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Immunometabolic signatures predict recovery from thyrotoxic myopathy in patients with Graves' disease

Daiki Setoyama[1], Ho Yeop Lee[2,3], Ji Sun Moon[2], Jingwen Tian[2,3], Yea Eun Kang[2], Ju Hee Lee[2], Minho Shong[2,3], Dongchon Kang[1,4] & Hyon-Seung Yi[2,3]*

[1]*Department of Clinical Chemistry and Laboratory Medicine, Kyushu University Hospital, Fukuoka, Japan;* [2]*Research Center for Endocrine and Metabolic Diseases, Chungnam National University Hospital, Chungnam National University School of Medicine, Daejeon, Korea;* [3]*Department of Medical Science, Chungnam National University School of Medicine, Daejeon, Korea;* [4]*Department of Clinical Chemistry and Laboratory Medicine, Graduate School of Medical Sciences, Kyushu University, Fukuoka, Japan*

Abstract

Background Thyroid hormone excess induces protein energy wasting, which in turn promotes muscle weakness and bone loss in patients with Graves' disease. Although most studies have confirmed a relationship between thyrotoxicosis and muscle dysfunction, few have measured changes in plasma metabolites and immune cells during the development and recovery from thyrotoxic myopathy. The aim of this study was to identify specific plasma metabolites and T-cell subsets that predict thyrotoxic myopathy recovery in patients with Graves' disease.

Methods One hundred patients (mean age, 40.0 ± 14.2 years; 67.0% female), with newly diagnosed or relapsed Graves' disease were enrolled at the start of methimazole treatment. Handgrip strength and Five Times Sit to Stand Test performance time were measured at Weeks 0, 12, and 24. In an additional 35 patients (mean age, 38.9 ± 13.5 years; 65.7% female), plasma metabolites and immunophenotypes of peripheral blood were evaluated at Weeks 0 and 12, and the results of a short physical performance battery assessment were recorded at the same time.

Results In both patient groups, methimazole-induced euthyroidism was associated with improved handgrip strength and lower limb muscle function at 12 weeks. Elevated plasma metabolites including acylcarnitines were restored to normal levels at Week 12 regardless of gender, body mass index, or age (P trend <0.01). Senescent $CD8^+CD28^-CD57^+$ T-cell levels in peripheral blood were positively correlated with acylcarnitine levels ($P < 0.05$) and decreased during thyrotoxicosis recovery ($P < 0.05$). High levels of senescent $CD8^+$ T cells at Week 0 were significantly associated with small increases in handgrip strength after 12 weeks of methimazole treatment ($P < 0.05$), but not statistically associated with Five Times Sit to Stand Test performance.

Conclusions Restoring euthyroidism in Graves' disease patients was associated with improved skeletal muscle function and performance, while thyroid hormone-associated changes in plasma acylcarnitines levels correlated with muscle dysfunction recovery. T-cell senescence-related systemic inflammation correlated with plasma acylcarnitine levels and was also associated with small increases in handgrip strength.

Keywords Graves' disease; Myopathy; Senescence; T cells; Metabolomics

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**Correspondence to: Hyon-Seung Yi, Research Center for Endocrine and Metabolic Diseases, Chungnam National University Hospital, Chungnam National University School of Medicine, Daejeon 35015, Korea. Phone: +82-42-280-6994, Fax: +82-42-280-7995, Email: jmpbooks@cnu.ac.kr*

Introduction

Thyroid hormones (THs) participate in contractile function, myogenesis, bioenergetics metabolism, and regeneration of skeletal muscle.[1–4] Cytoarchitecture and metabolic features of skeletal muscle are also regulated by circulating TH levels or local triiodothyronine (T3). Additionally, enhanced mitochondrial biogenesis by T3 treatments activates oxidative

pathways, leading to increased maximal oxygen consumption in skeletal muscle.[5] While intracellular T3 is mainly involved in the development of skeletal muscle and myogenic differentiation,[6] a decrease in TH signalling is linked with reduced myogenesis and type II fibres in skeletal muscle during ageing.[7]

In line with this, excess circulating THs, called thyrotoxicosis, induce loss of muscle mass, strength, and balance in humans.[8] Thyrotoxic myopathy, involving mainly proximal muscles, is an important clinical feature of patients with Graves' disease.[9] Hyperthyroidism increases Ca^{2+}-activated myosin ATPase activity in soleus muscle and produces atrophy of muscle fibres and conversion of type I (slow twitch) to type II (fast twitch) fibres in rats.[10] Increased protein catabolism caused by high levels of circulating TH is a critical factor in muscle dysfunction in patients with Graves' disease,[11] but the pathogenesis of thyrotoxic myopathy remains to be elucidated.

Muscle atrophy and weakness in various disorders is attributed to the catabolic effect of pro-inflammatory cytokines during inflammatory responses. Graves' disease, as an autoimmune disorder, induces not only local (e.g. eye) but also systemic (e.g. blood and muscle) inflammation. Pro-inflammatory cytokines produced by effector T cells play a critical role in mediating tissue injury,[12] which may be associated with loss of muscle mass and strength in patients with Graves' disease. In addition, systemic inflammation results in plasma metabolite changes, which may be derived from muscle wasting.[13] However, detailed immunophenotypic features of peripheral blood T cells and their relationship with thyrotoxic myopathy of Graves' disease have not been determined.

In the present study, we investigated whether specific plasma metabolites and different subsets of T cells were associated with recovery of muscle strength and function in patients with Graves' disease. Furthermore, we studied whether restoration of euthyroidism by treatment with methimazole altered immunophenotypes of peripheral inflammatory cells and plasma metabolites in patients with Graves' disease.

Methods

Study population

We initially recruited Koreans with newly developed or relapsed Graves' disease who visited the Department of Internal Medicine, Chungnam National University Hospital in Daejeon between January 2019 and December 2019. To determine the timing of muscle strength recovery in patients with Graves' disease, we evaluated handgrip strength and the time taken to perform the Five Times Sit to Stand Test (5XSST) in the participants treated with methimazole at Weeks 0, 12, and 24. Optimal sample size was determined by repeated measures analysis of variance, an effect size of 0.25, an α error probability of 0.01, and 95% power. The total sample size calculated was 80. Assuming a 20% dropout rate, 100 patients were required (calculated by G*Power 3.1.9.4).

Next, patients referred to Chungnam National University Hospital in Daejeon between May 2019 and April 2020 for a diagnostic workup or treatment of newly developed or relapsed Graves' disease were enrolled for a more intensive study. The Consensus Report of the Korean Thyroid Association recommends methimazole as the preferred drug for patients with Graves' disease[14]; therefore, all enrolled patients were maintained on methimazole or carbimazole. No patients were treated with radioiodine or thyroidectomy as an initial therapy. Beta-blockers were used for less than 1 week to reduce tachycardia or tremor. Patients with a thyroid storm were not included in the study. Measurement of muscle function and isolation of peripheral blood mononuclear cells (PBMCs) and plasma was conducted in all enrolled patients at an initial visit (Week 0) and a follow-up visit, which was scheduled 12 weeks later.

Inclusion criteria were as follows: (i) newly diagnosed or relapsed Graves' disease with thyrotropin (TSH) levels below the lower limit of the reference interval (0.25–4.0 μU/mL) and/or free thyroxine (free T4) above the upper limit of normal (ULN, 1.9 ng/dL), as well as plasma levels of TSH-binding inhibitor immunoglobulin (TBII) above the ULN ($>$15%), and (ii) age $\geq$18 years. Patients with any of the following conditions were excluded from the study: previous coronary heart disease, malignant hypertension, severe pulmonary disease, acute or chronic kidney disease (estimated glomerular filtration rate $<$45 mL/min/1.73 m^2), anaemia (haemoglobin $<$12 g/dL), history of any malignant or chronic inflammatory disease, current liver disease, drug or alcohol abuse, or pregnancy. Only patients without a previous history of musculoskeletal or joint disease were considered for inclusion in the study.

This study was reviewed and approved by the Institutional Review Board of Chungnam National University Hospital (CNUH 2019-02-012), according to the standards of the Declaration of Helsinki. Each participant gave informed consent, documented by the Department of Internal Medicine of Chungnam National University Hospital in Korea.

Handgrip strength, Five Times Sit to Stand Test, and short physical performance battery measurement

Experienced nurses were charged with collecting participant information, such as demographic characteristics and surgical or medical histories, through detailed interviews and reviewing medical records. Handgrip strength was measured

using an electronic hand dynamometer (Lavisen, Namyangju, Korea). Grip strength of the dominant hand was measured once in a sitting posture with 0° shoulder angle, 90° elbow angle, and a neutral wrist angle. In the 5XSST, the participants were placed in a chair with their arms crossed over their chest and their feet flat on the floor. The participants were asked to rise and sit five times in a row as fast as possible without using their hands. The time taken to perform the test was recorded for analysis. The short physical performance battery (SPPB) consists of measurements of gait speed, standing balance, and repeated chair stands.[15] In the standing balance test, participants were instructed to take a tandem stance, semi-tandem stance, and side-by-side stance, with each stance held for up to 10 s. Scores were recorded, which ranged from 0 to 12 points, with a higher SPPB score indicating better lower extremity function.

Sample preparation for plasma metabolomics

Metabolites in human plasma were prepared as described previously.[16] For whole metabolite extraction, 10 μL of plasma was added to 240 μL of water and 250 μL of ice-cold methanol, before being vortexed and centrifuged (14 000 *g*, 4°C, 15 min). The supernatant was collected in a 1.5 mL Eppendorf microtube, processed for the extraction of various types of compounds listed in the succeeding text, and used for liquid chromatography–mass spectrometry (LC–MS) measurement. For water-soluble metabolites, including amino acids and nucleotides, 25 μL of the supernatant was diluted three-fold with 0.1% formic acid. For acylcarnitines, 30 μL of the supernatant was added to 270 μL ice-cold methanol, vortexed, sonicated, and centrifuged (14 000 *g*, 4°C, 15 min), and the supernatant was collected. For free fatty acids, 25 μL of the supernatant was diluted two-fold with ice-cold methanol. For bile acids, 50 μL of the supernatant was evaporated and dissolved with 25 μL of 20% methanol. For phospholipids, 5 μL of the supernatant was diluted 200-fold with 0.1% formic acid in 20% acetonitrile.

Liquid chromatography–mass spectrometry measurements

Plasma samples were analysed using LC–MS on a LCMS-8060 instrument (Shimadzu Corp., Kyoto, Japan). To measure wide varieties of water-soluble metabolites, the prepared sample was separated on a Discovery HS-F5-3 column (150 × 2.1 mm, 3 μm particle size, Sigma-Aldrich, Dorset, UK) with mobile phases consisting of solvent A (0.1% formic acid) and solvent B (0.1% formic acid in acetonitrile). The column oven temperature was 40°C. The gradient elution programme was as follows: a flow rate of 0.25 mL/min: 0–2 min, 0% B; 2–5 min, 0–25% B; 5–11 min, 25–35% B; 11–15 min, 35–95% B; 15–25 min, 95% B; and 25.1–30 min, 0% B. The parameters for the heated electrospray ionization (ESI) source in negative/positive ion mode under multiple reaction monitoring (MRM) were as follows: drying gas flow rate, 10 L/min; nebulizer gas flow rate, 3 L/min; heating gas flow rate, 10 L/min; interface temperature, 300°C; DL temperature, 250°C; heat block temperature, 400°C; and CID gas, 270 kPa. For acylcarnitines, the sample was separated on a Luna HILIC column 200A (150 × 2.0 mm, 3 μm, Phenomenex) with mobile phases consisting of solvent A (10 mM ammonium formate) and solvent B [acetonitrile/10 mM ammonium formate (9/1, v/v)]. The column oven temperature was 40°C. The gradient elution programme was as follows: a flow rate of 0.3 mL/min, 0–2.5 min, 100% B; 2.5–4 min, 100–50% B; 4–7.5 min, 50–5% B; 7.5–10 min, 5% B; and 10.1–12.5 min, 100% B. Acylcarnitine profiles were detected in positive ESI mode under precursor ion scan for *m/z* 85.5 by changing collision energy according to the lengths of fatty acids; −20 for short-chain acylcarnitines (C0–C8); −35 for middle-chain acylcarnitines (C9–C12); and −45 for long-chain acylcarnitines (C12–C18), respectively. For free fatty acids, the sample was separated on a ACQUITY BEH Amide column (150 × 2.1 mm, 1.7 μm, Waters) with mobile phases consisting of solvent A (10 mM ammonium formate in 90% acetonitrile) and solvent B (0.1% formic acid in acetonitrile). The column oven temperature was 40°C. The gradient elution programme was as follows: a flow rate of 0.4 mL/min, 0–5 min, 0% B; 5.1–7.5 min, 100% B; and 7.6–11 min, 0% B. Free fatty acid profiles were detected in negative ESI mode under selected ion monitoring. For bile acids, the sample was separated on a ACQUITY BEH Amide column (150 × 2.1 mm, 1.7 μm, Waters) with mobile phases consisting of solvent A (0.1% formic acid) and solvent B (acetonitrile). The column oven temperature was 50°C. The gradient elution programme was as follows: a flow rate of 0.3 mL/min: 0–2 min, 20% B; 2–10 min, 80% B; 10–12 min, 80% B; and 12.1–15 min, 20% B. Bile acid profiles were detected in negative ESI mode under MRM. For phospholipids, the prepared sample was separated on a Kinetex C8 column (150 × 2.1 mm, 2.6 μm particle size, Phenomenex) with mobile phases consisting of solvent A (20 mM ammonium formate) and solvent B [acetonitrile/isopropanol (1:1, v/v)]. The column oven temperature was 45°C. The gradient elution programme was as follows: a flow rate of 0.3 mL/min: 0–1 min, 20% B; 1–2 min, 40% B; 2–25 min, 92.5% B; 25.1–35 min, 100% B; and 35.1–38 min, 20% B. Phospholipid profiles were detected in positive ESI mode under MRM.

Metabolomics data processing and analysis

Data processing was carried out using the LabSolutions LC–MS software program (Shimadzu), statistical analysis was

performed using GraphPad Prism 8 software, and volcano plots were visualized using the EnhancedVolcano package (Ver. 1.6.0) in R.

Isolation of peripheral blood mononuclear cells

Peripheral blood samples were obtained from all study participants, transferred aseptically into 50 mL polystyrene centrifuge tubes containing ethylenediaminetetraacetic acid (Sigma-Aldrich) as an anticoagulant, and gently mixed. Serum samples were prepared by centrifugation at 2000 *g* for 10 min at 4°C, and then PBMCs were isolated by centrifugation on a Ficoll-Paque density gradient (GE Healthcare Life Sciences, Buckinghamshire, UK) at room temperature. After centrifugation, the layer of PBMCs was collected and washed in Dulbecco's phosphate-buffered saline. The isolated and washed PBMCs were resuspended in 2 mL Roswell Park Memorial Institute 1640 medium (Welgene, Daegu, Korea), and trypan blue dye exclusion testing was used to determine the number of viable cells in the suspension. Samples were stained for flow cytometry analyses using direct fluorescence-conjugated monoclonal antibodies.

Flow cytometry analysis of human peripheral blood mononuclear cells

Peripheral blood mononuclear cells were pre-incubated with anti-mouse CD16/32 Fc blocker (BD Pharmingen, USA), followed by staining with anti-FVD-APC-Cy7 (all supplied by eBioscience, San Diego, CA, USA) to exclude dead cells from the analysis. After washing with FACS staining buffer, cells were treated directly with fluorochrome-conjugated monoclonal antibodies for 40 min at 4°C. The monoclonal antibodies used in this study were anti-CD3-PerCP-Cy5.5, anti-CD3-PE-Cy7, anti-CD4-AF700, anti-CD8-PE, anti-CD8-APC, anti-CD28-APC, anti-CD45RA-FITC, anti-CD45RO-PE-Cy7, anti-CD57-FITC, anti-TCR gamma/delta-FITC, fixable viability dye-APC-Cy7, anti-interferon (IFN)-γ-PE-Cy7, and anti-tumour necrosis factor (TNF)-α-APC (all supplied by eBioscience). For intracellular staining, surface-stained cells were stimulated with phorbol myristate acetate/ionomycin/brefeldin A/monensin for 5 h and then fixed and permeabilized using a Fixation/Permeabilization Buffer Kit (eBioscience) according to the manufacturer's instructions. The permeabilized cells were washed and resuspended in 1% formaldehyde and further stained for intracellular cytokines and cytotoxic molecules with anti-IFN-γ-PE-Cy7 and anti-TNF-α-APC. Multicolour flow cytometry was performed using a BD LSRFortessa flow cytometer (BD Biosciences, San Jose, CA, USA), and the data were analysed by FlowJo V10 software (FlowJo, LLC, Ashland, OR, USA).

Biochemical measurements

Peripheral blood was collected into heparin-coated tubes. Plasma levels of TSH, T3, free T4, and TBII were measured by standard methods on an automated analyser (Cobas 6000; Roche Diagnostics GmbH, Mannheim, Germany). Plasma lipid profiles, including low-density lipoprotein cholesterol, high-density lipoprotein cholesterol, total cholesterol, triglycerides, and creatine kinase, were evaluated using a blood chemistry analyser (Hitachi 47; Hitachi, Tokyo, Japan). Aspartate transaminase and alanine transaminase activities were measured using the International Federation of Clinical Chemistry Ultra Violet method without pyridoxal phosphate (TBA-2000FR; Toshiba, Tokyo, Japan).

Statistical analysis

All continuous variables are reported as mean ± standard error of the mean, except where otherwise stated. Statistical analyses were performed using GraphPad Prism 8 software (GraphPad, San Diego, CA, USA). All data were analysed by a one-way analysis of variance followed by Tukey's *post hoc* test or a two-tailed Student's *t*-test. Statistical correlations were evaluated using Spearman's correlation coefficient. P values <0.05 were considered statistically significant.

Results

Recovery of skeletal muscle strength and function in 100 patients with Graves' disease treated with methimazole

It is well known that patients with Graves' disease exhibit lower muscle strength than euthyroid controls.[8] However, it has not been established how quickly treatment with antithyroid drugs restores muscle function in patients with Graves' disease. To determine when muscle strength recovers in patients with Graves' disease, 100 patients were followed for 24 weeks. At Weeks 0, 12, and 24, handgrip strength and 5XSST performance were measured. Demographics and clinical characteristics of the enrolled participants at Weeks 0, 12, and 24 are summarized in Supporting Information, *Table* S1. At Week 12, TH levels were stabilized on methimazole treatment (*Table* S1) and most patients with Graves' disease regained handgrip power and lower extremity strength (*Figure* 1A and 1B). We also found that there was no further improvement in skeletal muscle function at Week 24 (*Figure* 1A and 1B and *Table* S1).

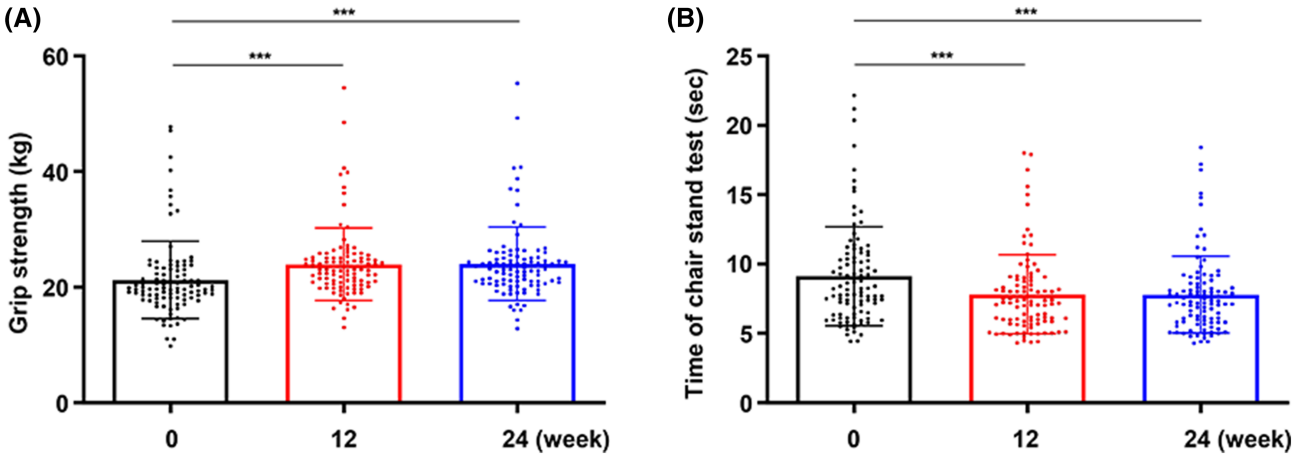

Figure 1 Serial measurement of handgrip strength and Five Times Sit to Stand Test performance in 100 patients with newly diagnosed or relapsed Graves' disease treated with methimazole. (A) Handgrip strength. (B) Five Times Sit to Stand Test performance. Data are expressed as mean ± standard error of the mean. $^{***}P < 0.001$ (A, B: one-way analysis of variance).

Changes in biochemical parameters in 35 patients with Graves' disease treated with methimazole

To further investigate the relationship between serum chemical characteristics and TH excess, 35 patients (12 men, 34.3%; 23 women, 65.7%) with newly diagnosed or relapsed Graves' disease were recruited. The demographics and baseline characteristics of the participants at the initial visit are summarized in *Table* S2. The mean age of the study population was 38.9 ± 13.5 years, and the mean baseline body mass index of the participants was 20.5 ± 2.4 kg/m^2. All enrolled patients showed high levels of serum free T4 and T3 as well as low levels of serum TSH prior to methimazole treatment (Week 0). Treatment with methimazole stabilized levels of serum free T4 and T3 in most patients (*Figure* S1A), but did not induce significant changes in the serum TSH concentrations between Weeks 0 and 12 (*Figure* S1A). At the follow-up visit, markers of liver injury were significantly decreased (*Figure* S1B), whereas lipid profiles, including total, low-density lipoprotein, and high-density lipoprotein cholesterol and triglycerides, were remarkably increased compared with Week 0 (*Figure* S1C). To assess the effect of thyrotoxicosis on bone turnover markers, we measured serum levels of alkaline phosphatase and C-telopeptide at the initial and follow-up visit; serum levels of C-telopeptide were significantly lower after treatment with methimazole (*Figure* S1D). These findings suggest that 12 weeks of methimazole treatment results in biochemical changes in patients with Graves' disease. To exclude the possibility of hypokalemic periodic paralysis in the study participants, we measured serum levels of potassium in all participants at initial visit as well as at 12 weeks after the initiation of methimazole treatment. Normal potassium levels were confirmed in all participants at initial and follow-up visits (*Figure* S1E), and there was no significant differences in potassium levels between initial visit and follow-up visits (*Figure* S1E). Serum levels of creatine kinase, a marker of muscle damage, in patients with Graves' disease did not change significantly between the initial visit and the follow-up visits, although three patients with Graves' disease showed markedly higher levels of serum CK at the follow-up visit than at the initial visit (*Figure* S1F). Fasting plasma glucose levels fell significantly after 12 weeks of antithyroid therapy (*Figure* S1G).

Recovery of skeletal muscle strength and function in 35 patients with Graves' disease treated with methimazole

Next, we investigated the recovery of muscle function in patients with Graves' disease using measurements of handgrip strength, the 5XSST, and the SPPB at the initial visit and at Week 12. As shown in *Table* S3, handgrip strength was remarkably increased in the patients with Graves' disease treated with methimazole for 12 weeks. Methimazole-induced euthyroidism resulted in a significant improvement in 5XSST performance and SPPB score between the initial and follow-up visit (*Table* S3). To determine associations between gender and recovery of physical performance in patients with Graves' disease, we divided the participants into male and female participants. We observed a significant improvement in grip strength and 5XSST performance in both men and women, although there were no significant differences in SPPB score in either gender (*Figure* 2A and 2B and *Tables* S4 and S5). Moreover, we observed improvements in grip strength and 5XSST, regardless of body mass index (low, 18.8 ± 0.89 kg/m^2 vs. high, 22.4 ± 1.98 kg/m^2) or age (young, 28.5 ± 5.26 years old vs. old, 51.4 ± 8.87 years old)

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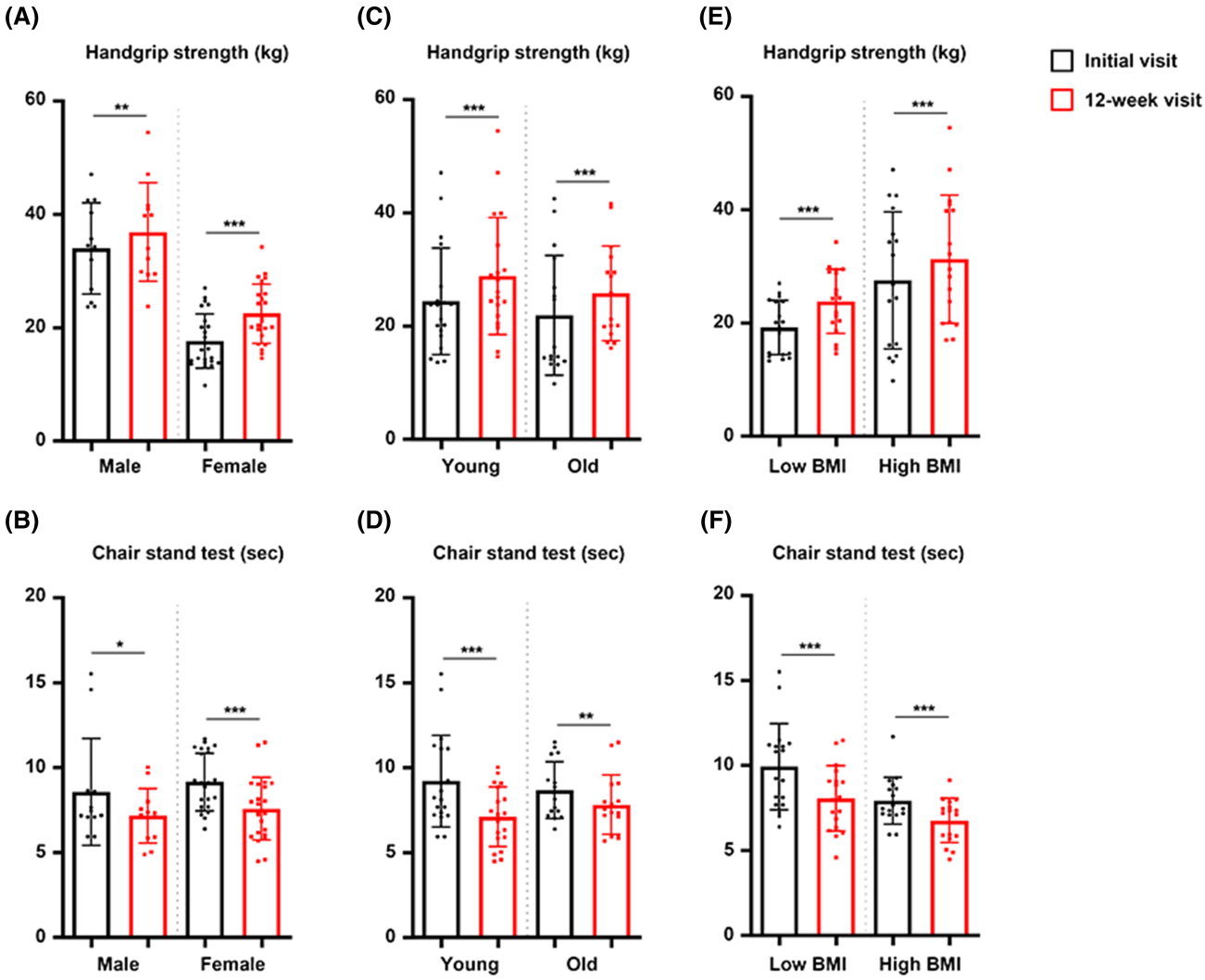


Figure 2 Evaluation of handgrip strength and Five Times Sit to Stand Test (5XSST) performance in 35 patients with Graves' disease at Weeks 0 and 12 after commencing methimazole treatment. (A,B) Handgrip strength and 5XSST performance according to gender. (C,D) Handgrip strength and 5XSST performance according to age (young, 28.5 ± 5.26 years old vs. old, 51.4 ± 8.87 years old). (E,F) Handgrip strength and 5XSST performance according to body mass index (BMI) (low, 18.8 ± 0.89 kg/m^2 vs. high, 22.4 ± 1.98 kg/m^2). Data are expressed as mean ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ compared with the corresponding controls by Student's *t*-test.

(*Figure* 2C–2F and *Tables* S6–S9). Taken together, these results suggest that restoring euthyroidism with methimazole treatment improves skeletal muscle function and performance in patients with Graves' disease at 12 weeks.

Plasma levels of acylcarnitines are associated with muscle dysfunction in 35 patients with Graves' disease

To identify markers of thyrotoxic myopathy, we measured plasma metabolites including amino acids and water-soluble metabolites, free fatty acids, acylcarnitines, bile acids, and phospholipids in patients with Graves' disease at the initial (Week 0) and 12 week visit. Several metabolites changed after methimazole treatment regardless of gender (*Tables* S10–S12), with changes in acylcarnitine species being the most prominent (*Figure* 3A and 3B). To find plasma metabolites associated with muscle wasting in patients with Graves' disease, Spearman's correlation coefficients were calculated (*Figure* 4). Many plasma metabolites correlated with recovery of motor function: aspartic acid and some bile acids, such as hyodeoxycholic acid and chenodeoxycholic acid, correlated with the recovery of muscle strength (*Figure* 4A and 4B), while many acylcarnitine species were associated with recovery of muscle endurance (*Figure* 4C and 4D). Taken together, these results suggest that TH-associated changes in plasma metabolite levels are associated with the recovery of muscle function in patients with Graves' disease.

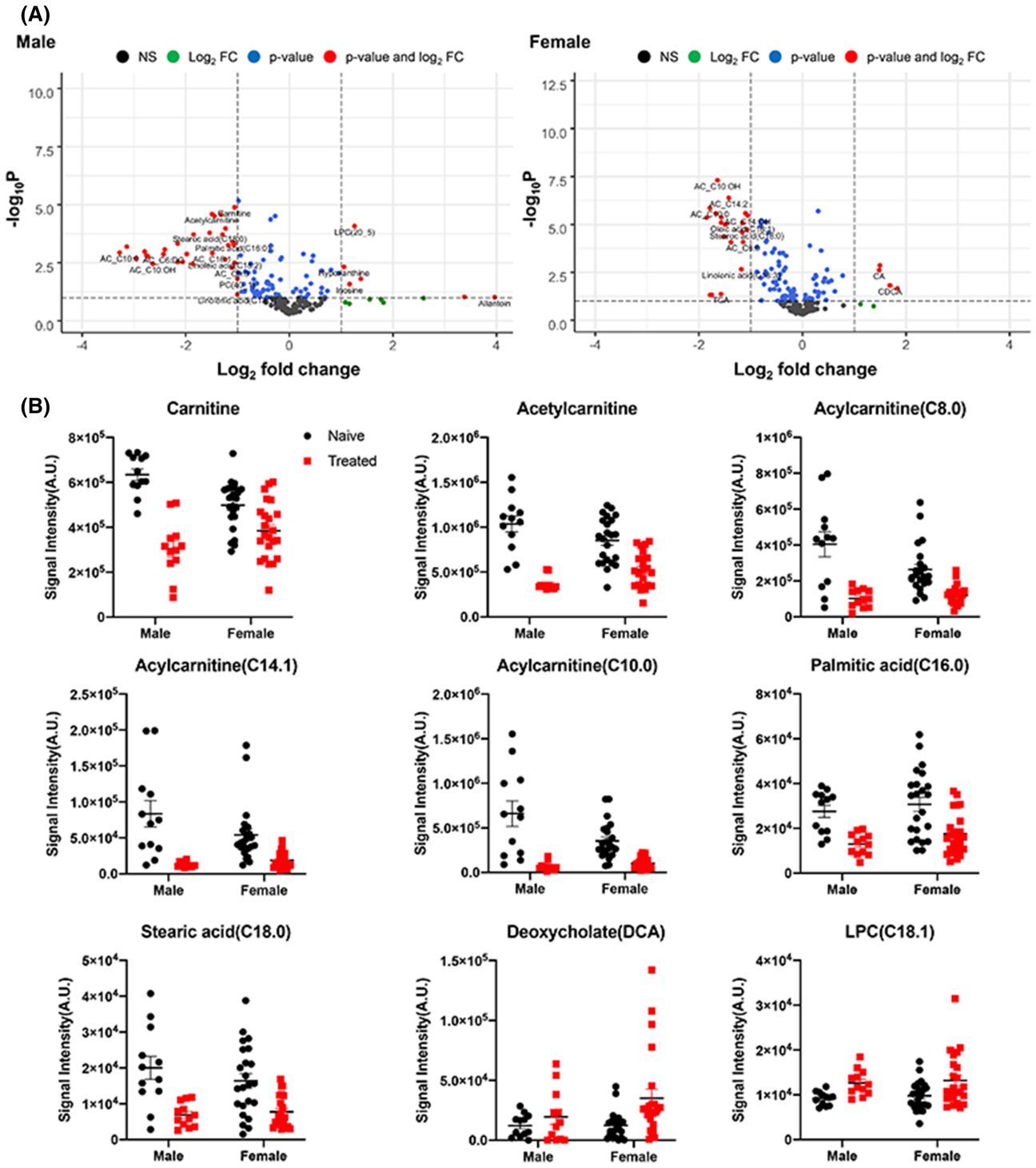
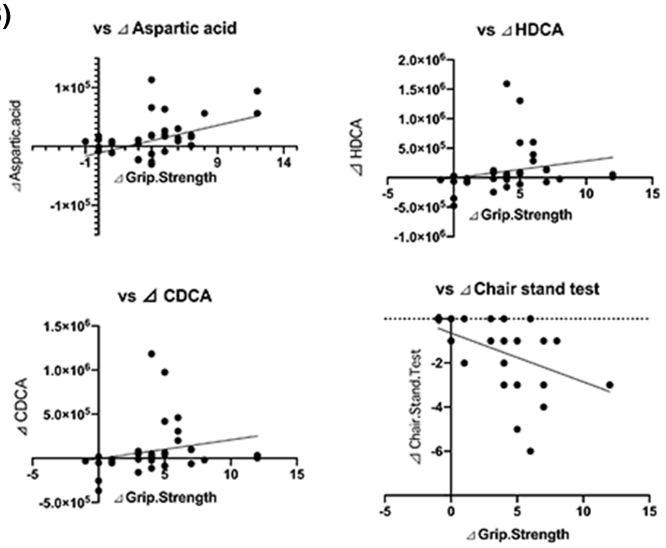


Figure 3 Plasma levels of metabolites before and after 12 week treatment of methimazole in 35 patients with Graves' disease. (A) Visualization of plasma metabolome data (200 metabolites) on volcano plots according to gender. Each dot indicates individual metabolites. Fold change denotes the ratio of the signal intensity after treatment to the value before treatment. *P* values were calculated by paired *t*-test. Colour of dot signifies significance: not significant (NS), black; changed but not significant (log2 FC), green; significantly weakly changed (*P*-value), blue; or significantly changed (*P*-value and log2 FC), red. (B) Signal intensity of significantly changed metabolites before (naïve) and after (treated) methimazole treatment according to gender. AC, acylcarnitine; CA, cholic acid; CDCA, chenodeoxycholic acid; LPC, lysoPC; PC, phosphatidylcholine; TCA, taurocholic acid.

(A)

Grip test			
Rank	ID	r	abs
1	Aspartic.acid	0.48	0.48
2	HDCA	0.48	0.48
3	CDCA	0.48	0.48
4	Chair.Stand.Test	-0.47	0.47
5	PC 36_2	0.46	0.46
6	UDCA	0.46	0.46
7	PC 30_2	0.43	0.43
8	PC 40_1	0.41	0.41
9	PC 30_1	0.39	0.39
10	PC 34_3	0.38	0.38
11	PC 36_3	0.37	0.37
12	PC 40_4	0.37	0.37
13	PC 30_0	0.37	0.37
14	CA	0.36	0.36
15	LCA	0.35	0.35

(B)



(C)

Chair stand test			
Rank	ID	r	abs
1	Acylcarnitine C12.0	0.42	0.42
2	Acylcarnitine C4.OH	0.42	0.42
3	Dimethylglycine	0.41	0.41
4	Acylcarnitine C10.OH	0.40	0.40
5	Acylcarnitine C14.0	0.40	0.40
6	Acylcarnitine C14.1	0.40	0.40
7	Acylcarnitine C12.OH	0.40	0.40
8	Acylcarnitine C12.1	0.39	0.39
9	Choline	0.39	0.39
10	Asparagine	0.39	0.39
11	Creatine	0.37	0.37
12	Asymmetric.dimethylarginine	0.36	0.36
13	Acylcarnitine C16.0	0.36	0.36
14	Acylcarnitine C18.1	0.36	0.36
15	Acylcarnitine C10.0	0.35	0.35

(D)

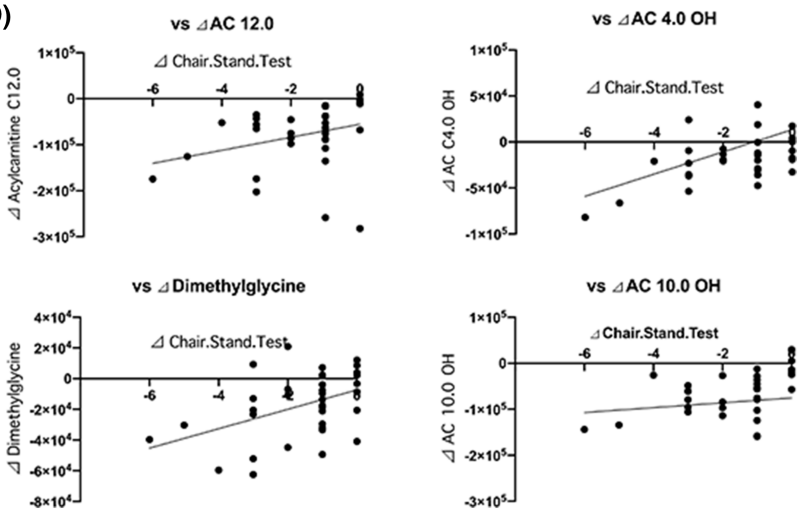


Figure 4 Plasma levels of metabolites before and after 12 week treatment of methimazole in 35 patients with Graves' disease. (A) Spearman's correlation coefficient was calculated between the change pre-methimazole and post-methimazole treatment in metabolite signal intensity and handgrip test performance. The Top 15 ranked metabolites with high correlation coefficients (r, rho) are listed. (B) Plot of representative correlation. (C) Spearman's correlation coefficient was calculated between the change pre-methimazole and post-methimazole treatment in metabolite signal intensity and Five Times Sit to Stand Test performance. (D) Plot of representative correlation.

Peripheral blood mononuclear cell immunophenotype changes in 35 patients with Graves' disease treated with methimazole

Loss of muscle mass and function can be attributed to the catabolic effect of pro-inflammatory cytokines during inflammatory responses. In addition, increased levels of TH lead to an amplification of the pro-inflammatory response of many kinds of immune cells.[17] Therefore, to observe systemic inflammation status in patients with Graves' disease, we investigated the immunophenotype of PBMCs at Weeks 0 and 12. Levels of PBMCs and lymphocytes were not significantly different between visits (*Figure* S2A and S2B). As expected, high monocyte populations were normalized by treatment with methimazole at Week 12 (*Figure* S2C), whereas neutrophil levels were remarkably increased by recovery of euthyroidism (*Figure* S2D). Low levels of haemoglobin and platelets caused by TH excess were also restored at 12 weeks (*Figure* S2E and S2F). Overall, complete blood cell counts were improved in patients with Graves' disease at 12 weeks of methimazole treatment.

Previously, it was reported that among T-cell subsets, the $CD28^{-}CD57^{+}$ senescent population of $CD4^{+}$ and $CD8^{+}$ T cells is significantly larger in drug-naïve patients with Graves' disease.[6] T-cell senescence is also associated with a systemic inflammatory response, which seems to be associated with age-related sarcopenia.[18] Thus, we assessed the frequency of $CD57^{+}$ and/or $CD28^{-}$ T cells among the $CD4^{+}$ and $CD8^{+}$ T

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cells in the PBMCs from the study participants at the initial and follow-up visit. Surface expression of CD4 and CD8 was then determined in this gated population (*Figure* S3). Although the population of senescent $CD4^+$ T cells was not different between the initial and follow-up visit (*Figure* 5A), senescent $CD8^+$ T cells were significantly decreased at Week 12 (*Figure* 5B). We also detected significant decreases in the production of IFN-γ in senescent $CD8^+$ T cells at Week 12 (*Figures* 5C and S4). Furthermore, recovery of euthyroidism attenuated IFN-γ and TNF-α production in memory $CD8^+$ T cells (*Figures* 5D, 5E, and S4).

Based on the high levels of plasma acylcarnitines in drug-naïve patients with Graves' disease, we also investigated the relationship between acylcarnitines and T-cell senescence. Senescent $CD8^+$ T cells exhibited a significant, positive correlation with plasma levels of acylcarnitines at the initial visit (*Figure* 5F and 5G). High frequencies of senescent $CD8^+$ T cells at Week 0 were significantly associated with smaller increases in handgrip strength in patients with Graves' disease at 12 weeks, but were not statistically associated with 5XSST performance (*Figure* 5H). This finding suggests that $CD8^+$ T-cell senescence may predict recovery of muscle strength in patients with Graves' disease.

Discussion

This study demonstrates that reduced muscle strength in Graves' disease can be restored by methimazole-induced euthyroidism after 12 weeks of treatment. Analysis of plasma metabolites in patients with Graves' disease revealed that elevated acylcarnitine levels were associated with thyrotoxic myopathy. In addition, we found that monocytes and senes-

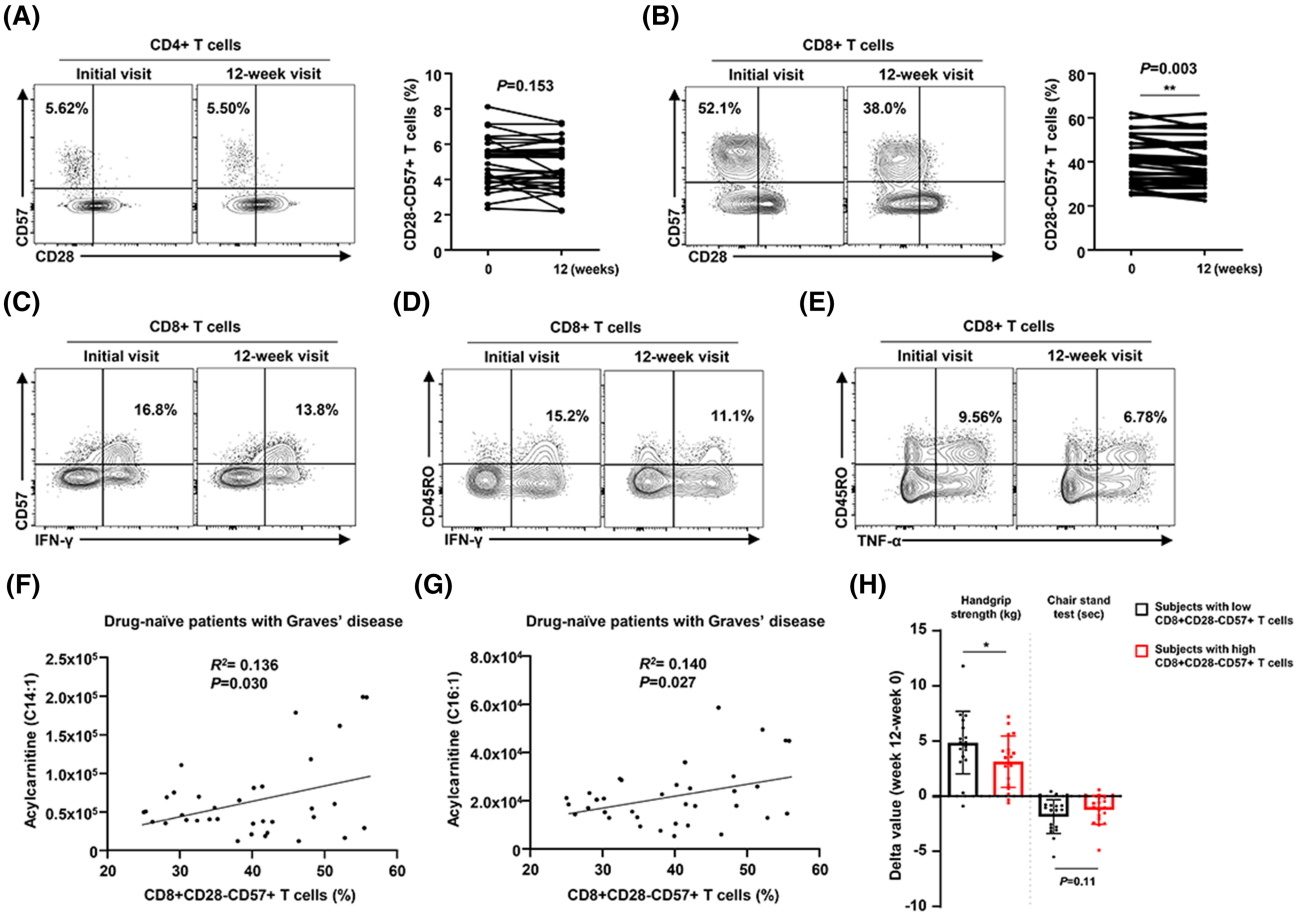

Figure 5 Immunophenotypes of peripheral blood mononuclear cells in 35 patients with Graves' disease at Weeks 0 and 12 after commencing methimazole treatment. (A,B) Representative flow cytometry plots are presented for CD57 and CD28 expression by $CD4^+$ or $CD8^+$ T cells in patients with Graves' disease at Week 0 and Week 12. (C) FACS analysis for interferon (IFN)-γ-producing cells in the population of $CD8^+$ $CD57^+$ T cells at Week 0 and Week 12. (D,E) Representative flow cytometry plots for IFN-γ or tumour necrosis factor (TNF)-α production in memory $CD8^+$ T cells of the patients at Weeks 0 and 12. (F,G) Correlation analysis of $CD8^+CD28^-CD57^+$ T cells and plasma acylcarnitine (C14:1) or (C16:1) in drug-naïve patients with Graves' disease. (H) Comparison of muscle function improvement according to frequency of senescent $CD8^+$ T cells (lower 50%, n = 17 vs. upper 50%, n = 18) in patients with Graves' disease at Week 0. Data are expressed as mean ± standard error of the mean. $^*P < 0.05$ and $^{**}P < 0.01$ compared with the corresponding controls by Student's t-test.

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cent $CD8^+$ T cells contributed to the progression of systemic inflammation by stimulating the release of pro-inflammatory cytokines, leading to the development of muscle dysfunction. We also showed that production of pro-inflammatory cytokines in senescent $CD8^+$ T cells was positively correlated with plasma levels of acylcarnitines. Furthermore, this study showed that changes in plasma metabolites and high levels of senescent $CD8^+$ T cells measured during an initial visit were associated with smaller increases in handgrip strength in patients with Graves' disease at 12 weeks of methimazole treatment (*Figure* 5).

It is well documented that muscle strength and endurance are decreased in patients with Graves' disease compared with euthyroid controls.[8] Previous investigations showed that thigh strength and cross-sectional area are reduced in patients with overt or subclinical hyperthyroidism at baseline compared with controls and are restored following treatment.[19,20] While euthyroidism mediates improvement of muscle weakness in Graves' disease, treatment with beta-blockers also contributes to attenuation of catecholamine-induced muscle wasting.[21] A recent large, population-based, age-matched and sex-matched case–control study (Graves' disease–euthyroid) suggests that postural stability and muscle strength are impaired by excess TH, which may increase falling risk in patients with Graves' disease.[8] Moreover, subclinical hyperthyroidism induced by treatment with levothyroxine in differentiated thyroid carcinoma deteriorates muscle function of upper limbs and health-related quality of life.[22]

As shown in *Figure* 1, the handgrip power and lower extremity strength of most patients with Graves' disease recovered by Week 12, although there was no further improvement in skeletal muscle function by Week 24. This finding is consistent with a previous study of a small Swedish cohort[23]; that study showed some recovery of skeletal muscle function and visceral adipose tissue during the initial 3 month period of recovery from hyperthyroidism, while near complete recovery was observed at 12 months after achieving a euthyroid state. This suggests that early recovery of skeletal muscle function in Asian patients with Graves' disease may occur more quickly (within the first 12 weeks after starting methimazole treatment) than in European patients. However, multinational clinical trials with long-term follow-up are required to determine ethnic differences with respect to recovery of skeletal muscle function after treatment of hyperthyroidism.

Although higher, 'supraphysiological' levels of TH contribute to deterioration of muscle strength and physical performance in Graves' disease patients, the association between systemic inflammation and thyrotoxicosis-mediated muscle dysfunction remains to be determined. Here, we found that pro-inflammatory, cytokine-senescent $CD8^+$ T cells were significantly increased in Graves' disease patients with high acylcarnitine levels. These findings suggest that systemic inflammation is a critical factor contributing to the elevation of plasma acylcarnitine levels, which may be derived from muscle wasting by TH excess.

Pro-inflammatory cytokines induced by Graves' disease can cause the progression of intrathyroidal autoimmune processes,[24] orbital inflammation,[25] and systemic inflammatory responses by changing the immune cell subsets of PBMCs.[26] T-cell senescence, which is associated with systemic inflammation, affects the development and progression of autoimmune and metabolic diseases.[27–29] However, it has not been determined whether T-cell senescence is related to the development and recovery of thyrotoxic myopathy in patients with Graves' disease. In this study, we found that high senescent $CD8^+$ T-cell levels recorded at an initial visit were associated with smaller increases of handgrip strength. This result indicates that although the effect of TH excess on immunosenescence in patients with Graves' disease has not been fully established, $CD8^+$ T-cell senescence may contribute to the progression of systemic inflammation-associated thyrotoxic myopathy. However, given the lack of significance of the 5XSST results, this study was unable to reveal an association between T-cell senescence and muscle endurance. We hypothesized that muscle strength, as measured by grip strength, recovers before the recovery of muscle endurance, as measured by 5XSST. However, a longer period of study including muscle tissue analysis will be required to prove this hypothesis.

We found that free carnitine levels were elevated in serum from patients with Graves' disease (*Figure* 2). This result is consistent with that reported by Pietzner *et al*., who showed that administration of levothyroxine to healthy young men increases free carnitine (as well as acylcarnitines) in association with an increase in blood free T4 levels.[30] Thus, it is plausible that the increase in serum free carnitine in Graves' disease is caused by an increase in free T4. Carnitine plays an important role in fatty acid metabolism and in beta-oxidation pathways in mitochondria; it is also utilized for production of acylcarnitines. By contrast, it acts as an antagonist that inhibits the function of TH by suppressing nuclear translocation of T3.[31] Thus, elevated levels of free carnitine in Graves' disease appear to serve as a source of acylcarnitines required for endurance exercise, while reducing excess TH levels via a feedback mechanism driven by elevated free T4. This may explain why carnitine therapy is effective at reducing muscle function-associated hyperthyroidism.[32–34]

Previous reports of serum metabolome analysis in patients with hyperthyroidism describe changes in acylcarnitine levels. Chng *et al*. reported that levels of short-chain, middle-chain, and long-chain acylcarnitines in serum from Chinese women with Graves' disease were higher than those in euthyroid women,[35] whereas Al-Majdoub *et al*. reported that only middle-chain acylcarnitines were increased.[36] Our results are consistent with those of Chng *et al*. in that all subjects were Asian, and all types of acylcarnitine were elevated in the serum of Graves' disease patients (*Figure* 2). Interestingly,

we found that a relatively large number of middle-chain acylcarnitines (C8, C10, and C12) were among the metabolites that correlated with recovery of lower extremity skeletal muscle function (*Figure* 3C and 3D), suggesting a substantial role for middle-chain acylcarnitines (at least a more important role than that of short-chain and long-chain acylcarnitines) in the reduced muscle endurance associated with Graves' disease. Supporting this, skeletal muscle is thought to release middle-chain acylcarnitines into the blood during endurance exercise.[37–39]

In the current study, broad, untargeted, LC–MS-based profiling of plasma metabolites was used to elucidate the changes that occur in biochemical metabolic networks in patients with Graves' disease. The present findings suggest that plasma acylcarnitines are closely associated with biological events related to muscle dysfunction. In fact, a previous study revealed that higher plasma acylcarnitines predict lower levels of objectively measured physical performance in older adults.[40] Although fluxes in acylcarnitines in humans are tissue and context dependent, the liver is a major source of short-chain acylcarnitines, whereas medium-chain acylcarnitines are derived from skeletal muscle during exercise.[39] Moreover, TH excess stimulates heart mitochondrial carnitine translocase activity by facilitating the entry of fatty acids through mitochondrial inner membranes in rats.[41] Thus, further studies are needed to establish fatty acid flux in skeletal muscle as well as the contribution of major organs to plasma acylcarnitines in thyrotoxic myopathy, which may provide insight into the relationship between immunosenescence and lipid metabolism in Graves' disease.

The main strength of this study is that it used serial data acquisition from LC–MS-based plasma metabolomics and FACS-based peripheral blood immunophenotyping, alongside assessments of functional parameters including grip strength, gait speed, 5XSST performance, and SPPB score, to obtain data that may have clinical value for the treatment of Graves' patients. However, this study has several limitations. Most importantly, as an observational study, it cannot determine a causal relationship between variables. Secondly, our study population was exclusively South Korean, and we cannot be certain that our results are applicable to other populations. Thirdly, various confounding factors could not be considered in multivariate analyses due to the relatively small sample size. Fourth, the follow-up period of 12 weeks is too short to assess fully euthyroid-induced recovery of biochemical metabolic networks and functional properties of immune cells. Extended follow-up may provide additional information on the long-term effects of thyrotoxicosis. Finally, metabolic changes induced by an excess of TH are a major factor that drives development of hyperthyroid myopathy. Although the populations and activation of circulating immune cells are altered in patients with Graves' disease, little is known about immune cell infiltration into muscle tissues. However, an increase in serum T3 levels, as seen in hyperthyroidism, can induce B-cell activation and plasma cell antibody secretion in the absence of antigens.[42] Interstitial myositis was found in nine post-mortem cases of Graves' disease. Myocardial degenerative lesions have been reported in thyrotoxicosis, with foci of cell necrosis and mononuclear and polymononuclear infiltrates in patients dying of thyrotoxicosis.[43] Because immune cell infiltration of muscle tissue is observed under extreme conditions, patients with various stages of Graves' disease should be investigated to validate the possibility of thyrotoxicosis-induced inflammatory myopathy. However, such studies come with ethical challenges. Thus, further studies using animal models of thyrotoxicosis are warranted to fully understand the role of immune cells in the development of hyperthyroid myopathy.

In conclusion, this study suggests that $CD8^+$ T-cell senescence is associated with smaller increases in handgrip strength in South Koreans with Graves' disease and immunosenescence is closely related to TH excess-mediated changes in plasma metabolites, including acylcarnitines (*Figure* S5). Further large-scale, prospective studies are needed to clarify the mechanism of TH excess-induced increases in plasma acylcarnitines and T-cell senescence, which may define a causal relationship between immunometabolism and muscle function in Graves' disease.

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Conflict of interest

None declared.

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Online supplementary material

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Figure S1. Thyroid function and serum chemistry in 35 patients with Graves' disease at week 0 and 12 after commencing methimazole treatment. (A) Measurement of free T4, T3, and TSH. (B) Serum levels of aspartate aminotransferase and alanine aminotransferase. (C) Serum lipid profiles including total cholesterol, triglyceride, high-density lipoprotein cholesterol, and low-density lipoprotein cholesterol. (D–F) Blood concentration of c-telopeptide, alkaline phosphatase, potassium, and creatine kinase (reference range: male, 56–244 U/L; female, 44–178 U/L). (G) Fasting plasma glucose levels. Data are expressed as the mean ± SEM. $*P < 0.05$, $**P < 0.01$, and $***P < 0.001$, compared with the corresponding controls (Student's *t*-test).

Figure S2. Comparison of complete blood counts in patients with Graves' disease at Week 0 and Week 12. Measurement of blood leukocytes (A), lymphocytes (B), monocytes (C), neutrophils (D), hemoglobin (E), platelet (F). Data are expressed as the mean ± SEM. $**P < 0.01$ and $***p < 0.001$, compared with the corresponding controls (Student's *t* test).

Figure S3. Gating strategy for analysis of senescent T cells, and naïve and memory T cells, within the peripheral blood $CD4^+$ and $CD8^+$ populations of patients with Graves' disease.

Figure S4. The number of IFN-γ- and TNF-α-producing cells in the population of peripheral blood senescent $CD8^+$ T cells and memory $CD8^+$ T cells of patients with Graves' disease at the initial visit and at the 12-week visit. Data are expressed as the mean ± SEM. $***p < 0.001$ compared with the corresponding controls (Student's *t* test).

Figure S5. Graphical summary of the study. Measurement of plasma metabolites, including acylcarnitines, and senescent peripheral $CD8^+$ T cells can be used to predict recovery of muscle dysfunction in patients with thyrotoxic myopathy.

Table S1. Clinical and biochemical characteristics of the study population at week 0, 12, and 24.

Table S2. Clinical and biochemical characteristics of the study population at initial visit.

Table S3. Muscle function measured by handgrip strength, chair stand test, and short physical performance battery.

Table S4. Muscle function measured by handgrip strength, chair stand test, and short physical performance battery in male patients with Graves' disease ($n = 12$).

Table S5. Muscle function measured by handgrip strength, chair stand test, and short physical performance battery in female patients with Graves' disease ($n = 23$).

Table S6. Muscle function measured by handgrip strength, chair stand test, and short physical performance battery in patients with lower BMI ($n = 18$).

Table S7. Muscle function measured by handgrip strength, chair stand test, and short physical performance battery in patients with higher BMI ($n = 17$).

Table S8. Muscle function measured by handgrip strength, chair stand test, and short physical performance battery in young patients with Graves' disease ($n = 19$).

Table S9. Muscle function measured by handgrip strength, chair stand test, and short physical performance battery in old patients with Graves' disease ($n = 16$).

Table S10. Global plasma metabolomics profiling in the study population.

Table S11. Global plasma metabolomics profiling in male patients with Graves' disease.

Table S12. Global plasma metabolomics profiling in female patients with Graves' disease.

References

1. Argov Z, Renshaw PF, Boden B, Winokur A, Bank WJ. Effects of thyroid hormones on skeletal muscle bioenergetics. In vivo phosphorus-31 magnetic resonance spectroscopy study of humans and rats. *J Clin Invest* 1988;**81**:1695–1701.
2. Dentice M, Ambrosio R, Damiano V, Sibilio A, Luongo C, Guardiola O, et al. Intracellular inactivation of thyroid hormone is a survival mechanism for muscle stem cell proliferation and lineage progression. *Cell Metab* 2014;**20**:1038–1048.
3. Simonides WS, van Hardeveld C. Thyroid hormone as a determinant of metabolic and contractile phenotype of skeletal muscle. *Thyroid* 2008;**18**:205–216.
4. Zhang D, Wang X, Li Y, Zhao L, Lu M, Yao X, et al. Thyroid hormone regulates muscle fiber type conversion via miR-133a1. *J Cell Biol* 2014;**207**:753–766.
5. Lombardi A, Moreno M, de Lange P, Iossa S, Busiello RA, Goglia F. Regulation of skeletal muscle mitochondrial activity by thyroid hormones: focus on the "old" triiodothyronine and the "emerging" 3,5-diiodothyronine. *Front Physiol* 2015;**6**:237.
6. Ambrosio R, De Stefano MA, Di Girolamo D, Salvatore D. Thyroid hormone signaling and deiodinase actions in muscle stem/progenitor cells. *Mol Cell Endocrinol* 2017;**459**:79–83.
7. Kramer IF, Snijders T, Smeets JSJ, Leenders M, van Kranenburg J, den Hoed M, et al. Extensive type II muscle fiber atrophy in elderly female hip fracture patients. *J Gerontol A Biol Sci Med Sci* 2017;**72**:1369–1375.
8. Malmstroem S, Grove-Laugesen D, Riis AL, Bruun BJ, Ebbehoj E, Hansen KW, et al. Muscle performance and postural stability are reduced in patients with newly diagnosed Graves' disease. *Thyroid* 2019;**29**:783–789.
9. Ramsay I. Thyrotoxic muscle disease. *Postgrad Med J* 1968;**44**:385–397.
10. Ianuzzo D, Patel P, Chen V, O'Brien P, Williams C. Thyroidal trophic influence on skeletal muscle myosin. *Nature* 1977;**270**:74–76.
11. DeMartino GN, Goldberg AL. Thyroid hormones control lysosomal enzyme activities in liver and skeletal muscle. *Proc Natl Acad Sci U S A* 1978;**75**:1369–1373.

12. Villalta SA, Deng B, Rinaldi C, Wehling-Henricks M, Tidball JG. IFN-γ promotes muscle damage in the mdx mouse model of Duchenne muscular dystrophy by suppressing M2 macrophage activation and inhibiting muscle cell proliferation. *J Immunol* 2011;**187**:5419–5428.
13. Pietzner M, Kaul A, Henning AK, Kastenmuller G, Artati A, Lerch MM, et al. Comprehensive metabolic profiling of chronic low-grade inflammation among generally healthy individuals. *BMC Med* 2017;**15**:210.
14. Moon JH, Yi KH. The diagnosis and management of hyperthyroidism in Korea: consensus report of the Korean Thyroid Association. *Endocrinol Metab (Seoul)* 2013; **28**:275–279.
15. Guralnik JM, Simonsick EM, Ferrucci L, Glynn RJ, Berkman LF, Blazer DG, et al. A short physical performance battery assessing lower extremity function: association with self-reported disability and prediction of mortality and nursing home admission. *J Gerontol* 1994;**49**:M85–M94.
16. Setoyama D, Kato TA, Hashimoto R, Kunugi H, Hattori K, Hayakawa K, et al. Plasma metabolites predict severity of depression and suicidal ideation in psychiatric patients—a multicenter pilot analysis. *PLoS ONE* 2016; **11**:e0165267.
17. van der Spek AH, Fliers E, Boelen A. Thyroid hormone metabolism in innate immune cells. *J Endocrinol* 2017;**232**:R67–R81.
18. Chou JP, Effros RB. T cell replicative senescence in human aging. *Curr Pharm Des* 2013;**19**:1680–1698.
19. Brennan MD, Powell C, Kaufman KR, Sun PC, Bahn RS, Nair KS. The impact of overt and subclinical hyperthyroidism on skeletal muscle. *Thyroid* 2006;**16**:375–380.
20. Norrelund H, Hove KY, Brems-Dalgaard E, Jurik AG, Nielsen LP, Nielsen S, et al. Muscle mass and function in thyrotoxic patients before and during medical treatment. *Clin Endocrinol (Oxf)* 1999;**51**: 693–699.
21. Olson BR, Klein I, Benner R, Burdett R, Trzepacz P, Levey GS. Hyperthyroid myopathy and the response to treatment. *Thyroid* 1991;**1**:137–141.
22. de Oliveira Chachamovitz DS, dos Santos Vigario P, Nogueira Cordeiro MF, de Castro CL, Vaisman M, dos Santos Teixeira PDF. Quality of life, muscle strength, and fatigue perception in patients on suppressive therapy with levothyroxine for differentiated thyroid carcinoma. *Am J Clin Oncol* 2013; **36**:354–361.
23. Lonn L, Stenlof K, Ottosson M, Lindroos AK, Nystrom E, Sjostrom L. Body weight and body composition changes after treatment of hyperthyroidism. *J Clin Endocrinol Metab* 1998;**83**:4269–4273.
24. Paschke R, Schuppert F, Taton M, Velu T. Intrathyroidal cytokine gene expression profiles in autoimmune thyroiditis. *J Endocrinol* 1994;**141**:309–315.
25. Bahn RS. Graves' ophthalmopathy. *N Engl J Med* 2010;**362**:726–738.
26. Sun Z, Zhong W, Lu X, Shi B, Zhu Y, Chen L, et al. Association of Graves' disease and prevalence of circulating IFN-γ-producing $CD28^{-}$ T cells. *J Clin Immunol* 2008;**28**: 464–472.
27. Giubilato S, Liuzzo G, Brugaletta S, Pitocco D, Graziani F, Smaldone C, et al. Expansion of $CD4^{+}CD28^{null}$ T-lymphocytes in diabetic patients: exploring new pathogenetic mechanisms of increased cardiovascular risk in diabetes mellitus. *Eur Heart J* 2011; **32**:1214–1226.
28. Thewissen M, Somers V, Venken K, Linsen L, van Paassen P, Geusens P, et al. Analyses of immunosenescent markers in patients with autoimmune disease. *Clin Immunol* 2007;**123**:209–218.
29. Yi HS, Kim SY, Kim JT, Lee YS, Moon JS, Kim M, et al. T-cell senescence contributes to abnormal glucose homeostasis in humans and mice. *Cell Death Dis* 2019;**10**:249.
30. Pietzner M, Engelmann B, Kacprowski T, Golchert J, Dirk AL, Hammer E, et al. Plasma proteome and metabolome characterization of an experimental human thyrotoxicosis model. *BMC Med* 2017;**15**:6.
31. Benvenga S, Lakshmanan M, Trimarchi F. Carnitine is a naturally occurring inhibitor of thyroid hormone nuclear uptake. *Thyroid* 2000;**10**:1043–1050.
32. Benvenga S. Effects of L-carnitine on thyroid hormone metabolism and on physical exercise tolerance. *Horm Metab Res* 2005; **37**:566–571.
33. Benvenga S, Amato A, Calvani M, Trimarchi F. Effects of carnitine on thyroid hormone action. *Ann N Y Acad Sci* 2004;**1033**: 158–167.
34. Benvenga S, Ruggeri RM, Russo A, Lapa D, Campenni A, Trimarchi F. Usefulness of L-carnitine, a naturally occurring peripheral antagonist of thyroid hormone action, in iatrogenic hyperthyroidism: a randomized, double-blind, placebo-controlled clinical trial. *J Clin Endocrinol Metab* 2001;**86**: 3579–3594.
35. Chng CL, Lim AY, Tan HC, Kovalik JP, Tham KW, Bee YM, et al. Physiological and metabolic changes during the transition from hyperthyroidism to euthyroidism in Graves' disease. *Thyroid* 2016;**26**: 1422–1430.
36. Al-Majdoub M, Lantz M, Spegel P. Treatment of Swedish patients with Graves' hyperthyroidism is associated with changes in acylcarnitine levels. *Thyroid* 2017;**27**: 1109–1117.
37. Lehmann R, Zhao X, Weigert C, Simon P, Fehrenbach E, Fritsche J, et al. Medium chain acylcarnitines dominate the metabolite pattern in humans under moderate intensity exercise and support lipid oxidation. *PLoS ONE* 2010;**5**: e11519.
38. Morville T, Sahl RE, Moritz T, Helge JW, Clemmensen C. Plasma metabolome profiling of resistance exercise and endurance exercise in humans. *Cell Rep* 2020; **33**:108554.
39. Xu G, Hansen JS, Zhao XJ, Chen S, Hoene M, Wang XL, et al. Liver and muscle contribute differently to the plasma acylcarnitine pool during fasting and exercise in humans. *J Clin Endocrinol Metab* 2016;**101**: 5044–5052.
40. Lum H, Sloane R, Huffman KM, Kraus VB, Thompson DK, Kraus WE, et al. Plasma acylcarnitines are associated with physical performance in elderly men. *J Gerontol A Biol Sci Med Sci* 2011;**66**: 548–553.
41. Paradies G, Ruggiero FM, Petrosillo G, Quagliariello E. Stimulation of carnitine acylcarnitine translocase activity in heart mitochondria from hyperthyroid rats. *FEBS Lett* 1996;**397**:260–262.
42. Bloise FF, Oliveira FL, Nobrega AF, Vasconcellos R, Cordeiro A, Paiva LS, et al. High levels of circulating triiodothyronine induce plasma cell differentiation. *J Endocrinol* 2014;**220**:305–317.
43. DeGroot LJ. In Feingold KR, Anawalt B, Boyce A, Chrousos G, de Herder WW, Dhatariya K, et al., eds. *Graves' Disease and the Manifestations of Thyrotoxicosis*. South Dartmouth (MA); 2000.
44. von Haehling S, Morley JE, Coats AJS, Anker SD. Ethical guidelines for publishing in the Journal of Cachexia, Sarcopenia and Muscle: update 2019. *J Cachexia Sarcopenia Muscle* 2019;**10**:1143–1145.