which there were 205 and 206 overlapping proteins between the three groups. The biological processes of all of the proteins identified in the three groups included protein-modifying enzymes (15.7%), metabolite interconversion enzymes (12.2%), nucleic acid metabolism (11.1%), transporters (10.2%), and gene-specific transcriptional regulators (9.9%) **(Figure 1B)**. There were 88 proteins that showed upregulated expression levels in the obesity groups compared to those in the healthy group at baseline. The top 20 of these DEPs, which exhibited relative fold changes ranging from 3.35 to 7.62, included interleukin-6 (IL-6), NADPH oxidase 4 (NOX4), apoptotic protease-activating factor 1 (APAF-1), and cholesterol transporter ATP-binding cassette sub-family A member 5 (ABCA5) **(Figure 2A)**. The top 20 of the 118 DEPs that showed downregulated expression in the obesity groups compared to those in the healthy group had relative fold changes ranging from −3.09 to −6.53, and included p53-activated protein-2 (PAP2), GATA binding protein 4 (GATA4), the renin/prorenin receptor ATPase H(+)-transporting lysosomal accessory protein 2 (renin receptor), p-glycoprotein (MDR1), glutathione S-transferase pi (GSTP1), DNA-directed RNA polymerase subunit (POLR1A), SEC31 homolog A (SEC31A), and amidophosphoribosyltransferase (GPAT).

Proteomic profiles in the obesity groups: comparison of DEPs between baseline and 3-month follow-up

Carotenoids are found in various fruits and vegetables and mainly function to regulate biological processes in cells. A Venn diagram of differential expression analysis of serum proteins in the obese-treatment group showed 1456 and

313 unique proteins at baseline and after completion of

(A)

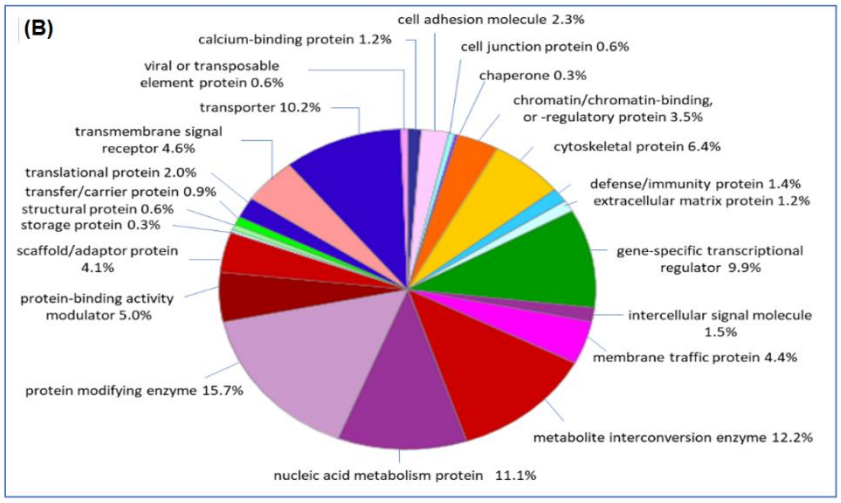

Figure 1. Venn diagram of the serum unique and overlapping proteins (A) and all their functions: biological process (B) at baseline in the healthy and obese-treatment and placebo groups.

β-carotene supplementation, respectively **(Figure 3A)**. The related biological processes for all identified proteins in the obese-treatment group included cellular processes (28.14%), biological regulation (18.64%), metabolic processes (17.66%), and response to stimulus (7.92%). There was overlap between baseline and 3-month follow-up in 377 of these proteins. The top 20 of the 354 upregulated DEPs between baseline and 3 months of supplementation included Rho GTPase activating protein 21 (ARHGAP21), transcription factor SOX-5 (SOX5), vacuolar protein sorting-associated protein 13C (VPS13C), ATP-binding cassette sub-family B member 6 (ABCB6), zinc finger protein 674 (ZNF674), GSTP1, and p53-activated protein-2 (PAP2) **(Figure 4A)**. The top 20 of the 210 downregulated DEPs between baseline and 3 months of supplementation included collagen alpha-2(VI) chain (COL6A2), transmembrane protein 200C (TMEM200C), disco interacting

protein 2 homolog A (DIP2A), apoptotic peptidase activating factor 1 (APAF1), metalloendopeptidase (TLL1), procollagen-proline 4-dioxygenase (P4HA2), and protein unc-45 (UNC45B) **(Figure 4B)**. In the obese-placebo group, 2,095 and 2,153 proteins were exclusively identified at baseline and 3-month follow-up, respectively, and 135 proteins

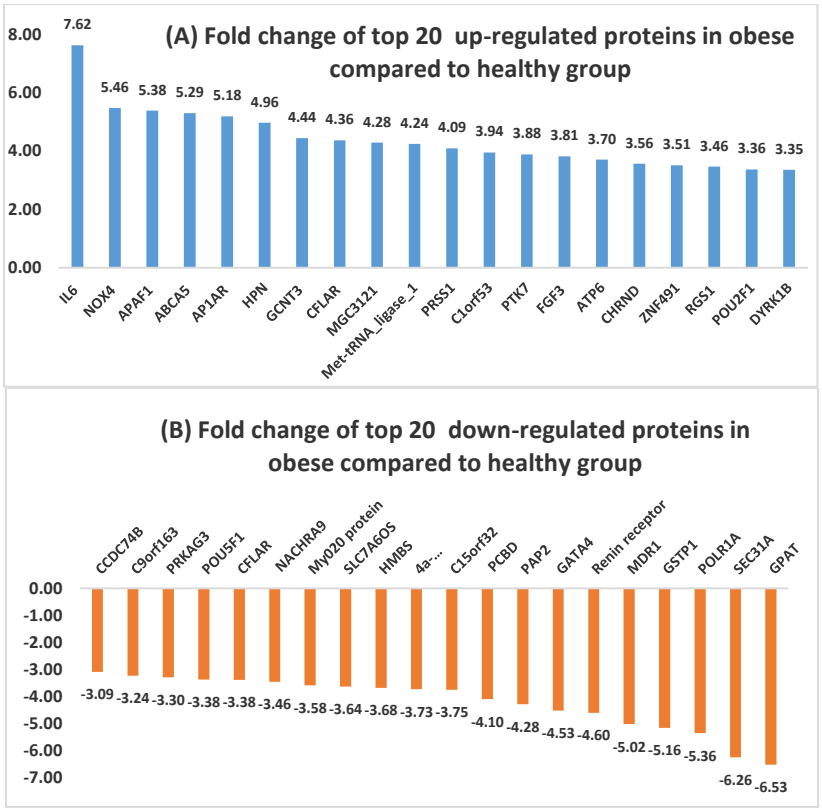

Figure 2. Fold change of top 20 upregulated (A) and downregulated proteins (B) in obese (treatment and placebo) groups compared to the healthy group.

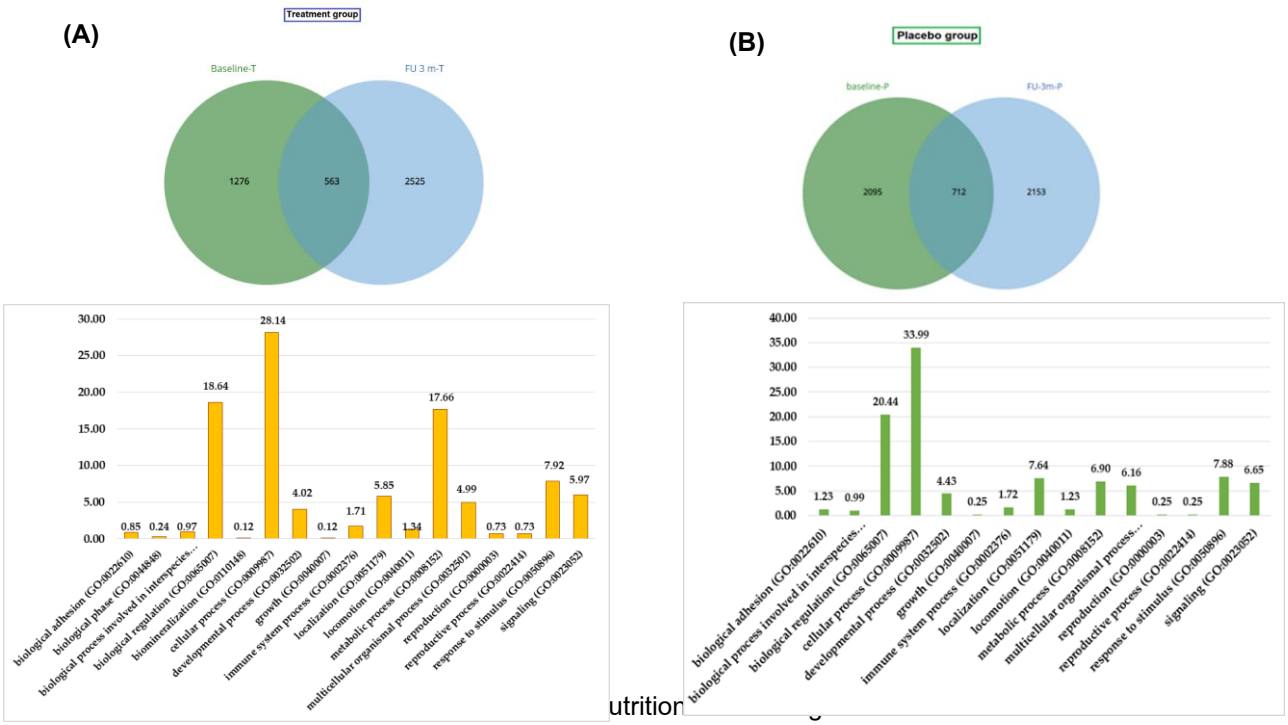

Figure 3. Venn diagram with unique and overlapping proteins and their biological process of the overlapping proteins in the treatment and placebo groups at baseline and 3-month follow-up.

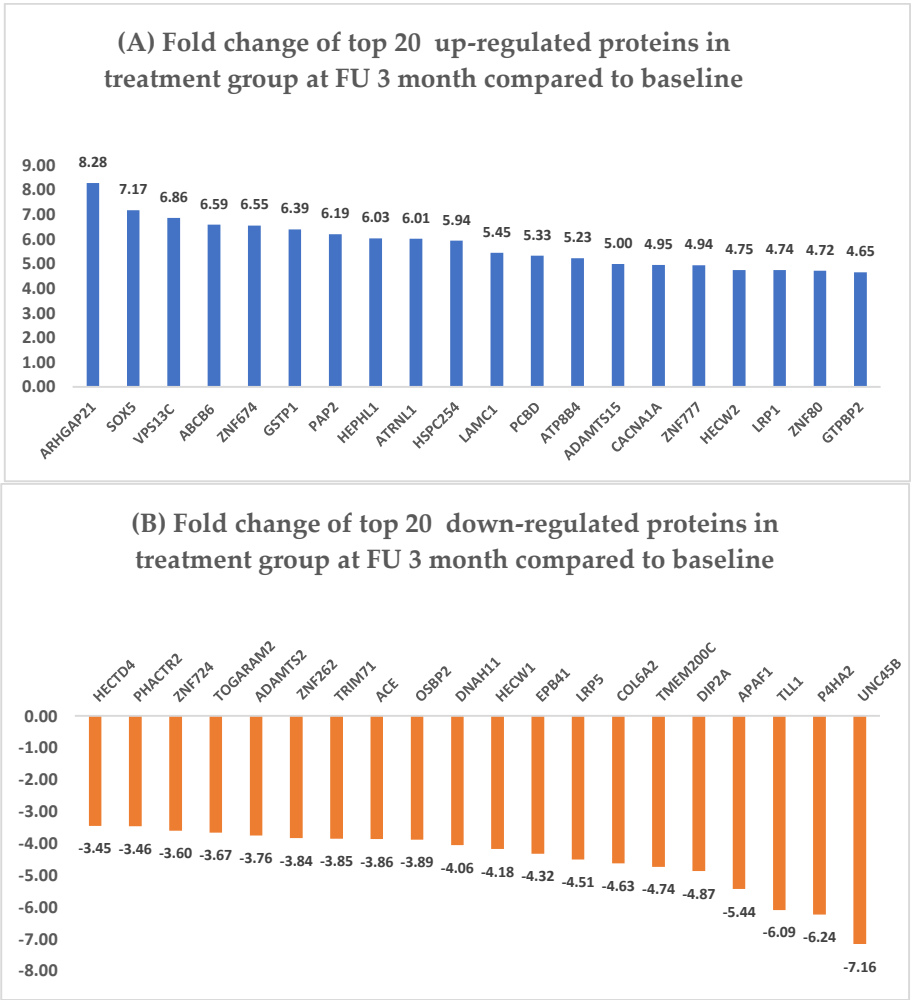

Figure 4. Relative fold changes of serum proteins from baseline and 3-month follow-up among the obese-treatment group

overlapped between the two time points. Of all proteins found in the placebo group, 33.99% and 20.44% were related to cellular processes and biological regulation, respectively **(Figure 3B)**. Among the 712 DEPs between baseline and 3-month follow-up, 400 were upregulated and 312 were downregulated. Figure 5A shows the top 20 upregulated proteins, which exhibited relative fold changes ranging from 4.82-11.83 and included collagen alpha-4 (IV) chain (COL4A4), mitochondrial pyruvate carrier 1-like protein (MPC1L), poly [ADP-ribose] polymerase, insulin-like growth factor-binding protein complex (IGFALS), kinesin-like protein KIF18A (KIF18A), and others. The top 20 downregulated proteins, which exhibited relative fold changes ranging from -4.49 to -9.36 included zinc finger protein 490 (ZNF490), Rho GTPase activating protein 8

(ARHGAP8), mitochondrial pyruvate carrier 2 (MPC2), and vacuolar protein sorting-associated protein 13D (VPS13D) **(Figure 5B)**. Upregulated proteins in the obese-treatment group were also analyzed to gain insights into protein-protein,

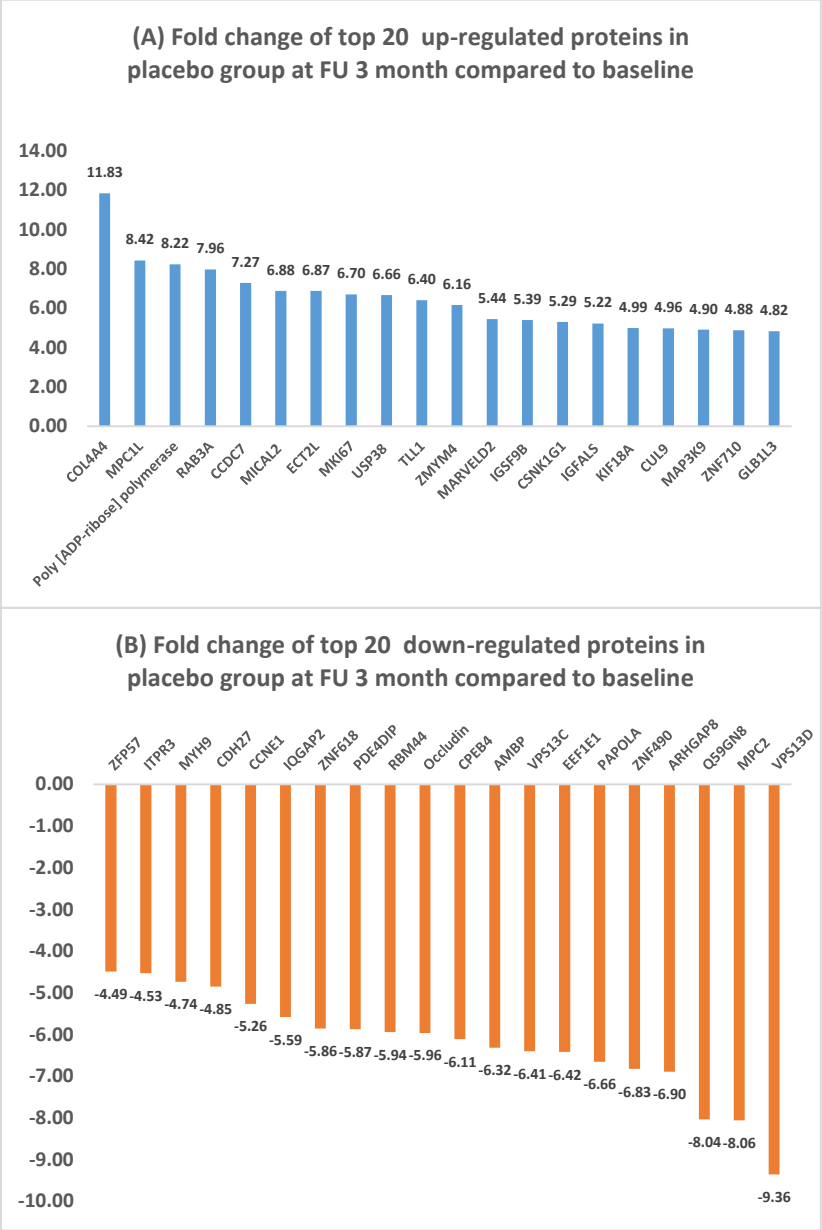

Figure 5. Relative fold changes of serum up- (A) and down-regulated proteins (B) from baseline and 3-month follow-up among the placebo group

protein-nutrient, and biomarker interactions using STITCH 4.0. For this analysis, we looked for functional interactions between DEPs (fold change > 1.5 from baseline to 3-month follow-up) and carotenoids (β-carotene, retinoic acid and lycopene), hs-CRP, and MDA. The analysis of upregulated proteins revealed their involvement in two important networks: cell surface receptor signaling pathways and the regulation of Wnt signaling pathway **(Figure 6)**. The downregulated

proteins were involved in the regulation of cytokine production and the regulation of cell proliferation **(Figure 7)**.

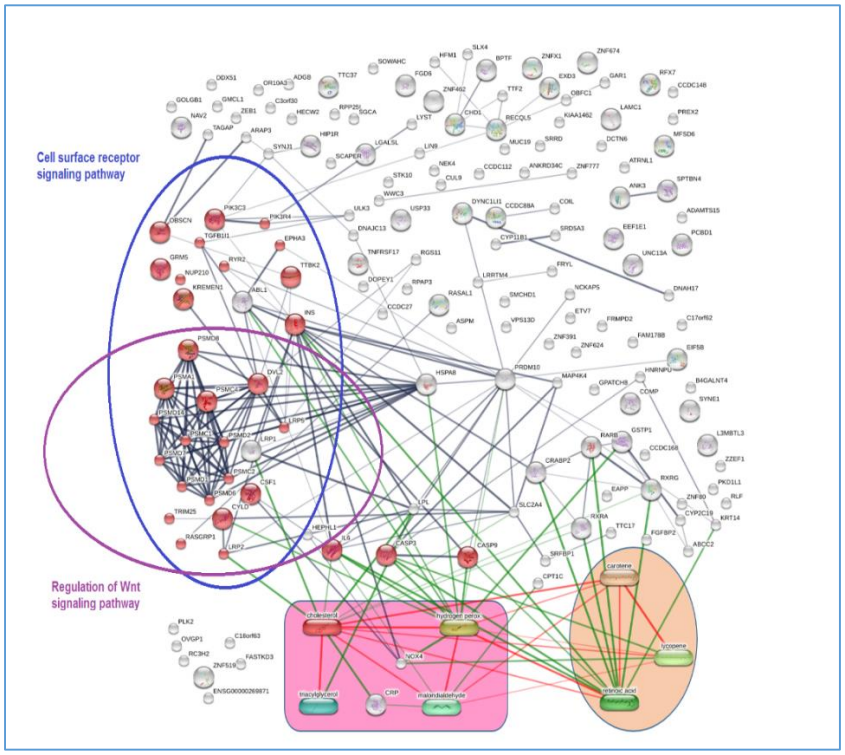

Figure 6. Interaction between serum up-regulated proteins with relative fold changes > 1.5 (from baseline and 3-month follow-up) among the treatment group. [Stronger associations are represented by thicker lines. Protein-protein interactions are shown in grey, chemical-protein interactions in green and interactions between chemicals in red.]

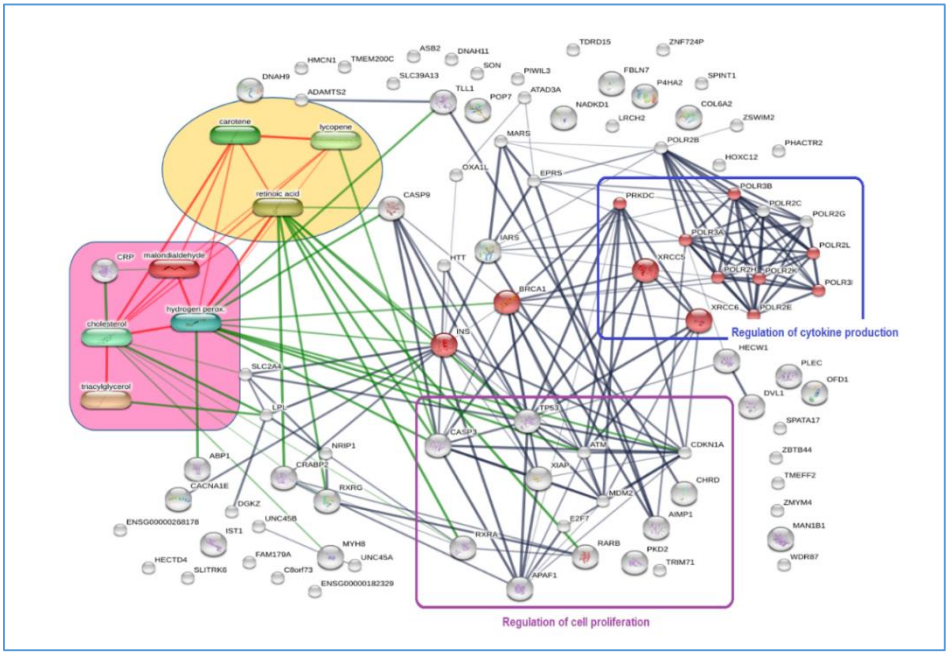

Figure 7. Interaction between down-regulated proteins with relative fold changes > 1.5 (from baseline and 3-month follow-up) among the treatment group. [Stronger associations are represented by thicker lines. Protein-protein interactions are shown in grey, chemical-protein interactions in green and interactions between chemicals in red.]

Discussion

A large number of phytochemical groups function in anti-inflammation, diminishing low-grade inflammation, and oxidative stress in obesity. Research on the protective roles of carotenoids, alone or in combination with vitamins, in maintaining cellular homeostasis has shown varying degrees of health benefit. However, the molecular pathways are still not completely understood. This study demonstrated the beneficial effects of carotenoid supplementation for 3 months via decreased levels of MDA, hs-CRP, and dyslipidemia. According to the findings of a comprehensive review and meta-analysis of randomized controlled trials, carotenoids have substantial impacts on inflammatory biomarkers, such as CRP ($p < 0.001$) and IL-6 ($p = 0.025$)[24]. The crucial link between obesity and systemic oxidative stress is partially supported by many mechanisms, including ROS-producing enzyme NOX, protein kinase C activation, and oxidative phosphorylation (OXPHOS). An increase in the number and size of adipocytes leads to the production of pro-inflammatory molecules, such as tumor necrosis factor (TNF)-α, IL-6, leptin, angiotensin (Ang) II, and plasminogen activator inhibitor 1. Subsequently, IL-6 can stimulate the acute-phase protein CRP in the liver to promote low-grade systemic inflammation[25]. Initiation of lipid peroxidation is activated by hydroxyl radicals with by-products comprising lipid hydroperoxides and many different aldehydes, such as MDA and 4-hydroxynonenal. A previous study showed that pro-oxidant damage by peroxyl radicals in human erythrocytes was prevented by β-carotene and zeaxanthin, but not by β-cryptoxanthin or lycopene[26]. This study found improvement in blood lipid profiles in line with the increase in plasma β-carotene levels after carotenoid supplementation in men with obesity. This finding is in accordance with those of a population-based cross-sectional study that revealed the role of serum carotenoids, especially β-carotene, as mediators for the inverse association between dietary carotenoid intake and CVD risk biomarkers, such as total homocysteine, CRP, and dyslipidemia[27]. Additionally, we found that carotenoid supplementation decreased both SBP and DBP levels in our obese participants. This finding was also reported in an analysis of data from 11,336 adults, with multivariate models, showing that all forms of carotenoid were independently and negatively associated with SBP and DBP (all $p < 0.05$), and trans-β-carotene was the most protective against high blood pressure[28]. Biological mechanisms underlying the protective role of carotenoids on hypertension involve diminishing ROS-induced vascular oxidative stress from NOX, as well as uncoupling endothelial nitric oxide synthase, xanthine oxidase, and endothelial dysfunction[29]. To assist in unraveling the mechanisms of obesity-associated metabolic phenotypes, we used a proteomics approach to characterize serum DEPs before and after supplementation with β-carotene in men with

obesity. This analysis revealed changes in a group of interesting proteins, including upregulation of factors related to inflammation (IL-6), mitochondrial oxidative stress (NOX4), and apoptosis (APAF1), and downregulation of factors associated with the synthesis of triacylglycerol (GPAT), endoplasmic reticulum (ER)-to-Golgi trafficking (SEC13A), and a phase II detoxifying enzyme (GSTP1). IL-6, a key cytokine released by macrophages and adipocytes, has been implicated in metabolic inflammation in obesity. A previous study revealed that obese participants had significantly higher levels of IL-6 protein expression in adipose tissue than their lean and overweight counterparts ($p=0.03$), and that the increased IL-6 expression and BMI were positively correlated ($r=0.58$, $p=0.008$)[30]. Complex redox biology indicates that increased levels of ROS production in adipose tissue by various enzymes, such as NOXs, cause impairment of insulin signaling, inflammation, and vascular dysfunction. Among the different NOX enzymes, Nox4 is the most extensively studied in adipocytes and has been linked to IR via oxidative inhibition of cellular protein-tyrosine phosphatases (PTPases), including PTP1B, downstream of the insulin signaling pathway[31]. High caloric intake can lead to mitochondrial dysfunction and potential susceptibility to apoptosis. Upregulation of APAF1 is involved in the intrinsic apoptosis signaling pathway that occurs in the mitochondria. Activation of caspase-9 is initiated by cytochrome C binding to the APAF1-induced apoptosome[32]. These findings highlight a strong relationship between ROS, mitochondrial apoptosis, and risk of metabolic consequences, such as IR, T2DM, and liver steatosis, in obese individuals. We also found downregulation of GPAT in our obese groups, but did not identify the specific GPAT isoform for synthesis of TG (GPAT1, GPAT2) or other acylglycerides (GPAT3, GPAT4). However, a previous study demonstrated that suppression of these GPAT activities resulted in reduced food intake among both lean and obese mice, together with decreased adipose tissue accumulation and increased fatty acid oxidation and insulin sensitivity[33]. Another downregulated protein, SEC13A, is an isoform of the SEC proteins, which transports biomolecules from the ER to the Golgi in a process mediated by coat protein complex II. *Sec13* knockout mice were used to illustrate its role as a negative modulator in transforming growth factor-β expression and a regulator of specific functions of immune cells[34]. Genetic polymorphisms in GSTP1, a detoxification enzyme, and/or other oxidative stress factors may lead to lower enzyme production and inefficient activity. Our obese groups showed lower expression of GSTP1 compared to the healthy group, implying that they had a greater potential for oxidative imbalance and thus the development of obesity-related adverse metabolic diseases. A previous study found that high-fat diet-fed mice were glucose intolerant, had downregulation of hepatic GSTP1 protein, JNK activation, increased cytokine mRNA expression, and impaired gluconeogenesis in the liver[35]. Additionally, plasma proteome data from three large cohorts of obese and overweight individuals showed differences from our findings in important proteins involving coagulation and complement cascades. These included

antithrombin-III (ANT3), kininogen-1 (KNG1), coagulation factor IX (FA9), and selenoprotein (SEPP1)[36]. The beneficial effects of carotenoid supplementation were further investigated for DEPs between baseline and follow-up in the obese-treatment group. We propose that there may be associations between the upregulated proteins and increased plasma β-carotene. ARHGAP21 plays an important role in various cellular metabolic processes, including insulin secretion, possibly through modulation of extracellular signal-related kinase (ERK)1/2[37], as well as control of body composition, energy expenditure, and glucose homeostasis[38]. SOX5 is a member of the SOX family of transcription factors, which bind to target DNA elements transactivate or transrepress genes. A study in a mouse model of T2DM found that SOX5 overexpression ameliorated expression of key beta-cell genes and improved glucose-stimulated insulin secretion[39]. The role of VPS13C on lipid homeostasis may impact glucose metabolism, and increased VPS13C expression following activation of lipolysis suggested a potential involvement in lipid droplet trafficking and fragmentation[40]. ABCB6 plays a role in porphyrin biosynthesis. Overexpression of this protein enhances the levels and activity of hemoproteins, including the heme-dependent antioxidant defense enzyme catalase, which reduces hydrogen peroxide-induced oxidative stress[41,42]. PAP2 unifies the functions of p53 and AP2 in the negative regulation of adipogenesis and also influences downstream networks, such as the excessive calorie intake-enhanced generation of ROS in adipose tissues, which can lead to DNA damage[43,44]. HEPHL1 is a Fe(II):oxygen oxidoreductase whose major function involves the oxidization of iron II to iron III. By oxidizing ferrous to ferric iron, it also serves an antioxidant function, preventing oxidative damage to proteins, lipids, and DNA, especially in obesity and metabolic syndrome[45]. LAMC1 is an isoform of laminins, which interact with various cell types through integrin receptors, thereby regulating intracellular signaling pathways, including PI3K/AKT and MAPK/ERK[46]. In brown or beige adipose tissue, these interactions increase thermogenic capacity and improve systemic energy metabolism and glucose homeostasis[47]. The disintegrin-like and metalloproteinase with thrombospondin type-1 motifs (ADAMTS) family member ADAMTS15 is closely associated with obesity through its metalloendopeptidase function. A study found that changes in ADAMTS15 resulted in mitochondrial biogenesis, lipolysis, fat oxidation, and adipogenesis[48]. Voltage-dependent P/Q-type calcium channel subunit alpha (CACNA1A) is an isoform of the high voltage-gated Ca^{2+} channel that functions in insulin secretion and β-cell mass maintenance and requires Ca^{2+} for signaling of the excitation-contraction coupling machinery[49]. LRP-1 is a member of the LDL receptor family whose roles in lipid metabolism involve clearance of chylomicron remnants from the circulation and glucose metabolism through insulin receptor trafficking. All of these mechanisms regulate lipoprotein and glucose homeostasis, preventing the development of metabolic syndrome and

CVDs[50]. ZNF674 and ZNF80 act as transcription factors through the zinc finger domain, which plays a significant role in multiple biological processes, including cell differentiation, apoptosis, autophagy, and cellular metabolism. Previous studies found that various ZNFs are involved in glucose metabolism through many mechanisms, including regulation of Glut4 transcription and the PPARγ signaling pathway[51]. ZNFs also mediate adipogenesis by regulating the expression of PPARγ[52]. Regarding the downregulated proteins in the obese-treatment group, most were indirectly associated with lipid or glucose mechanisms underlying obesity. However, decreased expression of these proteins seemed to reduce risks of metabolic phenotypes. An important link between obesity and hypertension involves activation of the renin-angiotensin system, which is characterized by increases in plasma renin activity, plasma angiotensinogen, angiotensin-converting enzyme (ACE) activity, and plasma Ang II levels[53]. Our obese-treatment group exhibited decreases in ACE expression as well as SBP and DBP, which resulted from downstream conversion of Ang I to Ang II. Furthermore, APAF1 was upregulated at baseline in our obese group, and showed downregulated expression following carotenoid supplementation. These results may be attributable to the antioxidant properties of carotenoids, diminishing ROS and mediating cell-signaling pathways through Erk, Akt, and NF-KB to enhance transcription of genes that promote antioxidant defense, anti-inflammation, and anti-apoptosis[54]. Other downregulated proteins were related to cytoskeletal organization, including phosphatase and actin regulator 2 (PHACTR2), TOG array regulator of axonemal microtubules protein 2 (TOGARAM2), zinc finger MYM-type protein 4 ZMYM4, and Dynein axonemal heavy chain 11 (DNAH11). Reduced levels of these proteins may limit cytoskeletal remodeling, preventing lipid storage of adipocytes, systemic lipid overload, and IR[55]. Additionally, adipose remodeling in obesity was partly determined by collagen cross-linking. Proteins related to the extracellular matrix (ECM) and collagen function in this study included ADAMTS2, COL6A2, and P4HA2, highlighting the importance of decreasing ECM remodeling in adipose tissue to prevent adipocyte dysfunction, chronic low grade inflammation, fibrosis, and IR[56]. The obese-placebo group, who did not receive carotenoid supplementation, also exhibited relative fold changes from baseline in serum proteins in different related pathways. Contrary to findings in the obese carotenoid supplementation group, there were upregulated proteins involved in ECM organization and cytoskeletal organization, such as COL4A4, F-actin-monooxygenase (MICAL2), zinc finger MYM-type protein 4 (ZMYM4), Kinesin-like protein (KIF18A), and Cullin-9 (CUL-9). Increased adiposity and IR are important causes of liver fibrosis, which is characterized by an excessive accumulation of ECM and excessive accumulation of collagens[57]. Other upregulated proteins related to cell signaling included immunoglobulin superfamily member 9B (IGSF9B), which belongs to a family that plays a central role in cell-cell adhesion in cancer progression and a metastatic phenotype[58], and IGFALS, which increases visceral adiposity and chronic inflammation and may lead to

disruption of the insulin-like growth factor system and IR[59]. Among the downregulated proteins in the obese-placebo group was an isoform of the mitochondrial pyruvate carrier, MPC2, for which impairment of pyruvate uptake results in abnormal hepatic gluconeogenesis and hyperglycemia[60]. Additionally, VPS13C was upregulated in the obese-treatment group but was downregulated along with VPS13D in the obese-placebo group. We did not find these associations in previous studies. Another identified protein related to cytoskeletal changes was ARHGAP8, which plays important roles in insulin secretion and glucose uptake in skeletal muscle and adipose tissue[61].

Our findings from the STITCH analysis indicated the presence of protein-protein and protein-carotenoid biomarker interactions among the obese-treatment group. These results supported the involvement of carotenoids with upregulated proteins related to mechanisms of the Wnt signaling pathway (adipocyte expansion and regulation of insulin secretion of the pancreatic beta-cell mass)[62], as well as cell surface receptor signaling pathways, including receptor tyrosine kinase (RTK), and Ras protein with the GTPase-activating protein (GAP), in obesity[63]. Furthermore, we found interactions between carotenoid-related markers and downregulated proteins related to regulation of cytokine production through the NF-KB pathway, thus inhibiting the downstream production of inflammatory cytokines and activating phase II enzymes and antioxidants, such as glutathione-S-transferases, via the Nrf-2 pathway[64]. Another pathway involved regulation of cell proliferation, which was similar to a previous study that found lutein inhibited adipogenesis, with blockage of early phase regulators of adipocyte differentiation, via repressed phosphorylation of AKT/ERK and activation of cyclin-dependent kinase[65]. This study's results also suggest a possible antioxidant-mediated improvement in inflammatory and oxidative markers. Few studies have investigated changes in proteome profiles in response to carotenoid intake in obese individuals. Our findings demonstrate a number of diverse serum proteins associated with plasma β-carotene that suggest involvement with lipid and glucose metabolism. Nonetheless, the small sample size and lack of serum analysis of carotenoid profiles, including β-cryptoxanthin, lutein/zeaxanthin, and lycopene, might be considered limitations of this study. To more accurately determine the effectiveness of β-carotene supplementation in obesity, clinical trials featuring a larger number of subjects together with individualized dietary assessment of carotenoid food intake should be designed.

Conclusion

Overall, this study's results demonstrated beneficial effects of carotenoid supplementation compared to placebo in men with obesity. Proteomics analysis revealed an abundance of DEPs between healthy and obese individuals, as well as between the obese groups with and without β-carotene-supplementation. The molecular pathways underlying the functions of these proteins are reportedly closely linked to adiposity-mediated metabolic diseases. Serum proteomics can be

applied not only to gain in-depth knowledge of the disease pathophysiology related to carotenoid status, but also to improve clinically relevant health outcomes in obesity.

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