Research Square

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Relationships Among Circulating TNF-α, Skeletal Muscle and Peripheral CD8+ T Cells During Cachexia in Gastric Cancer

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Abstract

Background: Cancer-associated cachexia is characterized by ongoing loss of skeletal muscle and has a high incidence in gastric cancer. Although studies have focused on the effect of inflammatory cytokines on skeletal muscle loss, it is not clear whether inflammatory cytokines affect other physiological functions, particularly immunity in gastric cancer. Thus, we aim to investigate the relationships between circulating TNF-α, skeletal muscle and peripheral immune status in gastric cancer.

Methods: Totally 392 gastric cancer patients in cohort A and 60 gastric cancer patients in cohort B were selected from the prospective cohort study NCT03115931 and ChiCTR1900026578 in West China Hospital, Sichuan University, respectively. Besides, human skeletal muscle myoblasts (HSMMs) and peripheral $CD8^+$ T cells completed about 7 to 9 days successive TNF-α intervention with different concentrations.

Results: From data in cohort A, multivariate analysis showed that preoperative weight loss ≥5% was associated with circulating TNF-α (P =0.001) and TNM stage (P =0.002). ROC curve indicated that cut-off point of 9.96 pg/ml for circulating TNF-α was an important warning of weight loss ≥5% in patient with gastric cancer. Before surgery, high circulating TNF-α (≥ 9.96 pg/ml) was associated with significantly lower body weight, skeletal muscle mass and handgrip strength but not fat mass. After radical surgery, high circulating TNF-α was also associated with significantly more loss of body weight and skeletal muscle mass instead of fat mass. In cohort B and vitro experiments, immunohistochemistry, western blot and flow cytometry identified that high TNF-α led to myofiber change, decreased expression of AMP-dependent protein kinase (AMPK) and phosphate-AMPK in skeletal muscle and increased myoblast apoptosis. Flow cytometry found that circulating TNF-α was not correlated with PD-1, LAG-3, TIM-3 and TIGIT expressed on peripheral $CD8^+$ T cells, and TNF-α not only promoted the proliferation of $CD8^+$ T cells but also increased their apoptosis ratio at higher concentration.

Conclusions: In our study, we found that TNF-α could result in myofiber change, interfered glycometabolism of skeletal muscle, whereas promoted the proliferation of $CD8^+$ T cells in gastric cancer during cachexia, and preoperative circulating TNF-α of more than 9.96 pg/ml could indicate a high risk of weight loss.

Background

Gastric cancer is the fifth most frequently diagnosed cancer and the third leading cause of cancer death according to global cancer statistics.[1] Cachexia, a common disorder in cancer, particularly has a high incidence of 35.8% to 60% in gastric cancer and usually leads to progressive functional impairment, treatment-related complications, and poor quality of life.[2-4] Cancer-associated cachexia is a multifactorial syndrome defined by an ongoing loss of skeletal muscle mass (with or without loss of fat mass) that cannot be fully reversed by conventional nutritional support, and patients who have more than

5% loss of stable body weight over the past 6 months can be classified as having cachexia.[5] Therefore, weight loss, particularly skeletal muscle loss is a key feature of cancer-associated cachexia.

Inflammatory cytokines such as interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF-α), also known as cachectin, play an important role in cachexia.[6-8] It is clear that TNF-α can directly promote skeletal muscle loss by activating the NF-κB pathway and inducing ubiquitinmediated proteasome degradation (UPR) of muscle protein[6], and IL-6 can suppress protein synthesis in muscle cells by Janus kinase (JAK) signal pathway.[6] However, many researches demonstrate that the targeting inflammatory factors alone cannot achieve the notable therapeutic effect on cachexia and prolong the survival of patients with tumors.[9] Cachexia is a multi-organ syndrome that, in addition to muscle, affects adipose tissues, heart, kidneys, liver and immune system.[10] The current studies, however, mainly focus on the effect of inflammatory factors on skeletal muscle loss, and rarely involve the role of inflammation in the immune system against cancer and other physiological changes. In our observation, normal circulating TNF-α was found in many of our patients with gastric cancer, but it was still unknown that, apart from the skeletal muscle loss, whether significant differences existed in other physiological functions between normal and high circulating TNF-α.

TNF-α can result in weight loss, but the characteristics of cachexia caused by TNF-α are rarely reported. The purpose of this study is to investigate the possible clinic parameters that were associated with weight loss, particularly skeletal muscle loss, which were caused by TNF-α in gastric cancer. Besides, we also aim to evaluate the relationships between circulatory TNF-α, skeletal muscle and peripheral immune status.

Methods

Patients and sample collection

Two independent cohorts (A and B) of patients diagnosed with gastric cancer were included in this study, and each diagnosis of gastric cancer was confirmed by the pathology. Excluded from other neoplasms and metabolic disorders such as diabetes, 392 patients in cohort A were randomly selected from a multicenter prospective cohort study (NCT03115931) at West China Hospital, Sichuan University between April 2017 and February 2019. Besides, there were 60 patients in cohort B recruited randomly from a prospective observational study (ChiCTR1900026578) between October 2019 and January 2020 in West China Hospital, Sichuan University. In cohort B, skeletal muscle sample (taken from the rectus abdominis) of each patient was collected during the open surgery and stored in liquid nitrogen for later experiments. Preoperative peripheral blood of each patient collected with heparin was also used for isolating the peripheral blood mononuclear cells (PBMCs). The tumor, node, and metastasis (TNM) staging system of the American Joint Committee on Cancer 2019 (AJCC 2019) was applied to tumor staging. All patients signed informed consent forms, and this study was supported by the Biomedical Ethics Subcommittee of West China Hospital, Sichuan University.

Clinic-pathological parameter assessment

The common clinic-pathological parameters, including the medical history, laboratory examination reports and pathology reports, were extracted from the Hospital Information System (HIS). For each patient in this study, circulating TNF-α and IL-6 were measured routinely during the perioperative and follow-up period in outpatient. Furthermore, by using bioimpedance analysis (BIA) and electronic hand dynamometer, we particularly measured the body composition and grip strength of the patients respectively in cohort A and cohort B before surgery, as well as one and six months after surgery.

Immunohistochemistry

Skeletal muscle samples embedded in paraffin were sectioned at 5 μm. Skeletal muscle sections were incubated with primary antibodies against AMP-dependent protein kinase (AMPK) (Thermo Fisher Scientific, Waltham, MA, USA). Then, sections were incubated with horseradish peroxidase (HRP)-labeled secondary antibodies (Servicebio, Hubei, China), and the diaminobenzidine was used for visualization of positive signals. At last, sections were counterstained with hematoxylin and dehydrated before sealed with non-aqueous mounting medium. Images were analyzed using Image-Pro Plus. Additionally, so as to investigate the ratio of type I and type II muscle fibers, we performed ATPase staining in frozen skeletal muscle sections using the ATPase staining kit (Solarbio, Beijing, China) according to the manufacturer's protocol.

Cell lines and cell cultures

Human skeletal muscle myoblasts (HSMMs) were obtained from the State Key Laboratory of Biotherapy of Sichuan University and cultured with SkGMTM-2 Skeletal Muscle Cell Growth Medium-2 BulletKitTM (LONZA, Basel, Switzerland). To investigate the impact of TNF-α on HSMMs, we cultured the HSMMs with 0 pg/ml, 5 pg/ml, 10 pg/ml and 15 pg/ml TNF-α (Novoprotein, Shanghai, China) for 7 days, respectively.

To evaluate the impact of TNF-α on $CD8^+$ T cells, five patients at a normal level of preoperative circulating TNF-α (< 8.1 pg/ml) were selected from cohort B. Fresh blood of each patient was collected before surgery, and PBMCs were isolated using density gradient centrifugation (800 g, 30 min, room temperature, with slow acceleration and deceleration) with human lymphocyte separation medium (DAKEWE, Guangdong, China). Then, $CD8^+$ T cells were sorted from PBMCs by FACSAria SORP (Becton, Dickinson and Company, NJ, USA). The PBMCs and isolated $CD8^+$ T cells were stimulated with soluble anti-CD3 plus anti-CD28 (Biolegend, San Diego, CA, USA) and respectively cultured in X-VIVO 15 (LONZA, Basel, Switzerland) containing 5% human AB serum (Sigma-Aldrich, Saint Louis, Missouri, USA) and 100 IU/ml IL-2 (Novoprotein, Shanghai, China) for 24 hours. Then, every 1×10^5 PBMCs and $CD8^+$ T cells were respectively cultured with 0 pg/ml, 10 pg/ml and 20 pg/ml TNF-α (Novoprotein, Shanghai, China) for 9

days. Particularly the number of $CD8^+$ T cells was calculated every two days. All cells were harvested for later analysis on the last day.

Western blot

HSMMs cultured with different concentrations of TNF-α were harvested to obtain cell lysates using RIPA lysis buffer (Servicebio, Hubei, China) supplemented with protease and phosphatase inhibitors. BCA Protein Assay Kit (Servicebio, Hubei, China) was used for protein concentration measurement. 30 μg of protein was mixed with sample-loading buffer (Servicebio, Hubei, China), separated by SDS-PAGE and transferred onto nitrocellulose membranes. After blocked with blocking buffer (5% nonfat milk and 0.5% Tris-buffered saline-Tween) for one hour, the membranes were incubated overnight with primary antibodies against AMPK (Thermo Fisher Scientific, Waltham, MA, USA) and phosphate-AMPK (Thermo Fisher Scientific, Waltham, MA, USA). The secondary HRP-conjugated antibodies (Servicebio, Hubei, China) were diluted 1:3000 in Tris-buffered saline-Tween and then incubated with membranes for 30 minutes. Immunoreactive signals were measured via ECL (Servicebio, Hubei, China), and western blots were analyzed through densitometry using alphaEaseFC (ProteinSimple, Silicon Valley, CA, USA).

Flow cytometry

PBMCs isolated from each patient in cohort B and PBMCs cultured with different concentrations of TNF-α as described earlier were all washed with PBS and then stained with the following monoclonal antibodies: anti-human CD8 (BD Pharmingen, San Diego, CA, USA); anti-human PD-1 (Biolegend, San Diego, CA, USA); anti-human LAG-3 (eBioscience, San Diego, CA, USA); anti-human TIM-3 (eBioscience, San Diego, CA, USA) and anti-human TIGIT (eBioscience, San Diego, CA, USA). Isotype controls were used in each antigen-specific antibody. The expressions of PD-1, LAG-3, TIM-3 and TIGIT on $CD8^+$ T cells were measured by ACEA NovoCyteTM (Agilent Biosciences, San Diego, CA, USA).

HSMMs and $CD8^+$ T cells cultured with different concentrations of TNF-α as described earlier were also harvested for apoptosis detection. Cell apoptosis was evaluated with Annexin V-FITC Apoptosis Detection Kit (KeyGEN BioTECH, Jiangsu, China). Cells were washed with PBS and incubated with Annexin V-FITC and propidium iodide (PI) at room temperature for 15 minutes, and cell apoptosis was detected by ACEA NovoCyteTM (Agilent Biosciences, San Diego, CA, USA). Additionally, cell cycle was also measured for $CD8^+$ T cells with the use of Cell Cycle Detection Kit (KeyGEN BioTECH, Jiangsu, China). $CD8^+$ T cells were fixed with 70% ethanol at 4°C for two hours and then washed with PBS. After incubated with PI/RNase A at room temperature for 60 minutes, cells were analyzed immediately by ACEA NovoCyteTM (Agilent Biosciences, San Diego, CA, USA).

Statistical analysis

Continuous variables were reported as mean (± standard deviation) or median with range. Categorical data were presented as the number of cases and percentages. Differences between continuous variables were analyzed through Student's two-tailed test, Mann-Whitney U test or one-way analysis of variance, and categorical variables were analyzed using Pearson's Chi-square test or Fisher's exact test. The body weight, skeletal muscle mass, fat mass and handgrip strength were categorized into high and low levels based on the median. Additionally, multivariate logistic regression was also performed to investigate the associations between clinic-pathological characteristics and weight loss before surgery. All P values were two-tailed, and a P value of < 0.05 was considered statistically significant. The software SPSS 25.0 and GraphPad Prism 6.0 were used for data analysis and image presentation.

Results

Patient characteristics

Among the 392 patients in cohort A, radical surgeries were performed in 376 patients, and 16 patients had non-radical surgeries. We divided the 392 patients into two groups according to the weight loss ≥ 5% within six months before the surgery (Table 1). Totally 167 patients in cohort A had weight loss ≥ 5% within the six months before surgery, indicating that at least 42.6% patients had cancer cachexia. There were no significant differences in age, sex and height between the two groups, while patients with weight loss ≥ 5% had significantly lower body weight, BMI, skeletal muscle mass and fat mass. The body weight of patients with weight loss < 5% was on average 4.39 kg higher than those with weight loss ≥ 5%, and particularly the skeletal muscle mass was on average 2.14 kg higher in patients with weight loss < 5%, illustrating that skeletal muscle loss could be a major cause of weight loss. Besides, both in male and female, lower skeletal muscle index (SMI) and handgrip strength were accompanied by weight loss ≥ 5%, suggesting cancer-associated cachexia was a disorder characterized by loss of body weight with particular losses of skeletal muscle mass and function. Particularly, compared to those with weight loss < 5%, women with weight loss ≥ 5% had more reduced SMI (0.21 kg/m^2 vs. 0.15 kg/m^2) and handgrip strength (2.84 kg vs. 2.67 kg) than men. For pathological parameters, significant differences were found in the TNM stage, depth of invasion and lymph node metastasis between the two groups, in which patients with weight loss ≥ 5% had a higher rate of TNM stage III + IV, T3 + T4 depth of invasion and positive lymph node metastasis. Moreover, the locations of gastric cancer were also different between the two groups, and the main difference was the incidence of entire gastric cancer (7.2% vs. 1.8%). Compared to those with weight loss < 5%, patients with weight loss ≥ 5% had significantly higher scores of NRS2002, indicating that more weight loss in gastric cancer was related to increased risk of malnutrition. As can be seen in Short Form 36 (SF-36) survey, the significant differences between the two groups were physical function, mental health and bodily pain (Table 1), saying that weight loss in gastric cancer may have an impact on the quality of life of patients, especially the physical function and mental health. Additionally, patients with weight loss ≥ 5% had a significantly higher level of preoperative circulating IL-6 and TNF-α.

Table 1
Participants' characteristics divided by weight loss within 6 months before surgery

Characteristics	**Weight loss < 5% (n = 225)**	**Weight loss ≥ 5% (n = 167)**	***P***
Age (years)	59.83 ± 10.00[a]	60.25 ± 9.18	0.67
Sex (Female/ Male)	78/147	69/98	0.18
Weight (kg)	61.52 ± 10.14	57.13 ± 11.97	0.001
Height (cm)	163.66 ± 8.09	162.69 ± 8.26	0.25
BMI (kg/m^2)	23.24 ± 3.29	21.18 ± 3.95	< 0.001
Body composition[b]			
Skeletal muscle mass (kg)	21.58 ± 4.32	19.44 ± 5.63	< 0.001
Fat mass (kg)	19.53 ± 2.73	18.35 ± 3.23	0.001
SMI (kg/m^2)			
Female	6.01 ± 0.19	5.80 ± 0.28	< 0.001
Male	7.38 ± 0.24	7.23 ± 0.20	< 0.001
Handgrip strength (dominant) (kg)			
Female	26.21 ± 4.71	23.37 ± 5.64	0.001
Male	44.19 ± 8.52	41.52 ± 9.36	0.02
Location			0.03
Esophagogastric junction	77 34.2	61 36.5	
Body	26 11.6	12 7.2	
Antrum	118 52.4	82 49.1	
Entire stomach	4 1.8	12 7.2	
TNM stage, I + II/III + IV	95/130	41/126	< 0.001
Depth of invasion, T1 + T2/T3 + T4	67/158	31/136	0.01

BMI, Body Mass Index; IL-6, interleukin-6; NRS 2002, Nutritional Risk Screening 2002; SF-36, Short Form 36; SMI, skeletal muscle index; TNF-α, tumor necrosis factor alpha; TNM, tumor, node, and metastasis.

[a]Mean ± standard deviation.

[b]Measurement results of human body composition analysis (InBody770)

Characteristics	Weight loss < 5% (n = 225)	Weight loss ≥ 5% (n = 167)	*P*
Lymph node metastasis, Negative/Positive	89/136	47/120	0.02
NRS2002	1.63 ± 0.14	3.65 ± 0.09	< 0.001
SF-36			
Physical function	80.18 ± 12.79	75.87 ± 14.05	0.002
Role-physical	80.78 ± 16.53	79.19 ± 18.28	0.39
Bodily pain	80.67 ± 13.89	77.66 ± 16.26	0.04
General health	64.11 ± 23.23	61.05 ± 22.40	0.19
Vitality	73.51 ± 17.06	71.08 ± 19.54	0.19
Social functioning	83.61 ± 13.23	81.59 ± 14.73	0.14
Role-emotional	74.96 ± 22.67	74.45 ± 24.73	0.83
Mental health	79.45 ± 12.37	74.49 ± 13.75	0.0002
Circulating IL-6 (pg/mL)	3.43 ± 4.43	6.16 ± 6.57	< 0.0001
Circulating TNF-α (pg/mL)	6.71 ± 1.84	11.21 ± 5.93	< 0.0001

BMI, Body Mass Index; IL-6, interleukin-6; NRS 2002, Nutritional Risk Screening 2002; SF-36, Short Form 36; SMI, skeletal muscle index; TNF-α, tumor necrosis factor alpha; TNM, tumor, node, and metastasis.

[a]Mean ± standard deviation.

[b]Measurement results of human body composition analysis (InBody770)

Circulating TNF-α, an independent important factors with weight loss

We further conducted a univariate analysis, which revealed that circulating IL-6, circulating TNF-α, TNM stage, location of tumors, depth of invasion and lymph node metastasis were significantly associated with weight loss ≥ 5%. However, in multivariate analysis, only circulating TNF-α and TNM stage had significant associations with weight loss ≥ 5% (Table 2). According to the laboratory examination reports, circulating TNF-α < 8.0 pg/ml was regarded as normal level. Thus we divide the circulating TNF-αinto four groups according to the multiple of normal TNF-α. When circulating TNF-α was more than two times of the normal level, the patients with weight loss ≥ 5% outnumbered those with weight loss < 5% (Fig. 1A).

The rate of weight loss ≥ 5% was also extremly increasing when circulating TNF-α was two to three times of the normal (Fig. 1B). Next, we used the ROC curve to test the sensitivity and specificity of TNF-α associated with weight loss ≥ 5% (Fig. 1C). Circulating TNF-α was a sensitive indicator of weight loss ≥ 5% (AUC = 0.66, $P < 0.001$). Through the ROC curve, the cut-off point value was calculated as 9.96 pg/ml, about 1.25 times higher than normal value. This is a reminder that circulating TNF-α, 1.25 times higher than normal was an important warning of weight loss in patient with gastric cancer. Then, the values of circulating TNF-α in different TNM stages were compared to clarify the relationship between TNF-α and TNM stage. The data showed that circulating TNF-α in stage III was significantly higher than that in stage I ($P = 0.01$). Although circulating TNF-α in stage IV was the highest and that in stage I was the lowest, the difference was not significant (Fig. 1D). This could be due to too small number of patients with stage I and stage IV. When patients were divided into two groups (I + II vs. III + IV), the difference of TNF-α was significant (Fig. 1E). To explore the effect of circulating TNF-α and TNM stage on weight loss, we divided circulating TNF-α into two groups according to the cut-off value of 9.96 pg/ml. In stage I and II, most patients with weight loss ≥ 5% were affected by high circulating TNF-α (≥ 9.96 pg/ml), while in stage III and IV, nearly half of patients with weight loss ≥ 5% had low circulating TNF-α (Fig. 1F,G,H,I). It meant that TNF-α and TNM stage were two independent factors causing weight loss, and the mechanisms of how TNF-α and TNM stage promoting weight loss might be different. Thus, we conjectured that the characteristics of weight loss induced by TNF-α and TNM stage should be variant.

Table 2
Univariate and multivariate analysis for the weight loss ≥ 5% before surgery

Variable	Univariate			Multivariate		
	HR	95% CI	*P*	HR	95% CI	*P*
Age (years)	0.98	0.96–1.01	0.29	0.98	0.95–1.02	0.29
Sex (Female/Male)	0.84	0.48–1.49	0.20	1.15	0.55–2.39	0.71
Circulating IL-6 (> median)	2.66	1.50–4.71	0.001	1.28	0.57–2.90	0.55
Circulating TNF-α (> median)	3.19	1.89–5.37	< 0.001	2.84	1.46–5.55	0.001
TNM stage (IV + III/II + I)	11.21	5.80-21.66	< 0.001	8.03	3.60-17.92	0.002
Location	2.07	1.18–3.64	0.01	1.44	0.63–3.28	0.45
Depth of invasion (T3 + T4/T1 + T2)	2.07	1.18–4.64	0.01	1.50	0.72–3.12	0.31
Lymph node metastasis (Yes/No)	3.45	1.93–6.18	< 0.001	1.97	0.99–3.89	0.28

95% CI, 95% confidence intervals; HR, hazard ratios; IL-6, interleukin-6; TNF-α, tumor necrosis factor alpha; TNM, tumor, node, and metastasis.

Clinic characteristics of patients in different TNM stage and circulating TNF-α

Next, through comparing the clinic parameters of patients in different TNM stage (stage Ⅰ + Ⅱ vs. Ⅲ + Ⅳ) and circulating TNF-α (TNF-α^L vs. TNF-α^H), many interesting different characteristics related to metabolism, nutrition and immune indexes were found, showing in Table 3 and Table 4. Among the 392 patients with gastric cancer, totally 104 patients (26.5%) had high level of circulating TNF-α (TNF-α^H ≥ 9.96 pg/ml), and those with TNF-α^H had significantly lower albumin (ALB), total protein (TP), transferrin (TRF) and prealbumin (PAB), which were important indicators of nutrition (Table 3). In stage Ⅲ and Ⅳ, the results were as same as those with TNF-α^H. It was consistent that circulating TNF-α and TNM stage could have an adverse impact on nunutrition. However, It was the opposite in globulin (GLB), a herd of the major proteins associated with immunity. Glucose (GLU), one of the important metabolic indexes, in TNF-α^H group was significant higher than it in TNF-α^L group, while it was lower in stage Ⅲ + Ⅳ than stage Ⅰ + Ⅱ. And lactate dehydrogenase (LDH) was as same as GLU between TNF-α^H group and TNF-α^L group or between stage Ⅲ + Ⅳ and stage Ⅰ + Ⅱ. But other metabolic indexes such as creatinine (CREA), triglyceride (TG), and cholesterol (CHOL), in TNF-α^H group and stage Ⅲ + Ⅳ were respectively lower than that in TNF-α^L group and stage Ⅲ + Ⅳ. In nutrition and metabolism, the clinic characteristics of circulating TNF-α and TNM stage were slightly different. However, in immunity, the divergences of circulating TNF-α and TNM stage were considerable. Even though, the number of lymphocyte (LYMPH) and percentage of CD4 were significant lowerer in TNF-α^H group compared to those in TNF-α^L group, and white blood cell (WBC), complement 3 (C3), CD3, CD8, immunoglobulin G (IgG) and IL-6 were all higher in TNF-α^H group. From Table 4, in TNM stage, immune indexes were decreased in Ⅲ + Ⅳ stage. By the data from clinic characteristics, we found that although both TNF-α and TNM stage could result in weight loss, the mechanisms should be diversity. Body weight was mainly composed of skeletal muscle mass and fat mass. And skeletal muscle mass loss has recently been identified as a risk factor for poor outcomes in various malignancies. Since clinic characteristics between circulating TNF-α and TNM stage were different, we speculated that the changes of body compositions were also different, despite identical weight loss.

Table 3
Participants' characteristics divided by level of TNF-α in peripheral blood

Characteristics	**TNF-α^L (n = 288)**	**TNF-α^H (n = 104)**	***P***
ALB (g/L)	42.08 ± 4.00	38.11 ± 5.25	< 0.001
TP (g/L)	67.01 ± 5.69	65.85 ± 7.52	0.10
GLB (g/L)	24.91 ± 4.01	26.71 ± 9.15	0.007
PAB (mg/L)	212.33 ± 55.30	201.33 ± 48.97	0.07
TRF (g/L)	2.32 ± 0.50	2.25 ± 0.46	0.21
GLU (mmol/L)	5.16 ± 1.29	5.66 ± 2.30	0.007
CREA (μmol/L)	66.98 ± 16.34	58.61 ± 11.76	< 0.001
TG (mmol/L)	1.25 ± 0.63	1.11 ± 0.44	0.04
CHOL (mmol/L)	4.33 ± 0.92	4.06 ± 0.98	0.01
LDH (IU/L)	154.01 ± 26.67	169.67 ± 51.24	< 0.001
RBC (× 10^{12}/L)	4.30 ± 0.68	4.26 ± 0.80	0.62
HGB (g/L)	126.33 ± 25.75	115.39 ± 29.25	< 0.001
WBC (× 10^9/L)	5.44 ± 1.42	6.19 ± 2.34	< 0.001
LYMPH# (× 10^9/L)	1.60 ± 0.54	1.49 ± 0.43	0.06
C3 (g/L)	0.81 ± 0.15	0.88 ± 0.20	< 0.001
CD3 (%)	68.52 ± 10.57	69.40 ± 10.83	0.47
CD4 (%)	41.05 ± 9.56	39.95 ± 9.14	0.31
CD8 (%)	22.79 ± 7.74	25.04 ± 10.39	0.02
CD4/CD8	2.08 ± 1.04	1.71 ± 0.86	0.001
IgG (g/L)	10.80 ± 2.33	13.36 ± 9.28	< 0.001
IL-6 (pg/ml)	5.64 ± 13.56	16.51 ± 29.76	< 0.001[a]

ALB, albumin; CD, cluster of differentiation; CHOL, cholesterol; CREA, Creatinine; C3, complement 3; GLB, globulin; GLU, glucose; HGB, hemoglobin; IgG, immunoglobulin G; IL-6, interleukin-6; LDH, lactate dehydrogenase; LYMPH, lymphocyte; PAB, prealbumin; RBC, red blood cell; TG, triglyceride; TP, total protein; TRF, transferrin; WBC, white blood cell.

[a]The Mann-Whitney Test was used.

Table 4
Participants' characteristics divided by TNM stage

Characteristics	I + II (n = 136)	III + IV (n = 256)	*P*
ALB (g/L)	42.53 ± 3.98	41.32 ± 4.44	0.008
TP (g/L)	67.57 ± 5.82	65.24 ± 6.30	< 0.001
GLB (g/L)	26.04 ± 3.96	24.92 ± 5.19	0.03
PAB (mg/L)	222.68 ± 49.06	201.49 ± 56.08	< 0.001
TRF (g/L)	2.44 ± 0.54	2.21 ± 0.48	< 0.001
GLU (mmol/L)	5.70 ± 1.80	5.01 ± 1.06	< 0.001
CREA (μmol/L)	70.31 ± 17.38	64.16 ± 14.20	< 0.001
TG (mmol/L)	1.41 ± 0.53	1.26 ± 0.63	0.02
CHOL (mmol/L)	4.48 ± 0.93	4.24 ± 0.93	0.02
LDH (IU/L)	161.74 ± 25.63	152.43 ± 32.43	0.004
RBC ($\times 10^{12}$/L)	4.49 ± 0.63	4.23 ± 0.73	< 0.001
HGB (g/L)	131.76 ± 23.08	122.27 ± 26.21	< 0.001
WBC ($\times 10^{9}$/L)	5.71 ± 1.91	5.37 ± 1.26	0.04
LYMPH# ($\times 10^{9}$/L)	1.70 ± 0.60	1.54 ± 0.48	0.004
C3 (g/L)	0.85 ± 0.14	0.81 ± 0.18	0.025
CD3 (%)	70.20 ± 11.24	66.33 ± 8.94	< 0.001
CD4 (%)	44.53 ± 9.34	38.71 ± 9.27	< 0.001
CD8 (%)	23.34 ± 8.33	19.27 ± 7.26	< 0.001
CD4/CD8	1.90 ± 1.21	2.00 ± 0.93	0.36
IgG (g/L)	11.07 ± 2.43	9.16 ± 7.47	0.004
IL-6 (pg/ml)	0.85 ± 0.14	0.81 ± 0.18	0.025

ALB, albumin; CD, cluster of differentiation; CHOL, cholesterol; CREA, Creatinine; C3, complement 3; GLB, globulin; GLU, glucose; HGB, hemoglobin; IgG, immunoglobulin G; IL-6, interleukin-6; LDH, lactate dehydrogenase; LYMPH, lymphocyte; PAB, prealbumin; RBC, red blood cell; TG, triglyceride; TP, total protein; TRF, transferrin; WBC, white blood cell.

The diversity of body composition and handgrip strength associated with circulating TNF-α and TNM stage

Our data showed that circulating TNF-α and TNM stage had significant associations with weight loss ≥ 5% before surgery, therefore, we firstly analyzed the preoperative body composition and handgrip strength of patients in cohort A with different circulating TNF-α and TNM stage. The body weight ($P = 0.02$), skeletal muscle mass ($P < 0.001$) and handgrip strength ($P < 0.001$) but not fat mass ($P = 0.06$) of patients in TNF-α^L group were significantly higher than those in TNF-α^H group, respectively (Fig. 2A, B, C, D). Meanwhile, patients of TNM stage I + II had significantly higher body weight ($P < 0.001$), skeletal muscle mass ($P = 0.007$), fat mass ($P < 0.001$) and handgrip strength ($P < 0.001$) than those of TNM stage III + IV (Fig. 2E, F, G, H). Except for fat mass, the trends of changes between circulating TNF-α and TNM stage were similar. We further analyzed the differences between TNF-α^H and TNF-α^L groups and found that the skeletal muscle mass accounted for 76.4% of the total body weight difference, while between TNM stage I + II and III + IV groups, this result was only 23.1%. Moreover, the average difference of handgrip strength between different TNF-α groups was higher than that between different TNM stage groups (3.80 kg vs. 1.96 kg). That was to say, circulating TNF-α seemed to have a greater effect on preoperative skeletal muscle than TNM stage.

Current studies suggested that TNF-α and IL-6, the important inflammatory cytokines, were related to cancer cachexia [8], thus we investigated the postoperative changes of circulating TNF-α and IL-6. Compared to those in TNF-α^L group before surgery, patients in TNF-α^H group tended to have higher postoperative circulating TNF-α (Fig. 3A). For patients with resected gastric cancer, the circulating TNF-α was elevated within the postoperative 3 days and then decreased slowly (Fig. 3B), and the circulating IL-6 was just increased within the first day after surgery and then decreased rapidly (Fig. 3C). Moreover, In patients with non-radical surgery, the changes of circulating IL-6 were similar to those with radical surgery (Fig. 3C), while the circulating TNF-α was elevated within the postoperative 3 days and still higher one month later (Fig. 3B), indicating that the existence of tumor after non-radical surgery might affect the circulating TNF-α continuously. Based on the findings mentioned above, we supposed that circulating TNF-α but not IL-6 might be associated with postoperative changes in body weight and composition in gastric cancer. This also reminded us that circulating TNF-α recovered to normal slowly after operation, and remained at a high level for a period of time. However, for patients with resected gastric cancer, tumor would have a weak effect on weight loss. This also showed that high circulating TNF-α should be caused by tumor but not all released by a tumor.

To further determine the different effects of circulating TNF-α and TNM on postoperative human body compositions, we followed up the patients with radical surgery (n = 376) in cohort A for half a year. From the analysis of postoperative body composition, we observed decreased body weight, skeletal muscle mass and fat mass at the first month after surgery, and all of them got a bit recovered in the sixth month after surgery (Fig. 3D). To determine whether preoperative circulating TNF-α was associated with postoperative changes in body composition, we next compared the patients between TNF-α^H (n = 93) and

TNF-α^L (n = 283). We found that patients in TNF-α^H group had significantly more loss of body weight (-3.37 kg vs. -2.02 kg, $P < 0.001$), skeletal muscle mass (-1.83 kg vs. -0.86 kg, $P < 0.001$) and fat mass (-0.85 kg vs. -0.25 kg, $P < 0.001$) than those in TNF-α^L group at first month after surgery (Fig. 3E), while at sixth month after surgery, patients in TNF-α^H group also had significantly more loss of body weight (-1.01 kg vs. -0.21 kg, $P < 0.001$) and skeletal muscle mass (-0.76 kg vs. -0.18 kg, $P < 0.001$) but not fat mass (-0.21 kg vs. -0.21 kg, $P > 0.9$) (Fig. 3F). Additionally, we also investigated the associations between TNM stage and postoperative changes of body composition. Interestingly, in the first month after surgery, patients of advanced stage (III + IV, n = 240) had significantly more loss of body weight, skeletal muscle mass and fat mass than those of early stage (I + II, n = 136) (Fig. 3G), whereas there was no significant difference in change of body composition between patients in early and advanced stage at sixth month after surgery (Fig. 3H). In summary, for postoperative changes of body composition, high circulating TNF-α that was mainly associated with skeletal muscle loss was further clarified. From the analysis, we surmised that circulating TNF-α mainly affected skeletal muscle loss to bring about weight loss, and TNM stage could influence both skeletal muscle and fat mass.

Low skeletal muscle mass and handgrip strength are associated with increased surgical adverse events

Given that current studies suggested that weight loss might be associated with increased risk of postoperative complications, we also evaluated whether the body composition and handgrip strength had associations with postoperative adverse events in patients of cohort A with radical surgeries. Based on the Common Terminology Criteria for Adverse Events (CTCAE) version 4, we found that low skeletal muscle mass and handgrip strength, but not body weight and fat mass, were associated with a higher risk of in-hospital grade 3–4 non-hematological surgical adverse events after radical surgery (Table 5). Thus patients with weight loss caused by high circulating TNF-α should deserves our due attention, especially that in advanced stage with high circulating TNF-α.

Table 5
In-hospital grade 3–4 non-hematological surgical adverse events for resection

CTCAE v4.0	Weight		Skeletal muscle mass		Fat mass		Handgrip strength	
	High (n = 196)	Low (n = 196)	High (n = 196)	Low (n = 196)	High (n = 196)	Low (n = 196)	High (n = 196)	Low (n = 196)
Overall	7	12	4	15**	9	10	2	17***
Pancreatic fistula	0	2	0	2	0	2	0	2
Anastomotic leak	0	1	0	1	0	1	0	1
Abdominal infection	2	3	1	4	2	3	1	4
Postoperative hemorrhage	0	1	0	1	1	0	0	1
Anastomotic stenosis	0	0	0	0	0	0	0	0
Cholecystitis	0	0	0	0	0	0	0	0
Dumping syndrome	0	0	0	0	0	0	0	0
Delayed gastric emptying	0	0	0	0	0	0	0	0
Gastroesophageal regurgitation	0	0	0	0	0	0	0	0
Bowel obstruction	0	0	0	0	0	0	0	0
Ileus	1	0	1	0	1	0	0	1
Thromboembolic event	0	0	0	0	0	0	0	0
Pneumonia	3	4	2	5	3	4	1	6
Chyle leakage	0	1	0	1	1	0	0	1
Wound infection	1	0	0	1	1	0	0	1
Wound dehiscence	0	0	0	0	0	0	0	0

CTCAE, Common Terminology Criteria for Adverse Events.

** $P < 0.01$

*** $P < 0.001$

High TNF-α leads to myofiber change, interfered glycometabolism of skeletal muscle and increased myoblast apoptosis

Based on clinical data from cohort A, we found that high circulating TNF-α was associated with skeletal muscle loss. To further investigate the effect of TNF-α on skeletal muscle, we next collected 60 skeletal muscle samples from patients in cohort B, in which 16 patients had high circulating TNF-α (> 9.96 pg/ml) and 21 patients were in TNM stage I + II. Immumohistochemical analysis of ATPase staining showed that the skeletal muscle of patients in the TNF-α^H group had a significantly decreased ratio of type II muscle fibers ($P < 0.001$) (Fig. 4A), which was a typical pathogenic characteristic of sarcopenia muscle [11]. Besides, immumohistochemical analysis also showed that skeletal muscles in TNF-α^H group had significantly lower expression of AMPK than those in TNF-α^L group ($P < 0.001$) (Fig. 4B). For validating this result, we cultured the HSMMs with different concentrations of TNF-α in vitro. Western blot showed that the expressions of AMPK and phosphate-AMPK (p-AMPK) in HSMMs were inversely associated with concentrations of TNF-α (Fig. 4C). Moreover, flow cytometry revealed a positive correlation between the apoptosis ratio of HSMMs and the concentration of TNF-α (Fig. 4D). These findings indicated that high concentrations of TNF-α could decrease the glycometabolism in skeletal muscle and induce HSMMs cells apoptosis, which might lead to the skeletal muscle wasting.

The relationships between TNF-α and $CD8^+$ T cells in vitro and in vivo

From data in cohort A, we found that patients with TNF-α^H had significantly higher CD3 and CD8 in peripheral blood. However, the relationships between TNF-α and $CD8^+$ T cells in peripheral blood were unclear. We collected the peripheral blood of patients in cohort B before surgery. PBMCs were isolated and then used for testing the expression of co-inhibitory receptors on $CD8^+$ T cells. Flow cytometry showed that the expressions of PD-1, LAG-3, TIM-3 and TIGIT on $CD8^+$ T cells had no significant differences between patients in TNF-α^H group (n = 16) and TNF-α^L group (n = 44) (Fig. 5A). However, patients of TNM stage I + II (n = 21) had significantly lower expressions of PD-1, LAG-3, TIM-3 but not TIGIT on $CD8^+$ T cells than those of TNM stage III + IV (n = 39) (Fig. 5B). In vitro, we cultured the PBMCs with different concentrations of TNF-α, whereas the expressions of PD-1, LAG-3, TIM-3 and TIGIT on $CD8^+$ T cells were not associated with the concentrations of TNF-α (Fig. 5C). Additionally, be consistent with the result in Fig. 1E, we also found that patients in cohort B with advanced TNM stage (III + IV) had significantly higher circulating TNF-α (Fig. 5D). Moreover, we also extracted the $CD8^+$ T cells from PBMCs. After being stimulated with soluble anti-CD3 plus anti-CD28 and cultured with different concentrations of TNF-α, flow cytometry showed that the apoptosis ratio and proportions of S phase of $CD8^+$ T cells were positively correlated with concentrations of TNF-α (Fig. 5E and 5F). Besides, $CD8^+$ T cells cultured with 10 pg/ml TNF-α had higher proliferation than those without TNF-α ($P = 0.04$). While no significant

difference was found between $CD8^+$ T cells cultured with 20 pg/ml and 10 pg/ml TNF-α, as well as between 0 pg/ml and 20 pg/ml (Fig. 5G). In summary, TNM stage instead of circulating TNF-α was associated with expressions of PD-1, LAG-3, TIM-3 and TIGIT on peripheral $CD8^+$ T cells, and vitro experiments showed that TNF-α promoted the proliferation of $CD8^+$ T cells but significantly increased their apoptosis ratio at higher concentration.

Discussion

Cancer-associated cachexia is a disorder characterized by the involuntary loss of body weight in addition to the loss of homeostatic control of both energy and protein balance, and depletion of skeletal muscle is a key feature.[4] Cancer-associated cachexia leads to progressive functional impairment, treatment-related complications, poor quality of life and cancer-related mortality.[12] The universally accepted diagnostic criterion for cancer-associated cachexia was weight loss greater than 5% within six months, or weight loss greater than 2% in individuals already showing depletion according to current body weight and height (BMI < 20 kg/m^2) or skeletal muscle mass (sarcopenia).[5] Therefore, weight loss is a key factor in identifying cancer-associated cachexia. In our study, patients with gastric cancer were divided by weight loss ≥ 5% in order to explore the main reasons for weight loss.

First, our data showed that the weight loss was accompanied by skeletal muscle loss, fat loss and decreased handgrip strength, and weight difference between weight loss ≥ 5% group and weight loss < 5% group were mainly due to difference of skeletal muscle (48.7%). In cancer-assciated cachexia, skeletal muscle loss is more creitical, which should be took seriously. Generally, the occurrence of cachexia is deemed in connection with age, while in our study, no significant difference in age was found between patients with weight loss ≥ 5% group and weight loss < 5%. Moreover, univariate analysis also found no effect of age on weight loss, which presented that young patients might occure weight loss as well. Current researches about cachexia mostly involve the elderly patients and ignore the young.[13–17] While this study reminds that young patients with gastric cancer are also at high risk of cancer-associated cachexia, thus preoperative nutritional assessment and even nutritional intervention should be required for young patients. Although the rate of weight loss ≥ 5% in women was higher than that in men, the difference between two groups was insignificant. Moreover, compared to those with weight loss < 5%, women with weight loss ≥ 5% had more reduced SMI and handgrip strength than men, which suggested that the women with weight loss would have more skeletal muscle loss than the men,consistent with many studies about cachexia.[18–22] Additionally, we found that weight loss could have an extreme impact on the quality of life, especially physical functioning. Interestingly, we also found that weight loss could produce an effect on mental health, which was rarely reported in cachexia.[23, 24] It has been reported that inflammatory factors are related to the mental health.[25–27] So we speculate that the result may be due to the high inflammatory factors in weight loss ≥ 5% group, which needs more studies to verify whether correcting inflammation can improve the quality of life of patients with cachexia. Through univariate and multivariate analysis, we found that tumor stage and circulating TNF-α were key

factors leading to weight loss, which was consistent with previous researches.[28] The incidence of weight loss ≥ 5% raised up with increased circulating TNF-α. Through ROC curve, we got the cut-off value of 9.96 pg/ml for TNF-α to determine weight loss ≥ 5%, which was reported rarely. The cut-off value could assist to assess the nutritions of patients with gastric cancer and estimate the risk of complications. Although TNF-α and tumor stage have similar effect on weight loss, they have different clinic characteristics, which illustrates that the mechanisms of TNF-α and TNM stage resulted in weight loss should be different. From Table 4, we could discover that in advanced gastric cancer, the changes in both metabolism and immunity were accompanied by decrease of nutritional status. This illustrated that cancer profoundly altered the normal homeostatic control of energy balance, resulting in internal environment disorder to promote sustained growth itself.[29] However, circulating TNF-α, commonly termed to be cachectin, is different from the tumor stage mainly in immune status and metabolism. In TNF-α^{H} group, the immune state and glucose were all elevated, which were opposite to the nutritional state. And This means that although TNF-α can lead to weight loss, it maybe boost the immunity against tumor, which is rarely noticed in cancer-associated cachexia.[30] Moreover, although circulating TNF-α and tumor stage were associated with the changes in body weight, skeletal muscle mass and handgrip strength before the surgery, TNF-α mainly affected the skeletal muscle mass and handgrip strength instead of fat mass. Besides, from Table 3, the increase of LDH also suggested that TNF-α could impaire the function of striated muscle, and postoperative changes of body compositions could confirm this result. This may indicate that characteristics of body composition changes in cancer-associated cachexia were heterogeneous and related to the mechanism of different participations. After tumor resection, high level of circulating TNF-α would return to normal, which suggested that high TNF-α might be induced by the tumor. But, the recovery process lasted for a period of time, which said that TNF-α not only came from tumor secretion. Thus, accelerating the recovery of TNF-α may be instrumental in preventing the loss of body weight, especially skeletal muscle.

To verify the mechanism of TNF-α on skeletal muscle, we first took the rectus abdominis from gastric cancer patients for immunohistochemistry. As it was shown in Fig. 4A, the ratio of type II muscle fibers, which are fast-twitch fibers with lower myoglobin content and more anaerobic glycolytic metabolism, significantly decreased in patients with high circulating TNF-α, explaining a remarkable decrease in the handgrip strength.[31] Thus, the fast-to-slow fiber type transition should make an impact on the metabolic and function of skeletal muscle, particularly glycometabolism. AMPK has a major role in modulation of energy balance and is activated in conditions of low energy, which increases energy production and reduces energy consumption.[32] Recent findings show that AMPK can sense glucose in the absence of any change in the cellular energy state.[33, 34] We also measured the expression of AMPK in skeletal muscle, and it showed that the expression of AMPK in patients with high circulating TNF-α was significantly lower, which was consistent with the changes in muscle fiber type transition. Based on these findings, we guess that TNF-α can regulate glucose metabolism of skeletal muscle by interfering AMPK. To verify this hypothesis, experiments in vitro with HSMMs cells were carried out, which not only demonstrated that TNF-α could induce apoptosis of HSMMs cells, a skeletal muscle stem cell line, but also explicitly explained why skeletal muscle mass in patients with high circulating TNF-α before surgery

was difficult to recover after surgery. This calls attention to cell therapy in cancer-associated cachexia. Now several clinical trials have been performed to treat skeletal muscle injuries in some models using myogenic progenitor cells or multipotent stromal cells, with promising outcomes.[35–38] Recent clinical trials on therapies of skeletal muscle loss such as physical exercise, nutraceutical, and pharmaceutical interventions have revealed that exercise is the only effective strategy, while nutraceutical or pharmaceutical interventions showed controversial results. However, the exercise has limited benefit to immobile patients.[39, 40] Thus, innovative cell-based solutions provide a positive outlook on future opportunities toward treating sarcopenia, resulted from cachexia.

The researches referring to cancer-associated cachexia, usually pay too much attention to the role of TNF-α in cachexia, regarded as cachectin, but its role in regulating immunity in cancer is often ignored. In our study, we found that TNF-α may be a double-edged sword for patients with gastric cancer. From clinical data, we found that high TNF-α was associated with the number of $CD3^+$ T cells and $CD8^+$ T cells, which was further verified in vitro. However, higher level of TNF-α didn't often result in a more favorable proliferation of $CD8^+$ T cells. And excessive high level of TNF-α could increase the apoptosis of $CD8^+$ T cells, which had also been reported in other studies.[41, 42] Interestingly, most patients with high circulating TNF-α were not above 20 pg/ml (2%). In majority of patients, circulating TNF-α can promote the proliferation of $CD8^+$ T cells in peripheral blood. In addition to proliferation, the expressions of immune checkpoints such as PD-1, TIM-3, LAG-3 and TIGIT are also characteristics of the killing function of $CD8^+$ T cells, which is well known.[43, 44] Therefore, we measured the expressions of PD-1, TIM-3, LAG-3 and TIGIT in patients with different circulating TNF-α, and the data showed that TNF-α was not significantly correlated with those expressions. And in vitro experiments, the result was as same as that in vivo.

It seemed that there were many contradictory findings in our study among TNF-α, $CD8^+$ T cells and skeletal muscle. However, these may be the relationships that actually exist in human body. When immune response caused by tumors is positive, it leads to increment of tumor-infiltrating immune cells in cancer patients. Then, more TNF-α was released into peripheral blood, which can promote the proliferation of $CD8^+$ T cells in peripheral blood to let more $CD8^+$ T cells enter the tumor. A lot of energy was needed in the rapid proliferation of $CD8^+$ T cells. But tumors compete with other organs and tissues for energy fuels and biosynthetic substrates and possess an intrinsic metabolic rate.[45] In order to provide energy for the proliferation of $CD8^+$ T cells, skeletal muscle, the largest energy-consuming organ, is sacrificed by TNF-α, which can lead to myofiber change, interfered glycometabolism of skeletal muscle and increased myoblast apoptosis. Persistent skeletal muscle loss is dangerous to maintain normal physiological function. The skeletal muscle, injured by high TNF-α, would reduce the secretion of cytokines to negatively regulate the immune function. This process may be a protection mechanism for patients with cancer to guarantee the organism in a relatively stable state. In the future, further experiments are required to verify our hypothesis.

Conclusion

Our study suggested that preoperative circulating TNF-α of more than 9.96 pg/ml could indicate a high risk of weight loss, particularly the skeletal muscle loss. Meanwhile, we found new relationships between circulating TNF-α, skeletal muscle and peripheral $CD8^+$ T cells in gastric cancer during cachexia. TNF-α could result in myofiber change, interfered glycometabolism of skeletal muscle to reduce the energy input to skeletal muscles. Whereas TNF-α promoted the proliferation of $CD8^+$ T cells and meanwhile created a high energy environment for the proliferation of $CD8^+$ T cells. Those can help us understand the inter-tissue communication in cancer-associated cachexia and unravel the mediators that drive communication between skeletal muscle and other tissues.[30] And inflammatory response such as high circulating TNF-α being the main driving force behind the metabolic alterations present.[46] However, the cross-talk between skeletal muscle wasting and immunity, for instance $CD8^+$ T cells, can regulate the system inflammatory response caused by multiple factors. For example, tumor cells release cytokines and other inflammatory mediators. It is known that now, apart from being a systemic disorder, cancer-associated cachexia is a multi-organ syndrome. Our research attempts to elucidate the relationship among circulating TNF-α, skeletal muscle and peripheral $CD8^+$ T cells in gastric cancer to reveal the inflammation involved in inter-organ communication that influences muscle metabolism and understand the sequence of events taking place in the involvement of the different organs and tissues during cancer-associated cachexia.

Declarations

Authors' contributions:

[#]Rui Zhao, [#]Qianyi Wan and **Lin Xia[#]** contributed equally to this study. Prof. Xiaoting Wu, together with Rui Zhao and Qianyi Wan, conceived the study, while all of those authors mentioned above have contributed to the research and development process of this article. Under the guidance of Prof. Xiaoting Wu and Hanshuo Yang, Rui Zhao, Qianyi Wan, Yong Wang and Qiqi Li have conducted the experiments with the clinical data collected by Lin Xia, Shuomeng Xiao, Yaping Cui, Xiangnan Su, Xiaoding Shen, Wen Zhuang, Yong Zhou and Yi Chen. The manuscript was completed by Rui Zhao, Qianyi Wan, Lin Xia and Yutao Wu. All the authors have read and approved the final manuscript.

Ethics approval and consent to participate：

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Competing interests:

There was no conflict of interest.

Availability of data and materials:

All data generated or analysed during this study are included in this published article

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Figures

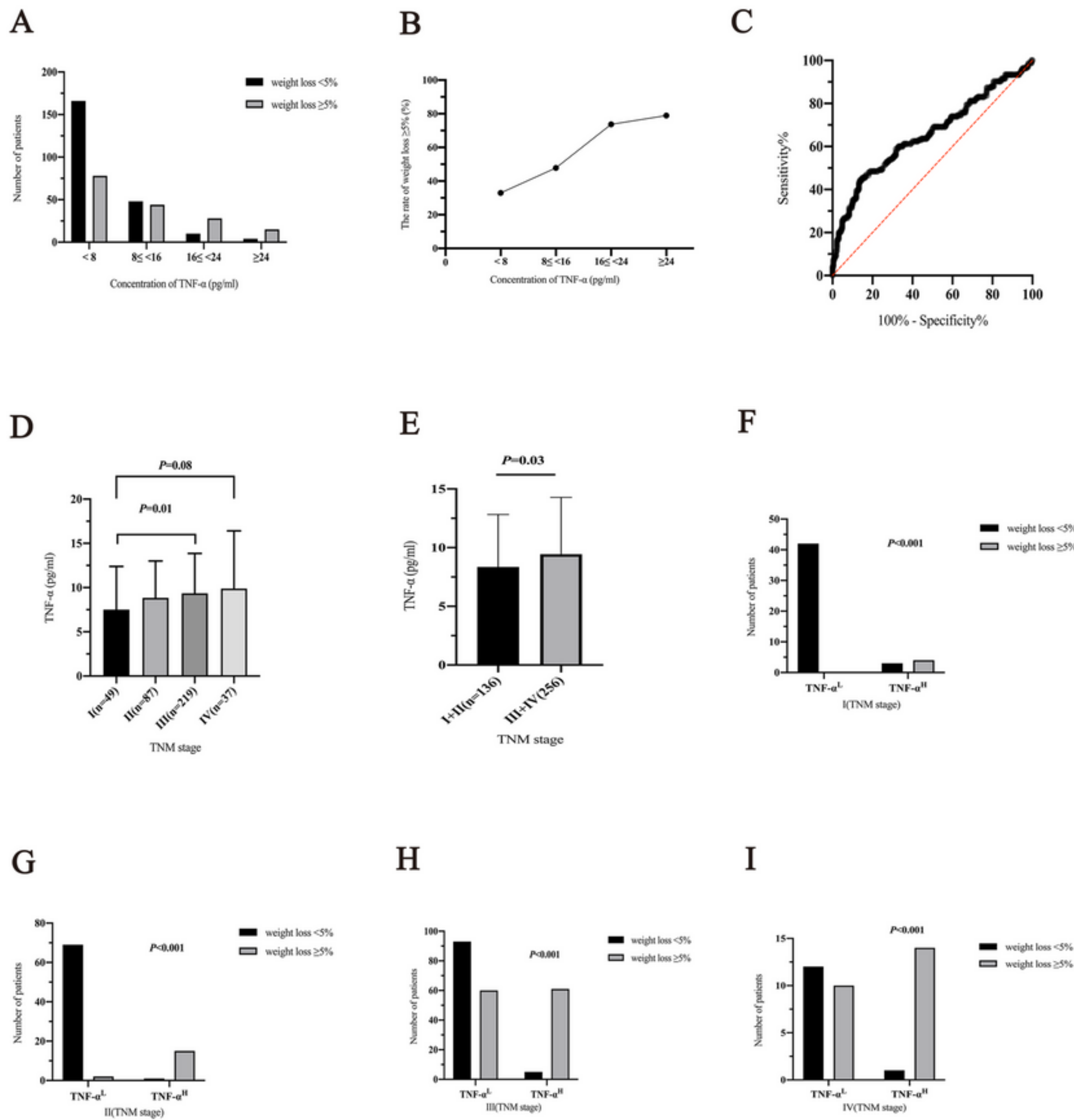

Figure 1

Comparison of preoperative weight loss in patients with different circulating TNF-α and TNM stage. (A) Relationship between circulating TNF-α and weight loss before surgery. (B) Relationship between circulating TNF-α and the rate of weight loss ≥5% before surgery. (C) ROC curve for testing the sensitivity and specificity of TNF-α associated with weight loss ≥5% (AUC=0.66, P <0.001). (D) Relationship between TNF-α and TNM stage. (E) Comparison of circulating TNF-α between patients of TNM stage I+II

(n =136) and stage III+IV (n =256). (F) Comparison of preoperative weight loss between stage I patients with TNF-αH (≥ 9.96 pg/ml) and TNF-αL (< 9.96 pg/ml). (G) Comparison of preoperative weight loss between stage II patients with TNF-αH (≥ 9.96 pg/ml) and TNF-αL (< 9.96 pg/ml). (H) Comparison of preoperative weight loss between stage III patients with TNF-αH (≥ 9.96 pg/ml) and TNF-αL (< 9.96 pg/ml). (I) Comparison of preoperative weight loss between stage IV patients with TNF-αH (≥ 9.96 pg/ml) and TNF-αL (< 9.96 pg/ml). Values are means ± SD.

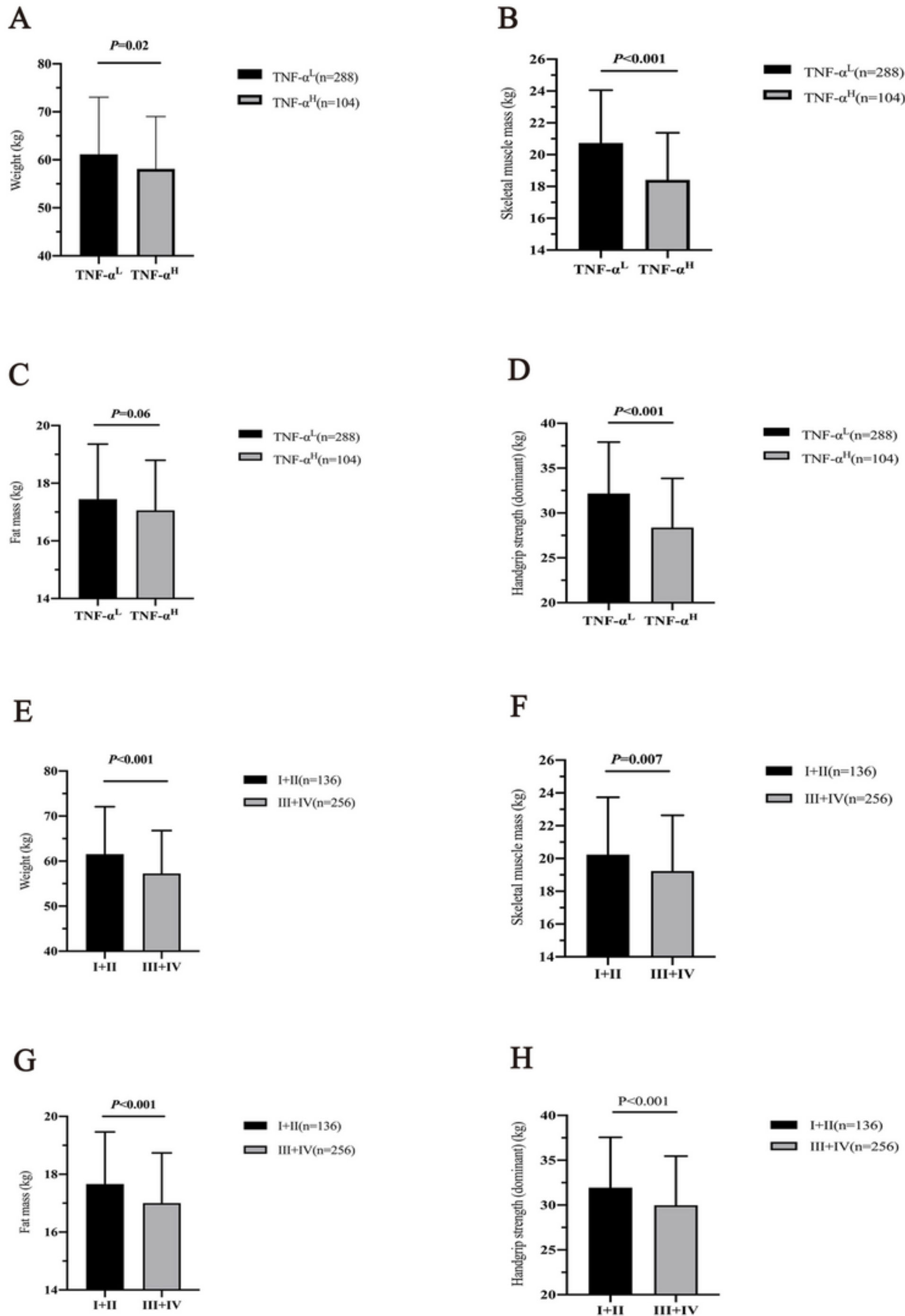

Figure 2

Comparison of preoperative weight loss in patients with different circulating TNF-α and TNM stage. (A) Relationship between circulating TNF-α and weight loss before surgery. (B) Relationship between circulating TNF-α and the rate of weight loss ≥5% before surgery. (C) ROC curve for testing the sensitivity and specificity of TNF-α associated with weight loss ≥5% (AUC=0.66, P <0.001). (D) Relationship between TNF-α and TNM stage. (E) Comparison of circulating TNF-α between patients of TNM stage I+II (n =136) and stage III+IV (n =256). (F) Comparison of preoperative weight loss between stage I patients with TNF-αH (≥ 9.96 pg/ml) and TNF-αL (< 9.96 pg/ml). (G) Comparison of preoperative weight loss between stage II patients with TNF-αH (≥ 9.96 pg/ml) and TNF-αL (< 9.96 pg/ml). (H) Comparison of preoperative weight loss between stage III patients with TNF-αH (≥ 9.96 pg/ml) and TNF-αL (< 9.96 pg/ml). (I) Comparison of preoperative weight loss between stage IV patients with TNF-αH (≥ 9.96 pg/ml) and TNF-αL (< 9.96 pg/ml). Values are means ± SD.

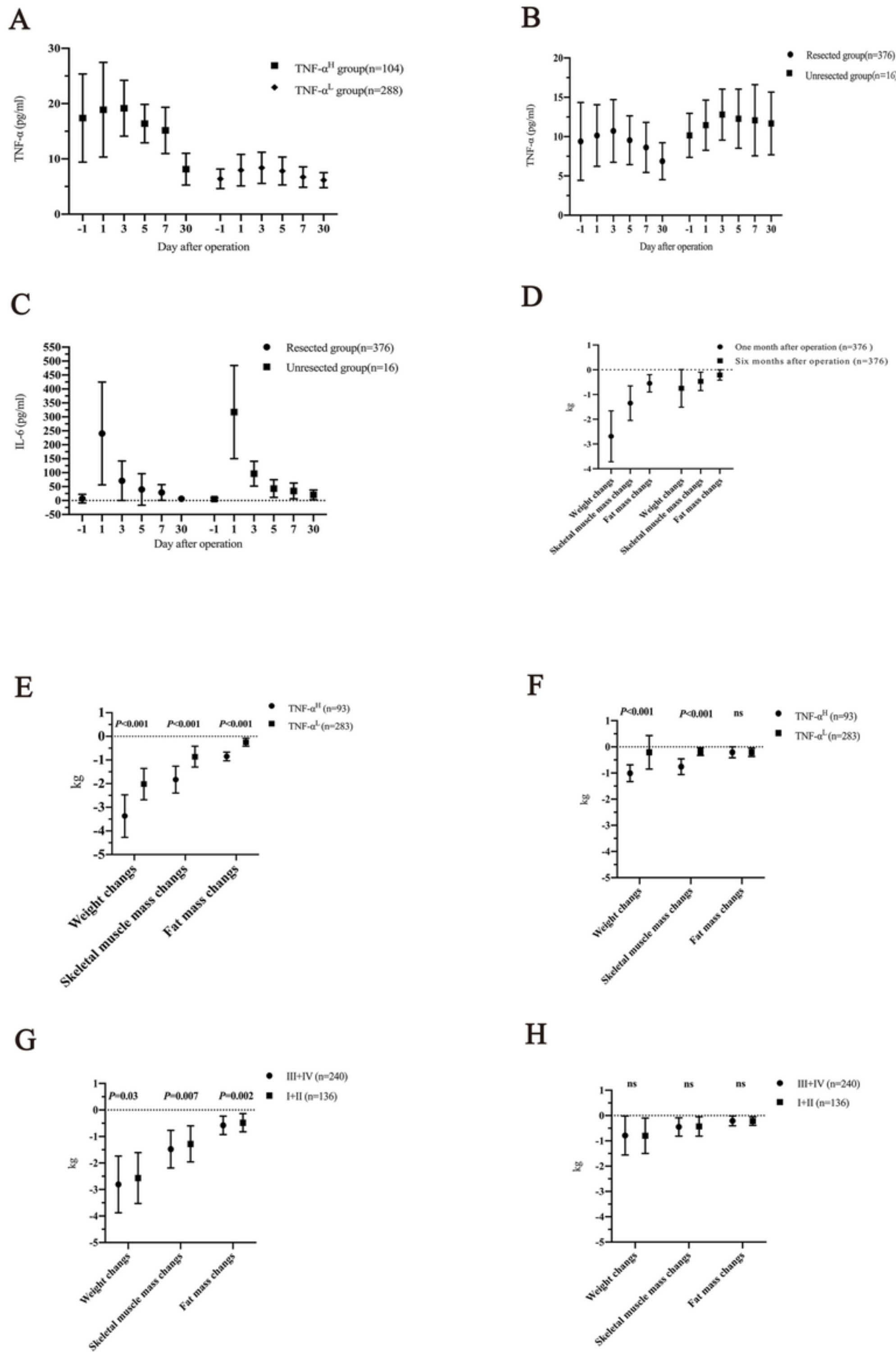

Figure 3

Analysis of postoperative body composition and inflammatory cytokines. (A) Postoperative changes of circulating TNF-α in patients with preoperative TNF-αH (n =104) and TNF-αL (n =288). (B) Postoperative changes of circulating TNF-α in patients with radical surgeries (n =376) and non-radical surgeries (n =16). (C) Postoperative changes of circulating IL-6 in patients with radical surgeries (n =376) and non-radical surgeries (n =16). (D) Changes of body composition one month and six months after surgery. (E)

Comparison of body composition changes between patients with preoperative TNF-αH (n =93) and TNF-αL (n =283) one month after surgery. (F) Comparison of body composition changes between patients with preoperative TNF-αH (n =93) and TNF-αL (n =283) six month after surgery. (G) Comparison of body composition changes between patients of TNM stage I+II (n =136) and stage III+IV (n =240) one month after surgery. (H) Comparison of body composition changes between patients of TNM stage I+II (n =136) and stage III+IV (n =240) six month after surgery. Values are means ± SD; ns, P ≥0.05.

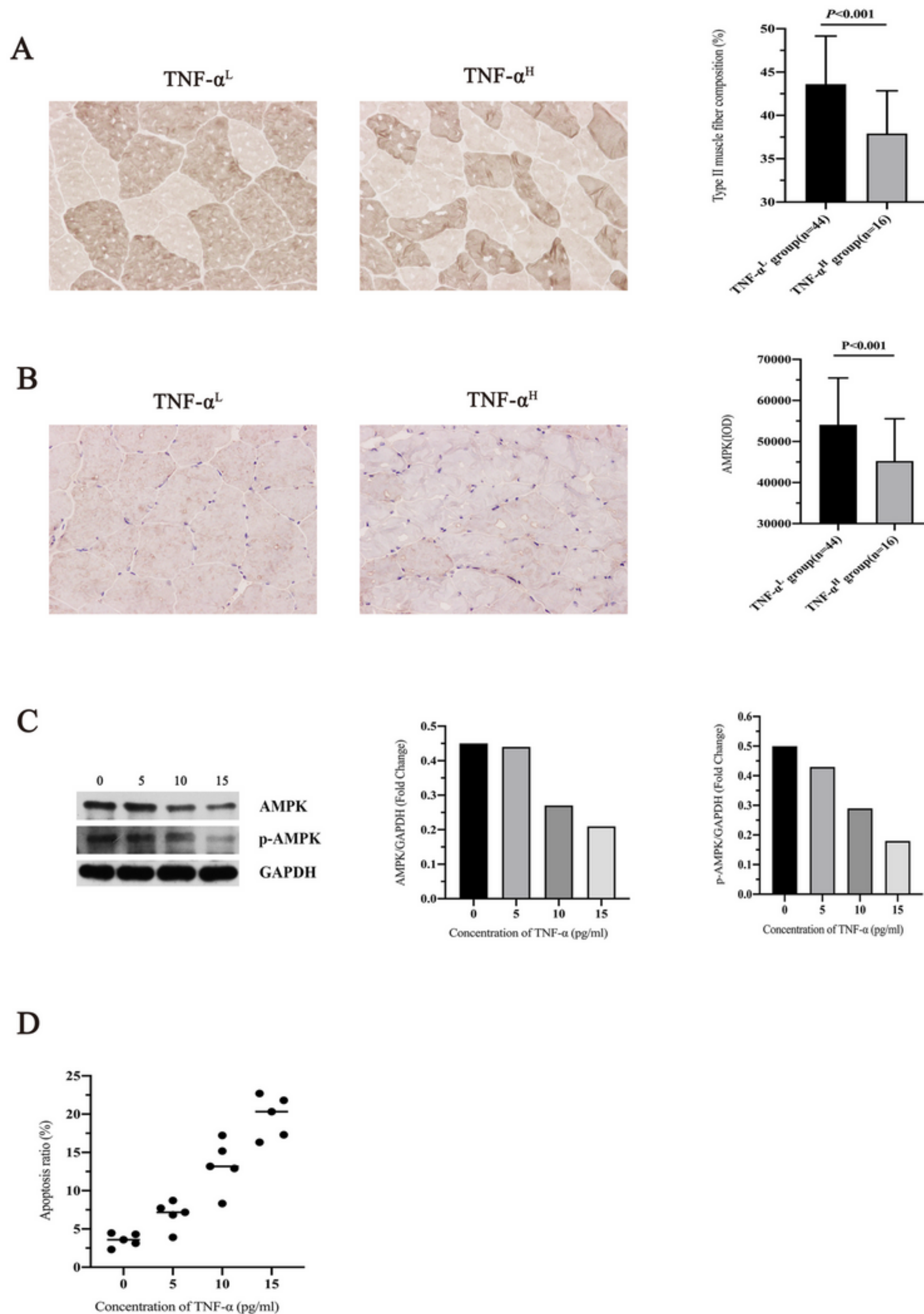

Figure 4

Analysis of the effect of TNF-α on skeletal muscle. (A) Comparison of type II muscle fiber composition between patients with TNF-αH (n =16) and TNF-αL (n =44). (B) Immumohistochemical analysis for comparing the expressions of AMPK in skeletal muscle between patients with TNF-αH (n =16) and TNF-αL (n =44). (C) Western blot analysis for comparing the expressions of AMPK and p-AMPK in HSMMs cultured with different concentrations of TNF-α. (D) Flow cytometry analysis for comparing the apoptosis of HSMMs cultured with different concentrations of TNF-α. Values are means ± SD.

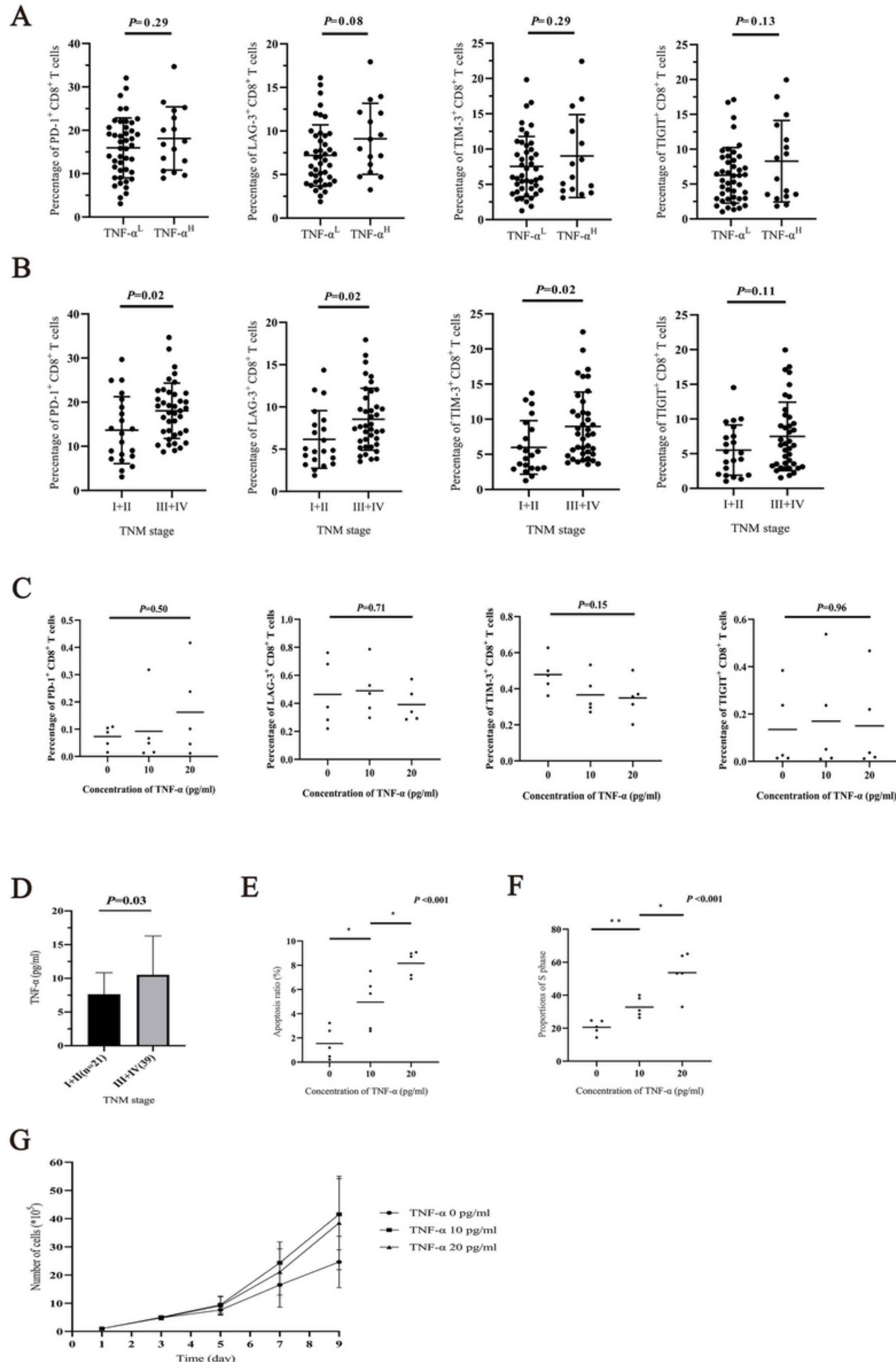

Figure 5

Analysis of the effect of TNF-α on circulating CD8+ T cells. (A) Comparison of the expressions of PD-1, LAG-3, TIM-3 and TIGIT in CD8+ T cells between patients with TNF-αH (n =16) and TNF-αL (n =44). (B) Comparison of the expressions of PD-1, LAG-3, TIM-3 and TIGIT in CD8+ T cells between patients of TNM stage I+II (n =21) and stage III+IV (n =39). (C) Comparison of the expressions of PD-1, LAG-3, TIM-3 and TIGIT in CD8+ T cells cultured with different concentrations of TNF-α. (D) Comparison of circulating TNF-α between patients of TNM stage I+II (n =21) and stage III+IV (n =39). (E) Comparison of the apoptosis of CD8+ T cells cultured with different concentrations of TNF-α. (F) Comparison of the proportions of S phase in CD8+ T cells cultured with different concentrations of TNF-α. (G) Comparison of the proliferation of CD8+ T cells cultured with different concentrations of TNF-α (n =5); Values are means ± SD.